

Probing Antigen Binding Characteristics Using Single Domain Antibodies

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Abstract

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Antibodies

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The variable heavy domains of heavy chain antibodies (VHH) possess unique characteristics which make them an attractive alternative to conventional antibody fragments for detailed NMR-based structural analysis. The small size offers an advantage over Fabs and the enhanced stability compared to scFvs is also beneficial. In addition, the elongated complementarity determining regions (CDRs) associated with VHH fragments may facilitate the characterisation of a more diverse range of antibody-antigen interactions as well as providing templates for studying the motion of CDRs.

The work described in this thesis illustrates the feasibility of using VHH fragments to gain structural insight into antibody-antigen binding. The successful generation of isotopically labelled protein allowed the acquisition of high quality NMR spectra from which the solution structures of two free VHH fragments were determined. The solution structures revealed significant differences in the conformational dynamics at the antigen-binding site with both flexible and structured CDR loops being identified. The binding sites of both the VHH and the antigen were mapped by NMR minimal shift analysis.

The work discussed in the latter chapters focuses on the structure of VHH 67, its interactions with IL-6 and how information gained from this antibody-antigen interaction may be applicable to drug discovery. The structure determination and binding site mapping showed that binding occurred through the long, structured CDR3 loop and also identified that VHH 67 bound to IL-6 through a novel binding site. This new binding site was confirmed by X-ray crystallography and the functional effects of the VHH 67:IL-6 interaction were ascertained. The relationship between binding affinity and the dynamics of CDR3 was also investigated.

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Abbreviations

° C	Degrees Centigrade
2D	Two-Dimensional
3D	Three-Dimensional
A	Amperes
Å	Angstrom
A ₆₀₀	Absorbance at 600 nm
AU	Absorbance Units
BP	Base Pairs
BSA	Bovine Serum Albumin
Buffer E	Equilibrium Buffer
Buffer L	Labelling Buffer
Buffer Q	Quench Buffer
C	Constant Domain
C'	Carbonyl Carbon
CANDID	Combined Automated NOE Assignment and Structure Determination Module
CCP4	Collaborative Computational Project Number 4
CD	Circular Dichroism
CDR	Complementarity Determining Region
CH	Constant Domain of the Heavy Chain
CHR	Cytokine Homology Region
CL	Constant Domain of the Light Chain
CNTF	Ciliary Neurotropic Factor
C _p	Heat Capacity
CSI	Chemical Shift Index
CYANA	Combined Assignment and Dynamics Algorithm for NMR Applications
D	Diversity
D1	Domain 1
D2	Domain 2
D3	Domain 3
Da	Daltons
dH ₂ O	Distilled Water
DNA	Deoxyribonucleic Acid
DSC	Differential Scanning Calorimetry
DTT	Dithiothreitol
<i>E.coli</i>	<i>Escherichia coli</i>
ECD	Extracellular Domain
ECL	Electrochemical Luminescence
EDTA	Ethylenediamine Tetra Acetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
ESI+	Electrospray Positive Ionisation
F ₁	NMR Dimension 1
F ₂	NMR Dimension 2
F ₃	NMR Dimension 3
Fab	Fragment, Antigen-Binding
Fc	Fragment Crystallisable

FDA	Food and Drugs Administration
FR	Framework Region
Fv	Fragment Variable
g	Grams
gp-130	Glycoprotein-130
gp-80	Glycoprotein-80 (Interleukin-6 Receptor)
GuHCl	Guanidine Hydrochloride
h	Human
H	Heavy Domain
H1	Hypervariable Region 1
H2	Hypervariable Region 2
H3	Hypervariable Region 3
HBS-EP	HEPES Buffered Saline with EDTA and P20
HcAb	Heavy Chain Antibody
hCG	Human Chorionic Gonadotropin Hormone
HDX	Hydrogen Deuterium Exchange
HEK	Human Embryonic Kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
hFc	Human Fragment Crystallisable
His ₆	Hexahistidine
HN	Amide Proton
HRP	Horseradish Peroxidase
hscFc	Human Single Chain Fragment Crystallisable
HSQC	Heteronuclear Single Quantum Coherence Spectroscopy
Hz	Hertz
IC ₅₀	Half Maximal Inhibitory Concentration
Ig	Immunoglobulin
IL-11	Interleukin-11
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-6Rα	See gp-80
IL-6Rβ	See gp-130
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
J	Joining
JAK	Janus-Activated Kinases
k	Kilo (10 ³)
k _a	Association Constant
k _d	Dissociation Constant
K _D	Equilibrium Dissociation Constant
k _{ex}	Exchange Rate
KOD	<i>thermococcus kodakaraensis</i>
l	Litre
L	Light Domain
LB	Luria-Bertani
LIF	Leukemia Inhibitory Factor
LLG	Log Likelihood Gain
M	Molar
m	Metres

m	Milli (10^{-3})
MeCN	Acetonitrile
MHz	Megahertz
MOPS	3-(N-morpholino)Propanesulphonic Acid
MPD	2-Methyl-2,4-Pentanediol
MS	Mass Spectrometry
MWCO	Molecular Weight Cut Off
n	Nano (10^{-9})
Ni-NTA	Nickel-Nitrilotriacetic Acid
NMR	Nuclear Magnetic Resonance
NOE	Nuclear Overhauser Effect
NOESY	Nuclear Overhauser Effect Spectroscopy
NUS	Non-Uniform Sampling
p	Pico (10^{-12})
P20	Polysorbate 20
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDB	Protein Data Bank
PEG	Polyethylene Glycol
pET	T7 Promoter Expression Vectors
PPA	Porcine Pancreatic α -Amylase
ppm	Parts Per Million
psi	Pounds Per Square Inch
REDAC	Redundant Dihedral Angle Constraints
RMSD	Root Mean Standard Deviation
rpm	Revolutions Per Minute
RSS	Recombination Signal Sequences
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
RU	Response Units
s	Seconds
scFc	Single Chain Fragment Crystallisable
scFv	Single Chain Fragment Variable
sd	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SLAM	Selected Lymphocyte Antibody Method
SOC	Super Optimal Catabolite
SPR	Surface Plasmon Resonance
STAT	Signal Transduction and Activators of Transcription
T1	Longitudinal Relaxation Time
T2	Transverse Relaxation Time
TAE	Tris-Acetate EDTA
TALOS	Torsion Angle Likelihood Obtained from Shift
TEV	Tobacco Etch Virus
THP1	Human Acute Monocytic Leukaemia Cell Line
T _m	Midpoint of Unfolding
TMB	3,3',5,5'-Tetramethyl Benzidine

TOCSY	Total Correlation Spectroscopy
TOF	Time-of-Flight
Tris	Tris(hydroxymethyl)aminomethane
TROSY	Transverse Relaxation-Optimised Spectroscopy
TSN	T-cell Supernatant
TY	Tryptone Yeast
UPLC	Ultra Performance Liquid Chromatography
UV	Ultra Violet
V	Variable Domain
VH	Variable Domain of the Heavy Chain
VHH	Variable Domain of a Heavy Chain Antibody
VL	Variable Domain of the Light Chain
w/v	Weight Per Volume
w/w	Weight Per Weight
WATERGATE	Water Suppression Through Gradient Tailored Excitation
WT	Wild Type
x g	Times Gravity
δ	Chemical Shift
μ	Micro (10^{-6})
ν	Frequency
Φ	Phi (Amide Nitrogen to Alpha Carbon Dihedral Angle)
Ψ	Psi (Alpha Carbon to Carboxyl Carbon Dihedral Angle)
ω_1	NMR Frequency 1
ω_2	NMR Frequency 2

Amino Acid Abbreviations and One Letter Codes

Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamic Acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

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Chapter 1 – Introduction

The work described in this thesis evaluates the use of VHH antibodies as an alternative to conventional antibody fragments for structural analysis, therefore, an introduction to both conventional antibodies and VHH fragments follows along with a review of a selection of VHH structures. The VHH antibodies characterised in this project were raised against the cytokine interleukin-6 so a brief background to this target is provided followed by a summary of the functional and structural analyses carried out prior to the start of this project.

1.1 – Conventional Antibodies and Heavy Chain Antibodies

1.1.1 – Conventional Antibodies

Conventional antibodies consist of two identical heavy (H) and two identical light (L) chains linked by disulphide bonds as shown in Figure 1.1 (Davies and Chacko, 1993). The lower portion of the antibody, known as the fraction crystallisable (Fc), determines effector functions and is joined via a flexible hinge to the fragment antigen binding (Fab), which is responsible for the affinity and specificity of the antibody to a particular antigen. The variable domains of the Fab fragment contain framework regions (FR) that are conserved between antibodies and regions known as hypervariable regions or complementarity determining regions (CDRs) in which the sequence varies significantly between antibodies. Each Fab contains six hypervariable loops (three per variable domain) that, together with a small number of framework residues, make up the antigen-binding site. The backbone structure of five of the six loops tend to adopt a known number of conformations (or canonical forms) which can be predicted based upon a few key interactions at specific positions between the CDRs and framework regions (Chothia *et al.*, 1989). The third CDR of the variable heavy domain (CDR-H3) shows greater variation and cannot be reliably predicted.

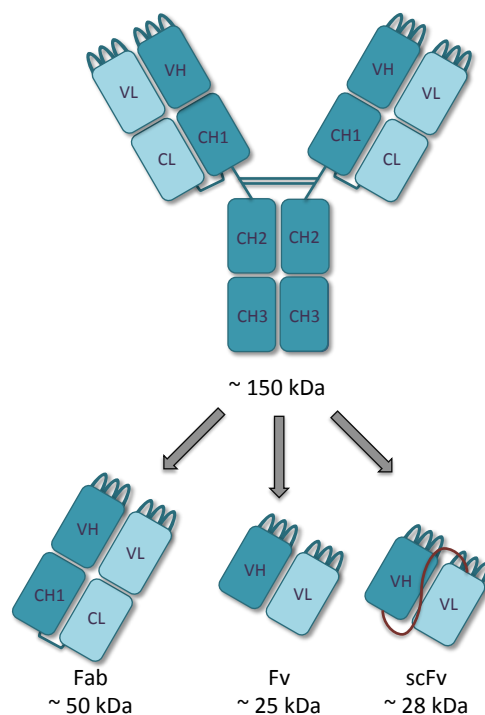


Figure 1.1 - Conventional Antibody Structure and Common Antibody Fragments

The typical structure of a conventional IgG antibody is shown and consists of two identical heavy (H) and light (L) chains. These chains are divided into constant (C) and variable (V) domains. Antigen affinity and specificity is determined predominantly by the three CDRs of each of the variable domains, which are shown as loops. Shown beneath the full size antibody structure are three common antibody fragments used in antibody research: the fragment antigen binding (Fab) consisting of the first constant and variable domains of the heavy and light chains; the fragment variable (Fv) consisting of just the variable domains; and the single chain fragment variable (scFv) produced by linking the variable domains of the Fv using a peptide linker.

The ability of antibodies to recognise and respond to the range of antigens that may be encountered requires huge diversity in the variable region. This diversity is introduced through a number of mechanisms including junctional diversity from the V(D)J recombination to form the V_H and V_L domains; combinatorial diversity from the random pairing of V_H and V_L domains; and by somatic hypermutation in which random point mutations are inserted to increase the affinity of the antibody to a specific antigen after initial exposure (Tonegawa, 1983).

The variable domain of the heavy chain (V_H) is formed by randomly selecting one diversity (D_H), one joining (J_H) and one variable (V_H) gene segment from a selection of gene segments clustered in the H locus (Flajnik *et al.*, 2001). Initially the D_H and J_H segments are combined followed by addition of the V_H segment (Oka and Kawaichi, 1995). Flanking each of these segments are recombination signal sequences (RSS). The cleavage and splicing at the RSS regions is imprecise and nucleotides can be added or deleted at the junctions resulting in further diversity of the V_H domain (Schroeder and Cavacini, 2010). For the variable domain of the light chain (V_L) a similar process occurs but only variable (V_L) and joining (J_L) segments are combined, selected from a pool of genes clustered on the L locus. The variable gene segments contain the exons that encode for framework regions 1, 2 and 3, CDRs 1 and 2 and the N-terminus of CDR3 while the joining segments contain the exons encoding for the C-terminus of CDR3 and Framework4 (Schroeder and Cavacini, 2010). The junction point between the V and J segments is located in CDR3 and accounts for increased diversity observed in CDR3 of both the heavy and light chains (CDR-H3 and CDR-L3). The inclusion of diversity segments in the formation of the heavy chain increases further the diversity and length of CDR-H3 as the D_H segments can be rearranged by inversion, deletion or can be spliced and translated into one of three potential reading frames (Schroeder and Cavacini, 2010).

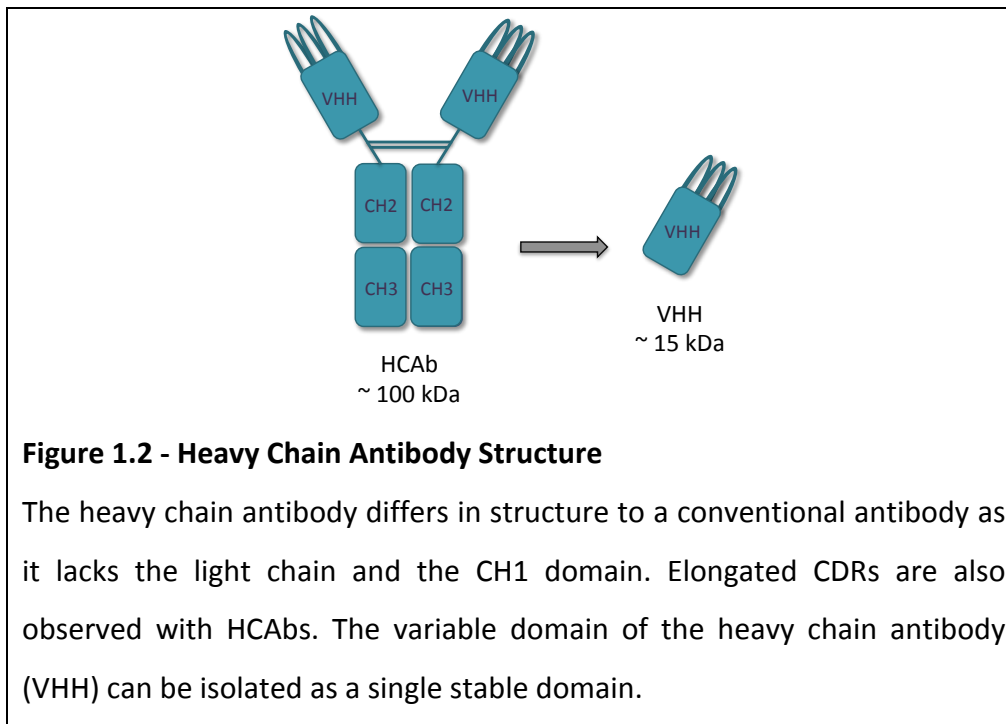
In the context of drug discovery, antibodies are large, complex molecules that often require mammalian expression systems to ensure the inclusion of the essential disulphide bonds and post-translational modifications during both industrial and commercial production (Chames *et al.*, 2009). This makes their production expensive, especially in comparison to small molecule drugs. Other properties of antibodies that, depending on the application, may be undesired are limited tissue penetration (Beckman *et al.*, 2007) prolonged half-life (Chames *et al.*, 2009) and Fc mediated responses (Chames *et al.*, 2009). Many of these negative effects can be attributed to the large size of antibodies and for this reason the general trend in the design of antibody therapeutics has been to reduce the size of the antibodies used in treatment from full length antibodies to smaller formats such as Fabs and single chain fragment variables (scFvs) (Bird *et al.*, 1988). In addition to reducing the size of antibodies for improved therapeutics, the development of smaller, stable and less complex antibody formats has advantages for structural methods, such as NMR, to solve antibody structures to allow the rational design of drug molecules.

The Fab consists of the first constant domain and the variable domain of both the heavy and light chains (see Figure 1.1). Fabs have been successfully developed into therapeutics, for example Abciximab or Reopro (Janssen Biologics) was approved by the Food and Drugs Administration (FDA) in 1994 for the prevention of blood clots in angioplasty and Ranibizumab or Lucentis (Genentech) was approved in 1998 for the treatment of macular degeneration (Reichert, 2010). However, with a size of approximately 50 kDa, these therapeutics are still costly to produce. Reducing the size further, by removing the constant domains, to produce the Fv (see Figure 1.1) retains the same antibody-antigen interface but dissociation of VH and VL domains at low concentrations is often observed (Glockshuber *et al.*, 1990). To stabilise the two domains of the Fv, a peptide linker can be added creating a scFv (see Figure 1.1). Unfortunately, scFvs are prone to a dimerisation process in which the domains of the scFv partially dissociate forming an open monomer that then associates with another open monomer resulting in a complex of approximately 56 kDa (Bennet *et al.*, 1995). This dimerisation process can be

reduced by optimising the linker length (Whitlow *et al.*, 1993) and examples of scFvs, such as Pexelizumab (Alexion Pharmaceuticals) used to reduce side effects during coronary bypass surgery, have been progressed into clinical trials although to date no scFv therapeutic has progressed successfully through clinical trials to be approved by the FDA. Reducing the antibody size further to a single VH domain has two main drawbacks. Firstly, the VH domain shows an affinity ten fold weaker than that of the Fv (Ward *et al.*, 1989) caused by the reduction in the antibody-antigen interface. Secondly, removing the VL domain exposes a large hydrophobic surface on the VH that would form the interface with the VL causing aggregation of the VH domain (Ward *et al.*, 1989). The removal of the Fc region in these smaller antibody formats may result in the loss of beneficial properties such as extended half-life and effector functions associated with the Fc region. To help overcome this alternative methods have been developed to maintain these features in the smaller antibody fragments. For example in the antibody therapeutic Certolizumab pegol (Cimzia, UCB), used to treat rheumatoid arthritis and Crohn's disease, the active Fab fragment was conjugated to polyethylene glycol (PEG) to increase the serum half-life (Choy *et al.*, 2002).

1.1.2 – Heavy Chain Antibodies

In 1993 a novel class of antibodies, termed heavy chain antibodies (HCAbs), was discovered in the serum of *Camelus dromedarius* (Hamers-Casterman *et al.*, 1993). HCAbs exist alongside conventional antibodies in the serum of camelid (camel, dromedary, llama and alpaca) and some species of cartilaginous fish (nurse shark (Greenberg *et al.*, 1995), wobbegong shark (Conrath *et al.*, 2003) and ratfish (Nguyen *et al.*, 2002)) and account for up to 75 % of the total circulating antibody content (Hamers-Casterman *et al.*, 1993). Heavy chain antibodies differ in structure to conventional antibodies as they lack both the light chains and the first constant domain of the heavy chain (CH1) as seen in Figure 1.2. Another feature associated with HCAbs are elongated CDR loops (Muyldermans, 2001). These unique structural features of HCAbs may provide advantages over current conventional antibody derived fragments as tools for drug discovery.



1.1.3 – The Origin of Heavy Chain Antibodies

As stated in Section 1.1.2, camelid and shark possess both conventional and heavy chain antibodies, the origin and the purpose of which has been widely debated. Initial hypotheses included that HCAs were the result of the somatic hypermutation of conventional antibodies but it was shown that the Framework2 substitutions associated with HCAs are imprinted in the dromedary genome (Nguyen *et al.*, 1998) and, therefore, could not be the result of somatic hypermutation. Another possible explanation is that HCAs emerged as a result of exposure to harsh conditions, such as elevated temperatures experienced by camelid or high urea concentrations in the blood of sharks, after which HCAs were preferentially selected over conventional antibodies due to their beneficial properties (Flajnik *et al.*, 2011). These properties may include the ability of the HCAs to access alternative binding sites to conventional antibodies, for example smaller antigen clefts; or the increased stability afforded to the HCAb by the additional disulphide bond of the variable domain (Flajnik *et al.*, 2011). However, emergence as a result of exposure to harsh conditions is unlikely to explain why HCAs are not observed in a greater number of species.

There is, however, agreement to the reasoning behind HCABs being found in mammals of the *Camelidae* family but not found in other ungulates (hoofed mammals). Phylogenetic analyses show the genes encoding for conventional antibodies emerged and diverged approximately 25 million years ago, after *Tylopoda* split from other mammals (*Artiodactyla*), approximately 60 to 80 million years ago, but before camel and llama speciation, approximately 11 million years ago (Conrath *et al.*, 2003).

1.1.4 – The VHH Domain

The variable domain of the HCAB is termed a VHH to distinguish it from the variable heavy (VH) domain of conventional antibodies. The backbone architecture of the VHH domain is identical to that of a conventional VH domain with a four-stranded β -sheet packed against a five-stranded β -sheet with a conserved disulphide bond linking the two sheets (Desmyter *et al.*, 1996). Due to amino acid substitutions in Framework2 region, at what would be the VH-VL interface, the hydrophobicity of this region is reduced (Muyldermans *et al.*, 1994). This means that, unlike the VH domain, the VHH domain does not aggregate and can be isolated as a single domain (Muyldermans *et al.*, 1994). Another outcome of VHHs having evolved without a light chain is that the antigen binding interface is restricted to a single domain meaning that, again unlike with the isolated VH domain, there is no loss in affinity between the parent antibody and single domain (Muyldermans, 2001).

The hypervariable regions of VHH domains are on average longer than those observed in conventional VH domains. This is especially true of CDR3. The average CDR3 length found in the VHH domains of dromedary is 15 residues (Vu *et al.*, 1997) compared to the CDR3 loops of human and mouse VH domains with an average length of 13 and 10 residues respectively (Wu *et al.*, 1993). Although elongated CDR3 loops are also found in llamas, a subfamily of llama VHHs were identified that have much shorter CDR3 loops with a length of around 6 residues (Harmsen *et al.*, 2000). The elongated loops found in VHH domains are thought to compensate for the absence of the VL domain (Muyldermans *et al.*, 1994), these

longer loops also deviate from the canonical structures observed in the hypervariable regions of conventional VH domains (Vu *et al.*, 1997). The combination of longer CDRs and the variation in the conformations of these CDRs increase the diversity and therefore the antigen binding repertoire. However, the loss of entropy associated with stabilising these more flexible, elongated CDRs would have a negative impact on the affinity of antigen binding. To overcome this, the occurrence of an additional cysteine pair is often incorporated into the CDRs. This allows formation of an inter-CDR disulphide bond that is proposed to stabilise the longer CDRs. The relationship between increased CDR3 length and the inclusion of the additional disulphide bond has been inferred for both camel and llama VHH domains (Muyldermans *et al.*, 1994, Vu *et al.*, 1997). As discussed further in Section 1.2, the elongated CDR3 of VHH domains appears to perform two functions. Firstly, the C-terminal portion of CDR3 has been seen to fold over the former VH-VL interface, partially shielding the hydrophobic region exposed by the absence of the VL, and creating an additional region of framework (Desmyter *et al.*, 1996). Secondly, the N-terminal portion, if long enough, can extend from the antigen binding site into antigen clefts not often accessible to the typically flat or concave paratopes of conventional antibodies (Desmyter *et al.*, 1996, Lauwereys *et al.*, 1998, De Genst *et al.*, 2006).

1.1.5 – Physical Properties of the VHH Domain

The physical characteristics of VHHs make them very attractive alternatives to Fab and scFv constructs. The literature reports high yields of VHH protein being expressed in *E.coli* (Ghahroudi *et al.*, 1997) and in *Saccharomyces* yeast (Frenken *et al.*, 2000). In contrast to isolated VH domain proteins, VHHs are highly soluble and stable (Muyldermans, 2001). The amino acid substitutions typically observed in Framework 2 of VHH domains render the hydrophobic former VH-VL interface more hydrophilic so VHHs are less prone to aggregation in solution than isolated VH domains (Davies and Riechman, 1994). Also the domain swapped dimer aggregation resulting from the incorporation of synthetic linkers in scFvs is negated (Muyldermans, 2001). Increased thermal stability compared to conventional antibodies has also been observed with VHHs being shown to retain

80 % to 100 % of their antigen binding affinity after incubation at 37 °C for 200 hours (Ghahroudi *et al.*, 1997) and to remain functional at temperatures up to 90 °C (Van der Linden *et al.*, 1999). This improved thermal stability has also been attributed to the Framework2 amino acid substitutions (Riechmann, 1996).

1.1.6 – Therapeutic Applications of VHHs

The properties of VHH domains offer advantages over conventional antibodies and antibody fragments for many therapeutic applications.

The successful use of antibodies as imaging agents to detect tumours relies on efficient penetration of the tumour tissue, high affinity to the target antigen and rapid clearance of any unbound imaging agent. Problems with immunogenicity, poor tumour penetration and slow clearance have been observed with full size antibodies (Muyldermans, 2001). The use of smaller scFv fragments showed rapid plasma and whole body clearance and faster penetration of the tumour, 0.5 hour post injection compared to 48 to 96 hours post injection for an IgG-based conjugate, with the imaging agent being more evenly distributed throughout the tumour (Yokota *et al.*, 1992). Reducing the size of the antibody fragment further, to a single VHH domain, has shown increased clearance and improved affinity over scFv-based conjugates without the instability often associated with scFvs (Muyldermans, 2001).

The small size, solubility, stability and ease of cloning make VHH domains an ideal building block for the generation of multivalent antibodies. Linking antibody fragments is often used to increase the avidity of an antibody or to allow simultaneous binding to more than one antigen (Harmsen and De Haard, 2007). The linking of conventional VH and VL antibody domains can lead to aggregation and reduced affinity due to the mispairing of the domains (Glockshuber *et al.*, 1990, Whitlow *et al.*, 1993) whereas the linking of VHHs domains allows more flexibility in both linker design and the format of the multivalent antibodies (Harmsen and De Haard, 2007).

In addition to combining multiple antibody domains, antibody-toxin fusions have been designed to allow site-specific delivery of a toxin, for example directed to tumours (Reiter and Pastan, 1996). It is believed that the smaller size, increased solubility and stability would result in improved penetration and clearance of a VHH-toxin fusion over Fab-toxin or scFv-toxin fusions (Muyldermans, 2001).

Another application of VHH domains could be as intracellular antibodies, or intrabodies. Intrabodies are expressed inside the cell and then directed to a particular compartment (for example mitochondria or secretory compartments) allowing inhibition of selected molecules within the cell (Kontermann, 2004). Intracellular expression of single VHH domains should be more efficient than the expression of more complex antibody fragments, such as scFvs, where problems with assembly of the domains and aggregation have been observed (Muyldermans, 2001).

Structural information gained from antibody fragments, such as Fabs and scFvs, may provide a basis for the rational design of small drug molecules (Lawson, 2012) which, with advantages such as the ability to administer orally, improved distribution and lower manufacturing costs, offer an attractive alternative to antibody therapeutics. Identification of the antibody residues involved in binding, or the paratope of the antibody, could allow peptides or small molecules to be designed that mimic the action of the antibody. The use of VHHs has physical benefits as VHHs are stable, less prone to aggregation and expression yields tend to be higher. They are also at least half the size of other commonly used antibody fragments, so gaining structural information should be less complex. VHHs may also offer unique information due to the differing dynamics of the elongated VHH CDRs, the inclusion of the additional disulphide bond constraining the CDRs and access to binding sites not accessible to the short CDRs of conventional antibody fragments.

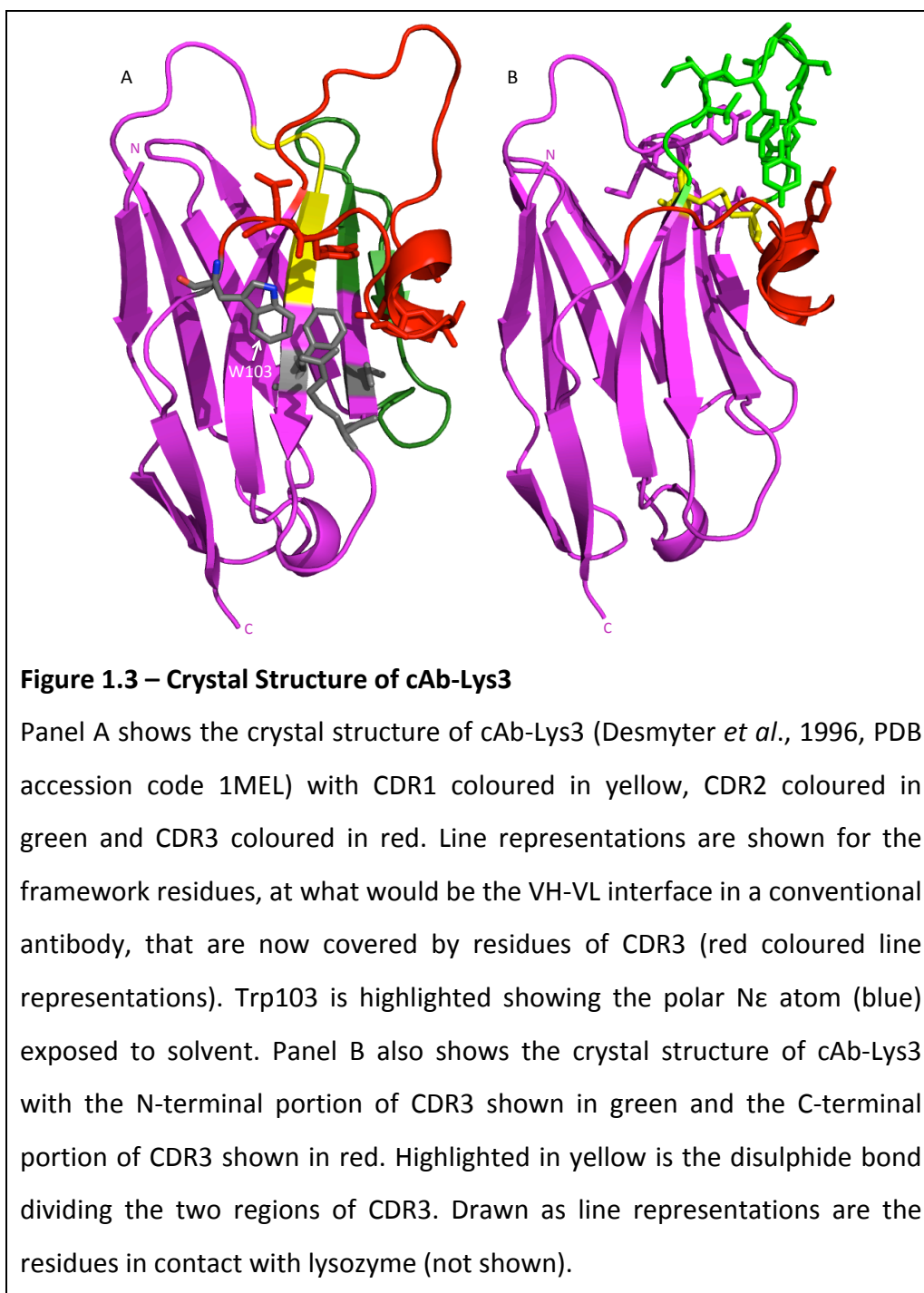
1.2 – Review of Literature VHH Structures

There are currently around 80 VHH structures submitted to the RCSB Protein Data Bank (PDB) (www.rcsb.org) and the vast majority were solved by X-ray crystallography. A review of some of the early VHH structural analyses gives insight into the differences between the VH domains of conventional antibodies and the VHH domains of heavy chain antibodies.

The first VHH structure to be solved was that of the lysozyme binder cAb-Lys3 (Desmyter *et al.*, 1996, PDB accession code 1MEL). This VHH contained the amino acid substitutions typically observed between VH and VHH domains, namely Leu11 to Ser, Val37 to Phe/Tyr, Gly44 to Glu, Leu45 to Arg and Trp47 to Gly. The crystal structure of cAb-Lys 3 was solved to a resolution of 2.5 Å and highlighted the reasons behind some of these substitutions. The substitution of Gly44 to Glu and Leu45 to Arg, on what would have been the VH-VL interface, leads to increased hydrophilicity, lessening the chance of the VHH domain aggregating in the absence of a VL domain. The polar to non-polar substitution of Val37 to Phe (or Tyr in some VHHs) appears to be counterintuitive to making the isolated VHH domain more soluble and stable but this substitution, along with the Trp47 to Gly substitution (and often also mediated by a His35 to Gly substitution (Spinelli *et al.*, 1996)), allows the shift of bulky residues towards Trp103. In stacking with Phe37, the Trp103 side chain can rotate exposing the polar Nε at the surface rather than the non-polar aromatic ring (highlighted in Figure 1.3 Panel A).

As is typical of many VHHs, the CDR3 loop of cAb-Lys3 is long, consisting of 24 residues, and this was shown to fold back over the framework of the VHH covering residues that would, in VH domains, form the interface with the VL domain. In total, a surface area of 150 Å² of framework residues (Phe37, Arg45, Gly47 and Trp103) were covered by the CDR3 residues (Gly100f, Leu100i, Ser100j, Gly110l, Trp100p and Asp101) as shown in Panel A of Figure 1.3. The substitutions that result in a shift of the bulky groups towards Trp103 (described above) provide the relatively flat surface onto which the long CDR3 can fold over.

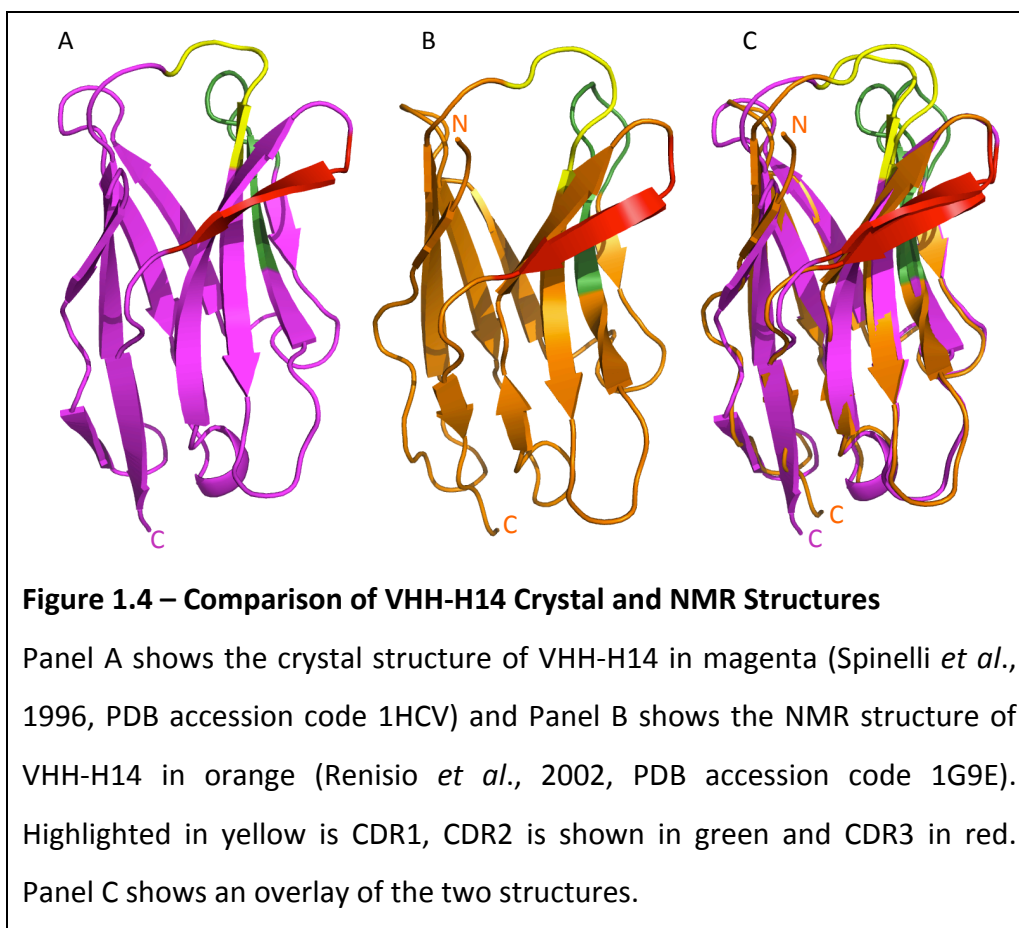
An interesting feature of the long CDR3 of cAb-Lys3 was that it appeared to be divided into two segments separated by a disulphide bond linking CDR3 to CDR1. The C-terminal portion of CDR3 folded over the VHH and made contact with the residues that would, in a conventional antibody, form the VH-VL interface. The N-terminal portion of CDR3 appeared, primarily, to be involved in antigen recognition and extended from the antigen binding site of the VHH penetrating the antigen cleft. The crystal structure did not suggest that this extended loop was flexible as it appeared to be stabilised by the disulphide bond linking CDR3 to CDR1 and by the interaction of three tyrosine side chains: Tyr99 and Tyr 100c of CDR3 and Tyr32 of CDR1. This stability was thought to reduce the number of conformations that CDR3 could adopt and therefore have a favourable entropic effect on binding. The residues at the interface with lysozyme are highlighted in Figure 1.3 Panel B.



For conventional antibodies, the conformation of five of the six hypervariable regions can be classified into a small number of known arrangements, termed canonical forms, and can be predicted from the sequence (Chothia *et al.*, 1989). The cAb-Lys3 structure showed that the five residue CDR1 deviates from the known canonical structures found in VH domains where as the 17 residue CDR2 adopted the predicted canonical conformation.

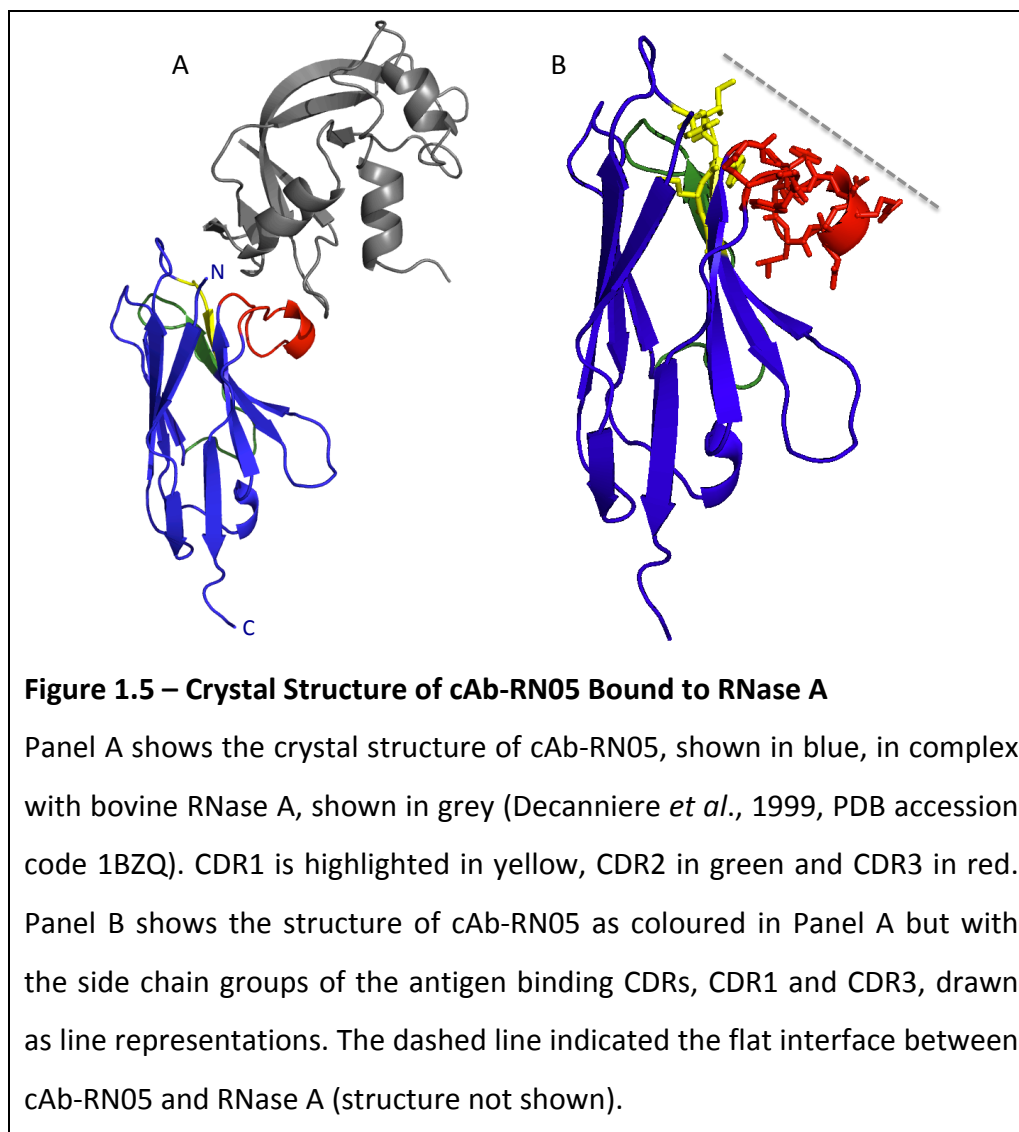
The next VHH structure to be solved was the crystal structure of VHH-H14, a VHH raised in llama against the α -subunit of the human chorionic gonadotropin hormone (hCG) (Spinelli *et al.*, 1996). The 1.85 Å crystal structure of the free llama VHH was solved and is shown in Figure 1.4 Panel A (PDB accession code 1HCV) but the VHH in complex with hCG could not be crystallised. Six years later Renisio *et al.* solved the NMR solution structure of free VHH-H14, which is shown in Figure 1.4 Panel B (Renisio *et al.*, 2002, PDB accession code 1G9E). This remains the only NMR structure of a VHH in the PDB. Similar VH to VHH substitutions at the VH-VL interface were observed with VHH-H14 to those described by Desmyter (Desmyter *et al.*, 1996). As with the previous example, the crystal structure of VHH-H14 showed good resolution of CDR2 and that it adopts one of the known canonical conformations. Poor definition was observed for CDR1 suggesting increased mobility of this loop. CDR3 of the llama derived VHH-H14 shows a shorter CDR3 than those seen in camels, consisting of just 5 residues so does not protrude from the antigen binding site in the same way as observed in cAb-Lys3. However, the paratope of VHH-H14 is not flat as CDR1 protrudes from the VHH into the antigen binding site (shown in Figure 1.4 Panel A)

Comparing the crystal structure and NMR structure of VHH-H14 shows that CDR1 is poorly defined in both structures; CDR2 is better defined in the crystal structure but shows poor resolution in the NMR structure as Asp54 and Ser55 appeared to be in exchange and could not be assigned; CDR3 showed good resolution in both the crystal and NMR structures but differences in conformation were observed between the two structures. Panel C of Figure 1.4 shows an overlay of the crystal and NMR structures.

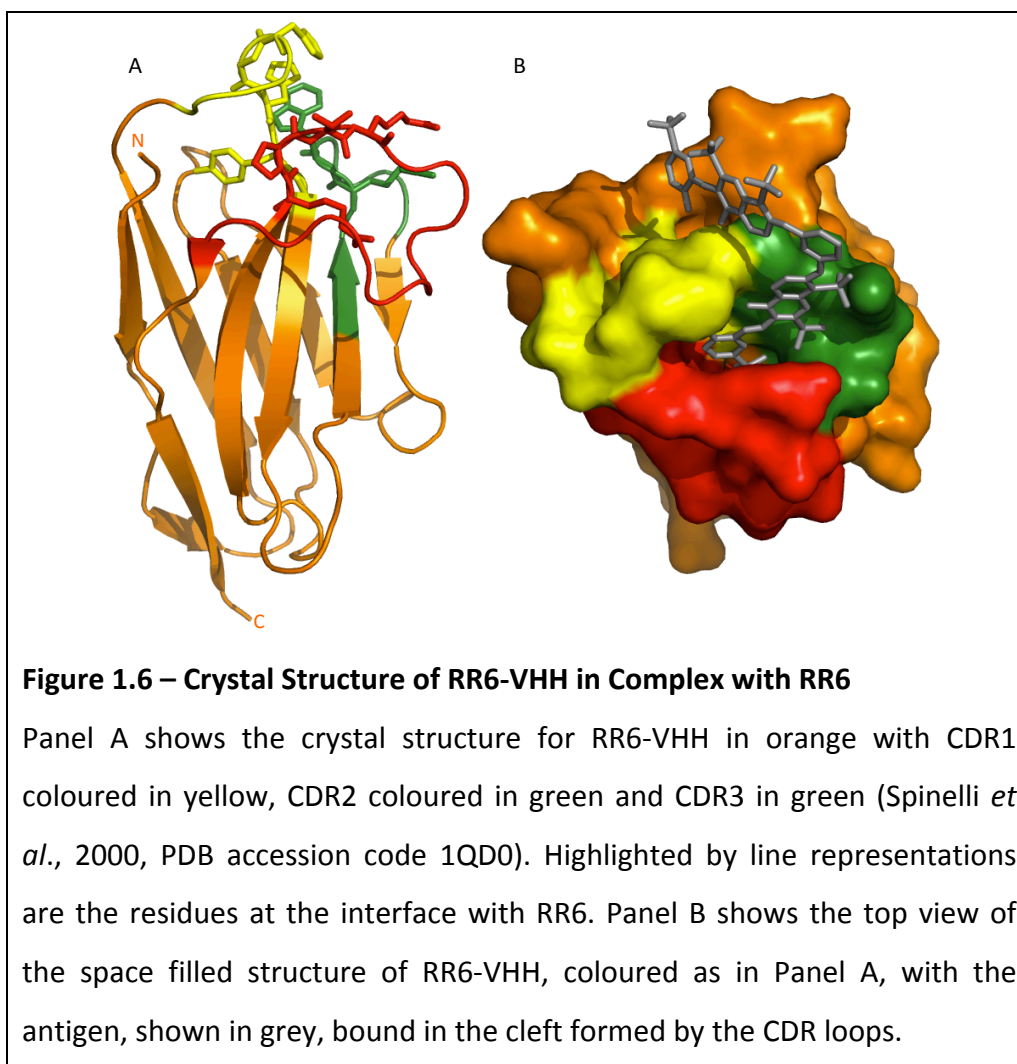


Another early example of a VHH crystal structure was that of cAb-RN05 (Decanniere *et al.*, 1999). The 2.8 Å crystal structure of the camel derived cAb-RN05 bound to bovine RNase A was solved and is shown in Figure 1.5 Panel A (PDB accession code 1BZQ). The CDRs showed structural similarities to those observed with cAb-Lys3 and VHH-H14. As with the previous two structures, CDR1 did not adopt one of the canonical forms associated with the H1 region of VH domains. It was suggested that an additional canonical form had been identified and this was reliant on the presence of a bulky, aromatic group in position 31, seen frequently in heavy chain antibodies. This new structure type has only been observed in VHH domains bound to antigen so it was suggested that this conformational change may be a result of antigen binding. The H2 region of cAb-RN05 also did not adopt the predicted canonical form and, as CDR2 is not involved in binding, cannot be the result of an antigen induced conformational change. The CDR3 of cAb-RN05 is shorter than that of cAb-Lys3, 12 residues compared to 24 residues, but shows similar behaviour with the C-terminal portion of CDR3 folding over the

hydrophobic framework residues. The CDR3 of cAb-RN05 differs in conformation from cAb-Lys3 as there is no disulphide bond stabilising the loop and the N-terminal portion is much shorter so does not protrude from the antigen binding site. The binding to RNase A occurs through CDR1 and CDR3 but neither of these antigen binding loops protrude resulting in a fairly flat paratope as highlighted in Figure 1.5 Panel B.



All VHH structures reported thus far have shown VHHs bound to protein molecules. Small hydrophobic antigens, such as haptens, tend to bind in the hydrophobic cleft at the VH-VL interface of conventional antibodies. To demonstrate that VHHs could be raised to these smaller molecules in the absence of the VL domain, Spinelli *et al.* solved the crystal structure of a VHH in complex with a copper containing azo dye, RR6 (Spinelli *et al.*, 2000). A llama was immunised with RR6 azo dye coupled to BSA and an anti-RR6 VHH was identified and isolated. The crystal structure of this VHH bound to RR6 was solved to a resolution of 2.5 Å and is shown in Figure 1.6 (PDB accession code 1QD0). All CDR loops were well defined in the crystal structure but CDR1 showed higher B factors than would normally be expected and binding to RR6 was shown to occur through all three CDRs. The H1 loop of RR6-VHH deviates from the known canonical structures and from other VHHs with non-canonical H1 structures, VHH-H14 for example. The H2 loop adopts the predicted type 3 canonical form. CDR3 of RR6-VHH consists of 14 residues and had no additional disulphide bond. The structure shows CDR3 folding over the framework region as seen in all previous examples of VHHs with long CDR3 loops.



Another interesting example of a VHH structure is that of camel derived VHH raised against bovine erythrocyte carbonic anhydrase (Desmyter *et al.*, 2001). Both the free and bound crystal structures of cAb-CA05 were solved to a resolution of 2.1 Å (PDB accession code 1F2X) and 3.5 Å (PDB accession code 1G6V) respectively. In the free structure the H1 loop, around CDR1, fits with the canonical type 1 structure expected of VH domains. This was the first example of a VHH H1 loop adopting a canonical form. As a result of somatic hypermutation, CDR2 is unusually short so does not fit with known structure types. CDR3 of cAb-CA05 consists of 19 residues and shows a similar structure to cAb-Lys3 in that the C-terminal region folds down over the framework of the VHH and the N-terminal portion is stabilised by interactions with CDR1 and CDR2. A comparison of the free and bound structures, shown in Figure 1.7, suggests there are no significant differences in the conformations of CDR1, CDR2 or CDR3 upon binding.

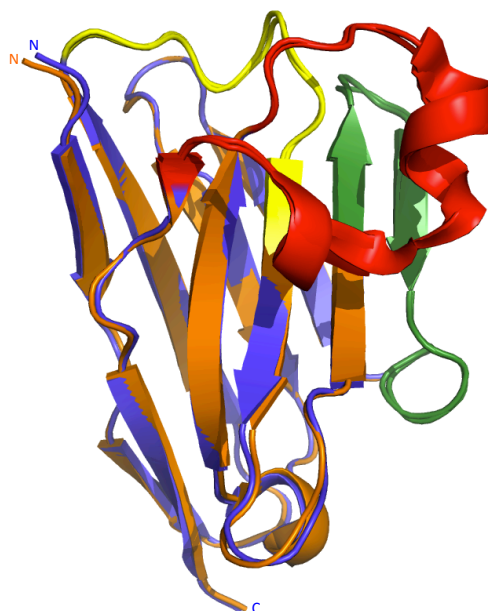
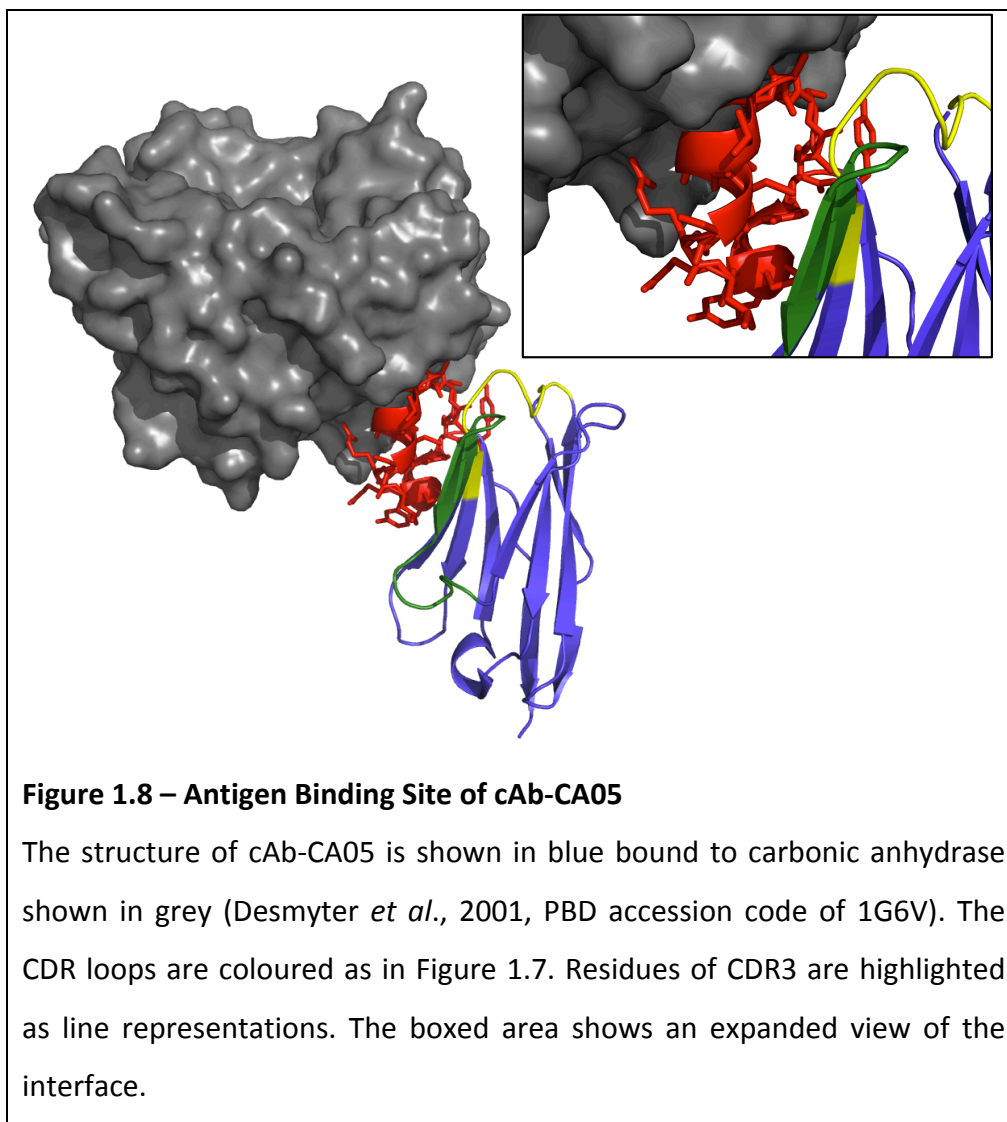


Figure 1.7 – Overlay of Free and Bound Crystal Structures of cAb-CA05

Shown in orange is the crystal structure of free cAb-CA05 (Desmyter *et al.*, 2001, PDB accession code 1F2X) overlaid with the crystal structure of cAb-CA05, shown in blue, bound to carbonic anhydrase (not shown) (Desmyter *et al.*, 2001, PDB accession code 1G6V). CDR1 is highlighted in yellow, CDR2 in green and CDR3 in red. The overlay shows very little change in conformation between the free and the bound structures.

Analysis of the bound crystal structure showed that cAb-CA05 binds solely through residues in CDR3, as highlighted in Figure 1.8. Residues in CDR1 and CDR2 make no contact with the antigen.

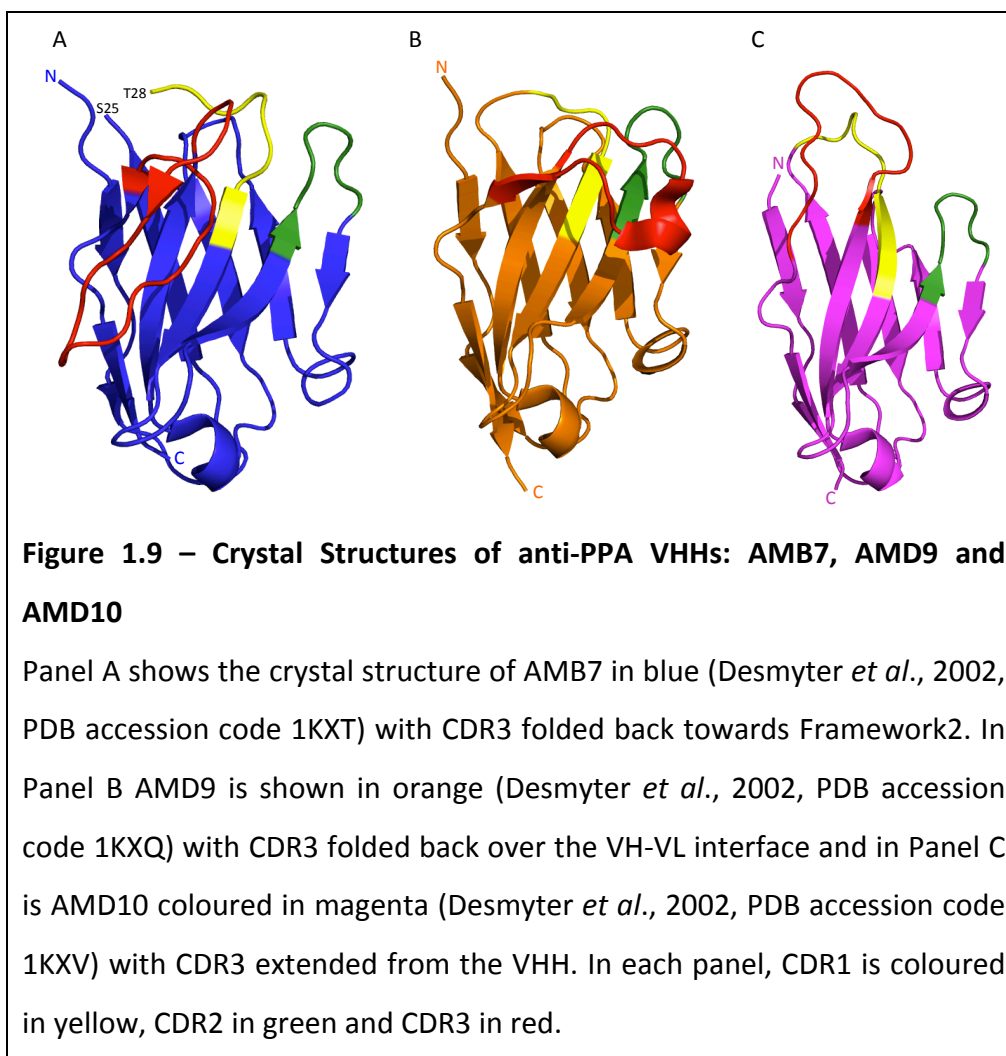


The differences in CDR conformation of VHHs, even when raised to the same antigen, were highlighted in work published by Desmyter *et al.* in which the crystal structures of three VHHs binding to porcine pancreatic α -amylase (PPA) were reported (Desmyter *et al.*, 2002). The crystal structures of AMB7, AMD9 and AMD10 were solved to a resolution of 2.0 Å (PDB accession code 1KXT), 1.8 Å (PDB accession code 1KXQ) and 2.7 Å (PDB accession code 1KXV) respectively.

The crystal structure of AMB7 (see Panel A of Figure 1.9) showed both CDR1 and CDR2 adopted the predicted canonical form. CDR3 consisted of 19 residues and folded back towards Framework2 as a result of an unusual disulphide bond between cysteines in CDR3 and Framework2. Figure 1.10 highlights AMB7 binding to PPA, through CDR2 and CDR3, away from the catalytic site at one end of the elongated cleft.

The crystal structure of AMD9 (see Panel B of Figure 1.9) showed that CDR2 adopts a canonical form but CDR1 deviated from any known canonical forms. CDR3 consisted of 14 residues which did not include a cysteine residue so could not form an additional disulphide bond. CDR3 can be observed folding over the region that, in a conventional antibody, would be the VH-VL interface. Binding occurs mostly through CDR2 and CDR3 and binds to the active site of PPA, blocking the catalytic site (see Figure 1.10)

AMD10 is similar to AMD9, as CDR2 adopts the predicted canonical structure whereas CDR1 does not. Binding occurs far from the catalytic crevice of PPA (see Figure 1.10) and predominantly binds through the 13 residue CDR3 which is shown to protrude from the antigen binding site of the VHH (see Panel C of Figure 1.9). The position of the cysteine in CDR3 is slightly unusual as it appears much closer to the C-terminus of CDR3 than in other VHHs. The disulphide bond formed between the cysteine in CDR3 and the cysteine in CDR1 may prevent CDR3 folding back over the framework as has been seen in structures such as AMD9, cAb-Lys3 and cAb-CA05.



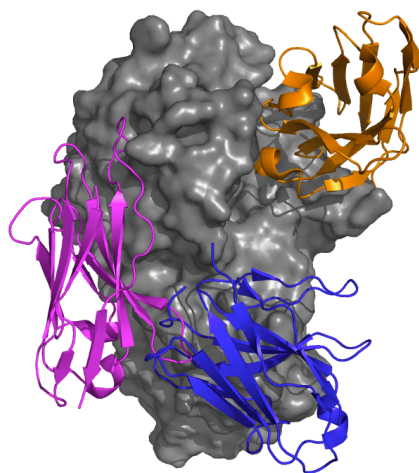


Figure 1.10 – Composite Crystal Structure Highlighting the Binding Sites of AMB7, AMD9 and AMD10 to PPA

The space filled structure of PPA is shown in grey. AMB7 is shown in blue binding to one end of the elongated active site. AMD9 is shown in orange binding to the catalytic site and AMD10 is shown in magenta binding far from the catalytic site.

The anti-PPA structures also showed a high contribution from framework residues to the binding interface. The involvement of both CDRs and framework residues in binding increases the area of the VHH binding interface to an area equivalent to that seen for the binding interface of the combined VH-VL domains of conventional antibodies.

Reviewing these early VHH structural papers highlights clear differences between heavy chain VHH domains and the VH domains of conventional antibodies.

VHHs have evolved without a light chain and have therefore adapted to prevent exposure to solvent of the hydrophobic surface that would make up the VH-VL interface. This has been achieved in two ways. First, by amino acid substitutions at the VH-VL interface which either replace non-polar residues with more polar residues or allow the reorientation of residues to increase hydrophilicity. Second, CDR3 is often observed extending down over the VH-VL interface shielding the hydrophobic surface.

The hypervariable regions of VHHs do not always adopt the canonical conformations observed in VH domains. It has been suggested that the appearance of these new conformations probably comes about as a result of some of the specific amino acid substitutions not typically seen in VH domains (Decanniere *et al.*, 1999).

Significant differences have been seen between the binding interactions of even the small number of structures reviewed here. The assumption that antigen binding occurs predominantly through CDR3 (Muyldermans, 2009) does not appear to be the case with many VHHs. The crystals structures suggest that binding can occur through all three CDRs as seen with cAb-Lys3 and RR6-VHH; through two of the CDRs as seen with cAb-RN05 and the PPA binders; or solely through CDR3 as observed with cAb-CA05. This probably reflects the fact that the conformation of CDR3 can differ significantly from one VHH to another. In the selection described there is an example of a very short CDR3, consisting of just five residues, in VHH-H14; CDR3 loops of varying lengths that fold back over the VH-VL interface as seen in the C-terminal portion of cAb-Lys3 (24 residue CDR3), cAb-CA05 (19 residue CDR3) and AMD9 (14 residue CDR3); and CDR3 loops that extend away from the antigen binding site as seen with the N-terminal portion of cAb-Lys3 and AMD10. It has also been shown, with RR6-VHH, that the CDRs of a VHH can assemble to form a hydrophobic cleft into which a small molecules can bind.

The potential conformations that the elongated CDR3 can adopt are further increased by the additional disulphide bond seen in some of the structures discussed above. This additional disulphide bond is typically seen linking CDR3 to CDR1 (as observed with cAb-Lys3, cAb-CA05 and AMD10) but has also been seen to link CDR3 to a cysteine in Framework2 (as in AMB7). No additional disulphide bond is present in the structures of VHH-H14, RR6-VHH or AMD9.

In the absence of a VL domain, one would expect the binding surface area of VHHs to be reduced in comparison to the binding surface area created by the combination of the VH and VL domains in a conventional antibody. In fact,

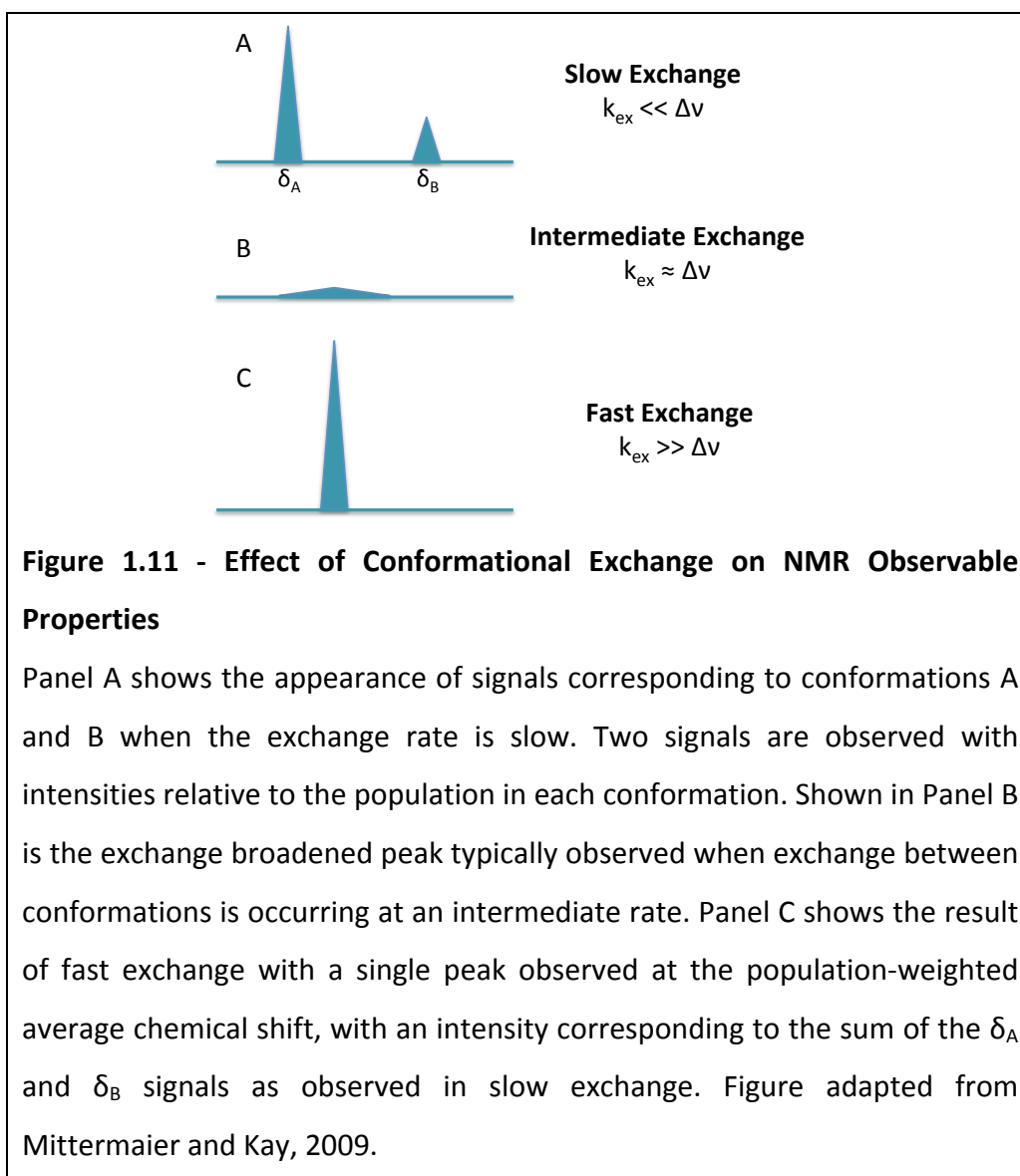
examples such the anti-PPA binders show that regions of the VHH framework, along with the CDRs, make up the antigen binding site increasing the binding area. The extended CDR3 loop seen in cAb-Lys3 also acts to increase binding surface by reaching up into the lysozyme cleft. Examples have also been seen in which a forth, non-hypervariable, loop in the antigen binding site interacts with antigen (see Section 4.4).

The structures available in the PDB provide a wealth of information regarding the structures of VHHs and the interactions made with antigens, however, all but one of the structures are of VHHs in the solid state so limited conclusions can be drawn as to the dynamics and flexibility of the antigen binding sites of VHH domains. The one NMR structure available is that of a llama VHH so has a shorter CDR3 than is typically seen in camel derived VHHs. No structure is available for the equivalent VHH bound to its antigen so we have no insight into structural changes upon binding.

1.3– Measuring Protein Dynamics by NMR

Proteins are not static molecules but are continually sampling different conformations and the function of the protein will be dependent upon the dynamics (Perutz and Mathews, 1966). The two main techniques used to determine protein structure are X-ray crystallography and NMR. Both techniques can elucidate the structure of a protein on an atomic level. With X-ray crystallography, proteins are presented in the solid state in the form of crystals whereas with NMR analyses the protein is in the solution state making it better suited to interpreting the dynamics. NMR methods can detect minor changes in the chemical environment for almost every atom within a protein through NMR observable properties such as frequency, an indication of the chemical environment of a nuclei; intensity, the population of nuclei in each environment; and linewidth, which is related to the transverse relaxation rate and therefore the dynamics of the protein (Kleckner and Foster, 2011).

Assuming a simple exchange mechanism between two conformations, conformation A and conformation B, two states are observed by NMR with frequencies ν_A and ν_B . The difference in chemical shift between these two frequencies is denoted as $\Delta\nu$. If the exchange rate, k_{ex} , is much slower than $\Delta\nu$, there will not be significant interconversion between the two conformations during the detection time of the NMR experiment and, therefore, two separate signals would be observed, at chemical shifts of δ_A and δ_B , with intensities relative to the populations in each conformation (see Figure 1.11). If k_{ex} is much faster than $\Delta\nu$, signal averaging occurs and a single peak is observed at the population-weighted average chemical shift. Intermediate exchange, when k_{ex} is approximately equal to $\Delta\nu$, results in an exchange broadened signal.



Dynamic processes in proteins occur over a wide range of timescales from some side chain rotations, which occur at picosecond rates, to protein folding, which can be as slow as many seconds (Bertini, 2012). To enable the study of this range of motions, a number of different NMR methods can be employed for example the determination of longitudinal relaxation times (T₁), the transverse relaxation times (T₂) and ¹⁵N-¹H heteronuclear nuclear Overhauser effects (NOEs).

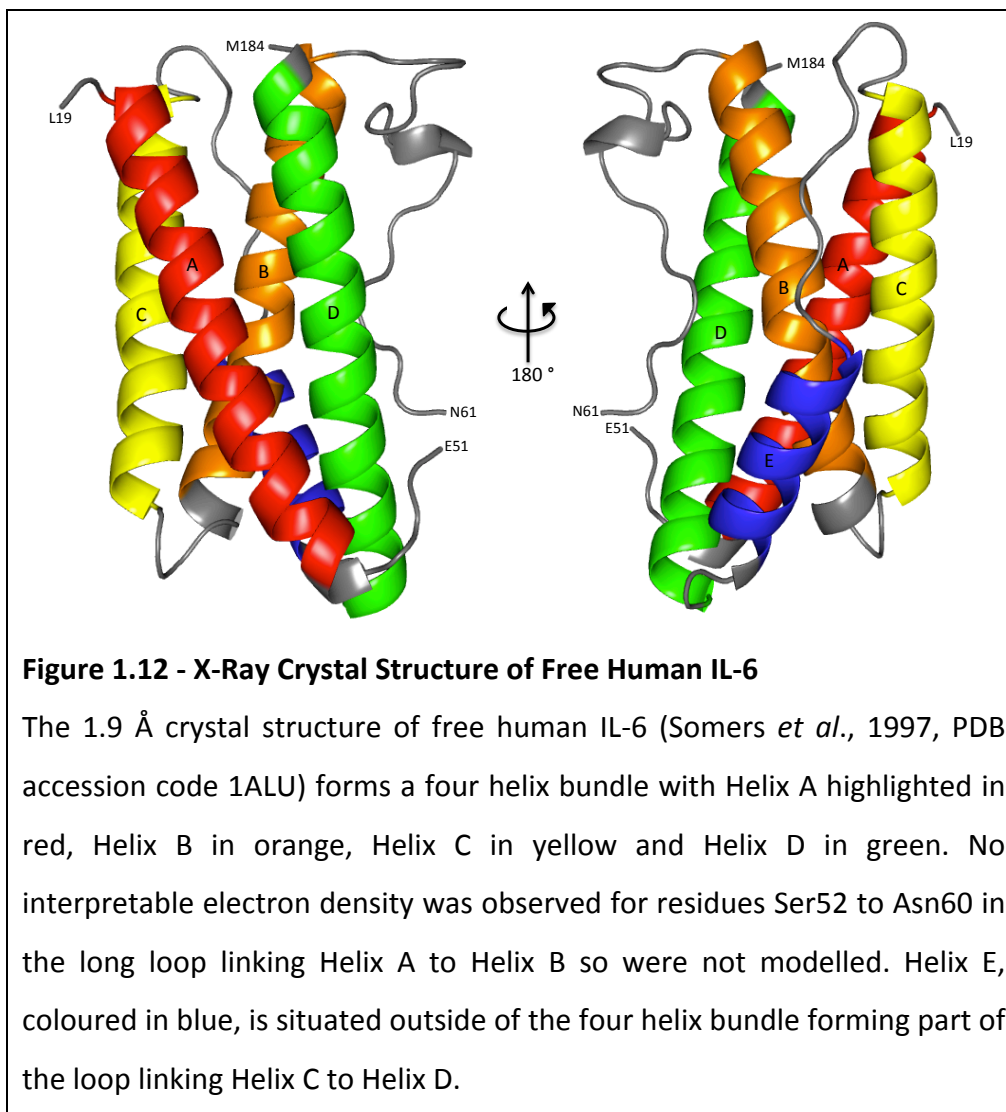
1.4 – Interleukin-6

The work described in this thesis has focused on VHHs raised to the cytokine interleukin-6 (IL-6). The behaviour and knowledge gained using the anti-IL-6 VHH antibodies should be applicable to VHHs raised against a wide range of target proteins.

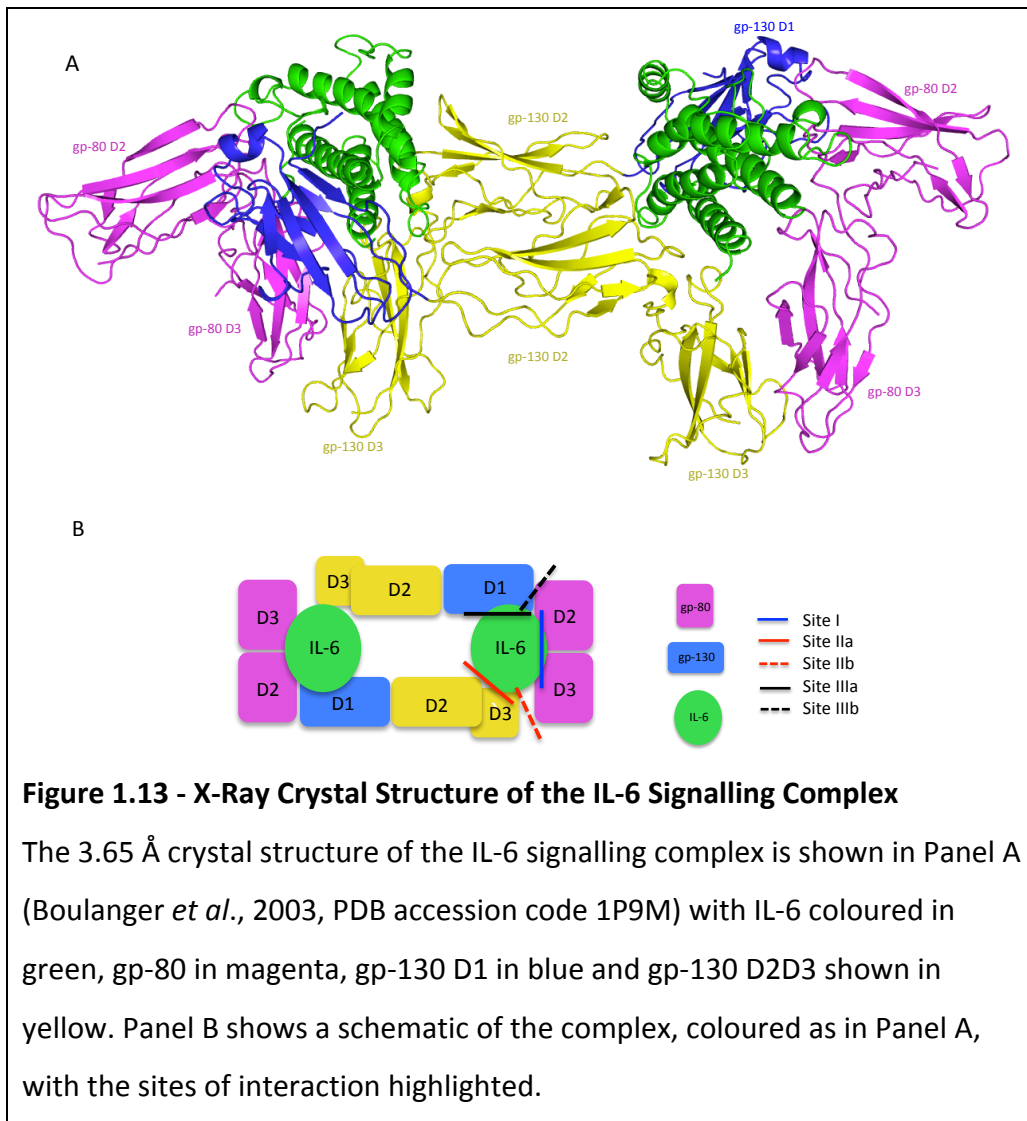
IL-6 forms part of a signalling complex responsible for inducing anti-inflammatory responses and also the pro-inflammatory responses associated with diseases such as rheumatoid arthritis (Ogata and Tanaka, 2012) and Crohn's disease (Gross *et al.*, 1992). A ternary complex comprising IL-6, gp-80 (also known as IL-6R α) and gp-130 (IL-6R β) is formed before two of the ternary structures dimerise to form the IL-6 signalling complex (Boulanger *et al.*, 2003). Phosphorylation of gp-130 activates signalling through the Janus-activated kinases (JAK) and signal transduction and activators of transcription (STAT) pathway (Heinrich *et al.*, 2003). The glycoprotein, gp-80, is an IL-6 specific receptor expressed in a limited number of cell types including hepatocytes, megakaryocytes and leukocytes (Wolf *et al.*, 2014) whereas gp-130 is a ubiquitous glycoprotein expressed in most tissue and cell types within the body (Wolf *et al.*, 2014) which plays a role in binding to many other cytokines including interleukin-11 (IL-11), leukemia inhibitory factor (LIF) and ciliary neurotropic factor (CNTF) (Heinrich *et al.*, 2003). Classic IL-6 signalling (or cis signalling) proceeds via the incorporation of membrane bound gp-80 into the signalling complex and is responsible for the anti-inflammatory activities of IL-6 (Gabay, 2006). However, gp-80 also exists as a soluble form, resulting from either alternative splicing (Lust *et al.*, 1992) or the cleavage of membrane bound gp-80 by the metalloprotease ADAM17 (Matthews *et al.*, 2003). This soluble gp-80 will also

complex with IL-6 and gp-130 to form the IL-6 signalling complex, resulting in trans signalling. The presence of soluble gp-80, coupled with the ubiquitous nature of gp-130, allows cells that do not naturally express gp-80 to become capable of responding to IL-6. It is believed that trans signalling is responsible for the pro-inflammatory actions of IL-6 (Chalaris *et al.*, 2011).

The 1.9 Å crystal structure of free human IL-6 was solved by Somers *et al.* showing the 185 residue protein forms a four helix bundle (see Figure 1.12) (Somers *et al.*, 1997). Helix A (Ser21 to Ala45) is joined to Helix B (Glu80 to Gln102) via a 25 residue loop, part of which showed poor electron density and was not modelled. A short crossover loop links Helix B to Helix C (Glu109 to Lys129) and another long loop links Helix C to Helix D (Gln156 to Arg182). Within this long loop a fifth helix, Helix E (Pro141 to Gln152) is observed. The four helices form a top-top-down-down bundle with Helices A and B running in one direction and Helices C and D running in the opposite direction, typical of structures seen in other related cytokines (Bazan, 1990).



The 3.65 Å crystal structure of the IL-6:gp-80:gp-130 signalling complex solved by Boulanger *et al.* is shown in Figure 1.13 Panel A (Boulanger *et al.*, 2003). The structure consists of a relatively planar top composed of IL-6, gp-80 domain 2 (gp-80 D2) and gp-130 domains 1 and 2 (gp-130 D1D2) with gp-80 domain 3 (gp-80 D3) and gp-130 domain 3 (gp-130 D3) extending beneath. A schematic of the hexameric IL-6 signalling complex is shown in Figure 1.13 Panel B.



The IL-6 signalling complex is formed as a result of ten protein-protein interactions, five within each trimer (see Figure 1.13 Panel B). The interaction between IL-6 and gp-80 is termed Site I and forms between Helix A and Helix D of IL-6 and predominantly domain 3 of gp-80. Site II binding occurs through two interaction sites: Site IIa which is between Helix A and Helix C of IL-6 and the elbow region between gp-130 domains 2 and 3 otherwise known as the cytokine homology region (CHR); and Site IIb between gp-130 D3 and gp-80 D3. Site III binding is, again, composed of two binding sites: Site IIIa is between one end of the IL-6 helix bundle and the lower β -sheet of gp-130 D1; and Site IIIb between the top of gp-130 D1 and one side of gp-80 D2.

The Site I interaction has a relatively low affinity (K_D of approximately 1 nM) but specific for IL-6 signalling (Yamasaki *et al.*, 1988) and is required before interactions with gp-130 can take place (Boulanger *et al.*, 2003). It was first proposed that the Site II interactions initiate the formation of the ternary complex before Site III interactions between the two trimers occurred (Boulanger *et al.*, 2003). However, more recent results suggest that the high affinity, IL-6 specific Site III binding drives formation of the ternary complex followed by formation of the hexameric complex through the less specific IL-6 and gp-130 CHR interaction (Site IIa) and the interaction between gp-130 D3 and gp-80 D3 (Site IIb) (Veverka *et al.*, 2012).

The inhibition of IL-6 trans signalling is an attractive target for the treatment of inflammatory diseases and due to the complicated nature of the interactions within the IL-6 signalling complex, the requirement for structural information during a drug discovery project is essential. This was one of the reasons IL-6 was selected as a target protein for the evaluation of VHH antibodies as tools in drug discovery process.

1.5 – Anti-IL-6 VHH Antibodies

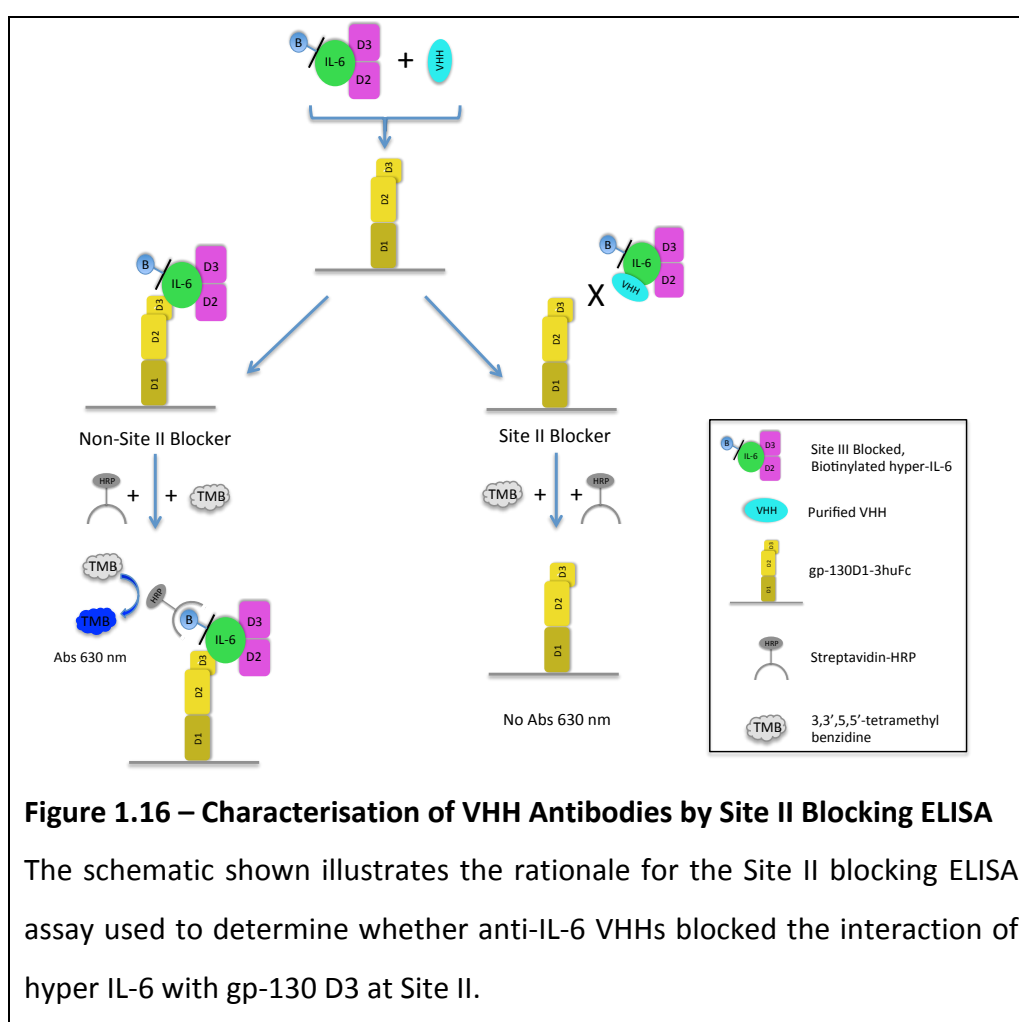
Work carried out by Dr. Laura Griffin née Hancock (UCB) focussed on isolating a panel of VHH antibodies raised to hyper IL-6 (a fusion protein of IL-6 and gp-80), characterising the anti-IL-6 VHH antibodies to give an indication of the sites of interaction with IL-6 before confirming these sites using structural methods (Hancock, 2010). A summary of the information obtained is shown in Figure 1.14 and a sequence alignment of the panel of anti-IL-6 VHH antibodies obtained is shown in Figure 1.15.

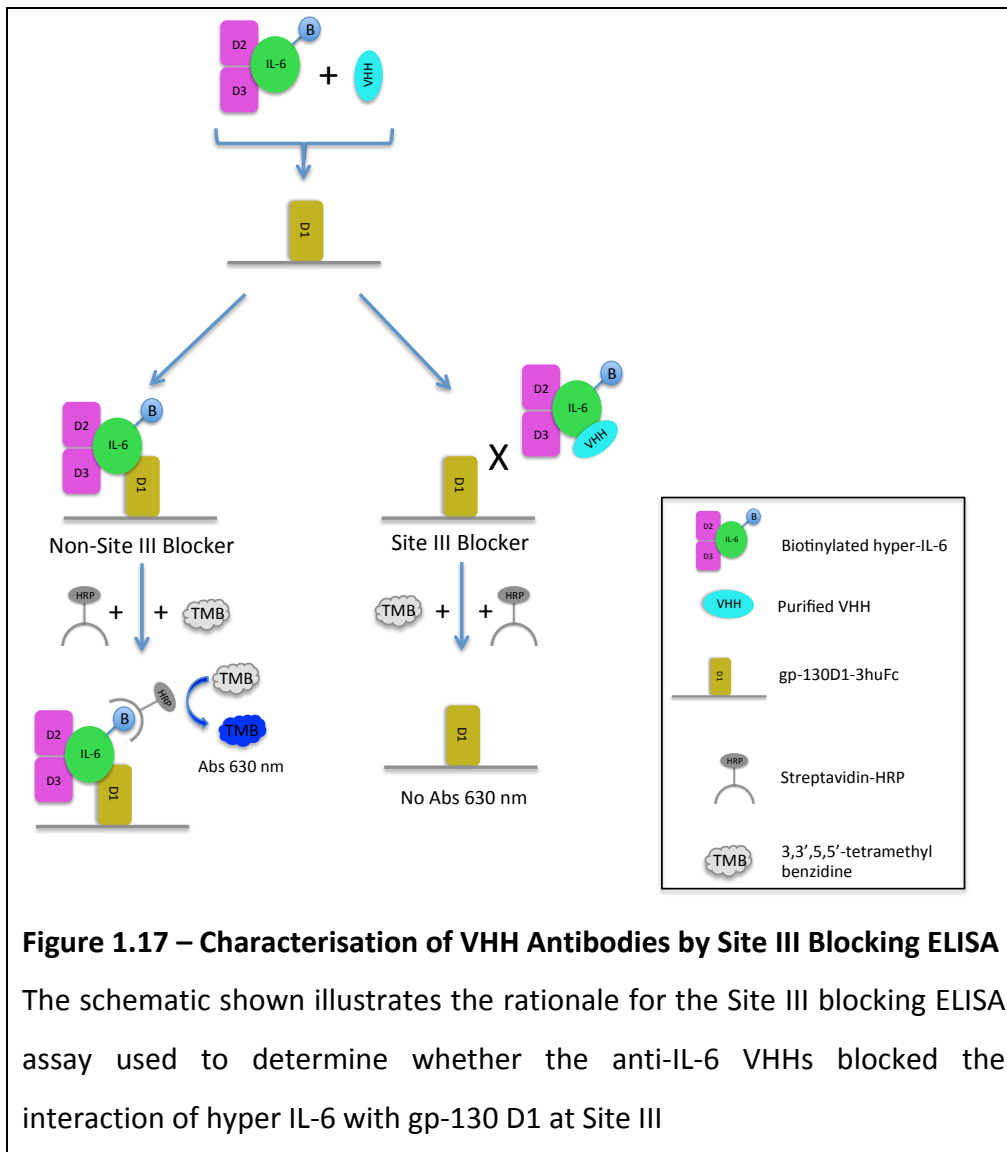
VHH	Binds			Blocks		Inhibits IL-6 Signalling	Affinity (nM)	Epitope Mapped by NMR	Crystal Structure
	hyper IL-6	IL-6	gp-80	Site II ELISA	Site III ELISA				
3	Y	Y	N	Y	N	Y	0.3	Y	
21	Y	Y	N	Y	N	Y	0.3		
22o	Y	Y	N	Y	N	Y	1.3		
27o	Y	Y	N	Y	N	Y	3.3		
29	Y	Y	N	Y	N	Y	8.4		Y
30	Y	Y	N	Y	N	Y	0.3	Y	
46	Y	Y	N	Y	N	Y	2.1		
63	Y	Y	N	Y	N	Y	1.8		
94	Y	Y	N	Y	N	Y	5.9		
103	Y	Y	N	Y	N	Y	0.7		
107	Y	Y	N	Y	N	Y	0.8	Y	
145	Y	Y	N	Y	N	Y	3.4	Y	
8	Y	Y	N	Site II blockers as transients, not tested as purified proteins				Y	
18	Y	Y	N						
19	Y	Y	N						
66	Y	Y	N						
22n	Y	Y	N	N	Y	N	1.8	Y	
26	Y	Y	N	N	Y	N	4.7		
56	Y	Y	N	N	Y	N	1.0	Y	Y
38	Y	Y	N	N	Y	Y	1.5	Y	Y
146	Y	Y	N	N	Y	Y	0.6	Y	Y
15	Y	Y	N	Y	Y	Y	1.6	Y	Y
40	Y	Y	N	Y	Y	Y	1.4		
2	Y	Y	N	Not tested as purified proteins, no blocking as transients					
12	Y	Y	N						
67	Y	Y	N	N	N	N	50.0		
97	Y	Y	N	N	N	N	2.1		
142	Y	Y	N	N	N	N	0.3		
17	Y		Y	Not tested as purified proteins due to gp-80 binding					
28	Y		Y						
27n	Y		Y						
134	Y		Y						
115	Y		Y						
158	Y		Y						

Figure 1.14 - Summary of the Biological and Structural Characterisation of Selected VHH Antibodies

The table summarises the information obtained from a series of binding and cell signalling assays conducted for a panel of anti-IL-6 VHH antibodies (Hancock, 2010). Also identified are the VHHs for which the epitope was mapped by NMR minimal shift and X-ray crystal structures were solved.

A total of 34 VHH antibodies were selected for characterisation. Proteins obtained from mammalian expression were assessed in an enzyme-linked immunosorbent assay (ELISA) to test for binding to IL-6 and gp-80. Of the 34 VHHs, 6 showed binding to gp-80 so were not studied further. Site II and Site III blocking ELISAs were developed to determine the binding sites of the anti-IL-6 VHHs to IL-6 and schematics of the site blocking ELISAs are shown in Figures 1.16 and 1.17. This tentatively identified 16 VHHs as Site II binders, 5 VHHs as Site III binders, 2 VHHs that appeared to inhibit both Site II and Site III binding and 5 VHHs for which binding sites could not be clearly resolved.



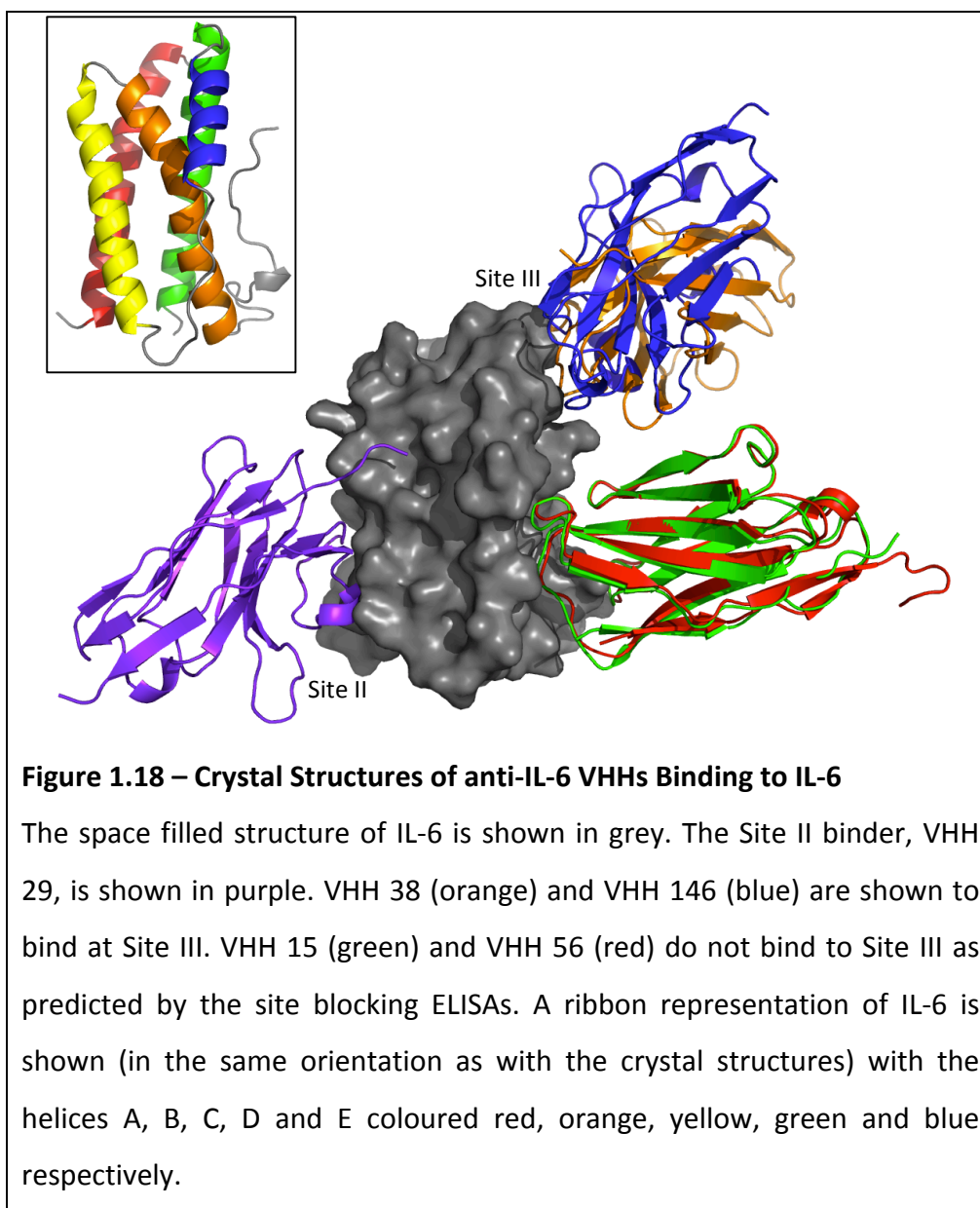


IL-6 signalling relies on the formation of the IL-6:gp-80:gp-130 signalling complex. VHH antibodies that block IL-6 at either Site II or Site III should inhibit the hyper IL-6:gp-130 binding therefore inhibiting formation of the signalling complex and ultimately IL-6 signalling. A cell based assay was used to determine the impact on IL-6 signalling by monitoring the STAT3 response in the presence of the VHHs. The results of the cell assay identified that, out of the 22 VHHs tested, 16 inhibited IL-6 signalling. Interestingly, some of the VHHs classified as Site III binders in the Site III blocking ELISA did not appear to inhibit formation of the signalling complex suggesting that although appearing to bind at Site III, they may not hinder the binding of hyper IL-6 to gp-130.

Surface plasmon resonance (SPR) was used to study the binding kinetics of the anti-IL-6 VHHs to IL-6 by titrating the VHHs over immobilised IL-6. The results obtained (see Figure 1.14) showed that all but one of the VHHs measured bound to IL-6 in the low nanomolar range, with K_D values between 0.3 nM and 8.4 nM being measured.

A number of the anti-IL-6 VHHs characterised by Dr. Laura Griffin (UCB) were subjected to structural analysis by X-ray crystallography and minimal shift mapping by NMR (Hancock, 2010). The IL-6 binding surface, as mapped by NMR minimal shift, identified VHH 3, VHH 18, VHH 30, VHH 107 and VHH 145 as Site II binders, consistent with the results of the site blocking ELISAs. VHH 38 and VHH 146 were identified as Site III binders from the site blocking ELISAs and the NMR results agreed with this. Results of the site blocking ELISAs suggested that VHH 15, VHH 22n and VHH 56 bound to IL-6 at Site III but the NMR epitope mapping suggested binding outside of the Site III cleft region.

The X-ray crystal structures of 5 VHHs were solved by Dr. Laura Griffin (UCB) and are shown in Figure 1.18 (Hancock, 2010).



The crystal structures confirmed that VHH 29 bound to IL-6 at Site II and that VHH 38 and VHH 146 both bound to IL-6 at Site III, agreeing with the conclusions of the site blocking ELISAs, the cell based assay and NMR epitope mapping. According to the site blocking ELISAs, VHH 15 and VHH 56 inhibit the binding of gp-130 D1 at Site III whereas the NMR identified the epitope as being remote to Site III. The crystal structures also agree with the NMR epitope mapping for VHH 15 and VHH 56.

The panel of anti-IL-6 VHH antibodies generated as a result of Dr. Laura Griffin's work provided a useful starting point for the work reported in this thesis. The profiling of the anti-IL-6 VHHs showed diversity both in the sites of interaction and in the structure of the VHHs, specifically in CDR3 length which, as outlined in the following section, will be one focus of the work described in the following chapters.

1.6 – Thesis Aims and Overview

The aims of the work presented in this thesis were to solve the NMR solution structures of VHHs to allow the behaviour of the antigen binding CDRs to be investigated; to gain an understanding of the fundamental benefits conferred by elongated CDR3 loops in VHH antibodies; and finally to evaluate the use of VHHs as tools for drug discovery.

As discussed in Section 1.2, the majority of structural data available for VHHs has been obtained using X-ray crystallography, limiting our understanding of the dynamics associated with VHH domains. The only example of a VHH structure solved by NMR was derived from a llama HCAb and does not include the elongated CDR3 typical of most HCABs (Renisio *et al.*, 2002, PDB accession code 1G9E). The work described in Chapter 3 encompasses the collection of NMR data and the calculation of the solution structures for two VHHs: VHH 18 with a short CDR3 loop and VHH 67 with a longer CDR3 loop. The solution structures obtained revealed the differences in the dynamics between the two VHHs with the short CDR3 loop of VHH 18 being identified as more dynamic than the longer CDR3 loop of VHH 67. In Chapter 2, the general cloning, expression and purification methods employed to generate the proteins required for the structural and functional studies are described.

A variety of experiments were carried out to identify the binding sites of both the VHHs and IL-6, which are reported in Chapter 4. This work allowed identification of the binding surfaces of both the VHH antibodies and IL-6.

The work described in Chapter 5 was undertaken to understand the behaviour of the elongated and structured CDR3 loop identified in VHH 67. Residues responsible for the stabilisation of this loop were identified and their impact on the binding to IL-6 was evaluated.

In Chapter 6 the crystal structure of VHH 67 in complex with IL-6 is presented identifying a novel binding site. The potential effects on IL-6 signalling of the VHH binding to this site were evaluated by SPR leading to the conclusion that VHH 67 modulates the activity of IL-6 via an allosteric mechanism. Another interesting observation was that a comparison of the free solution structure with the bound crystal structure of VHH 67 showed very little difference in the conformation of CDR3.

Chapter 7 discusses the structural and binding information obtained in the context of using VHHs as a tool for drug discovery.

Chapter 2 – Cloning, Expression and Purification of VHH Antibody Fragments

2.1 – Introduction

2.1.1 – VHH Expression Strategy

The VHHs used for the work described in Section 1.5 had previously been expressed using mammalian expression systems. Ideally, protein labelled with the NMR active nuclei of nitrogen and carbon (^{15}N and ^{13}C) would be expressed for structural analysis by NMR. Also, for larger proteins the replacement of the majority of hydrogen atoms for deuterium may also be required (Grzesiek *et al.*, 1993). It is not possible to express deuterated protein in mammalian systems and the inclusion of ^{15}N and ^{13}C is more efficiently achieved using *E.coli* expression methods, therefore, it was necessary to clone the VHH DNA into *E.coli* compatible vectors.

2.2 – Materials and Methods

2.2.1 – Construct Design

Primers were designed (as shown in Figure 2.1) to allow the insertion of the VHH DNA into commercially available, ampicillin resistant, isopropyl- β -D-1-thiogalactopyranoside (IPTG) inducible pET vectors (Novagen) (Studier and Moffat, 1986; Rosenberg *et al.*, 1987; Studier *et al.*, 1990) through the NdeI and XhoI restriction sites. An N-terminal hexahistidine-tag (His₆-tag) and tobacco etch virus (TEV) protease cleavage site were included to aid purification of the VHH protein.

Forward

QVQ:
GGC CCC CGC GCC CAT ATG CAC CAT CAT CAT CAC CAT GAA AAC CTA TAC TTC CAA GGA CAG GTG CAA CTG GTG GAG TCT GGG

DVQ:
GGC CCC CGC GCC CAT ATG CAC CAT CAT CAT CAC CAT GAA AAC CTA TAC TTC CAA GGA GAT GTG CAG CTG GTG GAG TCT GGG

EVQ:
GGC CCC CGC GCC CAT ATG CAC CAT CAT CAT CAC CAT GAA AAC CTA TAC TTC CAA GGA GAG GTG CAG CTG GTG GAG TCT GGG

Reverse

CGG GGG GGC CTC GAG CTA TTA CGA GGA GAC GGT GAC CTG GGT CCC

Figure 2.1 – Primers for Cloning of VHH DNA into the pET-21 Vector

The figure shows the primers used for the PCR amplification of the VHHs with a 5' NdeI restriction site and a 3' XhoI restriction site. The regions highlighted in blue code for the NdeI restriction site, regions in orange code for the XhoI restriction site, green for the His₆-tag and in purple the TEV protease cleavage site. The stop codon is shown in red. Three forward primers (QVQ, DVQ and EVQ) were required to allow cloning of all the anti-IL-6 VHH antibodies.

2.2.2 – DNA Analysis by Agarose Gel Electrophoresis

All PCR, ligation and digestion products were analysed by agarose gel electrophoresis using a 1 % (w/v) agarose gels in 1 x TAE buffer (40 mM Tris-acetate pH 8.0, 2.5 mM EDTA) run at 150 mA for approximately 3 hours. Ethidium bromide (0.5 µg ml⁻¹) was used to visualise the DNA under UV radiation at 366 nm. All gels were run with a reference lane containing an appropriate DNA ladder.

2.2.3 – Transformation of Vectors

50 µl of sub-cloning grade XL-1 blue competent *E.coli* cells (Agilent Technologies) were thawed on ice. 1 µl of 0.25 mg ml⁻¹ solution of pET-21 (Novagen) was added to the cells and incubated on ice for 20 minutes. The cells were then heat shocked at 42 °C for 45 seconds and transferred back to ice for a further 2 minutes. 200 µl Super Optimal Catabolite (SOC) medium was added and the mixture incubated at 37 °C for 1 hour on a shaking platform. The culture was then streaked onto LB-agar (1 % w/v Bacto-tryptone, 0.5 (w/v) Bacto-yeast extract, 171 mM sodium chloride, 1.5 % (w/v) Bacto-agar, pH 7.0) plates supplemented with 100 µg ml⁻¹ ampicillin and incubated overnight at 37 °C.

Single transformation colonies were picked into 100 ml of 2 x TY broth (2 % (w/v) Bacto-tryptone, 1 % (w/v) Bacto-yeast extract, 171 mM sodium chloride, pH7.0) supplemented with 100 µg ml⁻¹ ampicillin and incubated overnight at 37 °C on a shaking platform. Cell cultures were pelleted by centrifugation (2,500 g for 20 minutes). The vector DNA was isolated as per manufacturer's instructions using QIAgen plasmid plus maxi prep kits (Qiagen).

2.2.4 – PCR Amplification of DNA Fragments

PCR reactions were carried out using KOD Hot Start Polymerase (Novagen). PCR was performed on approximately 10 ng of template DNA with 1 mM MgSO₄, 0.5 µl KOD polymerase, 0.3 µM of each oligonucleotide, 0.2 mM dNTP mix (dATP, dCTP, dGTP, aTTP). PCR reactions were made up to a volume of 50 µl in PCR buffer (50 mM Tris.HCl, 1 mM DTT, 0.1 mM EDTA, pH 8.0) and carried out under the following conditions:

96 °C	2 minutes	} 29 Cycles
96 °C	15 seconds	
60 °C	15 seconds	
68 °C	1 minute	
68 °C	3 minutes	
4 °C	Constant	

Following PCR amplification, PCR products were purified using QIAquick PCR spin column purification kits (Qiagen) as per manufacturer's instructions.

2.2.5 – Restriction Enzyme Digests

The purified PCR products or approximately 10 µg plasmid DNA, were incubated with 20 units of each restriction enzyme (NdeI and XhoI) in an appropriate buffer overnight at 37 °C. One unit is defined as the amount of enzyme required to completely digest 1 µg of DNA in 1 hour. After digestion the vector DNA fragments were separated by gel electrophoresis (as described in Section 2.2.2). The DNA bands were excised and purified from the agarose gel as per manufacturer's instructions using QIAquick Gel Extraction Kit (Qiagen).

2.2.6 – Ligation of DNA Fragments

Ligation of the vector DNA and the insert DNA were performed using 1 µl T4 DNA ligase (New England Biolabs) with approximately 50 ng of the vector DNA and approximately 10 ng of the insert. Ligation mixtures were made up to a volume of 20 µl in T4 DNA ligase buffer (50 mM Tris.HCl, 10 mM MgCl₂, 1 mM ATP, 10 mM DTT, pH7.5) and incubated overnight at 16 °C.

2.2.7 – Transformation of DNA into Competent *E.coli* Cells

1 µl of the ligation reaction was transformed in to 50 µl of sub-cloning grade XL-1 blue competent *E.coli* cells (Agilent Technologies) as described in Section 2.2.3.

2.2.8 – Plasmid DNA Isolation for Sequencing

Single transformation colonies were picked into 10 ml of 2 x TY broth, incubated and harvested as described in Section 2.2.3. DNA was isolated as per manufacturer's instructions using mini prep kits (Qiagen).

2.2.9 – DNA Sequencing

DNA sequencing was carried out on approximately 250 ng of DNA as per manufacturer's instructions using a BigDye® Terminator sequencing kit (Applied Biosystems) on a 3100 automated DNA sequencer (Applied Biosystems).

2.2.10 – SDS-Polyacrylamide Gel Electrophoresis

SDS-PAGE was used to monitor the expression and purification of protein samples. 5 µg of each protein sample was heated to 100 °C for 3 minutes then loaded onto a 4 - 20 % acrylamide gradient pre-cast Tris-Glycine gel (Life Technologies). Electrophoresis was carried out at a constant 50 mA for 50 minutes in Tris-glycine SDS running buffer (25 mM Tris.Hcl, 200 mM glycine, 100 mM SDS). Gels were run with one lane containing SeeBlue pre-stained standard (Life Technologies) as a marker. Gels were stained with Coomassie Blue stain for 60 minutes and destained overnight with destain buffer (40 % v/v methanol, 10 % v/v acetic acid) to reveal protein bands.

2.2.11 – Western Blotting

Expression levels of VHHs were analysed by western blotting using a goat anti-llama IgG horse radish peroxidase (HRP) conjugated antibody (Bethyl Laboratories). An SDS-PAGE gel was prepared as described above but not stained with Coomassie Blue. Protein bands were transferred onto nitrocellulose transfer membrane overnight using Tris-glycine buffer (200 mM glycine, 25 mM Tris.HCl) at a constant current of 150 mA. After the transfer, the membrane was blocked for 1 hour with a 2 % (w/v) milk power in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4). Following the blocking, the membrane was washed with PBS for 10 minutes followed by a 1 hour incubation in a 0.5 µg ml⁻¹ goat anti-llama IgG HRP conjugated antibody in a 1 % (w/v) milk solution 0.1 % Tween-20. The membrane was then washed for 5 minutes in PBS five times before being treated, as per manufacturer's instructions, with Pierce Electrochemical Luminescence (ECL) Plus Blotting Substrates (Thermo Scientific). Protein bands were then observed by X-ray film exposure (0.5 to 5 minutes) and development.

2.2.12 – VHH Expression Trials

Expression trials were set up to determine, if possible, the optimum conditions for soluble expression of the VHH protein. Varying conditions including the IPTG concentration and incubation temperature did not encourage expression of soluble VHH protein, neither did testing a variety of *E.coli* cell types such as using BL21 DE3 (Novagen), Origami B DE3 (Novagen) and Tuner DE3 (Novagen) cells or the inclusion of solubilising tags such as thioredoxin and glutathione-S-transferase. As attempts to express soluble protein failed, it was decided that expression of the VHH protein as inclusion bodies followed by denaturation, purification and refolding should be evaluated to provide a source of protein for the NMR analyses. Given that the VHH proteins contain, at most, two disulphide bonds, it was considered likely that a successful refolding by dilution method (similar to that described by Williamson *et al.*, 1996) could be developed. Fluorescence spectroscopy was used to monitor the chemical denaturation (as described in Section 2.2.18) of the mammalian expressed VHH proteins to help predict the

guanidine hydrochloride (GuHCl) concentration required for the refolding of the *E.coli* expressed proteins.

2.2.13 – VHH Expression

The plasmids (prepared as described in Section 2.2.8) were transformed into *E.coli* BL21 DE3 competent cells (Novagen). To prepare unlabelled, ^{15}N labelled, $^{15}\text{N}/^{13}\text{C}$ labelled VHH samples and selectively labelled ^{13}C VHH samples, colonies were selected from LB agar plates, supplemented with $100\ \mu\text{g ml}^{-1}$ ampicillin, and used to inoculate six 10 ml cultures grown at $37\ ^\circ\text{C}$, 250 rpm overnight. These starter cultures were then pooled, centrifuged ($750 \times g$ for 20 minutes) and the pellets resuspended in either LB (1% (w/v) Bacto-tryptone, 0.5 % (w/v) Bacto-yeast, 171 mM sodium chloride, pH 7.0) containing $100\ \mu\text{g ml}^{-1}$ ampicillin for unlabelled samples; or minimal media (see Appendix A.1) containing $1\ \text{g l}^{-1}$ $>99\ \%$ ^{15}N ammonium sulphate and / or $2\ \text{g l}^{-1}$ $>99\ \%$ $^{13}\text{C}_6$ D-glucose and $100\ \mu\text{g ml}^{-1}$ ampicillin for the ^{15}N and $^{15}\text{N}/^{13}\text{C}$ labelled samples. The resuspended samples were then used to inoculate 500 ml expression cultures of the same minimal media to an A_{600} of 0.1. The selectively labelled ^{13}C samples were prepared as described above, but with the addition of non-isotopically labelled aromatic amino acids (His, Phe, Trp and Tyr at $50\ \text{mg l}^{-1}$).

Expression cultures were grown at 37°C , 250 rpm until an A_{600} of approximately 0.6 - 0.8 was reached. Cultures were then induced with IPTG to a concentration of 1 mM and left to express overnight (18 hours) at $37\ ^\circ\text{C}$, 250 rpm. Cells were harvested by centrifugation ($4,000 \times g$ for 30 minutes) and the cell pellets resuspended in PBS and lysed at a pressure of 30 kpsi using a cell disruptor (Constant Systems Ltd.) before being pelleted by centrifugation ($11,200 \times g$ for 30 minutes).

2.2.14 – VHH Purification and Refolding

The pelleted VHH inclusion bodies (obtained as described in Section 2.2.13) were resolubilised in approximately 7 mL of 6 M GuHCl with 10 mM dithiothreitol (DTT) and stirred for 60 minutes before being centrifuged ($45,000 \times g$ for 60 minutes). The supernatant was then diluted to 75 ml in the equilibration buffer (100 mM NaH_2PO_4 , 10 mM Tris.HCl, 6 M GuHCl, pH 8.0) and loaded at a flow rate of 3 ml min^{-1} on to a 10 ml Ni-NTA column equilibrated with the same buffer. Purification was achieved using a stepwise pH gradient with a volume of approximately 50 ml (5 column volumes) eluted during each step. Buffers were prepared by adjusting the pH of the equilibration buffer with HCl. The pH was initially decreased to pH 6.3 to elute non-specifically bound protein then reduced step-wise to pH 5.9 and then to pH 4.5 to elute the VHH protein. The gradient was run at a flow rate of 3 ml min^{-1} and 10 ml fractions were collected throughout. Pooled protein fractions were diluted in the equilibration buffer to approximately 2 mg ml^{-1} (as determined by absorbance at 280 nm) and the protein was refolded by dilution (1:100) into 50 mM Tris.HCl, 1 M GuHCl, 1 mM reduced glutathione, 0.2 mM oxidised glutathione, pH 8.5. The refolded protein was stirred for 90 minutes at room temperature and then left unstirred overnight also at room temperature. The refolded protein was concentrated to approximately 50 ml using a 5000 Da molecular weight cut off (MWCO) tangential flow device before being dialysed overnight in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , pH 7.4) and further concentrated to approximately 5 ml using an Amicon stirred cell with a 3000 Da MWCO membrane. The protein was then buffer exchanged into the NMR buffer (25 mM sodium phosphate, 100 mM sodium chloride, 10 μM EDTA and 0.02% (w/v) sodium azide buffer, pH 6.4) and concentrated by ultrafiltration using a 3000 Da MWCO Millipore Amicon Ultra 15 ml centrifugal filter unit.

2.2.15 – $^{15}\text{N}/^{13}\text{C}/^2\text{H}$ IL-6 Expression

IL-6 expression was carried out by Dr. Phil Addis (University of Leicester) based upon the method published by Veverka *et al.* (Veverka *et al.*, 2012). A human IL-6 construct including a His₆-tag with a TEV cleavage site was provided by UCB in a modified pET-21 vector containing an ampicillin resistant gene for selection. The plasmid was transformed into Origami B DE3 pLysS *E.coli* cells (Merck) and colonies selected from LB agar plates, supplemented with 12.5 $\mu\text{g ml}^{-1}$ tetracycline, 15 $\mu\text{g ml}^{-1}$ kanamycin, 34 $\mu\text{g ml}^{-1}$ chloramphenicol and 100 $\mu\text{g ml}^{-1}$ ampicillin, and used to inoculate 10 ml cultures which were grown at 37 °C, 200 rpm overnight. These cultures were then used to inoculate six 10 ml cultures of 30 % D₂O minimal medium grown for 8 hours at 37 °C, 200 rpm, which were in turn then used to inoculate another six 50 ml cultures of 70 % D₂O minimal medium that were grown overnight at 37 °C, 200 rpm. The six overnight cultures were pooled, centrifuged (750 x g, 20 minutes), resuspended in fresh 100 % D₂O minimal medium, containing 1 g l⁻¹ ^{15}N ammonium sulphate, 2 g l⁻¹ $^{13}\text{C}_6$ D-glucose and 100 $\mu\text{g ml}^{-1}$ ampicillin and used to inoculate ten 500 ml expression cultures of the same media to an A₆₀₀ of 0.1. The cultures were grown at 37 °C, 200 rpm for 8-10 hours until an A₆₀₀ of 0.2-0.3 was reached. Flasks were then transferred to an incubator pre-cooled to 17 °C and left to equilibrate for 30 minutes. After equilibration, the cultures were induced with IPTG to a concentration of 100 μM and left to express overnight (16 hours).

2.2.16 – $^{15}\text{N}/^{13}\text{C}/^2\text{H}$ IL-6 Purification

The purification of IL-6 was carried out based upon the method published by Veverka *et al.* (Veverka *et al.*, 2012). The pelleted cells harvested from the 5 l expression of $^{15}\text{N}/^{13}\text{C}/^2\text{H}$ IL-6 (described in Section 2.2.15) were resuspended in 100 ml high salt PBS (300 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4), with 100 μl lysonase (Novagen) and incubated on ice for 15 minutes. The cells were then passed through a cell disruptor at a pressure of 30 kpsi (Constant Systems Ltd.) before being centrifuged at 11,200 x g for 30 minutes.

The supernatant, containing IL-6, was diluted to 200 ml in high salt PBS and loaded at 3 ml min⁻¹ on to a 10 ml Ni-NTA column equilibrated in high salt PBS. The protein was washed with high salt PBS followed by a 10 mM imidazole wash. An imidazole gradient of 10 mM to 250 mM over 10 column volumes was run at a flow rate of 3 ml min⁻¹ with 5 ml fractions collected throughout. Fractions containing IL-6 were pooled and buffer exchanged back into high salt PBS by ultrafiltration. To remove the His₆-tag from IL-6, TEV protease (prepared at UCB) was added to the protein solution at a ratio of 1:50 (w/w) TEV protease to protein and incubated overnight at 4 °C. The TEV reaction was diluted 1 in 10 in high salt PBS and loaded on to a 10 ml Ni-NTA column at a flow rate of 3 ml min⁻¹ and the flow through collected. 250 mM imidazole was used to elute any bound protein at a flow rate of 3 ml min⁻¹ and 5 ml fractions were collected throughout. The flow through was concentrated to approximately 10 ml using a Millipore Amicon stirred cell with a 10,000 Da MWCO membrane. The protein was buffer exchanged into the NMR buffer (25 mM sodium phosphate, 100 mM sodium chloride, 10 µM EDTA and 0.02% (w/v) sodium azide buffer, pH 6.4) and concentrated to approximately 400 µM.

2.2.17 – Analytical Gel Filtration Chromatography

Analytical gel filtration was used to characterise the VHH and IL-6 samples. Approximately 30 µg of protein was injected onto either a Superdex S75 10/300 column (GE Healthcare) with a flow rate of 1 ml min⁻¹ PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4) using an Agilent 1100 HPLC. The Gel Filtration Standard containing thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), chicken ovalbumin (42 kDa), equine myoglobin (17 kDa) and vitamin B12 (1.3 kDa) (BioRad) was used as a guide for determining the molecular weights of the samples. Data processing was carried out using Chemstation software and peak areas at a UV absorbance of 214 nm were used to determine the purity of VHH, IL-6 and VHH : IL-6 complex samples.

2.2.18 – Fluorescence Spectroscopy

The intrinsic fluorescence profile of the VHH proteins was measured by fluorescence spectroscopy using a Cary Eclipse fluorescence spectrophotometer. Excitation was carried out at a wavelength of 280 nm and emission measured across a wavelength range of 300 nm to 400 nm at a scan rate of 600 nm min⁻¹. VHH samples were made up to a concentration of 30 µM in 10 mM Tris.HCl at increasing concentrations of GuHCl, from 0 M to 7 M in 1 M increments. Samples were left to incubate at room temperature for 1 hour before being analysed.

2.2.19 – Circular Dichroism Spectroscopy

Far UV circular dichroism (CD) spectroscopy was carried out using an Applied Photophysics ACD Chirscan Spectrophotometer. Triplicate samples were prepared at 0.5 mg ml⁻¹ in the NMR buffer (25 mM sodium phosphate, 100 mM sodium chloride, 10 µM EDTA and 0.02% (w/v) sodium azide buffer, pH 6.4). All experiments were carried out at a temperature of 20 °C. Data were collected across a wavelength range of 200 nm to 260 nm, at a resolution of 0.5 nm with a scan rate of 30 nm min⁻¹. Data were processed by subtracting the buffer contribution from the sample scans before normalising for sample concentration and converting to mean residue molar ellipticity.

2.2.20 – Surface Plasmon Resonance

The binding affinities of the purified, *E.coli* expressed VHHs to IL-6 were determined by surface plasmon resonance (SPR) using a Biacore 3000 (GE Healthcare). Goat anti-llama antibody (Bethyl Laboratories) was immobilised onto a CM5 sensor via amine coupling chemistry as per the manufacturer's instructions (GE Healthcare). The running buffer for the experiments was HBS-EP (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005 % (v/v) P20) and sensors were regenerated using 40 mM HCl. 30-50 µl of the VHH, at a concentration of approximately 20 µg ml⁻¹, was passed over the sensor at 10 µl min⁻¹ until a response of approximately 50 RU was reached. Affinity measurements were performed by titrating IL-6 over the captured VHH at 5 concentrations (20 nM, 10 nM, 5 nM, 2.5 nM and 1.25 nM). Double referenced binding curves were analysed

using the BIAEvaluation software and kinetic parameters calculated after fitting the data to a 1:1 model of binding and correcting for drifting baseline.

2.2.21 – Mass Spectrometry

The molecular weights of the VHH samples were confirmed using ultra performance liquid chromatography (UPLC) coupled with time-of-flight (TOF) mass spectrometry (MS) controlled via MassLynx 4.1 software. Data were acquired using a Waters bioQuaternary Acquity H-Class UPLC system with a Xevo G2 QTof detector. Samples were diluted to 90 μ M in 20 mM ammonium acetate pH 6.8 and 2 μ l injected onto an Acquity UPLC BEH300 C₁₈ 1.7 μ m (2.1 x 100 mm) column incubated at 40 °C. A gradient of 10 to 90 % mobile phase B was run over 5 minutes (mobile phase A: 0.1 % formic acid in water, mobile phase B: 0.1 % formic acid in acetonitrile) at a flow rate of 0.3 ml min⁻¹. The eluent from the column was diverted into the TOF-MS and data were acquired over a mass range of 50 to 2000 Da in the electrospray positive ionisation mode (ESI+). All data processing was carried out in MassLynx and the MS data deconvoluted using the Waters MaxEntTM and TransformTM algorithms (Cottrell *et al.*, 1991) to give the observed masses.

2.2.22 – NMR Spectroscopy

Initial ¹⁵N-¹H HSQC spectra (Bodenhausen and Ruben, 1980) of the VHH samples were collected at 35 °C on a 600 MHz Bruker Avance spectrometer. Samples were concentrated by ultrafiltration using a 3000 Da MWCO Amicon Ultra 4 ml centrifugal filter unit (Millipore) to a concentration of approximately 300 μ M in a 25 mM sodium phosphate, 100 mM sodium chloride, 10 μ M EDTA and 0.02% (w/v) sodium azide buffer at pH 6.4 containing 10 % D₂O / 90 % H₂O and transferred to 5 mm Shigemi NMR tubes. Typical acquisition times were 40 ms in F₁ (¹⁵N) and 60 ms in F₂ (¹H) with a total experiment time of 40 to 60 minutes. Solvent suppression was carried out using a WATERGATE pulse sequence (Piotto *et al.*, 1992) and data were processed using Topspin 3.1 software (Bruker BioSpin Ltd).

A ^{15}N - ^1H TROSY spectrum (Pervushin *et al.*, 1997) of the $^{15}\text{N}/^{13}\text{C}/^2\text{H}$ IL-6 was collected at 35 °C on an 800 MHz Bruker Avance II spectrometer. The sample was concentrated by ultrafiltration using a 3000 Da MWCO Amicon Ultra 4 ml centrifugal filter unit (Millipore) to a concentration of 380 μM in NMR buffer (25 mM sodium phosphate, 100 mM sodium chloride, 10 μM EDTA and 0.02% (w/v) sodium azide buffer, pH 6.4) containing 10 % D_2O / 90 % H_2O and transferred to 5 mm Shigemi NMR tubes. Typical acquisition times were 60 ms in F_1 (^{15}N) and 70 ms in F_2 (^1H) with a total experiment time of around 60 minutes. Solvent suppression was carried out using a WATERGATE pulse sequence and data were processed using Topspin 3.1 software.

2.3 – Results

Using the PCR method described in Section 2.2.4, the VHH DNA was amplified with the NdeI and XhoI restriction site. Successful amplification of the VHH DNA with the restriction sites was visualised by agarose gel electrophoresis (Figure 2.2). Both the insert DNA and the pET-21 plasmid DNA were digested, purified and ligated as described in Sections 2.2.5 and 2.2.6. Figure 2.3 shows the agarose gel of the pET-21 vector after incubation with the XhoI and NdeI restriction enzymes confirming successful digestion of the vector. The ligation products were transformed into *E.coli* competent cells, replicated, purified and then sequenced. Sequencing results confirmed the insertion of the VHH DNA into the plasmid DNA. Prior to purification and sequencing the insertion of the VHH DNA into the pET vector was also confirmed by PCR and the results are shown in Figure 2.4.

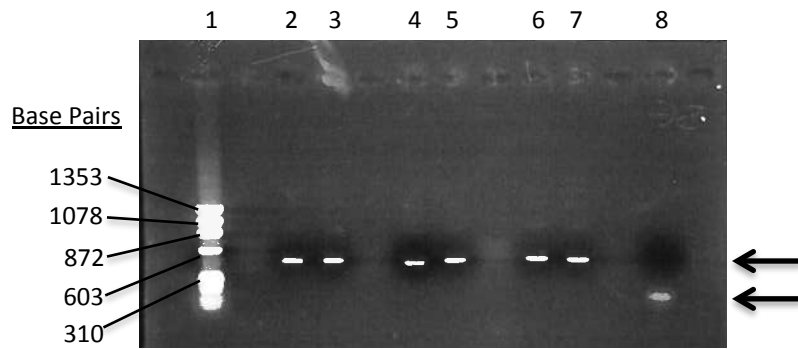
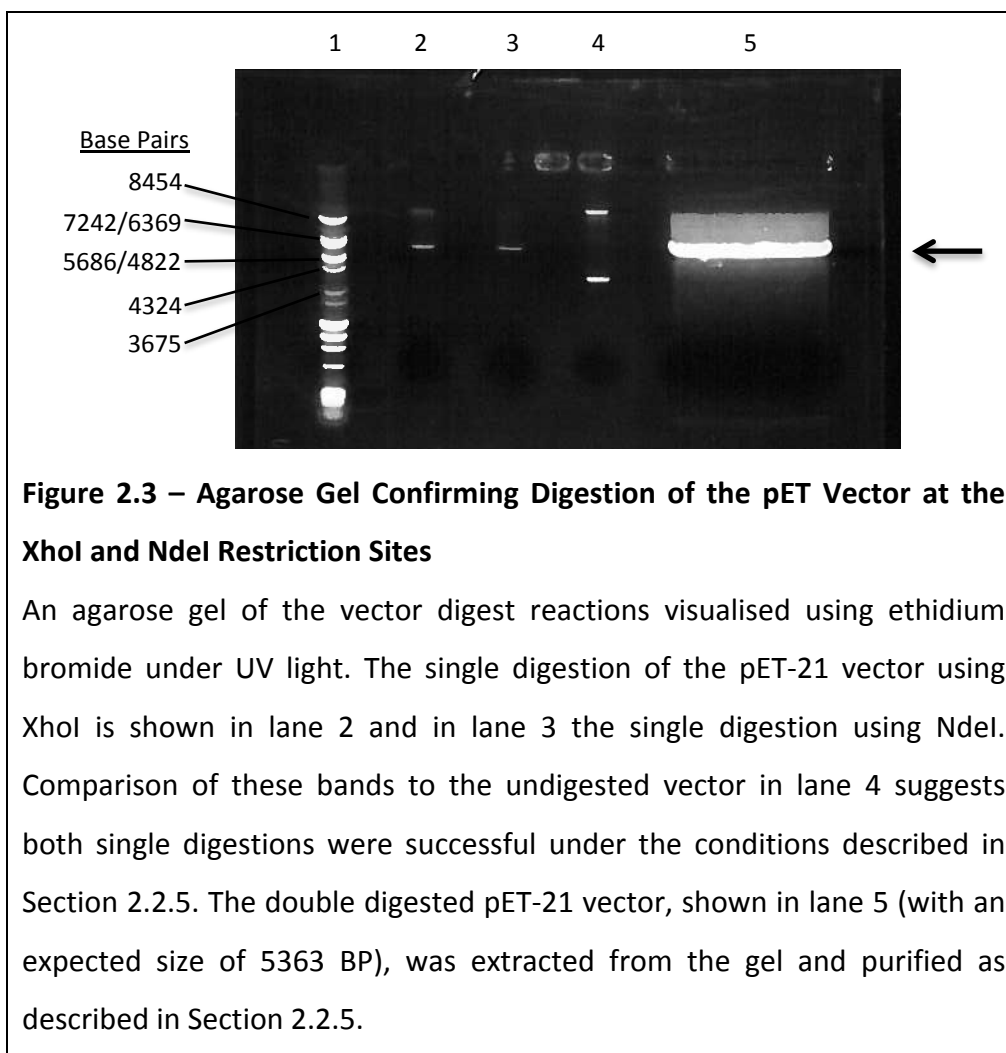


Figure 2.2 – Agarose Gel Confirming Amplification of VHH DNA with XhoI and NdeI Restriction Sites

An agarose gel analysing the products of the PCR reaction, used to amplify the VHH DNA with the XhoI and NdeI restriction sites, visualised using ethidium bromide under UV light. A DNA ladder was included in lane 1 and a negative control, containing the primers without the VHH DNA, was included in lane 8. Lanes 2 and 3 show VHH amplification using the DVQ primer, lanes 4 and 5 using the EVQ primer and lanes 6 and 7 using the QVQ primer. The position of the observed bands, between the 600 BP and 300 BP markers, is consistent with the expected 500 BP size of the amplified VHH DNA. This confirmed the successful amplification of the VHH DNA.



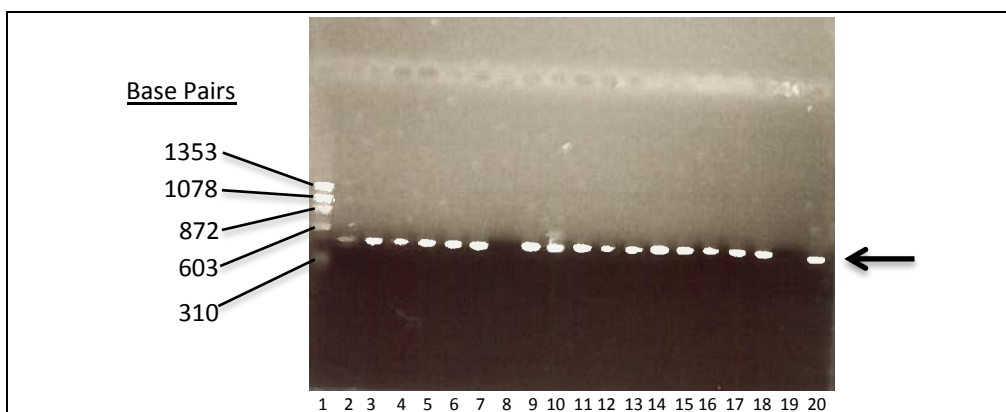


Figure 2.4 – Agarose Gel of PCR Screen used to Confirm Insertion of VHH DNA into pET Vector

An agarose gel of the PCR screen used to confirm the insertion of the VHH DNA into the pET-21 vector, visualised using ethidium bromide under UV light. Successful insertion of the VHH DNA into the pET-21 vector is confirmed with the appearance of a band at approximately 500 BP resulting from the amplification of the VHH DNA using the appropriate forward oligo. A positive control of VHH DNA was included in lane 20. Successful insertion can be observed for samples in lanes 2 to 7 and lanes 9 to 18. No band appears for the samples in lanes 8 and 19 suggesting the VHH DNA was incorporated into the vector.

2.3.1 – Expression Trials of VHHs

Small scale (5 ml) expression trials were set up using conditions described in Section 2.2.13 to confirm protein expression using the pET-21 vectors generated in Section 2.2.8. Figure 2.5 shows SDS-PAGE analysis of the expression trials confirming that all VHHs were expressed under these conditions. SDS-PAGE analysis of the total fraction compared to the soluble fraction (Figure 2.6) shows that little or no VHH protein is expressed in a soluble form.

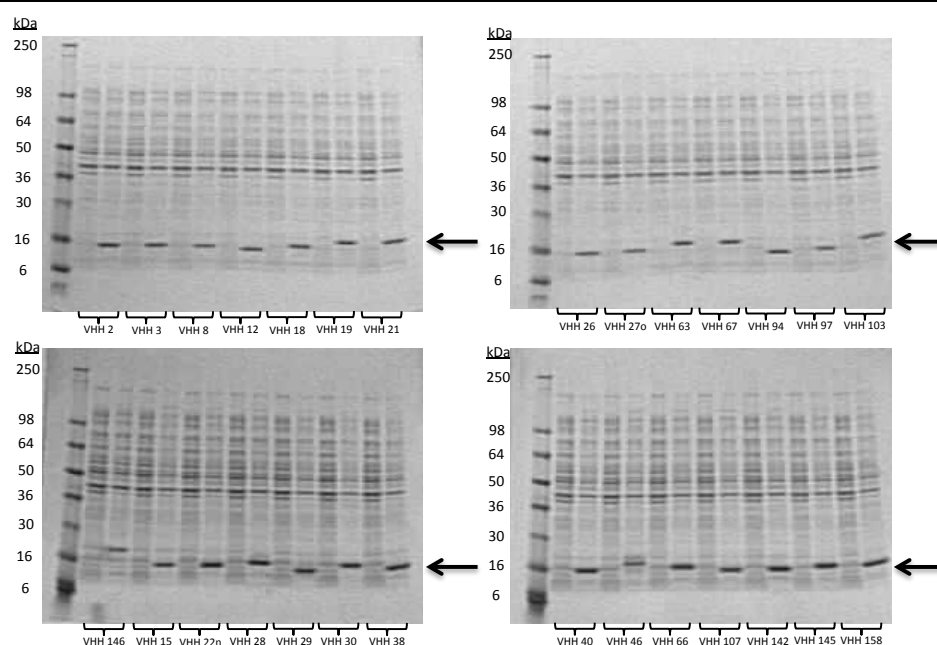
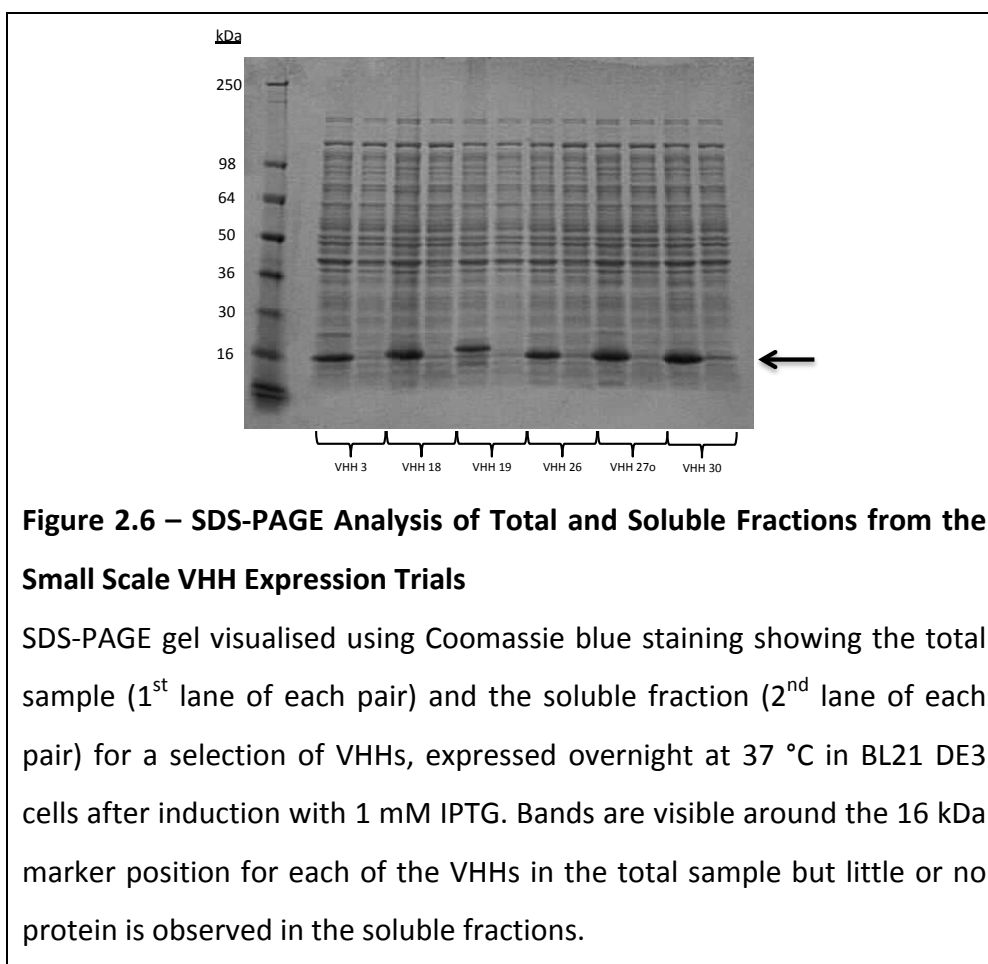


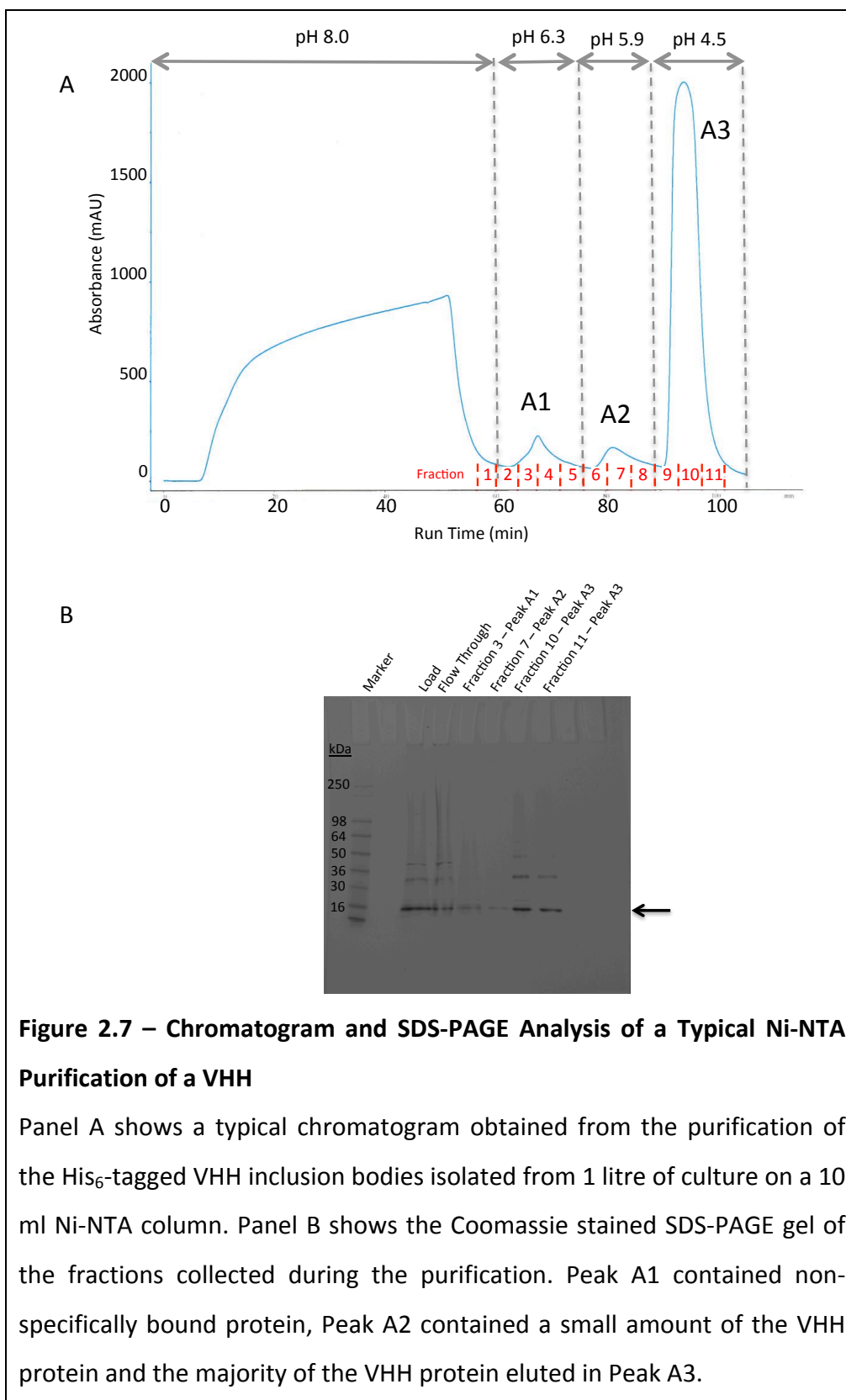
Figure 2.5 – SDS-PAGE Analysis of the Small Scale VHH Expression Trials

SDS-PAGE gels visualised using Coomassie blue staining showing the pre-induced (1st lane of each pair) and the post-induced (2nd lane of each pair) for the 28 VHHs cloned in to the pET-21 vector, expressed overnight at 37 °C in BL21 DE3 cells after induction with 1 mM IPTG. Bands are visible around the 16 kDa marker position for each of the VHHs in the post-induced sample.



2.3.2 – Expression and Purification of VHH 18 and VHH 67

VHH 18 and VHH 67 were expressed as described in Section 2.2.13. Overnight cultures grown in LB reached A_{600} values of between 2.2 and 3.0 and those grown in minimal media reached A_{600} values between 1.4 and 2.3. Figure 2.7 shows a typical chromatogram obtained from the Ni-NTA purification of the denatured VHH inclusion bodies, as described in Section 2.2.14, along with the SDS-PAGE gel of samples collected during the purification.



Pooled fractions were refolded and concentrated as described in Section 2.2.14. The refold efficiency, the percentage of unfolded protein successfully refolded, varied between 15 % and 70 % with an average of approximately 40 %. Precipitation of the protein was often seen during dialysis between the two concentration steps. When appropriate, this precipitate was collected by centrifugation, redissolved in denaturant and refolded to increase the VHH protein yield. The typical yields of both the unfolded and refolded VHH proteins prepared are detailed in Table 2.1.

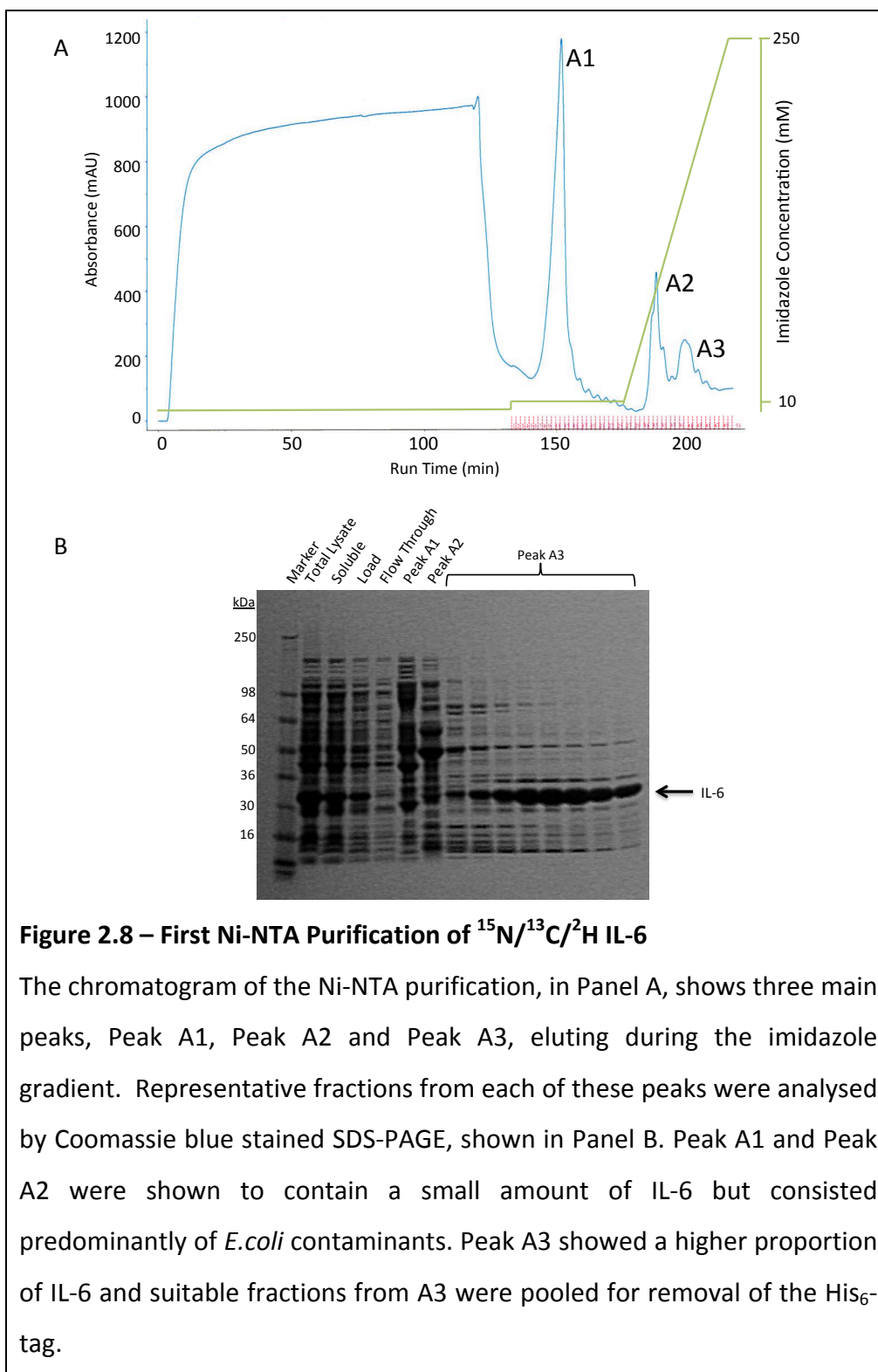
Protein Labelling	VHH 18 Yield (mg l ⁻¹)		VHH 67 Yield (mg l ⁻¹)	
	Unfolded	Refolded	Unfolded	Refolded
Unlabelled	69	22	82	56
¹⁵ N Labelled	55	26	59	12
¹⁵ N/ ¹³ C Labelled	43	21	43	18
¹³ C, Aromatics Unlabelled	62	19	46	6

Table 2.1 – Yield of Unfolded and Refolded Protein

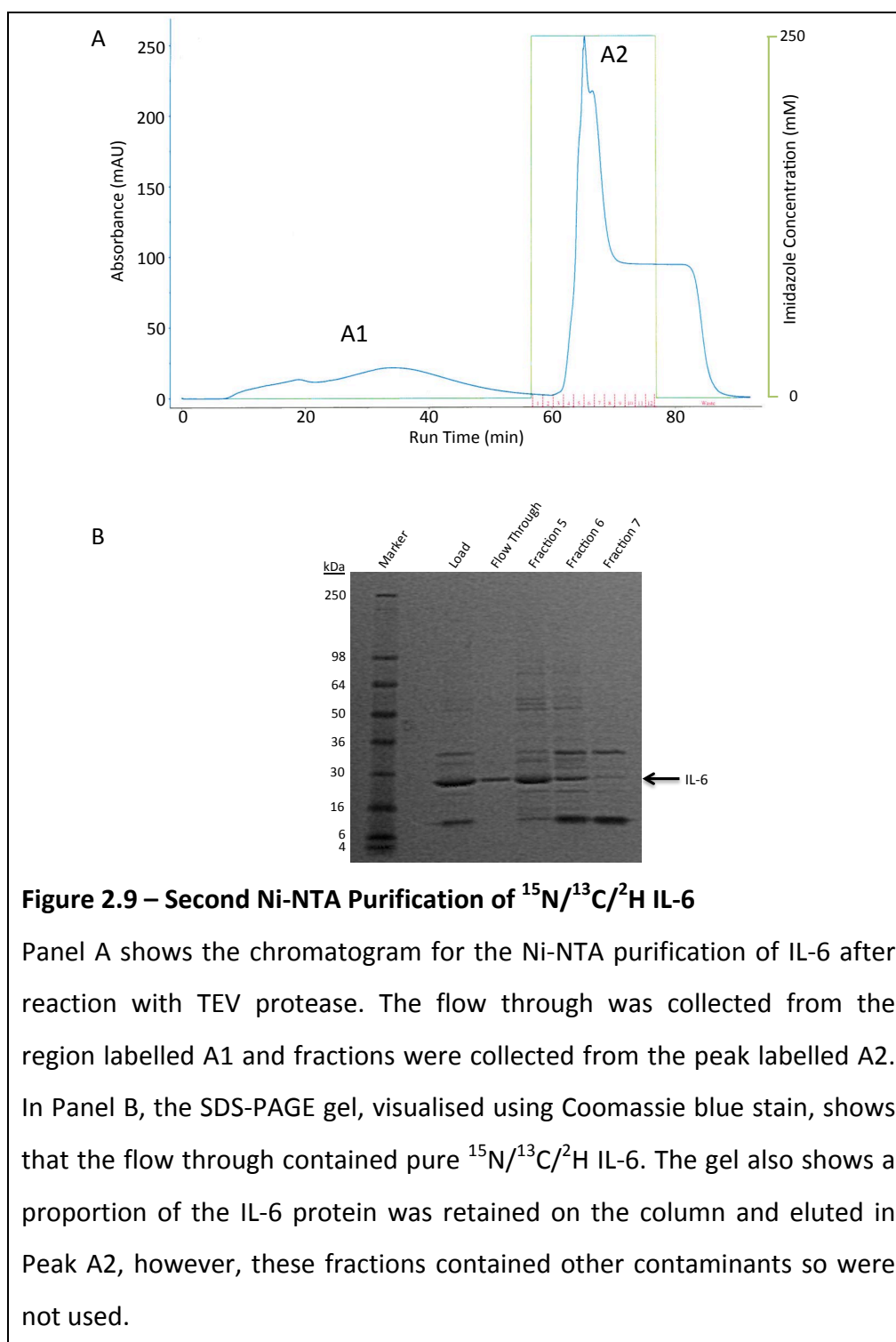
The yield of unfolded protein was measured directly after the Ni-NTA purification and the yield of refolded protein refers to the final quantity of VHH protein obtained after refolding, concentration and buffer exchange into the required buffer.

2.3.3 – Expression and Purification of ¹⁵N/¹³C/²H IL-6

A pellet of ¹⁵N/¹³C/²H IL-6 from a 5 l expression was provided by Dr. Philip Addis (University of Leicester) and purified as described in Section 2.2.16. The chromatogram of the Ni-NTA prep of IL-6 is shown in Figure 2.8 alongside the SDS-PAGE analysis of samples taken throughout the purification. Approximately 40 mg of IL-6 protein was obtained after the pooled protein fractions were buffer exchanged into high salt PBS and concentrated.

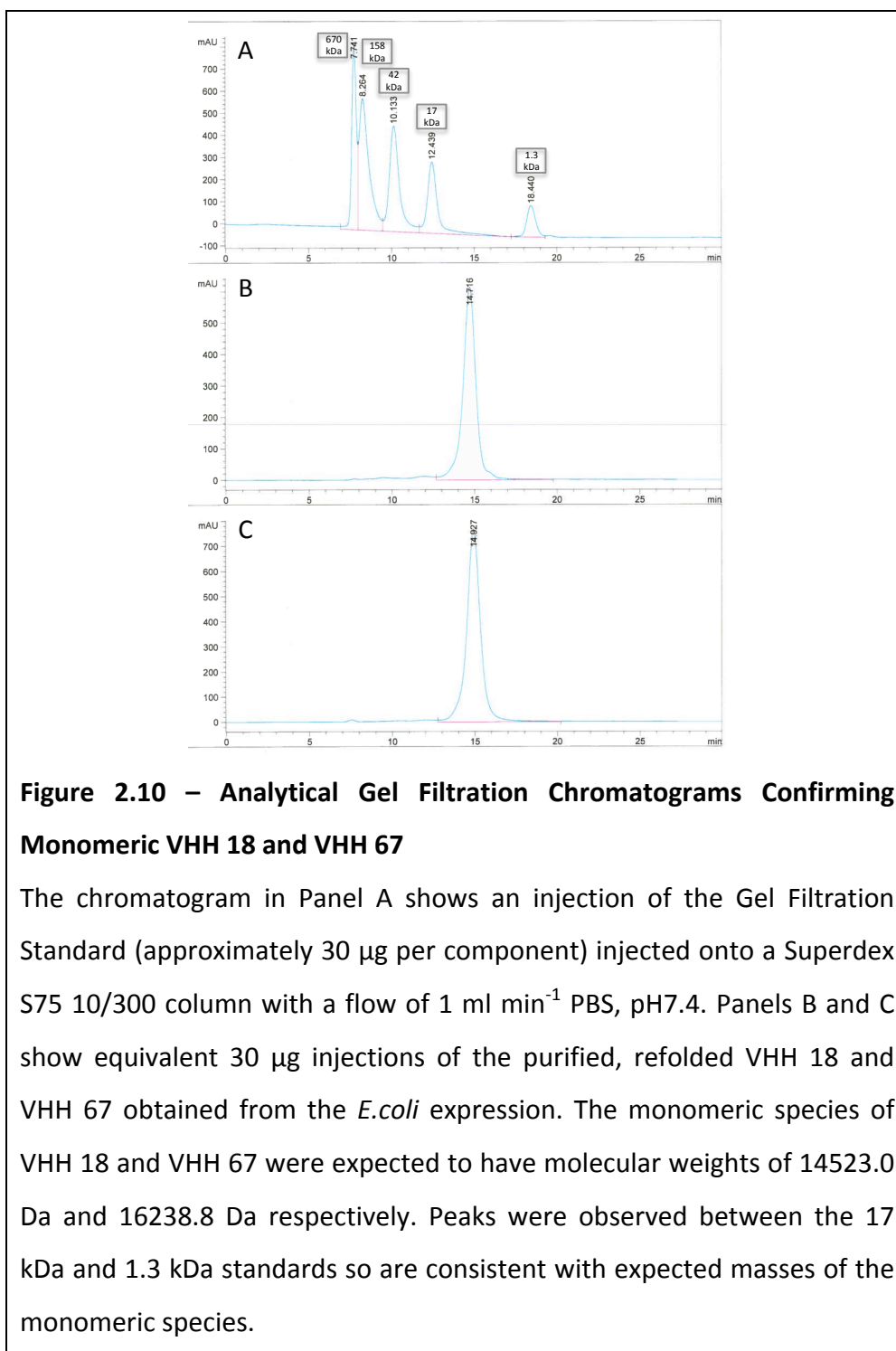


After cleavage of the His₆-tag a second Ni-NTA purification was carried out to isolate un-tagged IL-6. Figure 2.9 shows the chromatogram of the purification and SDS-PAGE analysis of samples collected during the purification. The final yield of ¹⁵N/¹³C/²H IL-6 was 11 mg or 2.2 mg l⁻¹ as determined by absorbance at 280 nm ($\epsilon = 10220 \text{ M}^{-1} \text{ cm}^{-1}$).



2.3.4 – Analytical Gel Filtration Chromatography

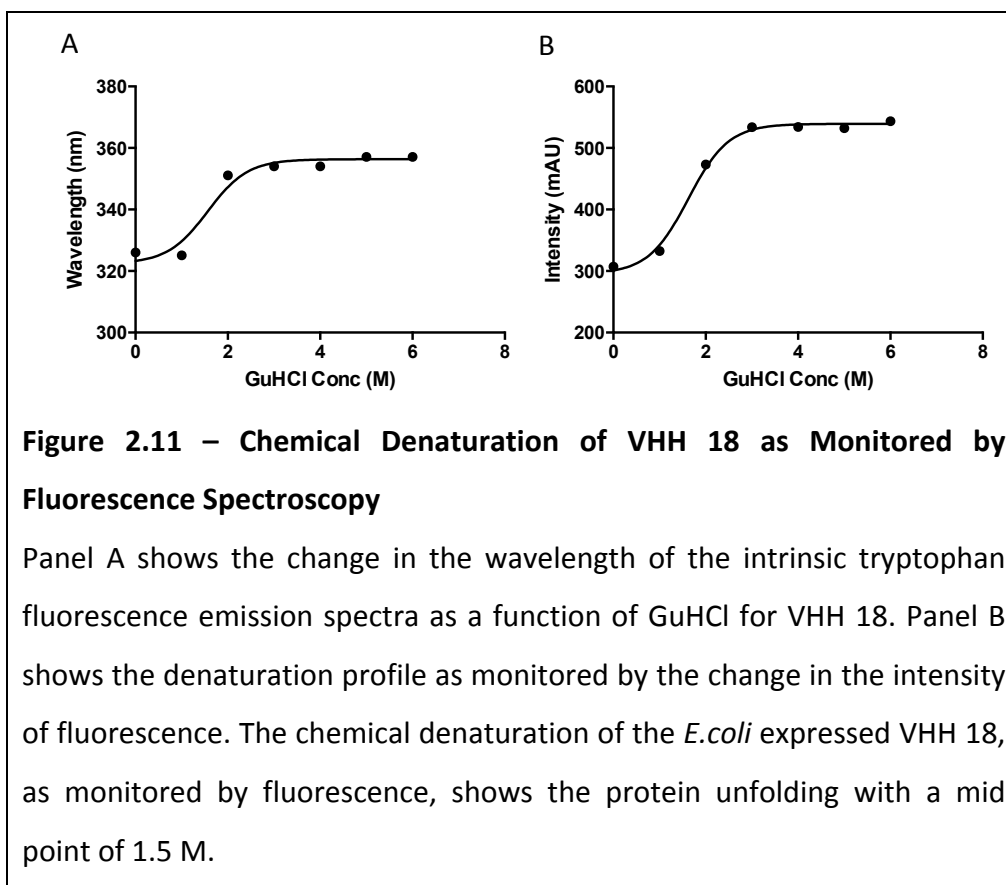
Analysis of the VHH samples by analytical gel filtration, as described in Section 2.2.17, showed that the VHH samples were pure and suggested a molecular weight between 17 kDa and 1.3 kDa which agrees with an expected molecular weight of approximately 16 kDa. Figure 2.10 shows examples of typical chromatograms.



2.3.5 – Fluorescence Spectroscopy

Fluorescence spectroscopy is commonly used to study the folding and conformational dynamics of proteins (Royer, 2006). Tryptophan and tyrosine are intrinsically fluorescent and the fluorescence signals from these residues (especially tryptophan) are sensitive to changes in tertiary or quaternary structure of the protein. Tryptophan residues are commonly found buried in the core of the protein or at the interface between protein domains and when partially buried will emit at a wavelength of 335 nm (Royer, 2006). As the protein unfolds and the tryptophan residues become exposed the emission wavelength increases through to a maximum of 355 nm when completely exposed to water (Royer, 2006). Along with a change in wavelength, the intensity can change, either increasing or decreasing, as the tryptophan comes into contact with water and interacts with quenching agents. This variation in fluorescence wavelength and intensity allows the unfolding of a native protein to be observed as a function of denaturant concentration.

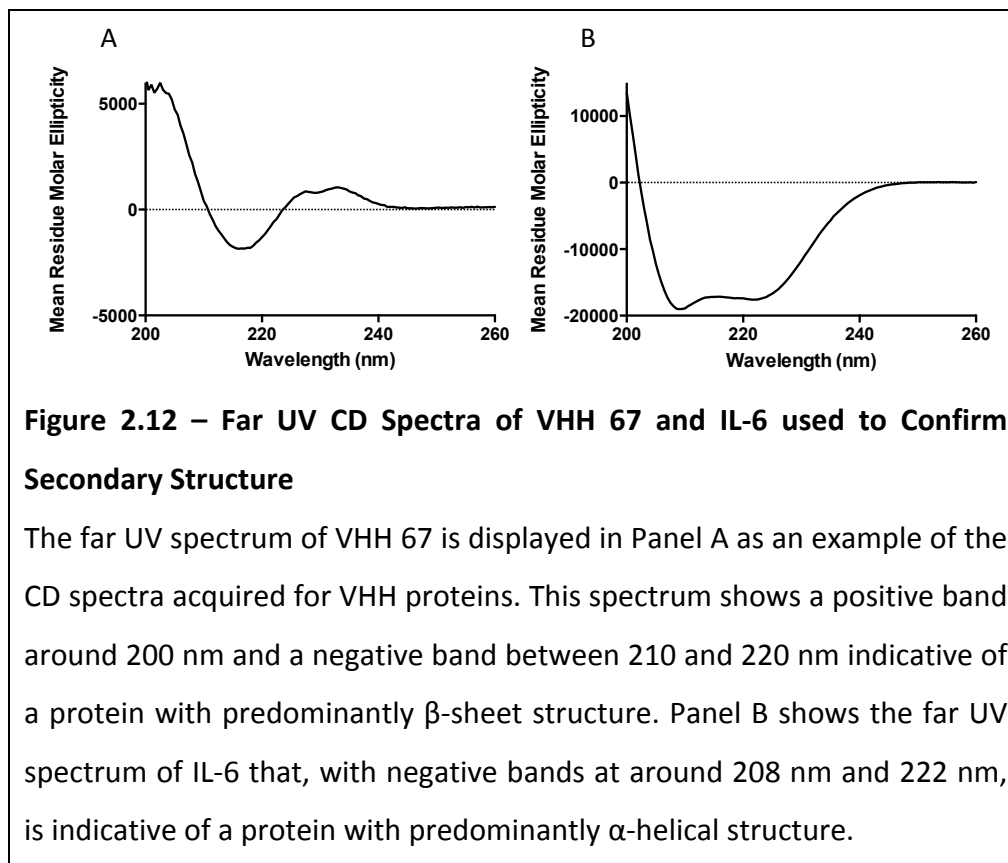
To confirm the presence of a stable protein fold for the VHH samples prepared, both the change in the fluorescence maxima and intensity were monitored over a range of GuHCl concentrations. The denaturation profile monitored by fluorescence for VHH 18 is shown as an example in Figure 2.11 and confirms the presence of secondary structure within the protein. VHH 18 unfolds with a mid point at a GuHCl concentration of 1.5 M and is completely unfolded when a GuHCl concentration of approximately 3 M is reached. Typical mid point values for the VHHs measured ranged from 1.2 M to 2.5 M.



2.3.6 – Circular Dichroism Spectroscopy

Analysis of the far UV CD spectrum allows characterisation of protein secondary structural features including determination of α -helical and β -sheet content (Kelly and Price, 2000). The CD spectra of α -helical proteins will show a positive peak around 190 nm and negative peaks at 208 nm and 222 nm; proteins with β -sheet character will show a positive peak between 195 nm and 200 nm and a negative peak between 210 nm and 220 nm; and disordered proteins show a negative band around 200 nm (Corrêa and Rammos, 2009).

Figure 2.12 shows the CD spectra collected for the *E.coli* expressed VHH 67 and IL-6. The results confirm that VHH 67 is predominantly β -sheet in character and IL-6 is an α -helical protein.



2.3.7 – Surface Plasmon Resonance

Association rate, dissociation rate and affinity of the purified VHHs were calculated from the data shown in Figures 2.13 and 2.14 and are displayed in Table 2.2. Both VHHs show K_D values in the low nanomolar range but the off rate of VHH 67 is an order of magnitude higher than VHH 18. The affinity measurement for the mammalian expressed VHH 67 (as detailed in Section 1.5) was 2.0 nM, slightly lower than measured here for the *E.coli* expressed sample.

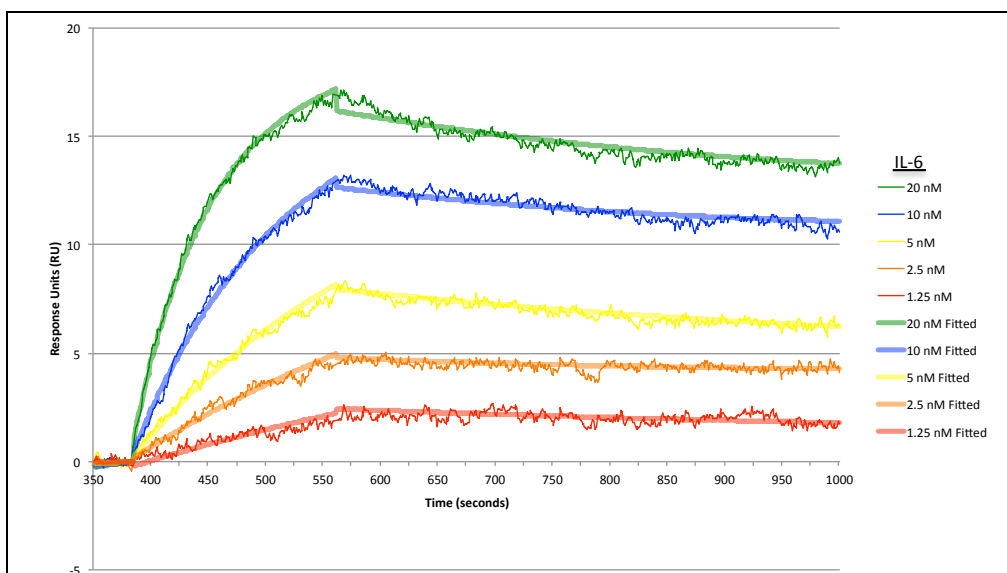


Figure 2.13 - SPR Analysis of IL-6 Binding to *E.coli* Expressed VHH 18

The sensorgram shows the titration of immobilised VHH 18 with IL-6 at concentrations of 20 nM (green), 10 nM (blue), 5 nM (yellow), 2.5 nM (orange) and 1.25 nM (red). The IL-6 injection begins at 380 seconds and continues until 560 seconds. Plotted alongside the raw data, as lighter lines, are the double referenced data fitted to a 1:1 model of binding and corrected for drifting baseline.

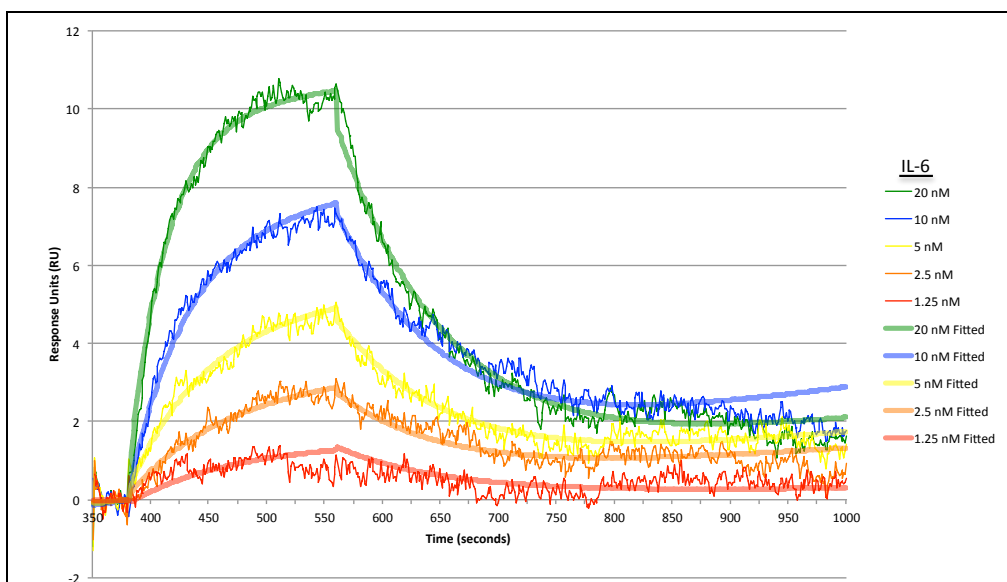


Figure 2.14 - SPR Results of IL-6 Binding to *E.coli* Expressed VHH 67

The sensorgram shows the titration of immobilised VHH 67 with IL-6 at concentrations of 20 nM (green), 10 nM (blue), 5 nM (yellow), 2.5 nM (orange) and 1.25 nM (red). The IL-6 injection begins at 380 seconds and continues until 560 seconds. Plotted alongside the raw data, as lighter lines, are the double referenced data fitted to a 1:1 model of binding and corrected for drifting baseline.

	k_a ($M^{-1} s^{-1}$)		k_d (s^{-1})		K_D (nM)
	Value	Std Error	Value	Std Error	
VHH 18	7.40×10^5	5.40×10^3	1.11×10^{-3}	3.50×10^{-5}	1.5
VHH 67	7.85×10^5	2.09×10^4	1.14×10^{-2}	1.40×10^{-4}	14.6

Table 2.2 - Binding Kinetics of VHH 18 and VHH 67 as Measured by SPR

The table lists the on rates and off rates, along with the standard error calculated for each result, for the *E.coli* expressed VHH antibodies. From these data K_D values of 1.5 nM and 14.6 nM were calculated for VHH 18 and VHH 67 respectively.

2.3.8 – Mass Spectrometry

The electrospray positive ionisation mass spectra acquired for the VHHs were of good quality showing a clear charge state distribution of ions from which the molecular weights could be calculated (see Figure 2.15). For VHH 18 the theoretical monoisotopic molecular weight was 14631.1 Da, assuming the formation of the two disulphide bonds. For VHH 67 the theoretical monoisotopic molecular weight was 16377.0 Da, assuming the formation of a single disulphide bond. The observed masses as calculated from the deconvolution of the MS data were 14631.85 ± 0.11 Da and 16378.57 ± 0.78 Da for VHH 18 and VHH 67 respectively.

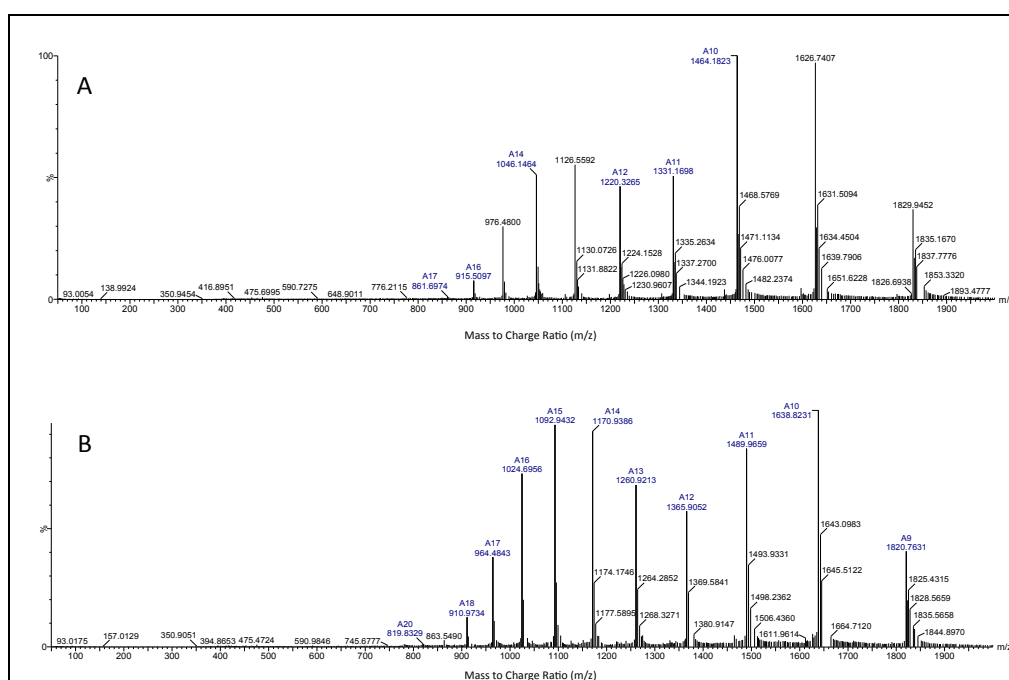


Figure 2.15 – Electrospray Positive Ionisation Mass Spectra of VHH 18 and VHH 67

Panel A shows the mass spectrum of VHH 18 and Panel B shows the mass spectrum of VHH 67. The different charge states from which the masses were calculated have been labelled, for example the peaks labelled A11 correspond to the $[M + 11H]^{11+}$ charge state. Using the Waters MaxEnt™ and Transform™ algorithms, the masses were calculated as 14631.85 ± 0.11 Da and 16378.57 ± 0.78 Da for VHH 18 and VHH 67 respectively.

2.3.9 – NMR Spectroscopy

Initial ^{15}N - ^1H HSQC spectra for VHH 18 and VHH 67 are shown in Figure 2.16. The spectra show well dispersed peaks with good signal to noise consistent with proteins of this size with β -sheet secondary structure (Vuchelen *et al.*, 2009). Of the 150 theoretical signals for VHH 18 (129 amide NHs, 6 pairs of asparagine side chain NH_2 s, 8 pairs of glutamine NH_2 s and 4 tryptophan indole NHs) approximately 136 signals were observed (90 %). For VHH 67, of the 174 theoretical signals for (143 amide NHs, 7 pairs of asparagine side chain NH_2 s, 7 pairs of glutamine NH_2 s and 3 tryptophan indole NHs) approximately 165 signals were observed (95 %).

To evaluate the stability of the VHH proteins, the samples were incubated at a temperature of 35 °C for three days and the ^{15}N - ^1H HSQC spectra were reacquired. No differences were observed between the spectra before and after incubation.

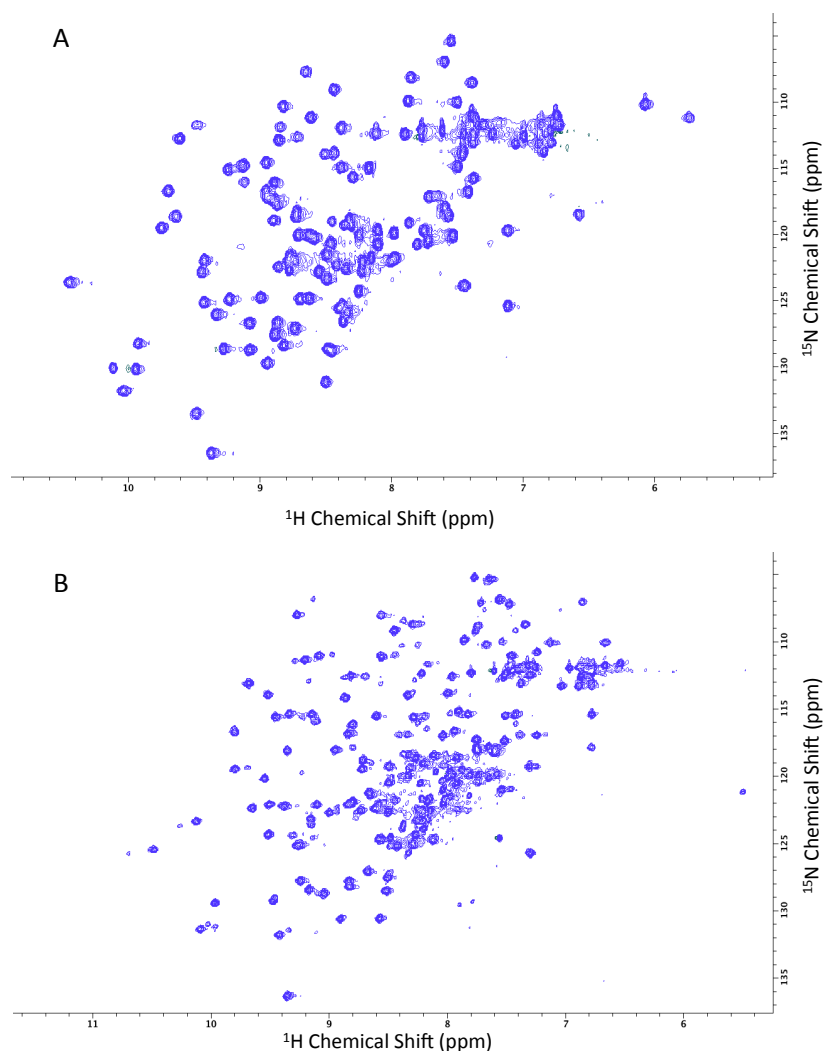


Figure 2.16 – ^{15}N - ^1H HSQC Spectra of VHH Proteins

Panel A shows a ^{15}N - ^1H HSQC spectrum of $^{15}\text{N}/^{13}\text{C}$ labelled VHH 18 at a concentration of 286 μM in 25 mM sodium phosphate, 100 mM sodium chloride buffer, pH 6.4 collected at 35 $^{\circ}\text{C}$ on a 600 MHz Bruker Avance spectrometer for 60 minutes. Panel B shows the equivalent spectrum for VHH 67 at a concentration of 312 μM collected for 40 minutes. The spectra show good signal to noise with little signal overlap.

The ^{15}N - ^1H TROSY spectrum collected for $^{15}\text{N}/^{13}\text{C}/^2\text{H}$ IL-6 (Figure 2.17) shows more spectral overlap than the VHH spectra due to the increased size of IL-6 (21 kDa compared to 16 kDa) and the α -helical content of IL-6. This is consistent with a previous example in the literature showing significant overlap of signals in the central region of the spectrum (Xu *et al.*, 1996). Of the 225 theoretical signals (176 amide NHs, 10 pairs of asparagine side chain NH_2 s, 14 pairs of glutamine NH_2 s and 1 tryptophan indole NH) approximately 210 signals were observed (95 %). This compares well to the published data in which 88 % of peaks were observed (Xu *et al.*, 1996).

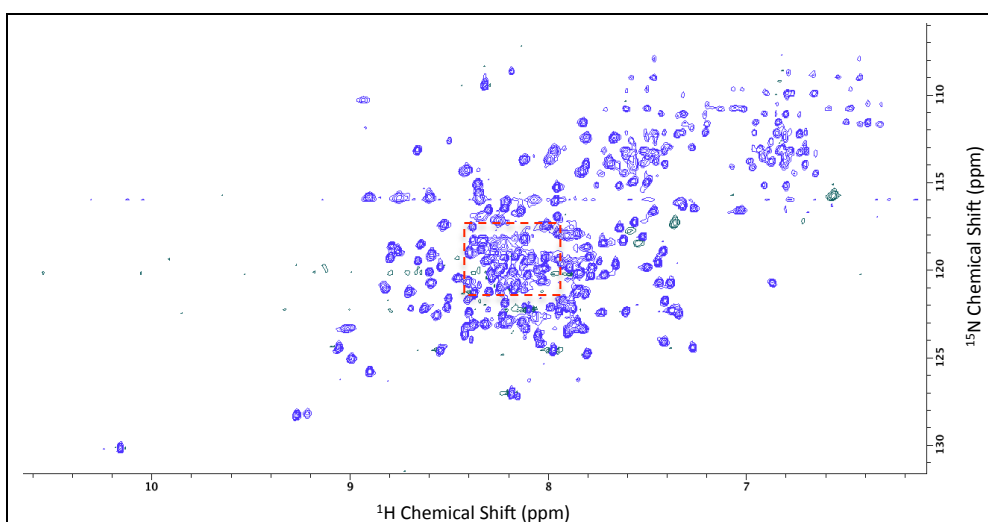


Figure 2.17 – ^{15}N - ^1H TROSY Spectrum of $^{15}\text{N}/^{13}\text{C}/^2\text{H}$ IL-6

The figure shows the ^{15}N - ^1H TROSY spectrum of $^{15}\text{N}/^{13}\text{C}/^2\text{H}$ labelled IL-6 at a concentration of 380 μM in 25 mM sodium phosphate, 100 mM sodium chloride buffer, pH 6.4 collected at 35 $^{\circ}\text{C}$ on an 800 MHz Bruker Avance II spectrometer for 60 minutes. A high degree of resonance overlap is observed in the central region of the spectrum (highlighted with a red box). Signals resulting from the arginine side chain $\text{H}\epsilon$ - $\text{N}\epsilon$ are aliased and appear as negative signals (shown in green).

2.4 – Discussion

The results presented in this chapter confirm the successful cloning, expression, refolding and purification of the VHH proteins. Although expression of soluble protein was not achieved, the purification and refolding of the insoluble protein was an effective strategy to generate high yields of pure VHH protein.

Analytical gel filtration confirmed the isolation of pure, monomeric VHH proteins. Intrinsic fluorescence spectroscopy confirmed the presence of a stable protein fold with mid points of unfolding around 1.5 M for the VHHs expressed for this project. Far UV CD analysis confirmed that the VHH proteins were predominantly β -sheet in character and binding kinetics, measured by SPR, show the *E.coli* expressed VHH proteins binding to IL-6 in the low nanomolar range, consistent with values measured for mammalian expressed VHHs. Finally, it was shown that isotopically labelled VHH proteins give rise to high quality NMR spectra with well dispersed signals and good signal to noise.

Also reported in this chapter was the expression and purification of $^{15}\text{N}/^{13}\text{C}/^2\text{H}$ IL-6, required for the VHH:IL-6 complex experiments carried out in Chapter 4. The final yield of purified IL-6 obtained using the method described in Sections 2.2.15 and 2.2.16 was 2.2 mg l⁻¹ compared to 2.75 mg l⁻¹ previously. The NMR spectrum acquired for IL-6 compared well to published data (Xu *et al.*, 1996) with approximately 95 % of the theoretical signals being observed in the ^{15}N - ^1H TROSY.

2.5 – Conclusions

This chapter shows that VHH proteins expressed in *E.coli* provide high quality NMR spectra. Yields of around 20 mg l⁻¹ are achievable for the unlabelled protein and for almost all varieties of the isotopically labelled proteins. Characterisation by chromatography, fluorescence spectroscopy, CD and SPR also confirmed that the VHH proteins were stable, folded, had the predicted secondary structure and binding affinities identical to those expressed in mammalian systems.

Chapter 3 – NMR Solution Structures of Free VHH Antibody Fragments

3.1 – Introduction

The aims of the work described in this chapter were to solve the free solution structures of VHH antibodies by NMR and in doing so gain an insight into the behaviour of the complementarity determining regions (CDRs). The VHH antibodies being investigated were raised to a IL-6:gp-80 fusion protein but it is hoped that conclusions drawn from this work will be applicable to VHH antibodies raised against a wide range of target proteins.

As discussed in Chapter 1, the length of the CDR loops of VHH domains are longer than those found in the VH domains of conventional antibodies (Desmyter *et al.*, 1996). These extended CDRs have also been shown to adopt alternative conformations to those observed in conventional antibodies (Muyldermans, 2001). The increased length and additional conformations may be responsible for maintaining VHH diversity despite having half the number of CDRs than conventional antibodies. The longer CDRs of VHH domains may be inherently more flexible than conventional antibodies as conformation is less restricted. Conversely, some VHHs have an additional disulphide bond linking CDR1 and CDR3 that may result in greater stability of CDR loops (Nguyen *et al.*, 2000).

Currently, around 80 VHH structures have been submitted to the RCSB Protein Data Bank (PDB) (www.rcsb.org). The vast majority of these structures have been solved by X-ray crystallography and around 80 % of them are VHH antibodies in complex with another protein or ligand. In order to understand the dynamics of the CDRs and the role that they play in binding, it is essential to first study the VHH antibodies free in solution before observing the VHH in complex with its antigen, where conformation and mobility of the VHH CDRs may be influenced by the presence of the antigen. NMR offers the opportunity to study the dynamics of the CDRs in solution.

Given the size and stability of VHH domains they should be ideally suited to structural studies by NMR but to date there is only one VHH structure solved by NMR. This single example of a VHH NMR structure available in the PDB is that of VHH-H14 which was raised against the α subunit of human chorionic gonadotropin (Renisio *et al.*, 2002, PDB accession code 1G9E). The X-ray crystal structure of VHH-H14 is also available (Spinelli *et al.*, 1996, PDB accession code 1HCV) both of which are discussed in Section 1.2. Comparison of the NMR structure and the crystal structure of VHH-H14 shows good agreement between the structures with the exception of the CDRs for which structural differences are observed. Renisio *et al.* go on to conclude, through measurement of NMR dynamics parameters, that the antigen recognition site is the most mobile region of the protein. With CDR1, CDR2 and CDR3 lengths of 6, 9 and 6 residues respectively, VHH-H14 has relatively short CDRs and therefore may not be representative of the majority of VHH domains. For this reason solving the NMR structures of VHH domains with longer CDRs and determining the dynamics of these extended CDRs could provide valuable insight into the behaviour of the antigen-binding regions of VHH antibodies.

Antigen binding is thought to occur predominantly through CDR3 (Muyldermans *et al.*, 2009) so to investigate the impact on CDR length on the dynamics of CDRs, VHH 18 was selected from the panel of VHH antibodies (described in Section 1.5) as an example of a VHH with a short CDR3 (10 residues) and VHH 67 was selected as an example of a VHH with a long CDR3 (19 residues). The hypothesis was that VHH 18 with a short CDR3, which also contained an additional disulphide bond linking CDR3 to CDR1, would be more rigid than the longer, untethered, CDR3 of VHH 67. Interesting and unexpected features of the structure of the long CDR3 in VHH 67 led to further work being carried on VHH 29 and VHH 146, two other examples of VHH antibodies with long CDR3 loops.

3.2 – Materials and Methods

3.2.1 – NMR Sample Preparation

The VHH samples were buffer exchanged into the NMR buffer (25 mM sodium phosphate, 100 mM sodium chloride, 10 μ M EDTA and 0.02 % (w/v) sodium azide buffer at pH 6.4) and concentrated using a 3000 Da molecular weight cut off (MWCO) Millipore Amicon Ultra 15 ml centrifugal filter unit as described in Section 2.2.14.

3.2.2 – NMR Data Acquisition and Processing for Sequence-Specific Assignments

NMR spectra were acquired from 0.35 ml samples of 300-400 μ M VHHs in a 25 mM sodium phosphate, 100 mM sodium chloride, 10 μ M EDTA and 0.02 % (w/v) sodium azide buffer at pH 6.4 containing 10 % D₂O / 90 % H₂O or 100 % D₂O as appropriate. NMR data were acquired at 35 °C on 600 MHz Bruker Avance, 600 MHz Bruker DRX or 800 MHz Bruker Avance II spectrometers. The 2D and 3D spectra recorded to make sequence-specific ¹⁵N, ¹³C and ¹H resonance assignments were: ¹H-¹H TOCSY (Braunschweiler and Ernst, 1983) with mixing times of 30 and 60 ms; ¹H-¹H NOESY (Macura and Ernst, 1980) with an NOE mixing time of 125 ms; ¹⁵N-¹H HSQC (Bodenhausen and Ruben, 1980); ¹⁵N-¹H TOCSY-HSQC (Marion *et al.*, 1989) with a mixing time of 55 ms; ¹⁵N-¹H NOESY-HSQC (Marion *et al.*, 1989) and ¹³C-¹H HSQC-NOESY (Majumdar and Zuiderweg, 1993) both with NOE mixing times of 125 ms; HCCH-TOCSY (Bax *et al.*, 1990) with a mixing time of 19.5 ms and ¹⁵N-¹³C-¹H HNCACB (Wittekind and Mueller, 1993), CBCA(CO)NH (Grzesiek and Bax, 1993) and ¹⁵N-¹³C-¹H HNCO (Kay *et al.*, 1990). 2D ¹⁵N-¹H HSQC spectra of the VHHs were also acquired in D₂O to identify non-exchanged amide protons, including hydrogen bonded amide protons. Typical acquisition times in F₁ and F₂ for the 3D experiments were 16.8-20 ms for ¹⁵N, 6.8-8 ms for ¹³C (20 ms in the HNCO) and 15-19 ms for ¹H, with an acquisition time of 50 ms in F₃ (¹H). Typical acquisition times in 2D experiments were 40 ms (¹⁵N), 9 ms (¹³C) or 35-40 ms (¹H) in F₁ and 100 – 200 ms in F₂ (¹H). 3D experiments were collected over approximately 72 hours, 2D ¹H experiments over approximately 24 hours, and 2D ¹⁵N-¹H or ¹³C-¹H spectra over 40 minutes. The WATERGATE method (Piotto *et al.*, 1992) was used for water suppression. 2D and 3D NMR data were processed using

NMRPipe (Delaglio *et al.*, 1995) with linear prediction used, when required, to extend the effective acquisition times by up to two fold in indirect dimensions. Non-uniform sampling (Schmieder *et al.*, 1993) was used to acquire data when necessary, with a subset of 25 % data sampled. The non-uniform sampled data were reconstructed using either istHMS (Hyberts *et al.*, 2012) or mddnmr (Tugarinov *et al.*, 2005). A series of ^{15}N - ^1H HSQC experiments were also carried out at temperatures between 5 °C and 40 °C to determine the temperature dependence of backbone amide signals.

3.2.3 – NMR Data Analysis for Sequence-Specific Assignments

Spectra were analysed using the Sparky package (T. D. Goddard and D. G. Kneller, SPARKY 3, University of California, San Francisco). Sequence-specific backbone assignments were made using the triple resonance ^{15}N - ^{13}C - ^1H HNCACB and CBCA(CO)NH spectra. In principle, the HNCACB shows the correlation between the resonances of the amide nitrogen, amide proton, $\text{C}\alpha$ and $\text{C}\beta$ atoms for each residue alongside the resonances of the $\text{C}\alpha$ and $\text{C}\beta$ atoms of the preceding residue. The CBCA(CO)NH links the amide nitrogen and amide proton resonances to the resonances of the $\text{C}\alpha$ and $\text{C}\beta$ atoms of preceding residue, allowing the inter-residue $\text{C}\alpha$ and $\text{C}\beta$ resonances to be distinguished from those of the intra-residue $\text{C}\alpha$ and $\text{C}\beta$ atoms. The characteristic resonances of $\text{C}\alpha$ and $\text{C}\beta$ atoms were used to identify a particular residue, or groups of residues, and then unique combinations of these characteristic $\text{C}\alpha$ and $\text{C}\beta$ chemical shifts allowed assignment of the signals to regions of the protein backbone (Sattler *et al.*, 1999). The remaining backbone assignments were then made by a process of elimination using the triple resonance spectra. Correlations observed in the ^{15}N - ^1H TOCSY-HSQC, ^{13}C - ^1H HCCH-TOCSY, ^{15}N - ^1H NOESY-HSQC and ^{13}C - ^1H HSQC-NOESY were used to help the assignment of backbone residues and make side chain assignments. The assignment of aromatic side chain protons was achieved using a combination of the ^1H - ^1H TOCSY and ^1H - ^1H -NOESY experiments alongside the ^{15}N - ^1H NOESY-HSQC and ^{15}N - ^1H TOCSY-HSQC experiments (Sattler *et al.*, 1999). Using the amide proton and amide nitrogen assignments, the ^{15}N - ^{13}C - ^1H HNCO spectrum was used to assign the carbonyl resonances for each residue.

3.2.4 – Secondary Structure Prediction from NMR Chemical Shifts

The locations of secondary structure elements were predicted by two methods, firstly by the Torsion Angle Likelihood Obtained from Shift method (TALOS+) (Cornilescu *et al.*, 1999, Shen *et al.*, 2009) and then by the Chemical Shift Index method (CSI) (Wishart *et al.*, 1992, Wishart and Sykes, 1994).

TALOS+ takes the ^{15}N (backbone amide), ^{13}C (C=O, C α and C β) and ^1H (H α) chemical shifts and searches for residue and chemical shift homology within triplets of residues against a database of proteins for which both NMR assignments and X-ray crystal structures are available. From this data the phi and psi backbone torsion angles, and subsequently, the secondary structure are predicted.

The CSI method predicts secondary structure by comparing ^{13}C (C=O, C α and C β) and ^1H (H α) chemical shifts to those of random coil values. The direction of the chemical shift difference, upfield or downfield of the random coil values, are indicative of atoms being located in areas of α -helix or β -strands. For the VHH proteins the C α and C β chemical shifts were used to predict secondary structure.

3.2.5 – NMR Structure Calculation

The VHH solution structures were calculated using the program CYANA (Guntert *et al.*, 1997). First, the combined automated NOE assignment and structure determination module (CANDID) (Herrmann *et al.*, 2002) was used to determine unique assignments for the NOEs identified in the two- and three-dimensional NOESY spectra that were used to produce initial structures. These structures then underwent several cycles of simulated annealing combined with redundant dihedral angle constraints (REDAC) to produce final converged VHH structures (Guntert and Wuthrich, 1991).

The inputs for CANDID included essentially complete ^{15}N , ^{13}C and ^1H resonance assignments for the VHHs; manually picked peak lists from the ^1H - ^1H , ^{15}N - ^1H and ^{13}C - ^1H NOESY spectra corresponding to all NOEs involving aromatic side chain protons, amide protons and aliphatic protons; together with torsion angle

constraints as predicted from the backbone dihedral angles in the program TALOS+ (Cornilescu *et al.*, 1999 and Shen *et al.*, 2009). From the initial CANDID structures, possible hydrogen bonds were identified, and when confirmed by the presence of non-exchanged amide protons, were included in the final CANDID run and in the REDAC refinement. The peaks lists were prepared using the Sparky package and CANDID calculations were carried out using the default settings of CYANA 2.1 with chemical shift tolerances set to 0.02 - 0.03 ppm (direct and indirect ^1H) and 0.2 - 0.3 ppm (^{15}N and ^{13}C). Analysis of the family of structures was carried out using the program MOLMOL (Koradi *et al.*, 1996).

3.3 Results

3.3.1 – NMR Data Acquisition and Processing for Sequence-Specific Assignments

3.3.1.1 – Sequence-Specific Assignments for VHH 18

VHH 18 gave rise to well resolved spectra as illustrated by the ^{15}N - ^1H HSQC shown in Figure 3.1.

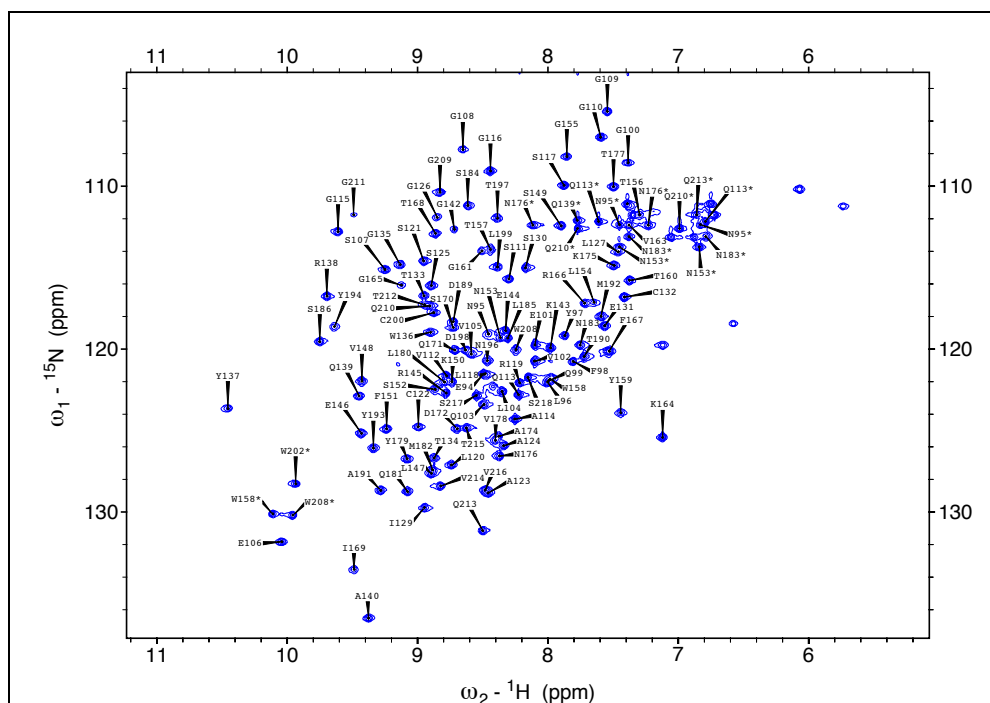


Figure 3.1 – ^{15}N - ^1H HSQC Spectrum of VHH 18

The figure shows the ^{15}N - ^1H HSQC spectrum of $^{15}\text{N}/^{13}\text{C}$ labelled VHH 18 at a concentration of 286 μM in 25 mM sodium phosphate, 100 mM sodium chloride buffer, pH 6.4 collected at 35 $^{\circ}\text{C}$ on a 600 MHz Bruker Avance spectrometer for 60 minutes. The assignments of the resonances from the backbone amide groups in VHH 18 are annotated by residue type and number. Residues numbered from 101 to 218 are the native VHH residues and those numbered 93 to 100 form the His₆-tag and TEV protease cleavage site. Peaks arising from side chain NH_2 groups or tryptophan indolic NH groups are highlighted with an asterisk.

The quality of the data acquired allowed for almost complete backbone resonance assignments as shown in Figure 3.2. Sequence-specific backbone amide assignments were obtained for all residues in VHH 18 with the exception of Arg128, Ser162, Gly188, Cys195 and Trp202 to Thr207 (corresponding to the assignment of 91 % of the backbone amides), and for C α and C β resonances apart from Trp202 to Asn206 (95 % assignment of C α and C β atoms). All H α and H β resonances were assigned with the exception of Glu101 (H β 2 and H β 3), Cys122 (H α), Ser125 (H β 2 and H β 3), Arg128, Ser162, Ser173, Gly188, Cys195 and Trp202 to Thr207 (corresponding to the assignment of 89 % of H α and H β atoms). Carbonyl carbons were assigned with the exception of Leu127, Ala140, Val148, Tyr159, Gly161, Gly165, Met182, Ser184, Ser186, Pro187, Tyr194, Cys200, Pro201 to Asn206, Gly211 and Ser218 (87% assignment of carbonyl carbons).

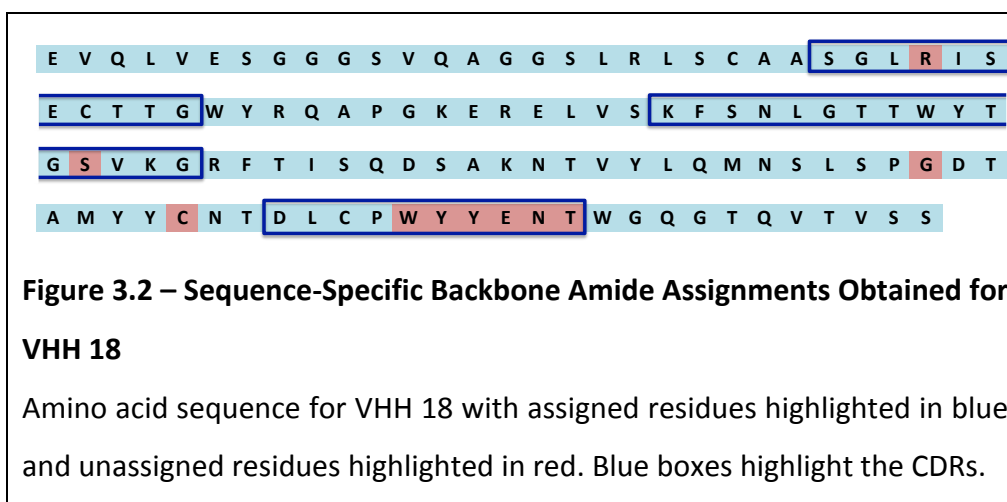


Figure 3.3 shows example ^{15}N strips from the triple resonance spectra that allowed the backbone assignments of VHH 18 to be made. For the non-exchangeable side chain atoms (^{15}N , ^{13}C and ^1H) assignments were obtained apart from Glu101, Gln103, Leu127 (C γ and H γ), Arg128, Gln139 (C γ), Phe151 (H ζ), Gln171, Leu180 (C γ and H γ), Gln181 (H ϵ 22 and N ϵ 2), Tyr193 (Q ϵ), Gln213 (C γ , H γ 2 and H γ 3) and Trp202 (H ϵ 3, H η 2 and H ζ 3). In addition, no side chain assignments were obtained for Tyr203 to Thr207.

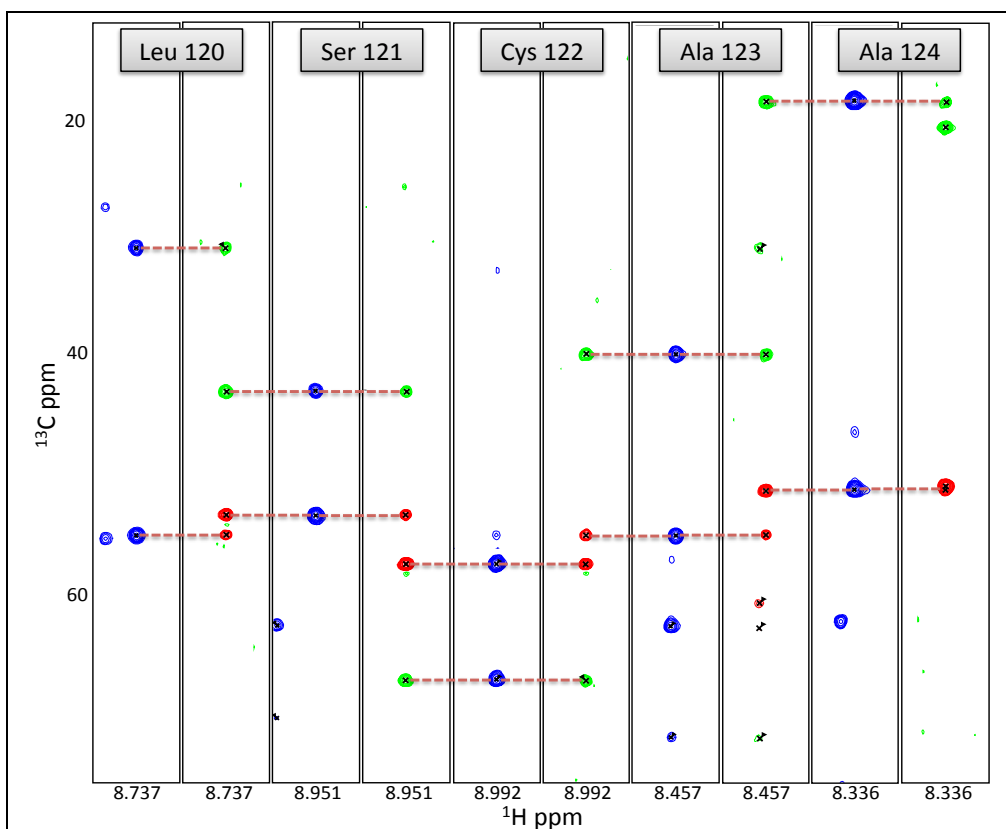


Figure 3.3 – Example ^{15}N Strips from the HNCACB and CBCA(CO)NH Spectra of VHH 18

The strips show the intra- and inter-residue connectivities for a string of five sequential residues of VHH 18. For each residue a strip from the CBCA(CO)NH (with $\text{C}\alpha$ and $\text{C}\beta$ peaks shown in blue) is shown adjacent to a strip from the HNCACB (with $\text{C}\alpha$ peaks shown in red and $\text{C}\beta$ peaks shown in green). Dashed red lines between the peaks show how the connectivities were made between each $\text{C}\alpha$ and $\text{C}\beta$ resonance in the HNCACB and the resonances from the $\text{C}\alpha$ and $\text{C}\beta$ atoms of the preceding residue in the CBCA(CO)NH allowing assignments to be made for the backbone of the VHH.

3.3.1.2 – Sequence-Specific Assignments for VHH 67

VHH 67 also gave rise to well resolved spectra as illustrated by the ^{15}N - ^1H HSQC shown in Figure 3.4.

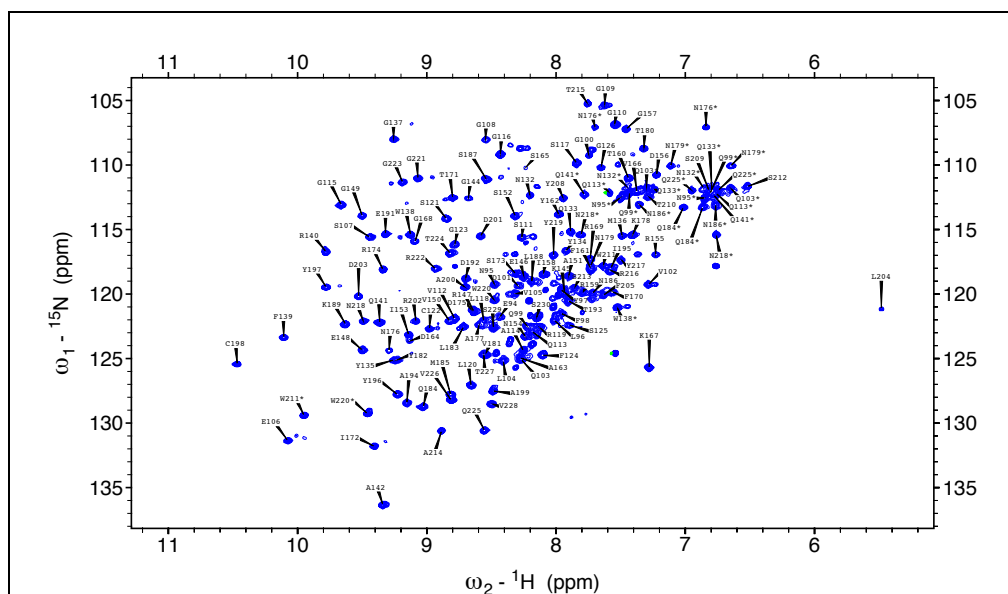
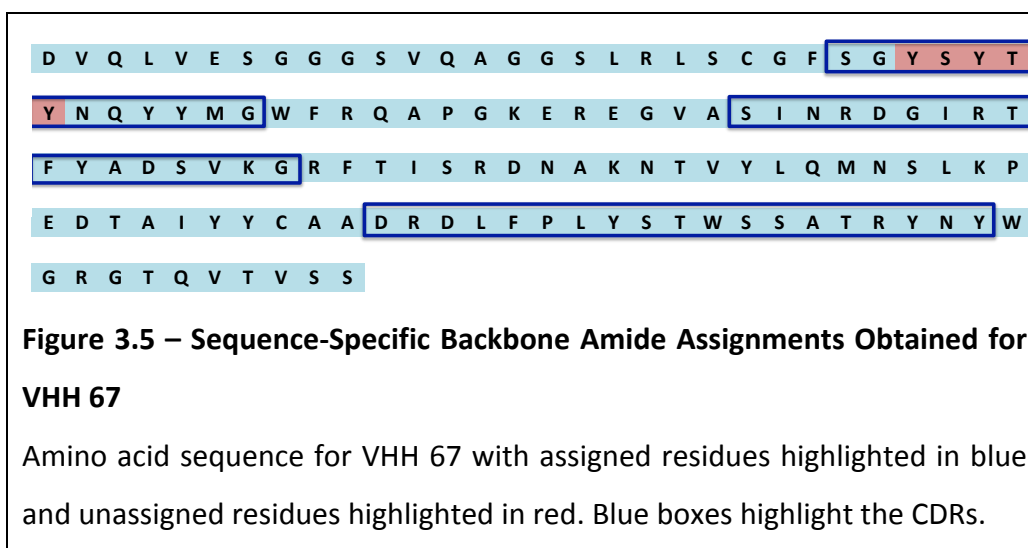


Figure 3.4 – ^{15}N - ^1H HSQC Spectrum of VHH 67

The figure shows the ^{15}N - ^1H HSQC spectrum of $^{15}\text{N}/^{13}\text{C}$ labelled VHH 67 at a concentration of 312 μM in 25 mM sodium phosphate, 100 mM sodium chloride buffer, pH 6.4 collected at 35 $^{\circ}\text{C}$ on a 600 MHz Bruker Avance spectrometer for 40 minutes. The assignments of the signals from the backbone amide groups in VHH 67 are annotated by residue type and number. Residues numbered from 101 to 230 are the native VHH residues and those numbered 93 to 100 form the His₆-tag and TEV protease cleavage site. Peaks arising from side chain NH_2 groups or tryptophan indolic NH groups are highlighted with an asterisk.

Again, the quality of the data acquired allowed for almost complete backbone resonance assignments, as shown in Figure 3.5. Sequence-specific backbone amide assignments were obtained for all residues in VHH 67 with the exception of Tyr127 to Tyr131 (corresponding to the assignment of 96 % of the amide backbone), and for C α and C β resonances apart from Tyr127 to Thr130 and Val228 (96 % assignment of C α and C β atoms). All H α and H β resonances were assigned with the

exception of Arg169 (H β 2 and H β 3), Ser173 (H β 2 and H β 3) and Tyr127 to Thr130 (95 % assignment of H α and H β atoms). Carbonyl carbons were assigned with the exception of Gly126 to Thr130, Ala142, Lys189, Phe205, Pro206 and Ser230 (93 % assignment of carbonyl carbons).



For the non-exchangeable side chain atoms (^{15}N , ^{13}C and ^1H) assignments were obtained apart from Val105 (C γ 2), Leu118 (C δ 1), Leu120 (C δ 1), Phe124 (H ζ), Phe139 (H ζ), Asn154 (H δ 21, H δ 22, N δ 2), Arg169 (C δ , C γ , H δ 2, H δ 3, H γ 2 and H γ 3) and Ile172 (C δ 1). In addition, no side chain assignments were obtained for Tyr127 to Thr130.

3.3.1.3 – Sequence-Specific Assignments for VHH 29

Figure 3.6 shows the ^{15}N - ^1H HSQC spectrum of VHH 29. As with the spectra of VHH 18 and VHH 67, the peaks are well resolved.

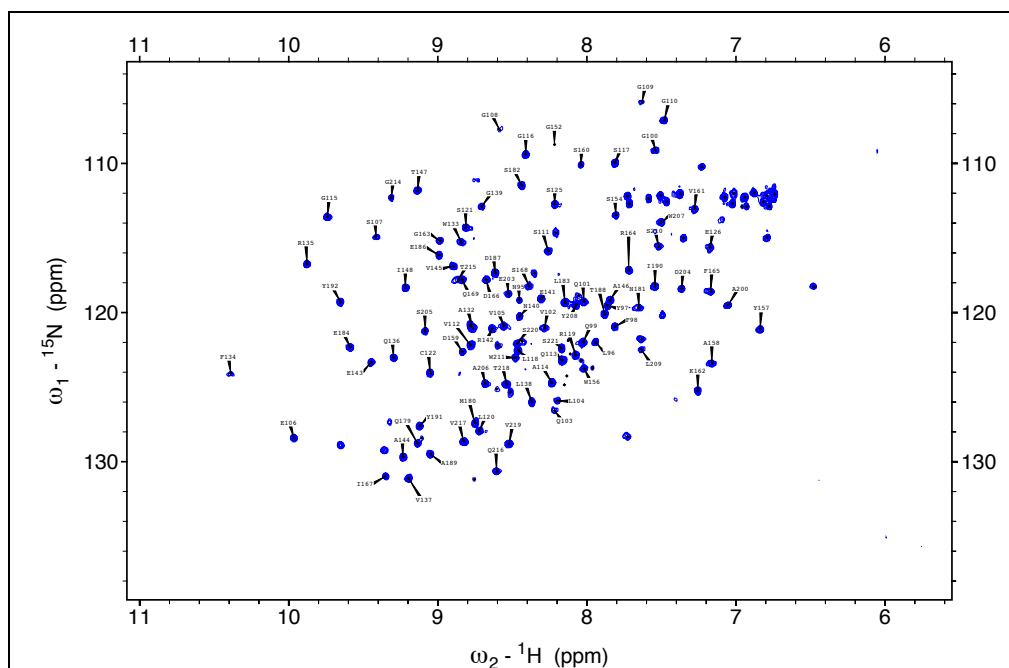
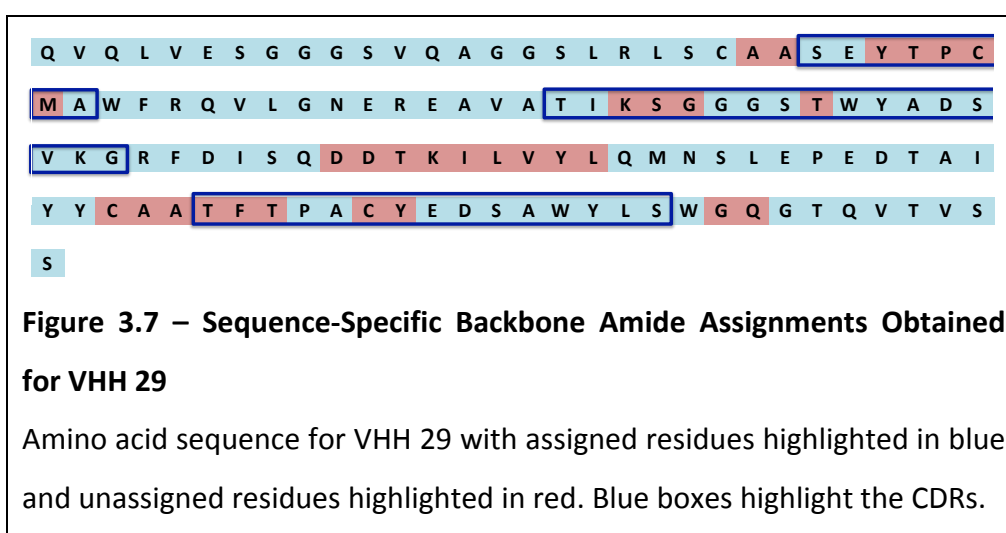


Figure 3.6 – ^{15}N - ^1H HSQC Spectrum of VHH 29

The figure shows the ^{15}N - ^1H HSQC spectrum of $^{15}\text{N}/^{13}\text{C}$ labelled VHH 29 at a concentration of 224 μM in 25 mM sodium phosphate, 100 mM sodium chloride buffer, pH 6.4 collected at 35 $^{\circ}\text{C}$ on a 800 MHz Bruker Avance II spectrometer for 40 minutes. The assignments of the signals from the backbone amide groups in VHH 29 are annotated by residue type and number. Residues numbered from 101 to 221 are the native VHH residues and those numbered 93 to 100 form the His₆-tag and TEV protease cleavage site.

As a full structural determination was not required for VHH 29, sequence-specific backbone assignments were made using ^{15}N - ^1H HSQC, ^{15}N - ^{13}C - ^1H HNCO, HNCACB and CBCA(CO)NH spectra only. Sequence-specific backbone amide assignments were obtained for all residues in VHH 29 with the exception of Ala123, Ala124, Tyr127 to Met131, Lys149 to Gly151, Thr155, Asp170 to Leu178, Cys193 to Thr198, Cys201, Tyr202, Gly212 and Gln213 (75 % assignment of backbone amides), and

for C α and C β resonances apart from Glu106, Ala123, Ala124, Tyr127 to Cys130, Lys149, Ser150, Asp170 to Tyr177, Cys193 to Thr198, Cys201, Gly212 and Gln213 (77 % assignment of C α and C β atoms). The completeness of the sequence-specific backbone assignments made for VHH 29 is highlighted in Figure 3.7 and shows 29 residues remain unassigned. The HSQC spectrum shows approximately 11 unassigned signals, too few to account for all of the unassigned residues of VHH 29. This implies a number of the signals are missing from the spectrum, which is mostly likely the result of exchange broadening (as described in Section 1.3).



3.3.1.4 – Sequence-Specific Assignments for VHH 146

The ^{15}N - ^1H HSQC spectrum of VHH 146 is shown in Figure 3.8. As with VHH 29, a full structural determination was not required, so sequence-specific backbone assignments were made using ^{15}N - ^1H HSQC, ^{15}N - ^{13}C - ^1H HNCO, HNCACB and CBCA(CO)NH spectra only. Sequence-specific backbone amide assignments were obtained for all residues in VHH 146 with the exception of Ala124 to Gly137, Ser155 to Leu160, Asp174 to Val180 and Ser201 to Pro211 (71 % assignment of backbone amides), and for C α and C β resonances apart from Ala124 to Met136, Ser155 to Asn158, Gln173 to Thr179 and Asp200 to Phe210 (72 % assignment of C α and C β atoms). Figure 3.9 shows the completeness of the sequence-specific backbone assignments made for VHH 146. A similar situation is seen to that of VHH 29 in that only approximately 15 signals are unassigned in the HSQC spectrum

but 37 residues remain unassigned in the amino acid sequence of VHH 146, again this is likely to be due to exchange broadening of signals in the HSQC.

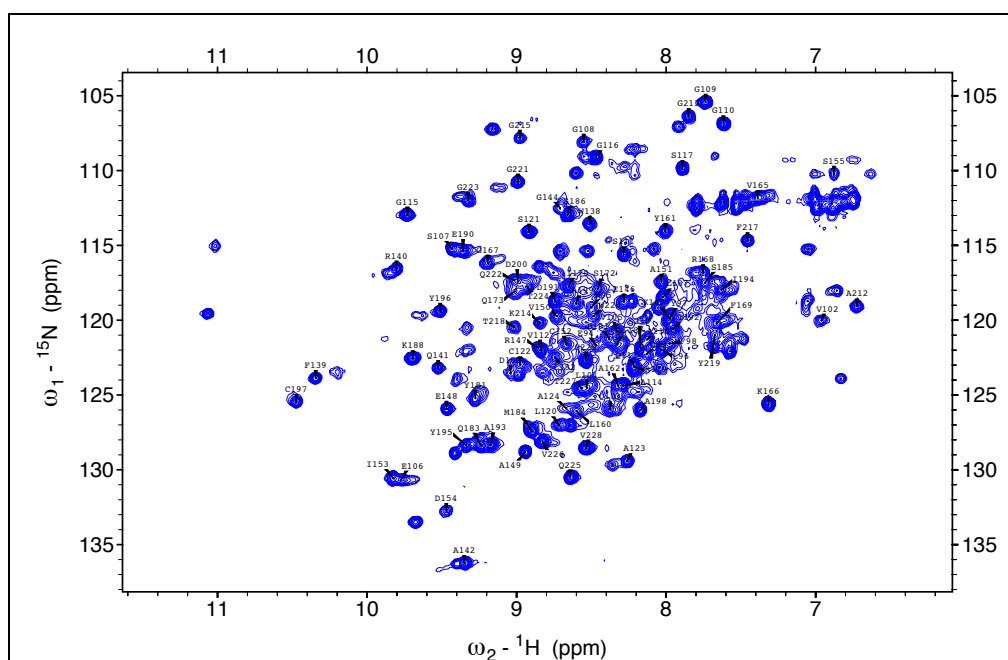


Figure 3.8 – ^{15}N - ^1H HSQC Spectrum of VHH 146

The figure shows the ^{15}N - ^1H HSQC spectrum of $^{15}\text{N}/^{13}\text{C}$ labelled VHH 146 at a concentration of 440 μM in 25 mM sodium phosphate, 100 mM sodium chloride buffer, pH 6.4 collected at 35 $^{\circ}\text{C}$ on a 600 MHz Bruker Avance spectrometer for 40 minutes. The assignments of the signals from the backbone amide groups of VHH 146 are annotated by residue type and number. Residues numbered from 101 to 230 are the native VHH residues and those numbered 93 to 100 form the His₆-tag and TEV protease cleavage site.

D V Q L V E S G G G S V Q A G G S L R L S C A A S G Y T D T
 T Y T Y C M G W F R Q A P G K E R E A V A C I D S G G N E L
 Y A D S V K G R F I I S Q D N A K K T V Y L Q M S S L K P E
 D T A I Y Y C A A D S N L Y T C R F R F P A G K G A F T Y W
 G Q G T Q V T V S S

Figure 3.9 – Sequence-Specific Backbone Amide Assignments Obtained for VHH 146

Amino acid sequence for VHH 146 with the assigned residues highlighted in blue and unassigned residues highlighted in red. Blue boxes highlight the CDRs.

3.3.2 – Secondary Structure Prediction from NMR Chemical Shifts

3.3.2.1 – Secondary Structure Prediction for VHH 18

The locations of secondary structure elements for VHH 18, as determined from the chemical shift data by the programs TALOS+ (Cornilescu *et al.*, 1999 and Shen *et al.*, 2009) and CSI (Wishart *et al.*, 1992, Wishart and Sykes, 1994) are shown in Figure 3.10. As expected, this analysis revealed that the VHH 18 structure is comprised predominantly of β -sheets, with TALOS+ suggesting that the domain is composed of 10 separate β -strands corresponding to about 75 % of the protein backbone. CSI predicts a similar pattern of 10 β -strands corresponding to nearly 60 % of the protein backbone. The psi and phi angles predicted by TALOS+ from the chemical shift data of VHH 18 are listed in Appendix A.2.1.

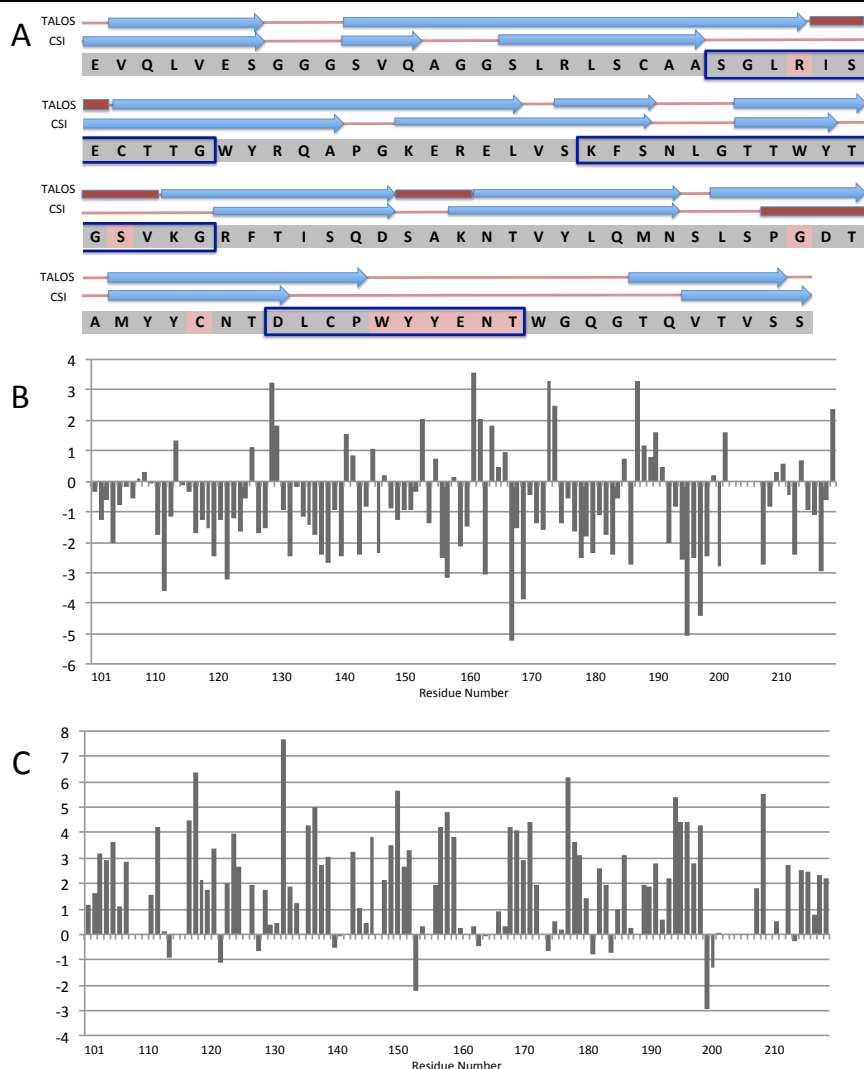


Figure 3.10 – Secondary Structure Elements Identified for VHH 18.

The location of regions of regular secondary structure, as determined by the programs TALOS and CSI, are highlighted above the protein sequence in Panel A. β -strands are shown as blue arrows and α -helical regions as red blocks. Unassigned residues are highlighted in red. Panel B shows the deviation of the observed $C\alpha$ chemical shifts from the expected random coil values (Wishart and Sykes, 1994). Residues within β -sheet regions are characterised by negative secondary shifts of greater than 0.7 ppm and the helical regions by positive shifts of greater than 0.7 ppm (Wishart and Sykes, 1994). An equivalent plot showing the $C\beta$ secondary shift is shown in panel C. Residues within β -sheet regions are characterised by positive secondary shifts of greater than 0.7 ppm and the helical regions by negative shifts of greater than 0.7 ppm (Wishart and Sykes, 1994).

3.3.2.2 – Secondary Structure Prediction for VHH 67

The locations of secondary structure elements for VHH 67, as determined from the chemical shift data by the programs TALOS+ and CSI, are shown in Figure 3.11. Both TALOS+ and CSI predict that the VHH 67 structure is comprised predominantly of β -sheets, with both TALOS+ and CSI suggesting that the domain is composed of 10 separate β -strands corresponding to between 55 % and 66 % of the protein backbone. The psi and phi angles predicted by TALOS+ from the chemical shift data of VHH 67 are listed in Appendix A.2.2.

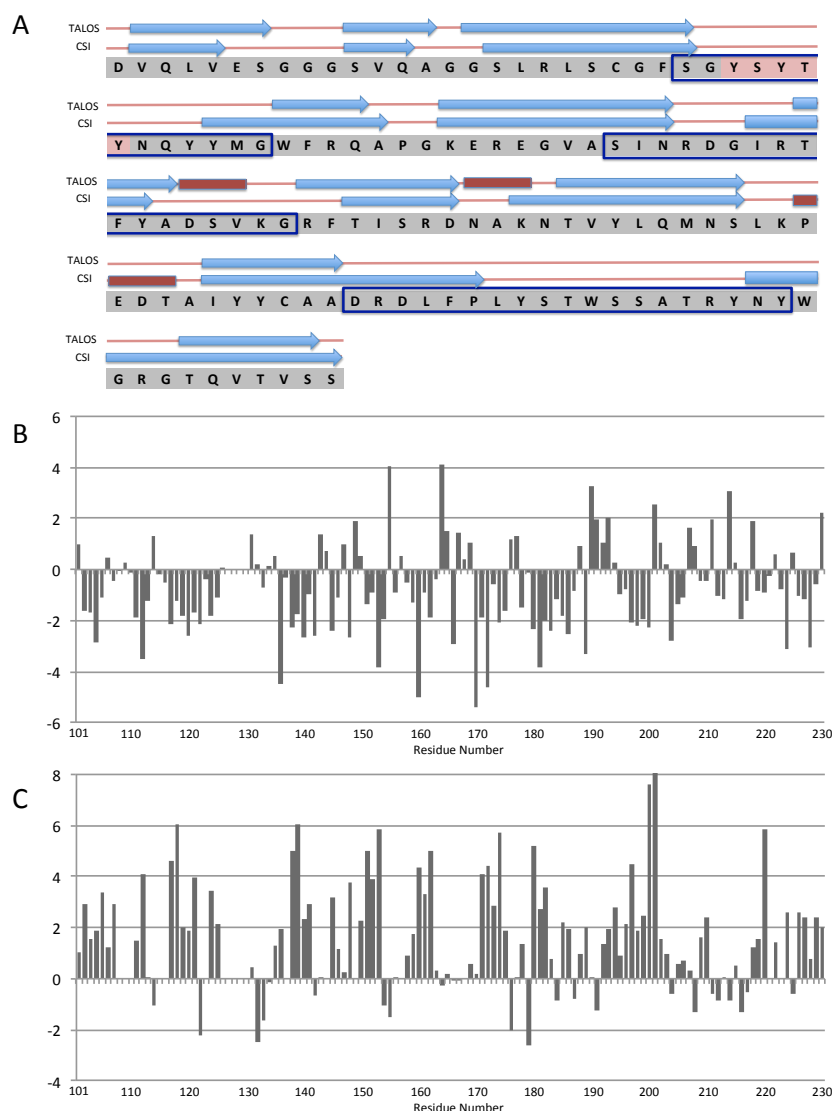


Figure 3.11 – Secondary Structure Elements Identified for VHH 67

The location of regions of regular secondary structure, as determined by the programs TALOS and CSI, are highlighted above the protein sequence in Panel A. β -strands are shown as blue arrows and α -helical regions as red blocks. Unassigned residues are highlighted in red. Panel B shows the deviation of the observed $C\alpha$ chemical shifts from the expected random coil values. Residues within β -sheet regions are characterised by negative secondary shifts of greater than 0.7 ppm and the helical regions by positive shifts of greater than 0.7 ppm. An equivalent plot showing the $C\beta$ secondary shift is shown in panel C. Residues within β -sheet regions are characterised by positive secondary shifts of greater than 0.7 ppm and the helical regions by negative shifts of greater than 0.7 ppm.

3.3.2.3 – Secondary Structure Prediction for VHH 29

As with VHH 18 and VHH 67, the locations of secondary structure elements for VHH 29 were determined from the chemical shift data by the programs TALOS+ and CSI and the results are shown in Figure 3.12. TALOS+ predicts 8 β -strands corresponding to 51 % of the protein backbone. Only 3 β -strands were predicted using the CSI method corresponding to 14 % of the protein backbone. These figures are lower than were predicted for VHH 18 and VHH 67 reflecting the lower proportion of $C\alpha$ and $C\beta$ atoms assigned. Also, the consensus between predictions from the $C\alpha$ and $C\beta$ chemical shifts were not as good as with VHH 18 and VHH 67. Interestingly, both TALOS+ and CSI predict α -helical character in the C-terminal portion of CDR3, which was seen in the crystal structure of VHH 29 when bound to IL-6 (Hancock, 2010).

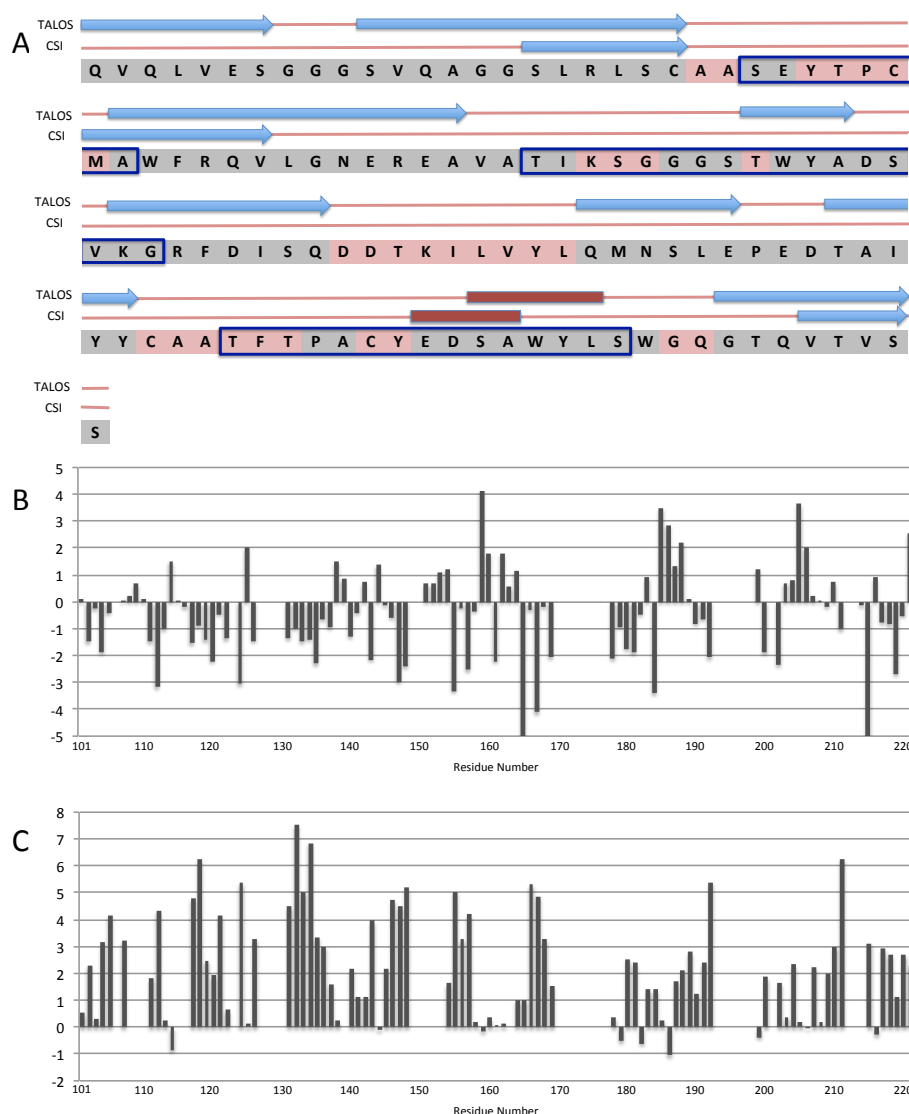


Figure 3.12 – Secondary Structure Elements Identified for VHH 29

The location of regions of regular secondary structure, as determined by the programs TALOS and CSI, are highlighted above the protein sequence in Panel A. β -strands are shown as blue arrows and α -helical regions as red blocks. Unassigned residues are highlighted in red. Panel B shows the deviation of the observed $C\alpha$ chemical shifts from the expected random coil values. Residues within β -sheet regions are characterised by negative secondary shifts of greater than 0.7 ppm and the helical regions by positive shifts of greater than 0.7 ppm. An equivalent plot showing the $C\beta$ secondary shift is shown in panel C. Residues within β -sheet regions are characterised by positive secondary shifts of greater than 0.7 ppm and the helical regions by negative shifts of greater than 0.7 ppm.

3.3.2.4 – Secondary Structure Prediction for VHH 146

Figure 3.13 shows the locations of secondary structure elements for VHH 146 as determined by TALOS+ and CSI. For VHH 146, TALOS+ predicts 7 β -strands corresponding to 42 % of the protein backbone and CSI also predicts 7 β -strands but these are shorter so only correspond to 27 % of the protein backbone. As with VHH 29, the proportion of the VHH assigned is slightly lower than seen for VHH 18 and VHH 67 explaining why fewer regions of secondary structure can be predicted. Again, similar to VHH 29, TALOS+ and CSI predicted some helical character in the C-terminal region of CDR3.

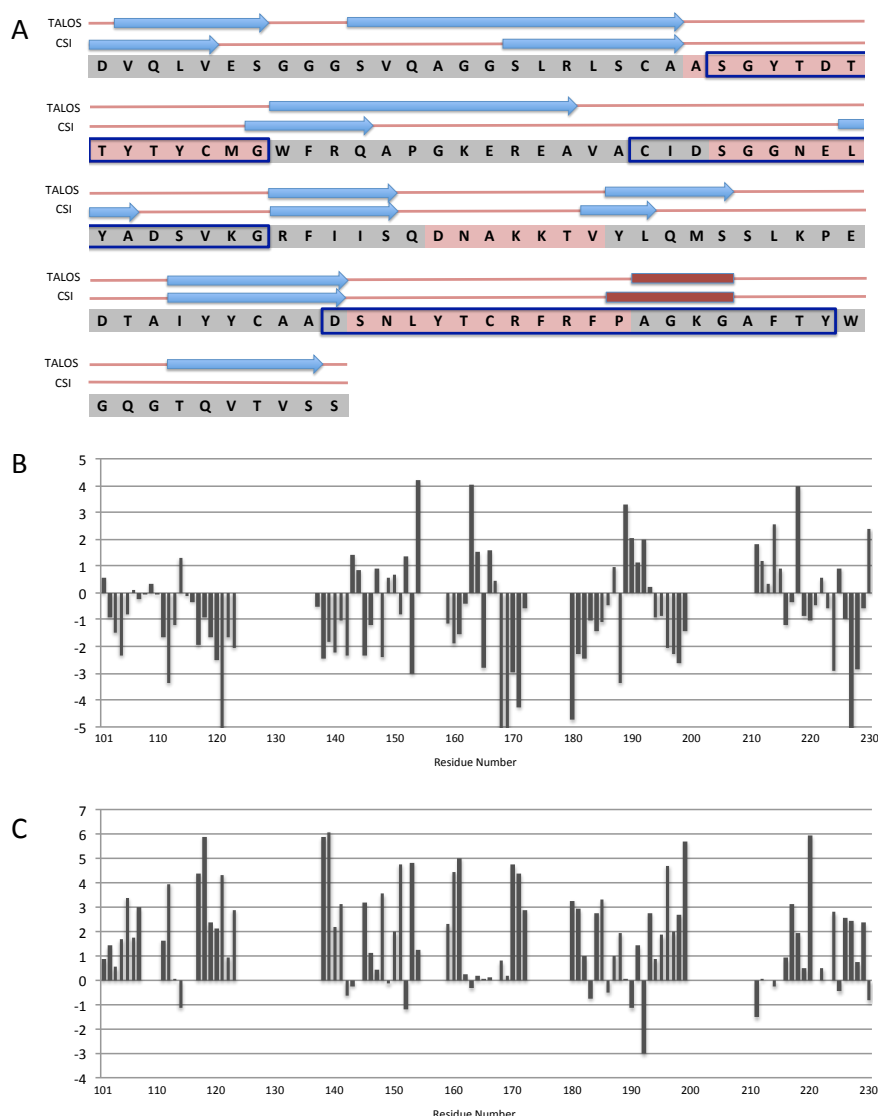


Figure 3.13 – Secondary Structure Elements Identified for VHH 146

The location of regions of regular secondary structure, as determined by the programs TALOS and CSI, are highlighted above the protein sequence in Panel A. β -strands are shown as blue arrows and α -helical regions as red blocks. Unassigned residues are coloured in red. Panel B shows the deviation of the observed $C\alpha$ chemical shifts from the expected random coil values. Residues within β -sheet regions are characterised by negative secondary shifts of greater than 0.7 ppm and the helical regions by positive shifts of greater than 0.7 ppm. An equivalent plot showing the $C\beta$ secondary shift is shown in panel C. Residues within β -sheet regions are characterised by positive secondary shifts of greater than 0.7 ppm and the helical regions by negative shifts of greater than 0.7 ppm.

3.3.3 – NMR Structure Calculation

3.3.3.1 NMR Structure Calculation for VHH 18

The data used as the input for CANDID included 1244 peaks corresponding to NOEs involving amide protons, 1416 peaks corresponding to aliphatic proton to aliphatic proton NOEs and 329 peaks corresponding to aliphatic proton to aromatic proton NOEs along with 200 torsion angle constraints as determined by TALOS.

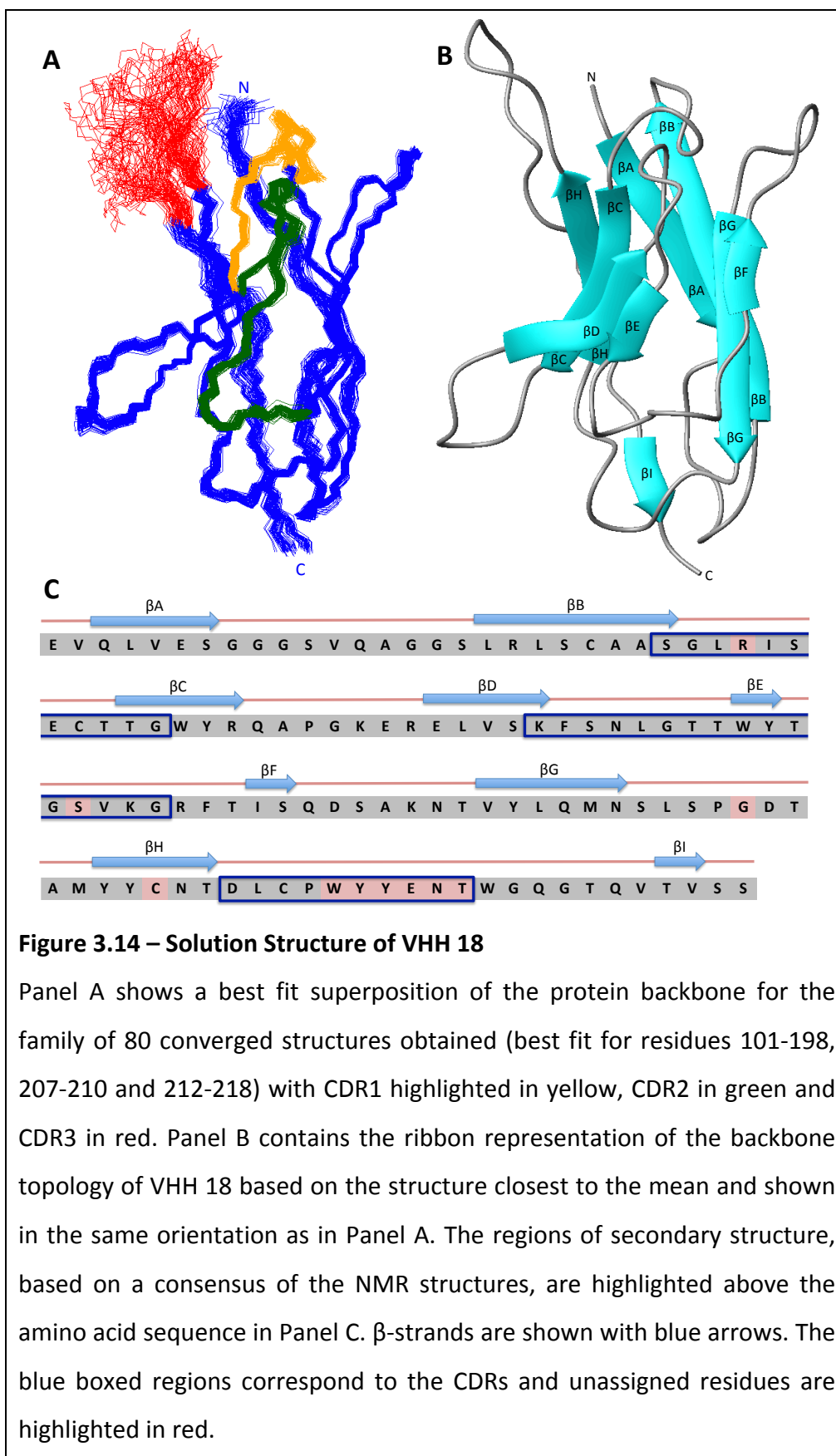
CANDID resulted in unique assignments for 90.1 % (1121/1244) of the NOE peaks picked in the ^{15}N - ^1H NOESY-HSQC spectrum, 93.2 % (1320/1416) in the ^{13}C - ^1H HSQC-NOESY spectrum and 88.4 % (291/329) in the ^1H - ^1H NOESY spectrum, which resulted in 1692 non-redundant ^1H to ^1H upper distance constraints. The final family of VHH 18 structures was determined using a total of 1932 NMR-derived structural constraints (an average of 14.7 per residue), including 1692 NOE-based upper distance limits, 197 backbone torsion angle constraints (98 phi and 99 psi angle constraints predicted by TALOS+ as described in Section 3.3.2.1 and listed in Appendix A.2.1.) and 48 hydrogen bond constraints (four constraints for each of the 12 hydrogen bonds). Following the final round of CYANA calculations, 80 satisfactorily converged structures were obtained from 100 random starting structures. The converged structures contain no distance or van der Waals violation greater than 0.5 Å and no dihedral angle violations greater than 5 °, with an average value for the CYANA target function of $0.62 \pm 0.31 \text{ Å}^2$. The sums of the violations for the upper distance limits, lower distance limits, van der Waals contacts and torsion angle constraints were $2.60 \pm 1.06 \text{ Å}$, $0.00 \pm 0.00 \text{ Å}$, $3.13 \pm 0.98 \text{ Å}^2$ and $17.5 \pm 7.10 \text{ °}$ respectively. Similarly, the maximum violations for the converged structures were $0.18 \pm 0.06 \text{ Å}$, $0.01 \pm 0.01 \text{ Å}$, $0.18 \pm 0.05 \text{ Å}^2$ and $2.55 \pm 1.27 \text{ °}$ respectively. The NMR constraints and structural statistics for VHH 18 are summarised in Table 3.1.

The solution structure of VHH 18 is determined to a high precision, which is evident from the superposition of the protein backbone shown for the family of converged structures in Figure 3.14 (best fit for residues 101-198, 207-210 and 212-218) and is reflected in the low root mean standard deviation (RMSD) values to the mean structure for both the backbone and all heavy atoms of $0.70 \pm 0.13 \text{ \AA}$ and $1.37 \pm 0.16 \text{ \AA}$ respectively.

Figure 3.14 reveals one region of VHH 18 for which the solution structure is poorly defined. This region is CDR3 and the poor definition is due to the lack of NMR constraints obtained. The absence of resonances for CDR3 in the NMR spectra is thought to be due to exchange between conformations on an intermediate timescale (Kleckner and Foster, 2010). This result was unexpected as the original hypothesis had been that the short CDR3 of VHH 18, with the additional disulphide linking CDR3 to CDR1, would be a fairly rigid loop.

(A) No. of Constraints used in Final Calculation		
Intraresidue NOEs	429	
Sequential NOEs (i, i + 1)	417	
Medium-range NOEs (i, i ≤ 4)	143	
Long-range NOEs (i, i ≥ 5)	703	
Torsion Angles	196	(98 ϕ and 99 ψ)
Hydrogen Bonding	48	(4 per hydrogen bond)
(B) Maximum and Total Constraint Violations in 80 Converged VHH 18 Structures		
Upper Distance Limits (Å)	0.18 ± 0.06	2.60 ± 1.06
Lower Distance Limits (Å)	0.01 ± 0.01	0.00 ± 0.00
van der Waals Contacts (Å ²)	0.18 ± 0.05	3.13 ± 0.98
Torsion Angle Ranges (°)	2.55 ± 1.27	17.50 ± 7.10
Average CYANA Target Function (Å ²)	0.62 ± 0.31	
(C) Structural Statistics for the Family of Converged VHH 18 Structures		
Residues within the Regions of the Ramachandran plot (%):		
favourably allowed	71.3	
additionally allowed	26.1	
generously allowed	2.1	
disallowed	0.4	
Backbone Atom RMSD to the Mean for Structured Region		
(Residues 101-198, 207-210 and 212-218 of VHH 18)		
	0.70 ± 0.13 Å	
Heavy Atom RMSD to the Mean for Structured Region		
(Residues 101-198, 207-210 and 212-218 of VHH 18)		
	1.37 ± 0.16 Å	

Table 3.1 – NMR Constraints and Structural Statistics for VHH 18



3.3.3.2 NMR Structure Calculation for VHH 67

The assignments used as the input for CANDID included 1198 peaks corresponding to NOEs involving amide protons, 2636 peaks corresponding to aliphatic proton to aliphatic proton NOEs and 275 peaks corresponding to aromatic proton to aliphatic proton NOEs along with 202 torsion angle constraints as determined by TALOS.

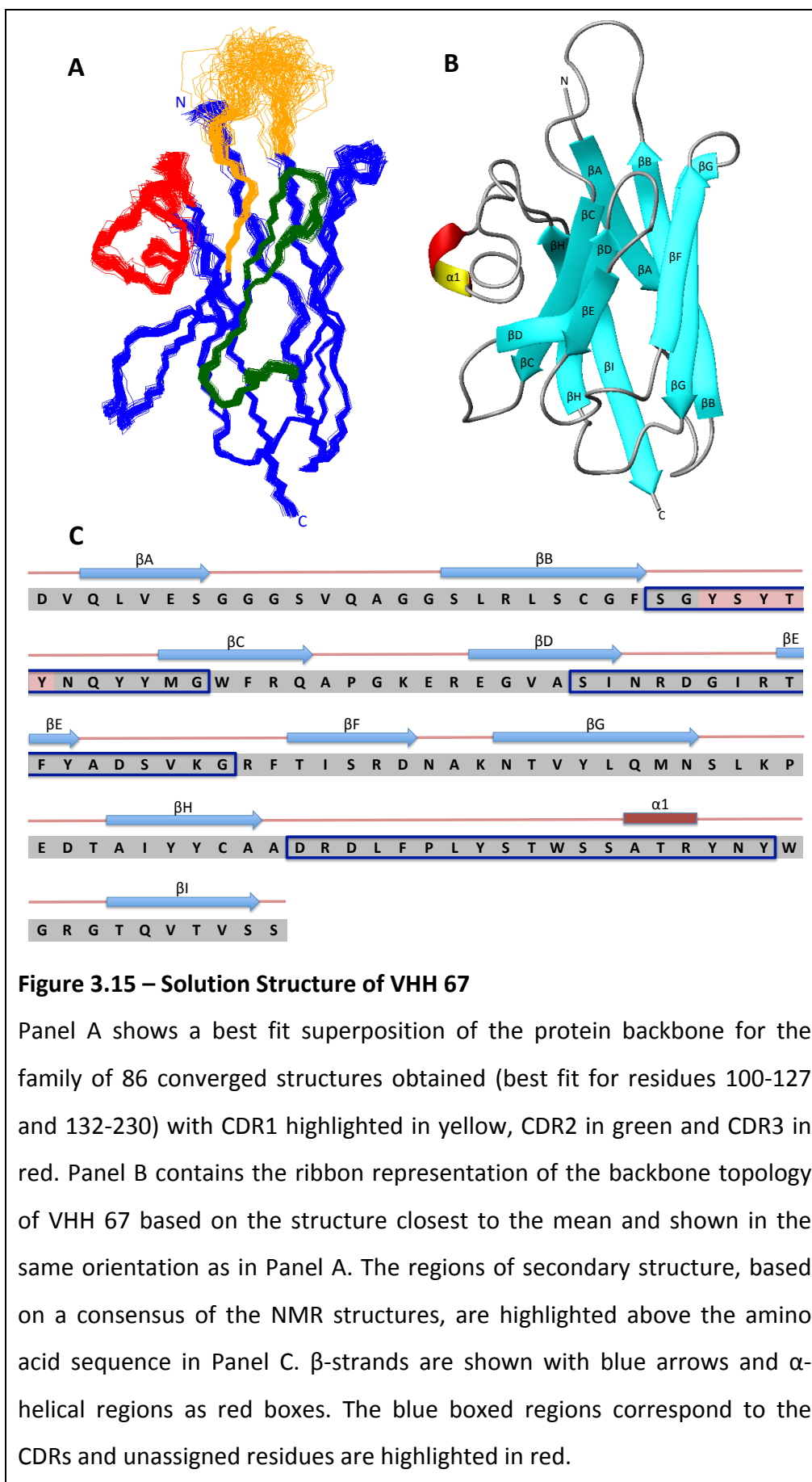
CANDID resulted in unique assignments for 84.5 % (1012/1198) of the NOE peaks picked in the ^{15}N - ^1H NOESY-HSQC spectrum, 80.9 % (2133/2636) in the ^{13}C - ^1H NOESY-HSQC spectrum and 82.5 % (227/275) in the ^1H - ^1H NOESY spectrum, which resulted in 2101 non-redundant ^1H to ^1H upper distance limits. The final family of VHH 67 structures was determined using a total of 2343 NMR-derived structural constraints (an average of 16.2 per residue), including 2101 NOE-based upper distance limits, 202 backbone torsion angle constraints (101 phi and 101 psi angle constraints predicted by TALOS+ as described in Section 3.3.2.2 and listed in Appendix A.2.2.) and 40 hydrogen bond constraints (four constraints for each of the 10 hydrogen bonds). Following the final round of CYANA calculations, 86 satisfactorily converged structures were obtained from 100 random starting structures. The converged structures contain no distance or van der Waals violation greater than 0.5 Å and no dihedral angle violations greater than 5 °, with an average value for the CYANA target function of $1.02 \pm 0.20 \text{ Å}^2$. The sums of the violations for the upper distance limits, lower distance limits, van der Waals contacts and torsion angle constraints were $2.59 \pm 0.53 \text{ Å}$, $0.00 \pm 0.00 \text{ Å}$, $4.47 \pm 0.65 \text{ Å}^2$ and $25.39 \pm 3.69 \text{ °}$ respectively. Similarly, the maximum violations for the converged structures were $0.20 \pm 0.02 \text{ Å}$, $0.01 \pm 0.01 \text{ Å}$, $0.27 \pm 0.05 \text{ Å}^2$ and $3.24 \pm 0.36 \text{ °}$ respectively. The NMR constraints and structural statistics for VHH 67 are summarised in Table 3.2.

The solution structure of VHH 67 is determined to a high precision, which is evident from the superposition of the protein backbone shown for the family of converged structures in Figure 3.15 (best fit for residues 100-127 and 132-230) and

is reflected in the low RMSD values to the mean structure for both the backbone and all heavy atoms of $0.70 \pm 0.15 \text{ \AA}$ and $1.46 \pm 0.19 \text{ \AA}$ respectively.

(A) No. of Constraints used in Final Calculation		
Intraresidue NOEs	440	
Sequential NOEs (i, i + 1)	471	
Medium-range NOEs (i, i ≤ 4)	214	
Long-range NOEs (i, i ≥ 5)	939	
Torsion Angles	202	(101 ϕ and 101 ψ)
Hydrogen Bonding	40	(4 per hydrogen bond)
(B) Maximum and Total Constraint Violations in 86 Converged VHH 67 Structures		
Upper Distance Limits ($\text{\AA}$)	0.20 ± 0.02	2.59 ± 0.53
Lower Distance Limits ($\text{\AA}$)	0.00 ± 0.00	0.00 ± 0.00
van der Waals Contacts ($\text{\AA}^2$)	0.27 ± 0.05	4.47 ± 0.65
Torsion Angle Ranges ($^\circ$)	3.24 ± 0.36	25.39 ± 3.69
Average CYANA Target Function ($\text{\AA}^2$)	1.02 ± 0.20	
(C) Structural Statistics for the Family of Converged VHH 67 Structures		
Residues within the Regions of the Ramachandran plot (%):		
favourably allowed	72.1	
additionally allowed	26.1	
generously allowed	1.5	
disallowed	0.2	
Backbone Atom RMSD to the Mean for Structured Region (Residues 100-127 and 132-230 of VHH 67)		
	$0.70 \pm 0.15 \text{ \AA}$	
Heavy Atom RMSD to the Mean for Structured Region (Residues 100-127 and 132-230 of VHH 67)		
	$1.46 \pm 0.19 \text{ \AA}$	

Table 3.2 – NMR Constraints and Structural Statistics for VHH 67



The solution structure of VHH 67 shows CDR1 to be relatively poorly defined as few NMR constraints were observed for this region. This is most likely due to exchange between conformations on an intermediate timescale (as explained in Section 1.3). The most interesting feature of VHH 67 is that the long CDR3 is seen in a single conformation and is structured, folding back onto the framework of the VHH. The structure closest to the mean (Figure 3.15, Panel B) shows a portion of helical structure in the C-terminal region of CDR3 that is not predicted by either TALOS+ or CSI. Of the 86 satisfactorily converged structures, just over half (56 %) include this region of helical structure.

3.3.4 – NMR Temperature Study of VHH 67

The NMR spectra collected to complete the sequence-specific assignments and structure calculation of VHH 67 highlighted a lack of observable amide signals for residues Tyr127 to Tyr131 in CDR1. In an attempt to change the dynamics of this region and visualise these signals, ^{15}N - ^1H HSQC spectra of VHH 67 were acquired at temperatures between 5 °C and 40 °C in 5 °C increments (as described in Section 3.2.2). The results of the temperature study are shown in Figure 3.16. Reducing the temperature resulted in increased linewidth along with progressive changes in chemical shift but no additional amide signals were observed that could be associated with the slowing down of the exchange rate between conformations for the missing residues of CDR1.

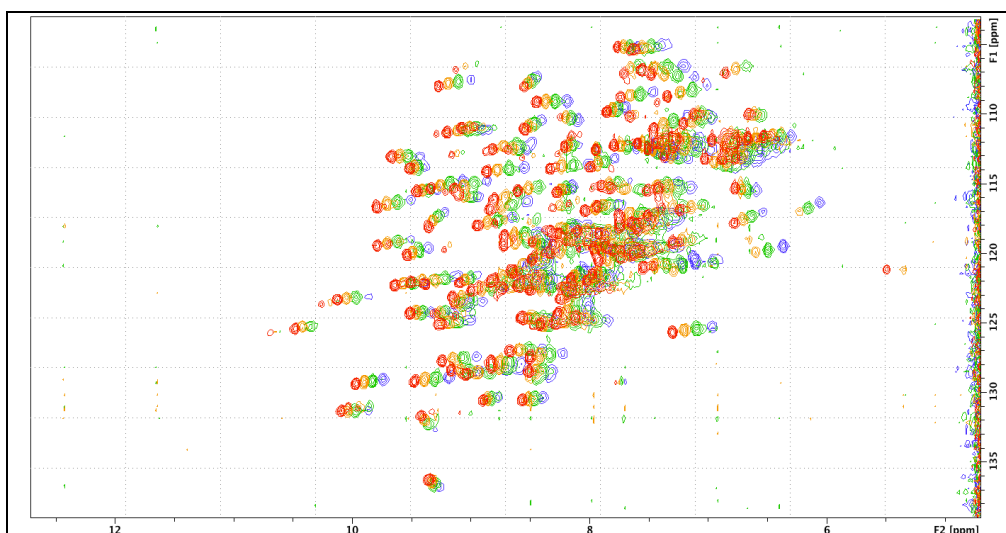


Figure 3.16 – HSQC Spectra of VHH 67 Collected at 5 °C to 35 °C

Overlaid are the ^{15}N - ^1H HSQC spectra of VHH 67 at a concentration of 312 μM in 25 mM sodium phosphate, 100 mM sodium chloride buffer, pH 6.4 collected on a 600 MHz Bruker Avance spectrometer at 5 °C (blue), 15 °C (green), 25 °C (orange) and 35 °C (red) for 40 minutes. An upfield shift in the resonances and an increase in linewidth were observed with the reduction in temperature but no additional amide signals were observed. Spectra were collected in 5 °C increments but to simplify the figure only 10 °C increments are shown.

3.4 – Discussion

The work detailed in this chapter showed that high quality 2D and 3D NMR spectra could be obtained from the VHH samples prepared as described in Chapter 2. The NMR experiments gave rise to high quality, well-resolved spectra from which virtually complete backbone assignments could be made for both VHH 18 (91 % complete) and VHH 67 (96 % complete).

With the addition of side chain assignments and the assignment of NOEs, solution structures of VHH 18 and VHH 67 were determined. The results of the structure calculations show a family of well-defined structures for both VHH 18 and VHH 67 with low RMSD values to the mean structure. However, the structure of VHH 18 shows poor definition for CDR3 due to a lack of NMR constraints and, for the same

reason, CDR1 of VHH 67 is also poorly defined. The absence of NMR resonances for these loops is likely to be a result of exchange broadening brought about by the exchange between two or more conformations on an intermediate timescale (see Figure 1.11). Slow exchange between conformations would result in peaks being observed for each distinct conformation. At the other extreme, with fast exchange between conformations, a single peak is observed with a chemical shift that corresponds to the population-weighted average chemical shift of all the conformations. Conformational exchange on an intermediate timescale results in a single, averaged peak with a linewidth so broad that it can no longer be observed (Kleckner and Foster, 2010).

Interactions that may be responsible for the stabilisation of CDR3 in VHH 67 have been identified. The aromatic residues Phe139 in Framework2; Tyr217 in CDR3; and Trp220 in Framework4 appear to be making a network of hydrophobic interactions maintaining CDR3 in a single conformation (shown in Panel A of Figure 3.17). The phenylalanine involved in this interaction is one of the characteristic VH to VHH substitutions discussed in Section 1.2. The valine to phenylalanine substitution on, what would be, the VH-VL interface is thought to require shielding by the elongated CDR3 to prevent aggregation of the VHH domains. This is exactly what is observed with VHH 67.

Another possible interaction stabilising CDR3 of VHH 67 could be between Phe161 of CDR2 and Leu207 of CDR3 (shown in Panel B of Figure 3.17). Interestingly, this interaction between CDR2 and CDR3 occurs in almost the same position as the additional disulphide bond formed between CDR2 and CDR3 in VHH 146 (shown in Panel C of Figure 3.17). This interaction, although may be weaker than a disulphide bond, could explain why CDR3 of VHH 67 behaves like the other VHHs with long CDR3 loops despite not having the additional disulphide bond.

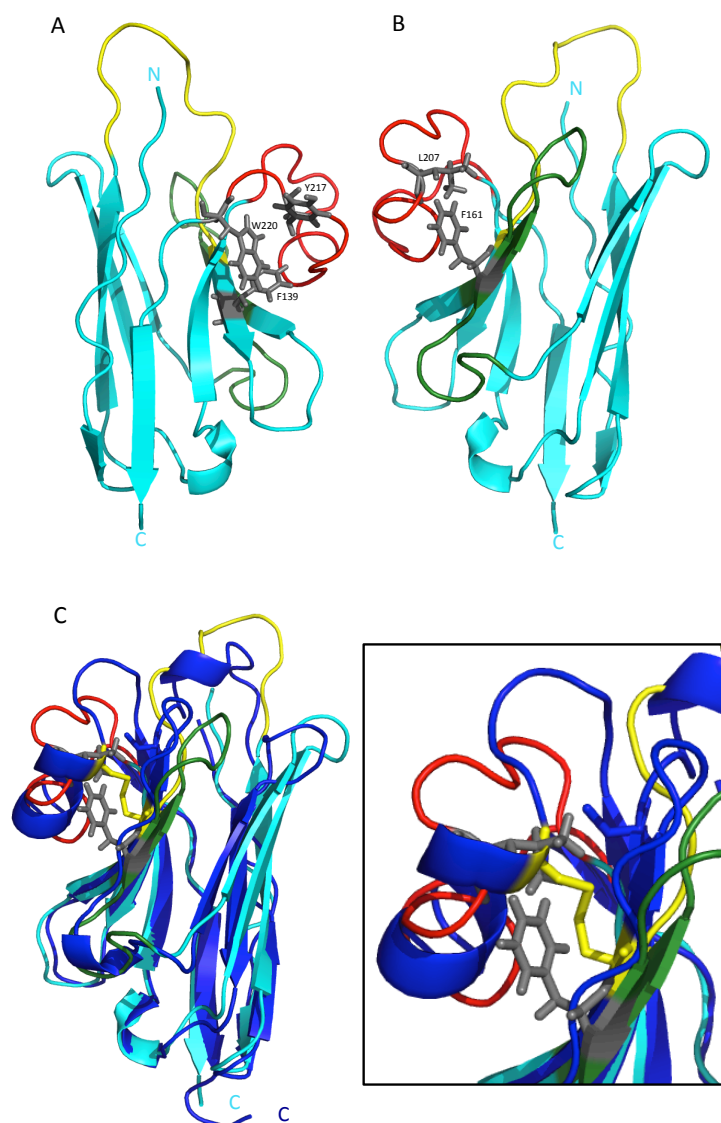
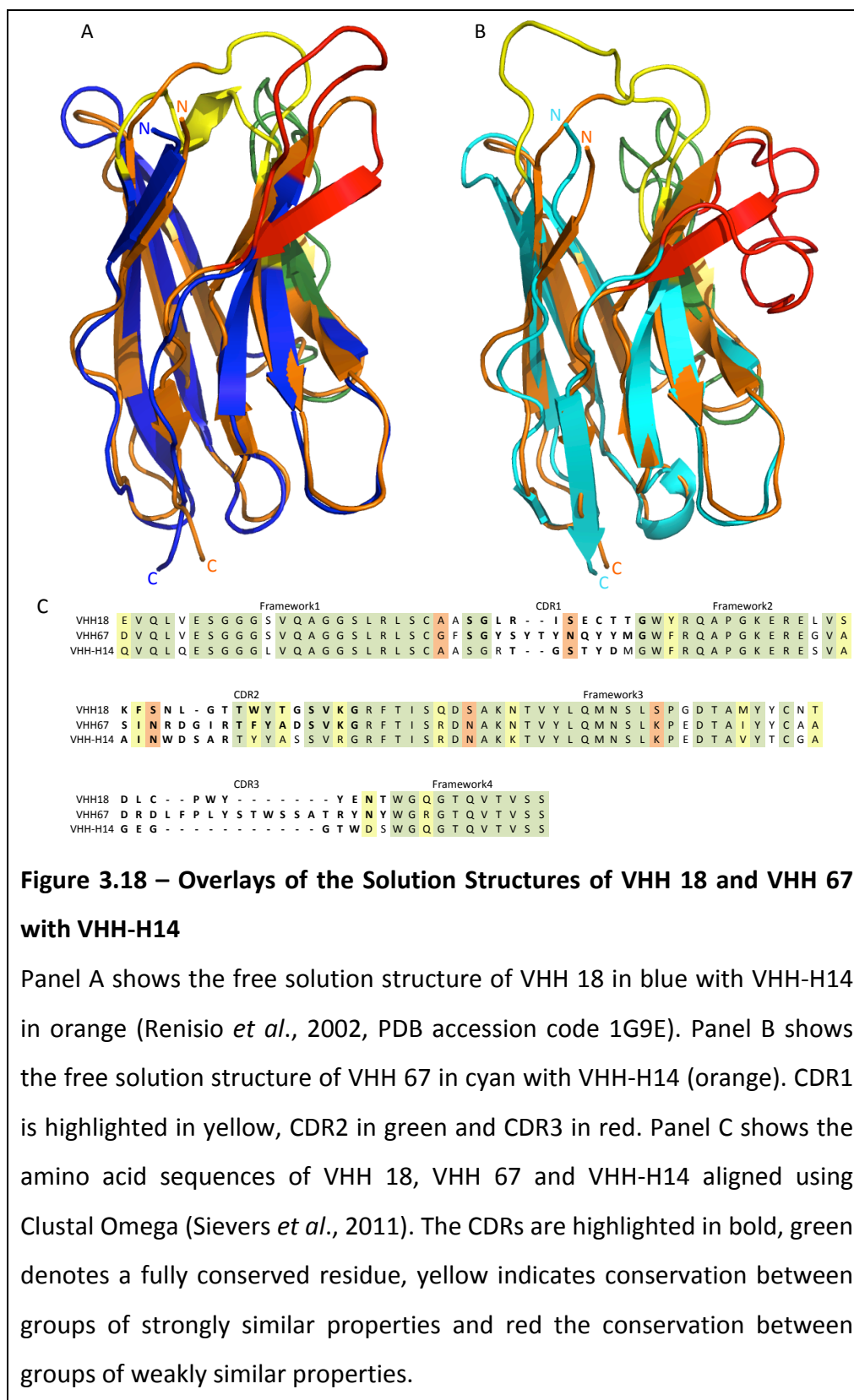
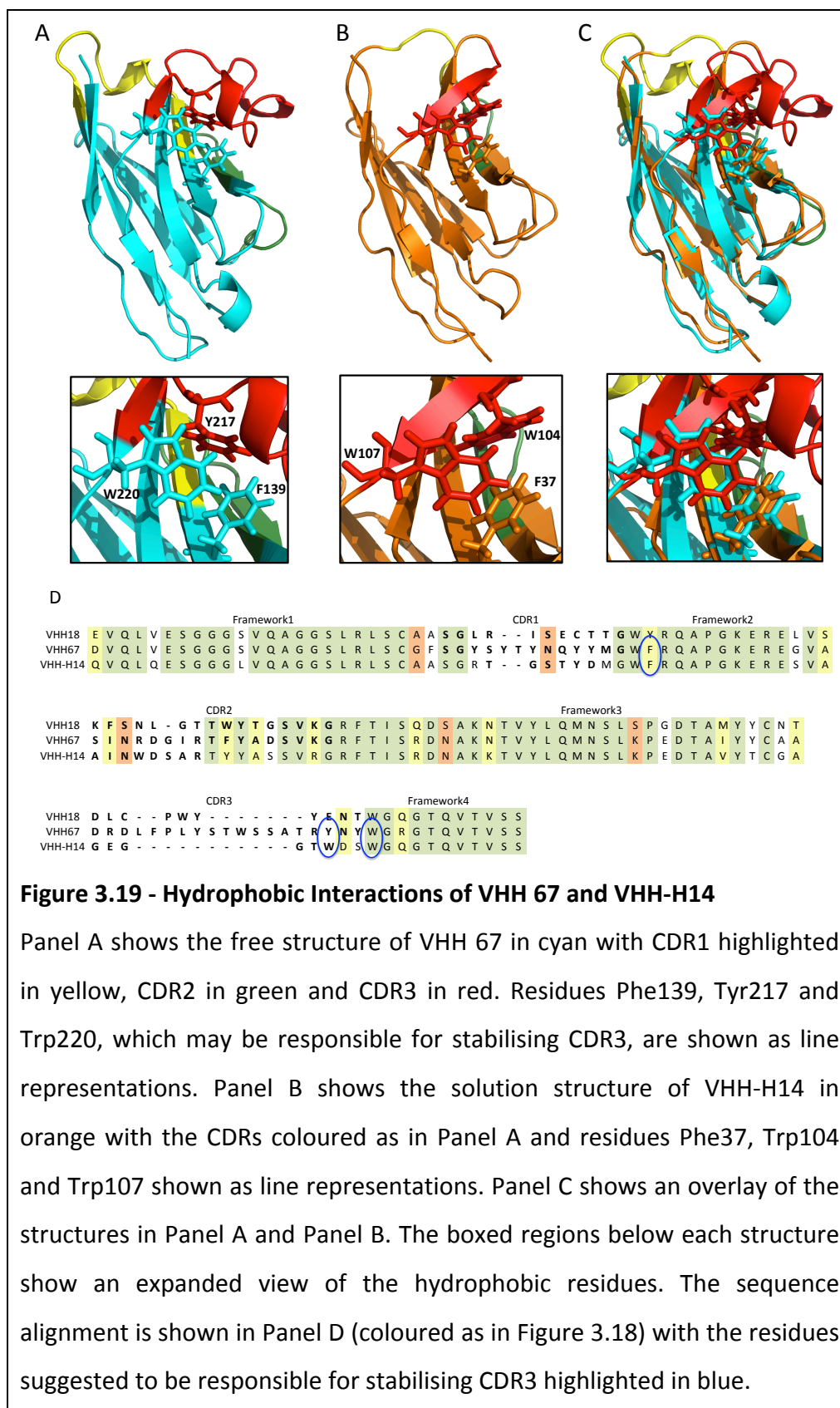


Figure 3.17 – Residues Responsible for the Stabilisation of CDR3 of VHH 67

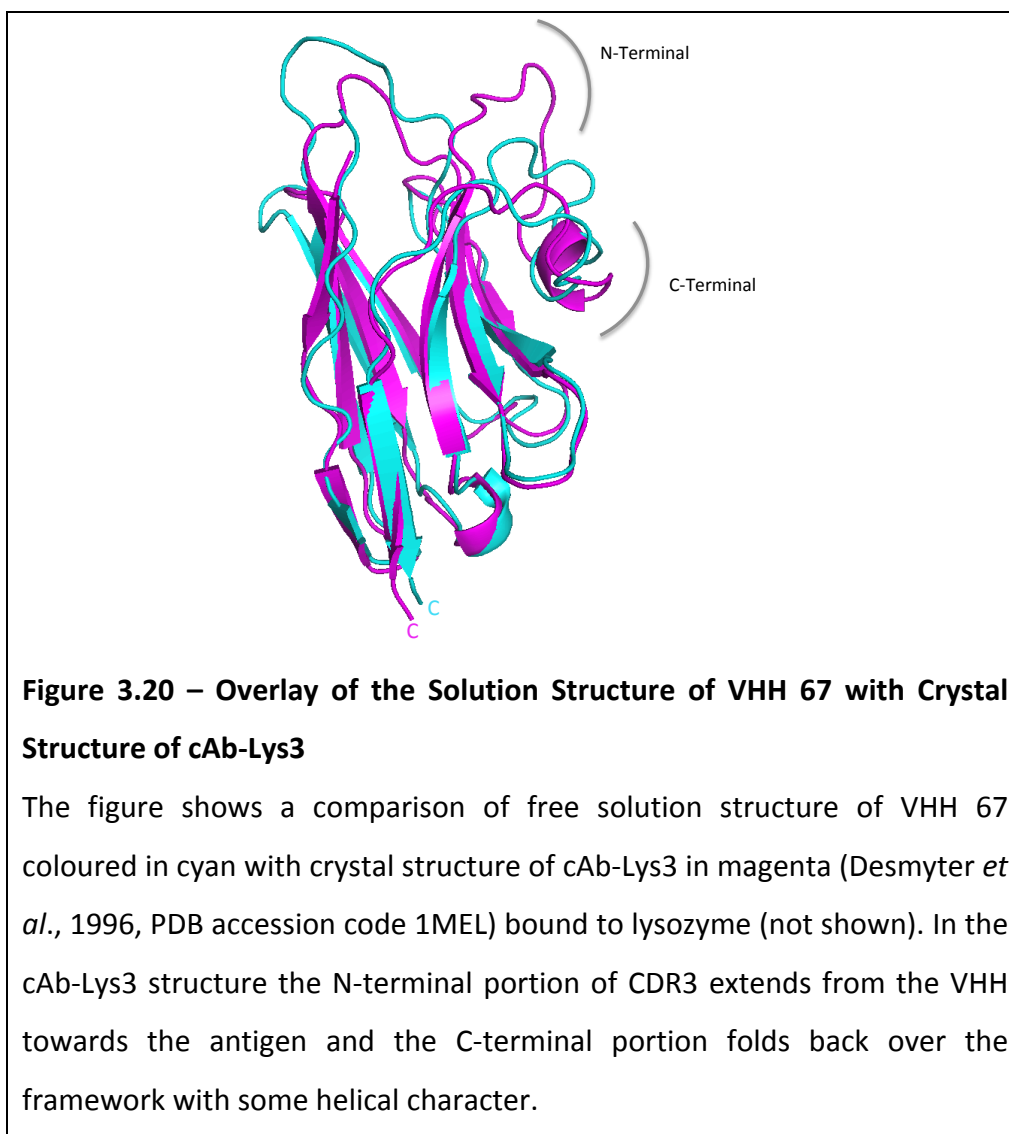
VHH 67 is shown in cyan with CDR1 coloured in yellow, CDR2 in green and CDR3 in red. The residues of interest are highlighted in grey. Panel A shows CDR3 may be stabilised by the interaction between Phe139 of Framework2, Tyr217 of CDR3 and Trp220 of Framework4. Panel B shows another possible stabilising interaction between Phe161 of CDR2 and Leu207 of CDR3. Panel C shows VHH 67 in cyan overlaid with the crystal structure VHH 146 in blue (Hancock, 2010). The boxed region shows an expanded view and highlights the Phe161 CDR2 to Leu207 CDR3 interaction in VHH 67 is almost identically positioned to that of the disulphide bond in VHH 146.

Figure 3.18 shows the overlays of the solution structures of VHH 18 and VHH 67 with the only example of a VHH solution structure in the PDB, VHH-H14 a VHH raised against the α subunit of the human chorionic gonadotropin hormone (hCG) (Renisio *et al.*, 2002, PDB accession code 1G9E). The framework regions of both VHH 18 and VHH 67 overlay well with VHH-H14, but significant differences can be seen in the CDRs of the structures. CDR1 of VHH 18 folds back towards CDR2 and shows some helical structure. In contrast, CDR1 of VHH 67 appears to be in more than one conformation with residues Tyr127 to Tyr131 unassigned. In the solution structure of VHH-H14, all residues in CDR1 were assigned but poor definition was seen in the N-terminal region of CDR1. This poor definition of CDR1 was also observed in the crystal structure of VHH-H14 (Spinelli *et al.*, 1996, PDB accession code 1HCV). CDR2 of VHH 18 and VHH 67 were well both defined although differences in the structure are observed in the turn of the loop. CDR2 of VHH-H14 was not well defined as residues Asp54 and Ser55 could not be assigned and the assumption was made that this part of the loop was in exchange. CDR3 is where most differences between the structures are observed. CDR3 of VHH 18 appears to be in conformational exchange so is not well defined. CDR3 of VHH 67 is much longer than that of VHH-H14 (19 residues compared to 6) and folds back over the framework region of the VHH. CDR3 of VHH-H14 may be stabilised by hydrophobic interactions between Phe37 of Framework2 and Trp104 and Trp107 of CDR3, in a similar way to the Phe139, Tyr217 and Trp220 interaction in VHH 67, despite having a short CDR3 (see Figure 3.19).





The solution structure of VHH 67 is another example of an elongated CDR3 loop that folds over the framework region of the VHH. Desmyter *et al.* describe the CDR3 of the lysozyme binder cAb-Lys3 as being divided into two regions, the C-terminal portion which interacts with the framework of the VHH and the N-terminal portion that extends from the VHH and interacts with the antigen (Desmyter *et al.*, 1996). Figure 3.20 shows a comparison of the solution structure of free VHH 67 with the crystal structure of the bound cAb-Lys3. Although it is not ideal to compare free with bound structures, the bound structure of VHH 67 does not alter significantly upon binding to IL-6 (described in Chapter 6). The overlay highlights the fact that the C-terminal region of CDR3 in both structures folds over the framework to the same extent. The difference occurs in the structure of the N-terminal region of CDR3 where the extra five residues of cAb-Lys3 extend further from the VHH into the antigen cleft mimicking the structure of the carbohydrate substrate of lysozyme (Decanniere *et al.*, 1998). The extent to which the CDR3 protrudes from the VHH is, therefore, likely to be antigen dependent and may suggest that VHH 67 does not bind to a deep cleft on IL-6.



X-ray crystal structures of three other anti-IL-6 VHHs bound to IL-6 showed a similar arrangement of long CDR3 loops (Hancock, 2010). The CDR3 of VHH 29, VHH 38 and VHH 146 are made up of 15, 16 and 20 residues respectively and all three include an additional cysteine that forms a disulphide bond with a cysteine in CDR1 (or CDR2 in VHH 146). Figure 3.21 shows an overlay of the crystal structures alongside the NMR structure of VHH 67. Despite the CDR3 length varying from 15 residues (VHH 29) to 20 residues (VHH 146) the CDR, in the bound form, CDR3 folds back over the framework region to the same extent with the proportion of helical character increasing with the length.

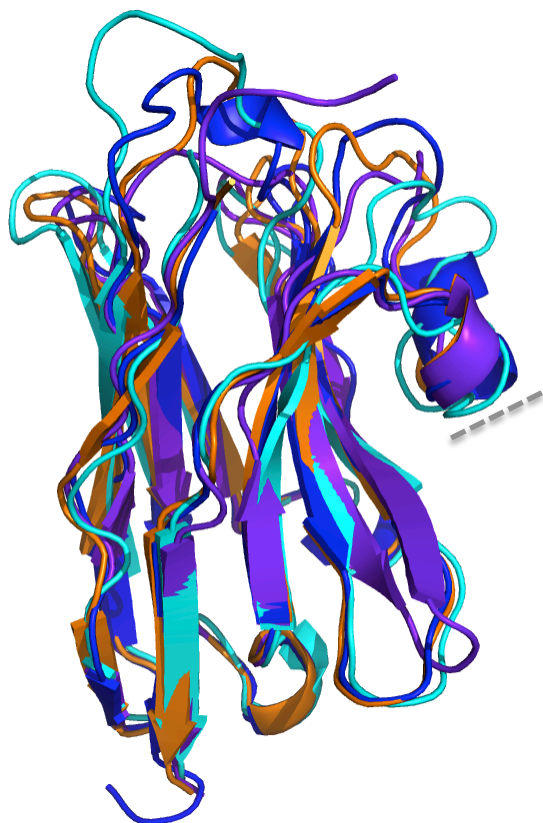


Figure 3.21 – Overlay of Crystal and NMR Structures of anti-IL-6 VHHs with Long CDR3 Loops

The overlay shows the crystal structures of VHH 29 coloured in purple, VHH 38 in orange and VHH 146 in blue, all in complex with IL-6 (not shown) (Hancock, 2010), overlaid with the NMR solution structure of free VHH 67 in cyan. The dashed line indicates the extent to which each CDR3 folds back onto the framework of the VHH.

As reported above, VHH 29, VHH 38 and VHH 146 all contain the additional disulphide bond linking CDR3 to either CDR1 or CDR2. If the CDR3 of these VHH antibodies were divided into two portions (the N-terminal and C-terminal region) as described by Desmyter *et al.*, it can be seen that the residues in the C-terminal region fold back against the framework of the VHH and the N-terminal region extends away from the VHH. The extension of the N-terminal region is then dependent on the number of residues in this region, for example VHH 146 extends furthest from the VHH and has the greater number of residues in the N-terminal portion of the CDR. Figure 3.22 details the results.

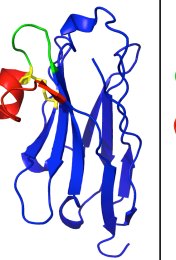
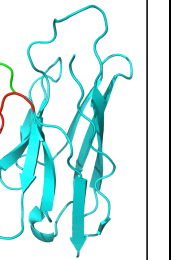
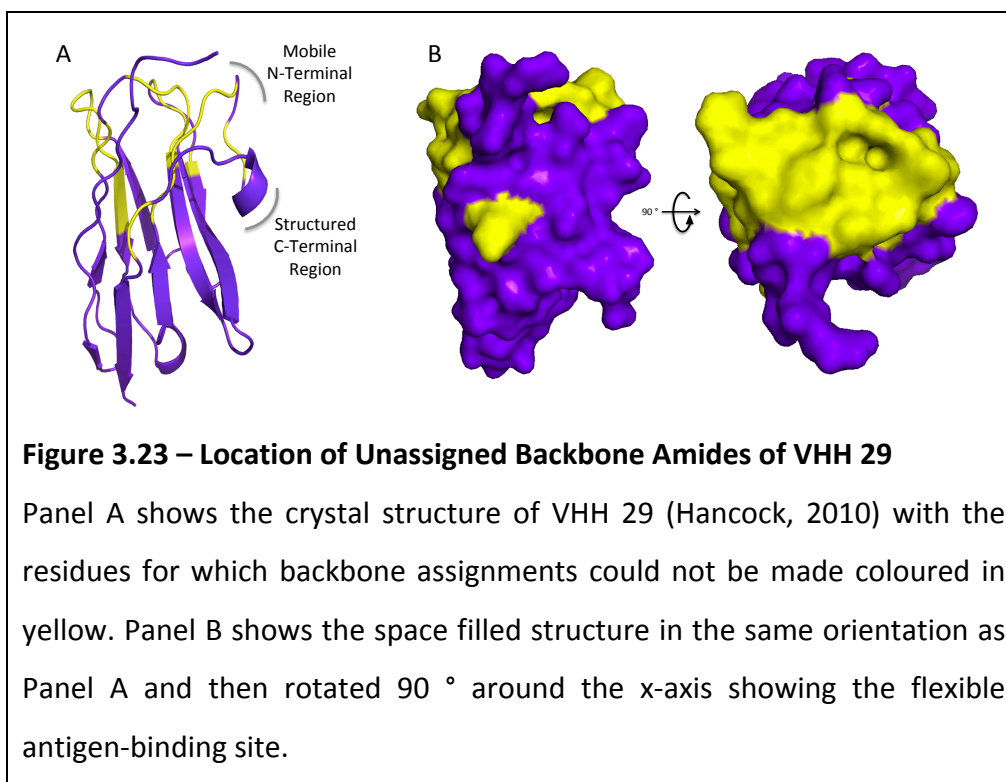
	VHH 29	VHH 38	VHH 146	VHH 67
				
CDR3 Length	15	16	20	19
Additional Disulphide	Yes	Yes	Yes	No
N-terminal Length	5	3	6	-
C-terminal Length	9	12	13	-

Figure 3.22 – Comparison of the Features of the Long CDR3 Loops of anti-IL-6 VHs

The crystal structures of VHH 29 coloured in purple, VHH 38 in orange and VHH 146 in blue are shown with the N-terminal region of CDR3 coloured in green and the C-terminal region coloured in red. The disulphide bond between CDR3 and CDR1 (or CDR2) is shown in yellow and indicates the division between the N- and C-terminal regions. The NMR solution structure of VHH 67 is shown in cyan.

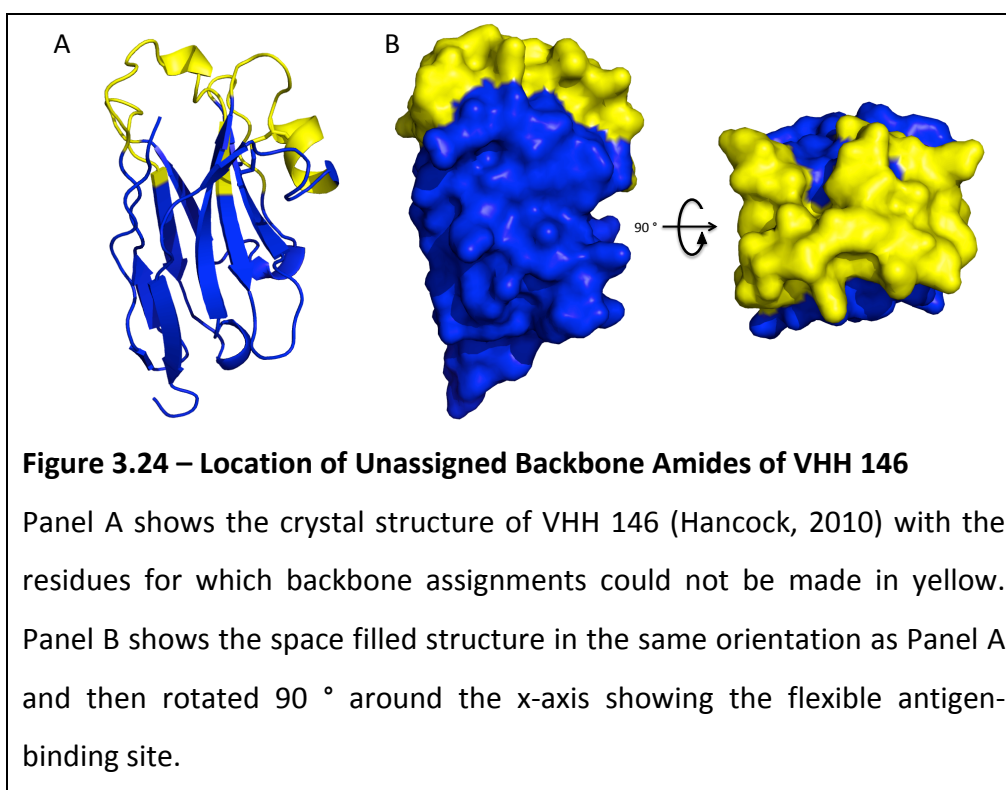
VHH 67 shows a similar structure to VHH 29, VHH 38 and VHH 146 with a portion of the CDR3 folding back but, in this example, there is no cysteine in the CDR3 so no disulphide bond is formed to either CDR1 or CDR2. It has been suggested that the additional disulphide bond increases the stability of the CDRs (Nguyen *et al.*, 2000) but as CDR3 of VHH 67 appears structured in its free form without having this extra disulphide there is likely to be other factors stabilising CDR3 such as the aromatic residues identified in Figure 3.19.

To the best of our knowledge VHH 67 is the only free solution structure of a VHH that shows a long, structured CDR3. As described above, this characteristic is seen in VHH domains in complex with antigens and has also been seen in an example of a crystal structure of a free VHH, cAb-CA05 (Desmyter *et al.*, 2001, PDB accession code 1F2X), but in both circumstances the conformation of CDR3 may be influenced by the crystal packing. To better understand the behaviour of the long CDR3 when free in solution, another example of a VHH needed to be studied. The crystal structure of VHH 29 shows the residues of CDR3 folding back over the framework region (as seen with VHH 67) but this may not be the conformation of unbound VHH 29. To investigate whether the CDR3 residues of VHH 29 are structured when free in solution the NMR backbone assignments were made. Sequence-specific assignments were made for 75 % of the backbone amides (see Section 3.3.1.3). The completeness of assignment for VHH 29 was lower than seen with VHH 18 and VHH 67, most likely due to the structure being in conformational exchange. Figure 3.23 shows the unassigned residues highlighted on the crystal structure of VHH 29 bound to IL-6 (Hancock, 2010). The unassigned residues are all located in the antigen binding region of VHH 29 and the absence of the NMR signals for these residues suggests that this whole region may be in conformational exchange on an intermediate timescale. When looking at CDR3 of VHH 29, signals were not observed for the amide groups of the N-terminal residues suggesting that this region of CDR3 is in exchange whereas signals for the C-terminal residues are present suggesting this region is structured. Interestingly, both CSI and TALOS+ predicted helical character in the C-terminal region of VHH 29. In the absence of a full structure determination it cannot be established whether the structured part of CDR3 is in the same orientation as is seen in the crystal structure.



The backbone assignments of VHH 146 were also made to gain a wider understanding of the behaviour of the long CDR3 loops. The crystal structure of VHH 146 bound to IL-6 shows CDR3 folds back over the framework of the VHH, as seen with VHH 67 and VHH 29. VHH 146 has the longest N-terminal portion of all the anti-IL-6 VHHs studied with six residues prior to the cysteine. As discussed in Section 3.3.1.4, 71 % of the backbone amide assignments were made, again this figure is lower than for VHH 18 and VHH 67 and is most likely due to exchange broadening. Figure 3.24 shows the majority of the unassigned residues are in the antigen-binding region. Assignments could not be made for CDR1, a six residue string of CDR2 and the N-terminal region of CDR3. In addition to this, seven residues that make up the fourth loop in the antigen-binding region could not be assigned. Figure 3.24 shows these results highlighted on the crystal structure of VHH 146. The free form of VHH 146 appears to be the most dynamic of all the anti-IL-6 VHHs with almost the entire antigen binding region in intermediate exchange, the only part of the region that could be assigned was the C-terminal region of CDR3. Although, from the crystal structure we know these residues are at the IL-6

interface, they make contact with the surface of IL-6 rather than reaching into the cleft like the N-terminal residues of CDR3.



The work described in this chapter has highlighted the differences in dynamics between the anti-IL-6 VHH antibodies. At one extreme is VHH 67 for which all but five residues in CDR1 have been assigned, to VHH 146 for which the majority of the antigen-binding site is missing from the NMR spectra. Figure 3.25 compares the proportion of unassigned residues for the four free VHH antibodies that were studied in this chapter.

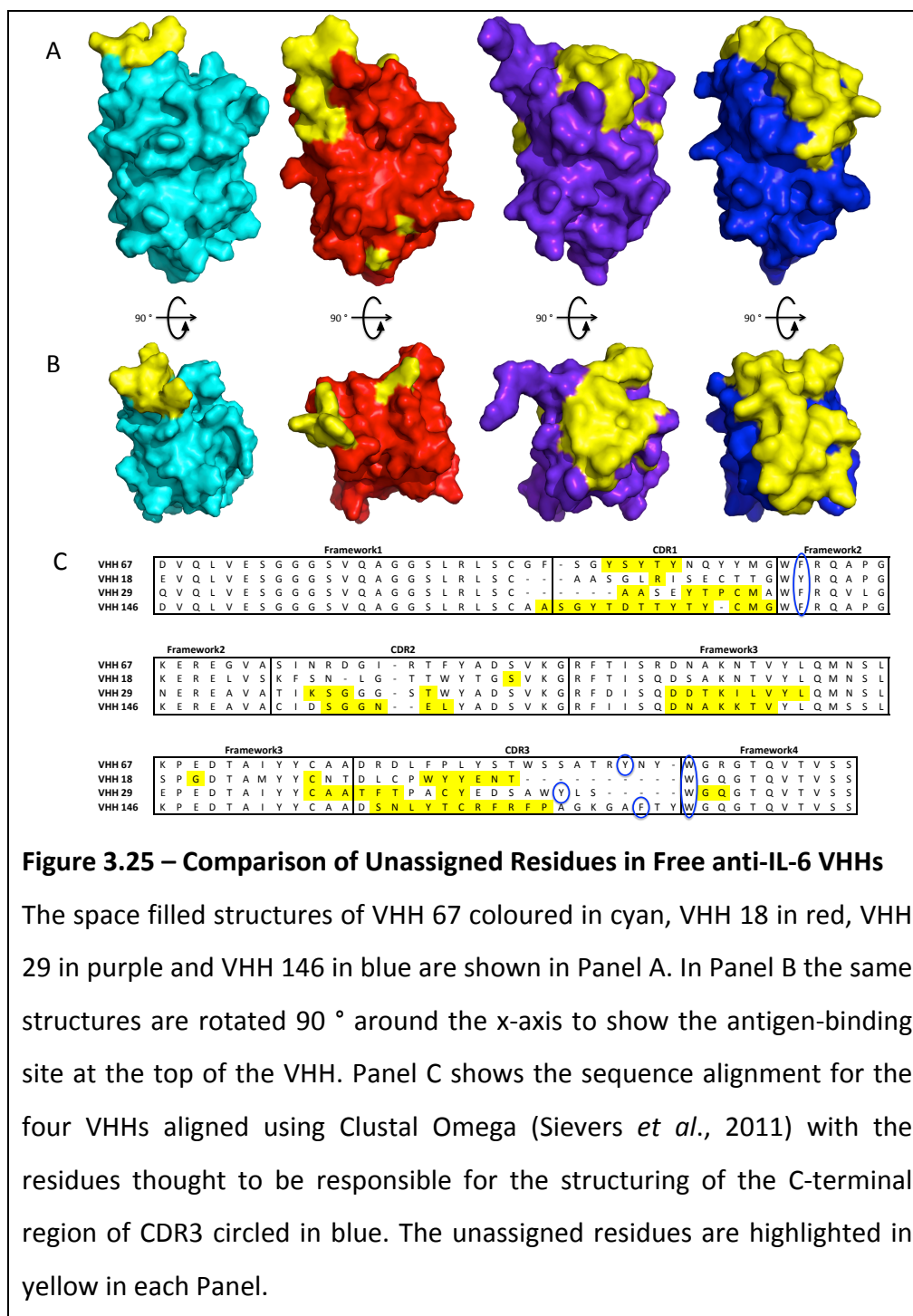


Figure 3.25 – Comparison of Unassigned Residues in Free anti-IL-6 VHHs

The space filled structures of VHH 67 coloured in cyan, VHH 18 in red, VHH 29 in purple and VHH 146 in blue are shown in Panel A. In Panel B the same structures are rotated 90 ° around the x-axis to show the antigen-binding site at the top of the VHH. Panel C shows the sequence alignment for the four VHHs aligned using Clustal Omega (Sievers *et al.*, 2011) with the residues thought to be responsible for the structuring of the C-terminal region of CDR3 circled in blue. The unassigned residues are highlighted in yellow in each Panel.

3.5 – Conclusions

In contrast to the original hypothesis that the short CDR3 and an additional disulphide bond in VHH 18 would be more rigid than VHH 67, it was shown that the longer CDR3 of VHH 67 allows it to fold down over the VHH creating, what appears to be, an additional region of framework while the shorter CDR3 of VHH 18 remains flexible.

The solution structures of VHH 18 and VHH 67 show the CDR loops behaving differently with two out of the three loops being structured and one appearing more dynamic, exchanging between conformations on an intermediate timescale. The more dynamic CDR is not conserved between the two VHs and is most likely dependent on characteristics of the antigen binding site.

The position of the structured CDR3, as observed in VHH 67, has been seen previously in VHs with long CDR3 loops in complex with antigens, however, there are no previous examples in the literature of this being observed with a VHH free in solution. To identify whether VHH 67 might be a unique example, the backbone assignments of VHH 29 and VHH 146 were made. In both cases, the majority of the antigen-binding site remained unassigned suggesting that the whole of this region is in intermediate exchange. The only exception was the C-terminal region of CDR3 that appears to be stabilised, probably by forming interactions with residues in the framework. The residues believed to be responsible for stabilising the C-terminal region of CDR3 are highlighted in Figure 3.25 Panel C and will be discussed further in Chapter 5.

Chapter 4 will focus on the complexes formed by VHH 18 and VHH 67 with IL-6 to determine the role these flexible CDR loops play in binding. Following on from this, Chapter 5 will look into the biological impact of the structured CDR3 observed in VHH 67.

Chapter 4 – Mapping the Binding Sites of VHH : IL-6 Complexes

4.1 – Introduction

4.1.1 – Aims

The aims of the work described in this chapter were to investigate VHH:IL-6 complexes focusing on any differences in the dynamics between the free and bound forms. NMR minimal shift mapping and hydrogen deuterium exchange monitored by mass spectrometry (HDX by MS) have been utilised to map the VHH and IL-6 binding sites. The value of using VHHs to map the potential antibody binding sites, as an alternative to the more commonly used Fab and scFv fragments, will also be evaluated. To simplify further discussion, from here on in the binding site of the antibody (the VHH) onto the antigen (IL-6) will be termed the epitope and the binding site of the IL-6 onto the VHH will be termed the paratope.

The VHH domains that were structurally characterised by the work described in Chapter 3 showed varied behaviour of the CDR loops with all the VHH domains showing some degree of conformational flexibility. With VHH 18 and VHH 67 one out of the three CDR loops appeared to be in conformational exchange but with VHH 29 and VHH 146 the majority of the antigen-binding site appeared to be in exchange. This type of conformational heterogeneity has been seen in a number of other antibody-antigen complexes leading to the view that this flexibility may be critical to effective protein-protein binding (Addis *et al.*, 2014). The work described in this chapter seeks to explore further the rationale that some CDR loops are structured and other have the potential to adopt multiple conformations by identifying the regions of the VHH antibodies that are involved in binding and how their behaviour alters upon binding.

4.1.2 – NMR Minimal Shift Mapping

NMR minimal shift mapping is a method used to identify regions of a protein that undergo changes in chemical environment upon binding. The method described here is adapted from methods described by Farmer *et al.*, when used to map the binding site of NADP+ on the MurB enzyme (Farmer *et al.*, 1996), and Williamson *et al.* when used to map the binding site of TIMP-2 (Williamson *et al.*, 1997). Firstly an assigned 2D or 3D spectrum of the free protein is required, then the equivalent 2D or 3D spectrum is collected for the bound protein. The combined shift ($\Delta\delta$) of each free peak to every peak in the bound spectrum is calculated using the equation below:

$$\Delta\delta = \sqrt{(\Delta\delta_{HN}\alpha_{HN})^2 + (\Delta\delta_N\alpha_N)^2 + (\Delta\delta_{C'}\alpha_{C'})^2}$$

The scaling factors α_{HN} , α_N and $\alpha_{C'}$ are applied to each term to account for the difference in the spectral dispersion of the ^1H , ^{15}N and ^{13}C dimensions. The lowest combined shift value is termed the minimal shift for that peak. Residues most affected by binding will be those with the greatest minimal shift values.

4.1.3 – Hydrogen Deuterium Exchange Monitored by Mass Spectrometry

HDX by MS is a method that can be used to explore the conformational changes and dynamics of proteins (Engen, 2009). When exposed to D_2O , the amide protons of the protein backbone exchange for deuterons. This exchange results in a mass increase of 1 Da per exchanged amide and this change in mass can be monitored by MS. The exchange will be most rapid for solvent exposed protons that are not hydrogen bonded. Any changes in solvent exposure will alter the rate and extent of deuterium uptake of the protein and this includes the binding of ligands or proteins to the protein of interest. Therefore, we can use this method to map the binding interface by comparing the rates of deuterium uptake for the free protein with that of the bound protein.

The HDX by MS method consists of four main steps: incubation of the protein in D₂O; quenching the deuterium exchange; digestion of the protein; and analysis of the digestion products. Incubation of the protein in D₂O initiates the exchange of amide protons for deuterons and is repeated with different incubation times prior to the quench step which involves reducing the temperature and the pH to stop any further deuterium exchange and prevent back exchange of deuterons for protons during analysis. Digestion of the protein is then carried out using pepsin to fragment the protein into peptides of varying length. Ideally, the digestion results in the generation of multiple overlapping peptide fragments covering the entire protein sequence. Analysis of the peptide fragments by ultra performance liquid chromatography (UPLC) coupled with time-of-flight (TOF) MS allows each peptide to be identified and the deuterium incorporation into each fragment determined, with the ultimate goal being to measure the rate of deuterium uptake per residue of the protein. This process is carried out for the free protein and then repeated with the protein complex so the differences can be calculated and the binding interface defined.

4.2 – Materials and Methods

4.2.1. – Complex Formation for Minimal Shift Mapping

Minimal shift mapping was carried out to define both the paratope of the VHH and the epitope of IL-6. To prepare the samples for paratope mapping of IL-6 onto the VHH, a 10 % excess of unlabelled IL-6 was mixed with ¹⁵N/¹³C labelled VHH (both samples having been buffer exchanged into the NMR buffer (see Section 4.2.3) and concentrated to approximately 250 μM) then incubated at 25 °C for 2 hours. To allow epitope mapping of the VHH onto IL-6, the same method was followed but a 10 % excess of the unlabelled VHH was mixed with ¹⁵N/¹³C/²H IL-6. All samples were analysed by analytical gel filtration chromatography using a Superdex S75 column (GE Healthcare), as described in Section 2.2.17, to ensure that all the isotopically labelled protein was complexed.

4.2.2 – Preparation of Samples for HDX by MS

To prepare the free IL-6 sample, IL-6 was diluted to 90 μ M in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , pH 7.4). For the complexed samples, both the IL-6 and VHH were diluted to 90 μ M in PBS and then mixed in a ratio of 5:1 of VHH to IL-6.

4.2.3 – NMR Acquisition and Data Analysis for Minimal Shift Mapping

NMR spectra were acquired from 0.35 ml samples of 150-400 μ M VHH, IL-6 or VHH:IL-6 in a 25 mM sodium phosphate, 100 mM sodium chloride, 10 μ M EDTA and 0.02 % (w/v) sodium azide buffer at pH 6.4 containing 10 % D_2O / 90 % H_2O . NMR data were acquired at 35 °C on 600 MHz Bruker Avance, 600 MHz Bruker DRX or 800 MHz Bruker Avance II spectrometers. For each analysis a reference spectrum was collected of the free VHH or the free IL-6 prior to the complex sample being acquired. The 2D and 3D spectra recorded to allow paratope mapping of IL-6 onto the VHHs were: ^{15}N - ^1H HSQC (Bodenhausen and Ruben, 1980) and HNC0 (Kay *et al.*, 1990) and for the epitope mapping of the VHHs onto IL-6 were ^{15}N - ^1H TROSY (Pervushin *et al.*, 1997) and ^{15}N - ^{13}C - ^1H TROSY-HNC0 (Salzmann *et al.*, 1998). Typical acquisition times in 2D experiments were 70 ms for ^1H and 40 ms for ^{15}N . For the 3D experiments typical acquisition times were 70 ms in ^1H , 22-26 ms in ^{15}N and 20-25 ms in ^{13}C . 3D experiments were collected over approximately 48-60 hours and 2D experiments over approximately 40 minutes. The WATERGATE method (Piotto *et al.*, 1992) was used for water suppression. 2D and 3D NMR data were processed using NMRPipe (Delaglio *et al.*, 1995), with linear prediction used, when required, to extend the effective acquisition times by up to two-fold in the indirect dimensions.

4.2.4 – HDX by MS Data Acquisition and Data Analysis

The following buffers were prepared: Equilibrium Buffer (Buffer E): 10 mM potassium phosphate in H₂O, pH 7.0; Labelling Buffer (Buffer L): 10 mM potassium phosphate in D₂O, pD 7.0; and Quench Buffer (Buffer Q): 100 mM potassium phosphate in H₂O, pH 2.66.

All liquid handling was carried out on a LEAP HDX Automation Manager in tandem with a Waters nanoACQUITY UPLC HDX Manager and a Waters SYNAPT G2 mass spectrometer. Samples were digested using a Waters Enzymate™ BEH Pepsin Column (2.1 x 30 mm, 5 µm) at 20 °C and the products were trapped onto a Waters ACQUITY VanGuard® BEH C₁₈ Pre-Column (2.1 x 5 mm). The peptides were then eluted onto a Waters ACQUITY BEH C₁₈ Column (1.0 x 100 mm, 1.7 µm) and analysed using a 40 µl min⁻¹ H₂O and MeCN gradient + 0.1 % formic acid (6 % MeCN to 40 % MeCN over 6 minutes). The eluent from the column was then directed to the MS where it was analysed in electrospray positive ionisation mode (ESI+) scanning 50 Da to 1700 Da. Data analysis was carried out using Waters DynamX™ 2.0.

A control digest of the IL-6 was performed by diluting 1.6 µl of the IL-6 stock into 25 µl of Buffer E, adding an equal volume of Buffer Q and then injecting the sample onto the UPLC for analysis.

The HDX experiments for unbound IL-6 were carried out by diluting 1.6 µl of the IL-6 stock into 25 µl of Buffer L, incubating for 1 minute and then quenching with 25 µl of Buffer Q. The sample was then injected onto the UPLC for analysis. This process was repeated with incubation times of 10 minutes, 30 minutes, 120 minutes and 240 minutes. Peptide fragments were identified using the TOF-MS data and the deuterium uptake for each peptide was calculated. Providing good coverage of the protein sequence from the protein fragments is observed, the average deuterium uptake per residue can be calculated.

The HDX experiments for the VHH:IL-6 complexes were then performed as described above but using the 5:1 VHH to IL-6 complex samples in place of the free IL-6.

The reverse experiments were also carried out in an attempt to map the regions of the VHHs interacting with IL-6. Unfortunately the peptide fragments resulting from the digest of the VHHs did not give good coverage across the protein so the rate of HD exchange per residue could not be determined.

4.3 – Results

4.3.1 – NMR Minimal Shift Mapping of the IL-6 Paratope on VHH 18

Minimal shift mapping was carried out to identify the paratope of IL-6 on VHH 18. Both ^{15}N - ^1H HSQC and ^{15}N - ^{13}C - ^1H HNCOC spectra were collected for free VHH 18 and for VHH 18 bound to IL-6. Panel A of Figure 4.1 shows an overlay of the HSQC spectrum of free VHH 18 and the equivalent spectrum of VHH 18 bound to IL-6. Panel B of Figure 4.1 shows an expanded region of the HSQC spectra in which the chemical shift differences between the free and bound forms of VHH 18 can be clearly seen for a number of residues, for example the signals for residues Arg138 and Thr197 have significantly shifted. The shifts in these signals arise from a change in their chemical environment once bound to IL-6, so they are likely to be at the binding interface or influenced by structural changes as a result of binding. The signals for residues such as Gly115 and Ser184 have only shifted a small amount suggesting the chemical environment has not altered significantly with the binding of IL-6. Without reassignment of the bound spectrum one cannot be certain where each peak has shifted to, but the minimal shift method offers a conservative estimate of the shifts upon binding by assuming the free signal and corresponding bound signal are the peaks closest together in the NMR spectra (Farmer *et al.*, 1997).

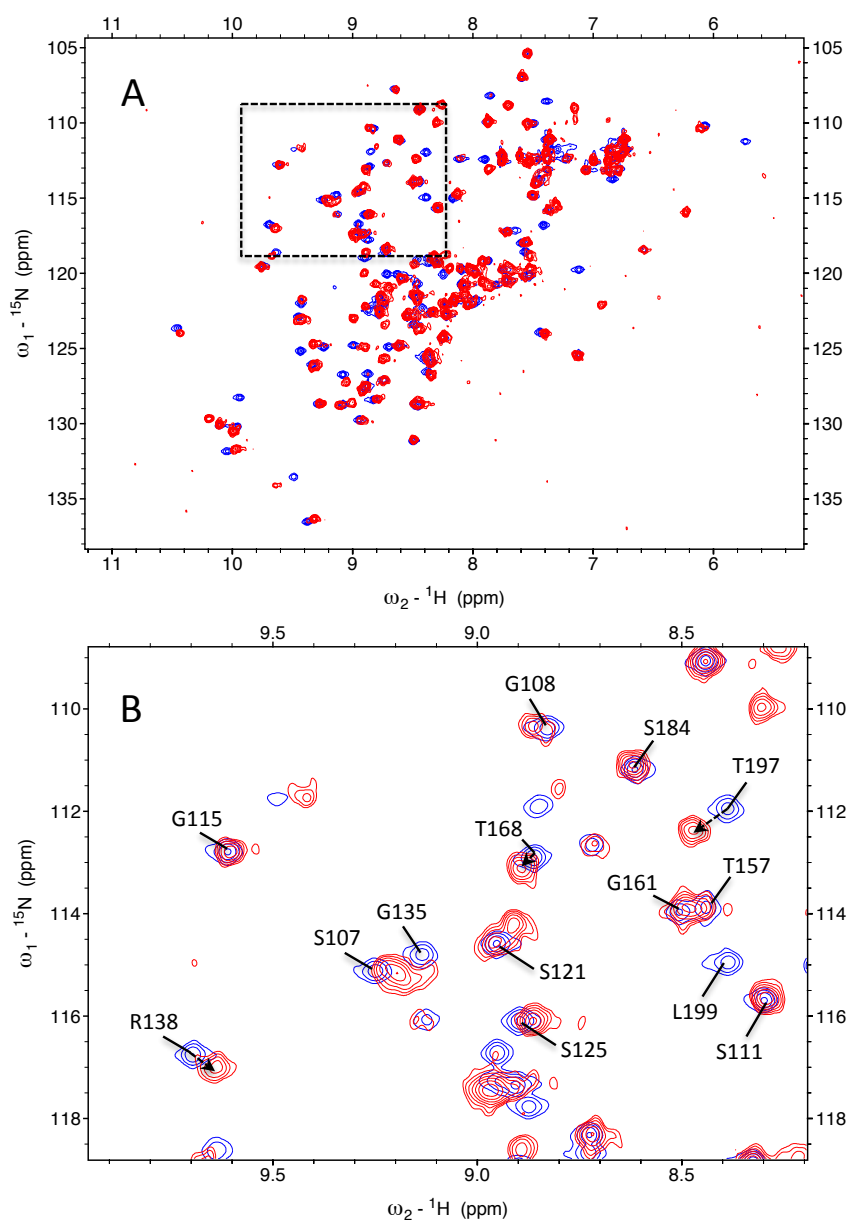


Figure 4.1 – Comparison of the Spectra for Free VHH 18 and VHH 18 Bound to IL-6

Panel A shows in blue the ^{15}N - ^1H HSQC spectrum of $^{15}\text{N}/^{13}\text{C}$ labelled free VHH 18 at a concentration of 286 μM in 25 mM sodium phosphate, 100 mM sodium chloride buffer, pH 6.4 collected at 35 $^{\circ}\text{C}$ on a 600 MHz Bruker Avance spectrometer for 60 minutes. Overlaid in red is the equivalent spectrum for VHH 18 bound to unlabelled IL-6 at a concentration of 151 μM . The boxed area is expanded in Panel B showing that the backbone amide signals of residues such as Arg138 and Thr197 shift upon binding to IL-6.

Chemical shift changes observed in the HNCO spectra rather than the HSQC spectra were used to calculate the combined minimal shift values, as the extra dimension results in less signal overlap. The combined minimal shift values for each residue were calculated as described in Section 4.1.2 using the scaling factor values of $\alpha_{\text{HN}} = 1$, $\alpha_{\text{N}} = 0.2$ and $\alpha_{\text{C}'} = 0.35$. The minimal shift values are plotted in Figure 4.2 and clearly show that CDR1, CDR2 and the visible signals of CDR3 are affected by the binding of IL-6. Residues Ile169 to Ser173 also show large shifts in signals between the free and bound samples. These residues make up the fourth loop that is situated alongside the three CDR loops. For VHH 18 it appears that this fourth loop plays a role in binding to IL-6. No additional backbone amide signals were observed in the bound spectra that may account for the missing CDR3 peaks. The signals for the peaks at the N-terminal of CDR3 do show large shifts upon the binding of IL-6 and this may suggest that CDR3 is involved in binding, but residues Trp202 to Thr207 still remain in intermediate exchange when bound to IL-6, possibly due to the plasticity of IL-6 (discussed in Section 4.4).

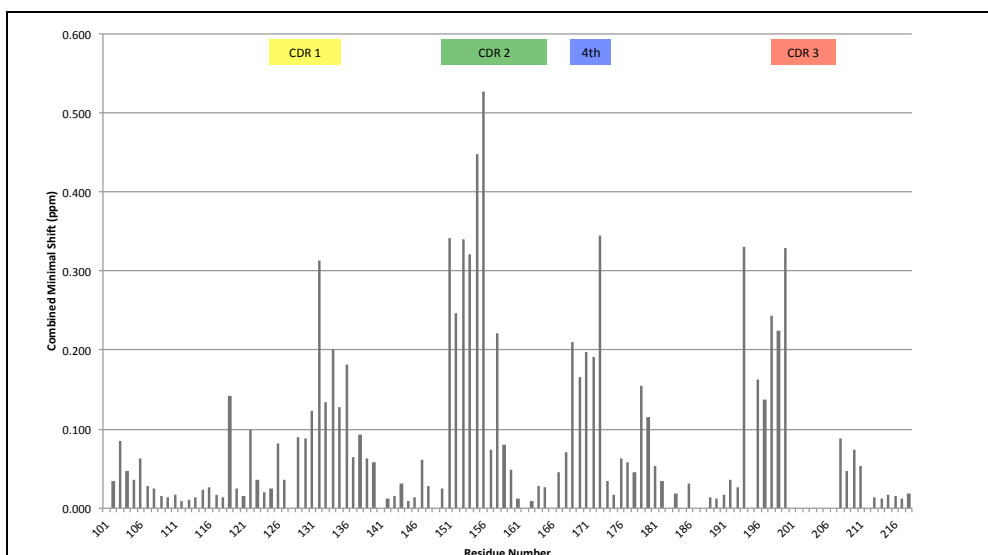
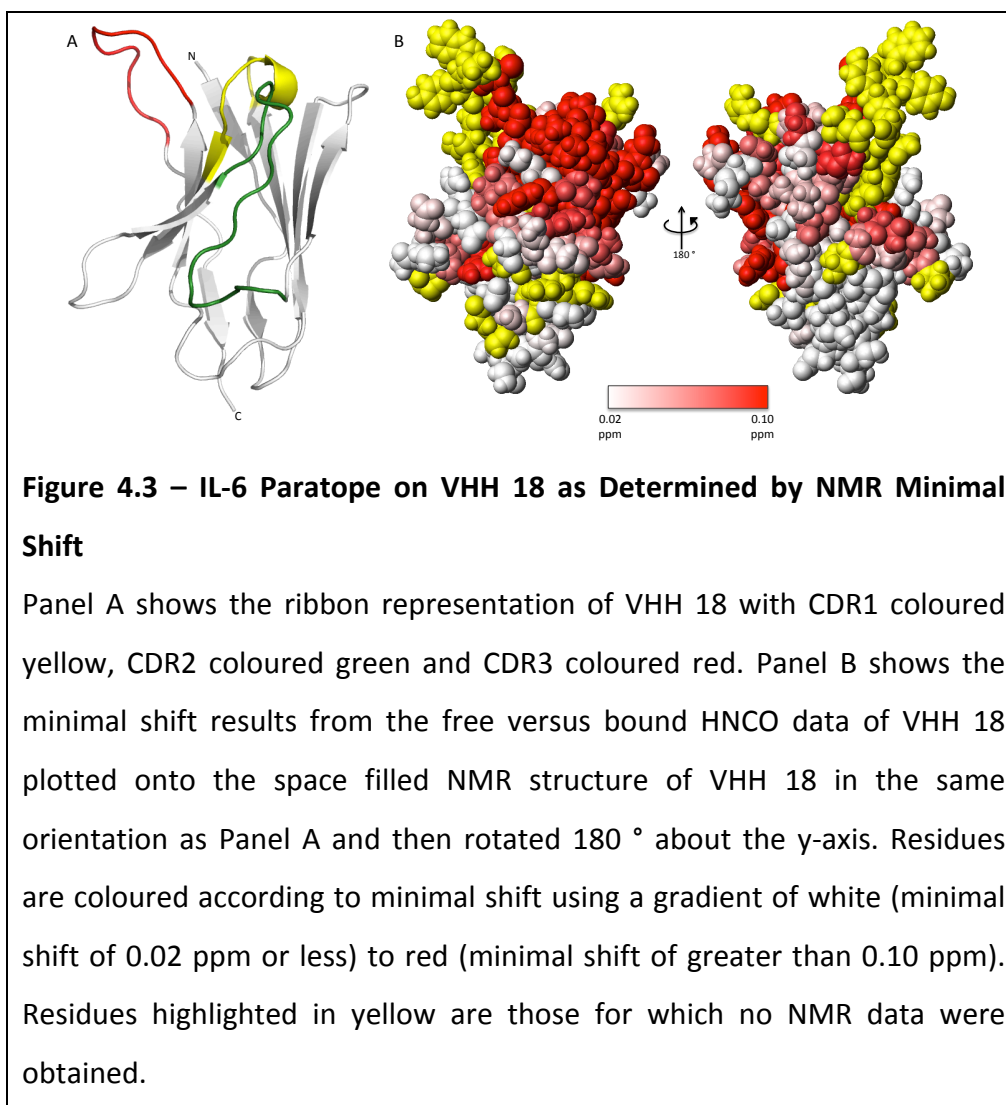


Figure 4.2 – Backbone Amide Minimal Shift Values for VHH 18 Induced upon Binding to IL-6

The graph shows the backbone amide minimal shift values calculated from the HNCO spectra of the free and bound VHH 18 plotted against residue number, and highlights the regions most affected upon binding to IL-6. CDR1, CDR2 and CDR3 are highlighted in yellow, green and red respectively. The region highlighted in blue is the fourth loop which is located in the vicinity of the CDRs. Large shifts can be seen for the amide groups of CDR1, CDR2, residues Ile169 to Ser173, that make up the fourth loop, and the residues preceding the missing backbone amide signals in CDR3.

The backbone amide minimal shift results were mapped onto the NMR solution structure of VHH 18 and are shown in Figure 4.3. A clear binding site can be seen on the side of VHH 18 with most of the residues in CDR1 and CDR2 showing large shifts upon binding to IL-6.



4.3.2 – NMR Minimal Shift Mapping of the VHH 18 Epitope on IL-6

To map the binding site of VHH 18 on IL-6, ^{15}N - ^1H TROSY and ^{15}N - ^{13}C - ^1H TROSY-HNCO spectra of free IL-6 and of IL-6 bound to VHH 18 were collected. Shown in Figure 4.4 is an overlay of the TROSY spectra of free IL-6 and IL-6 bound to VHH 18. As with the mapping of the IL-6 binding site onto VHH 18, clear shifts in the backbone amide signals for a number of residues were observed.

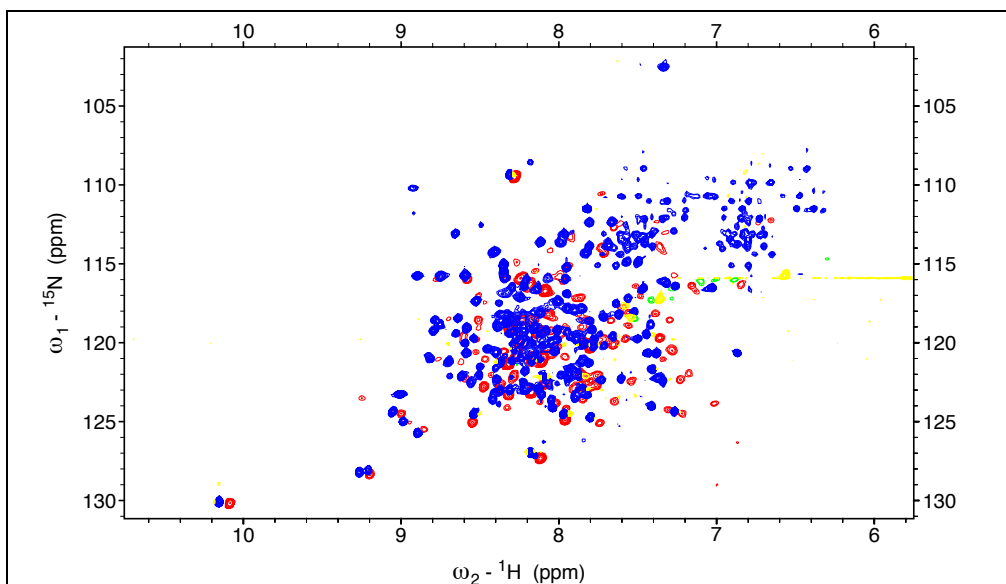
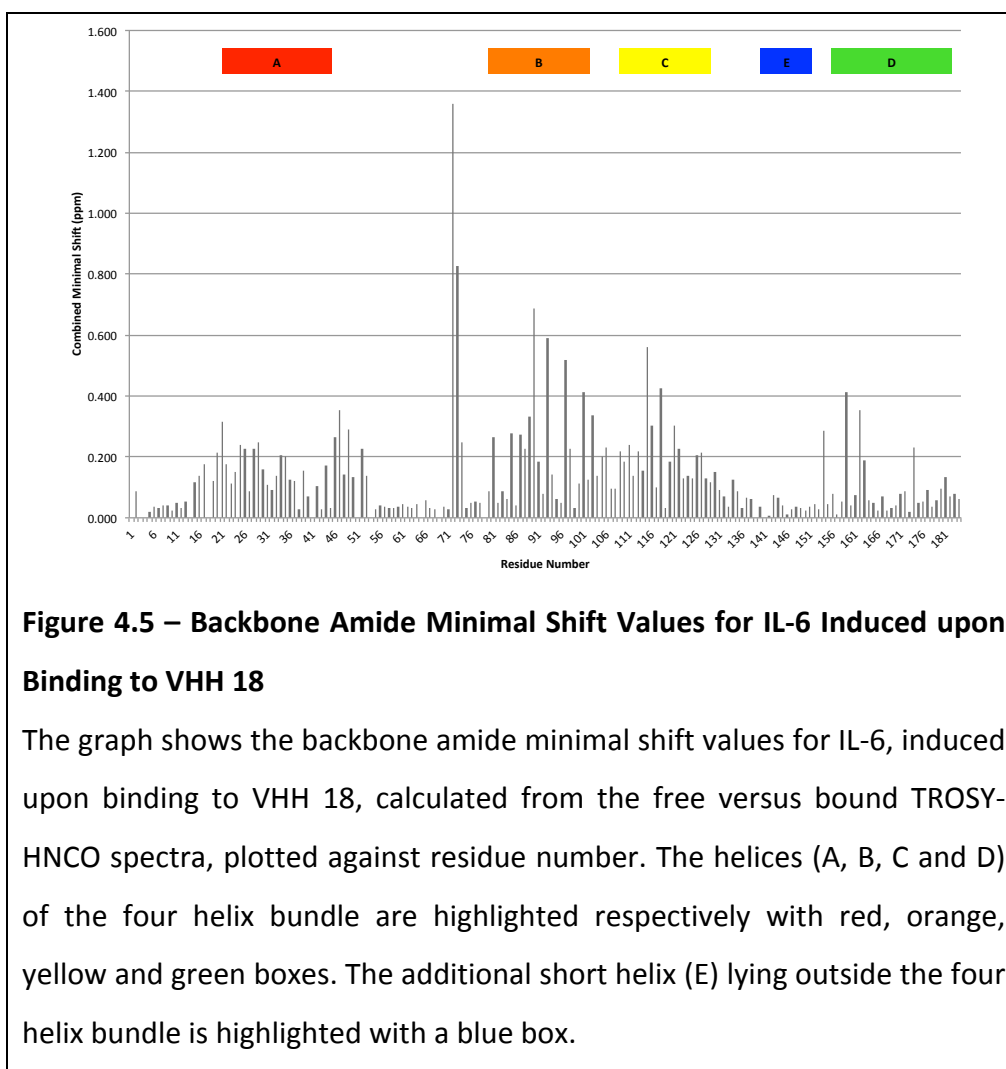


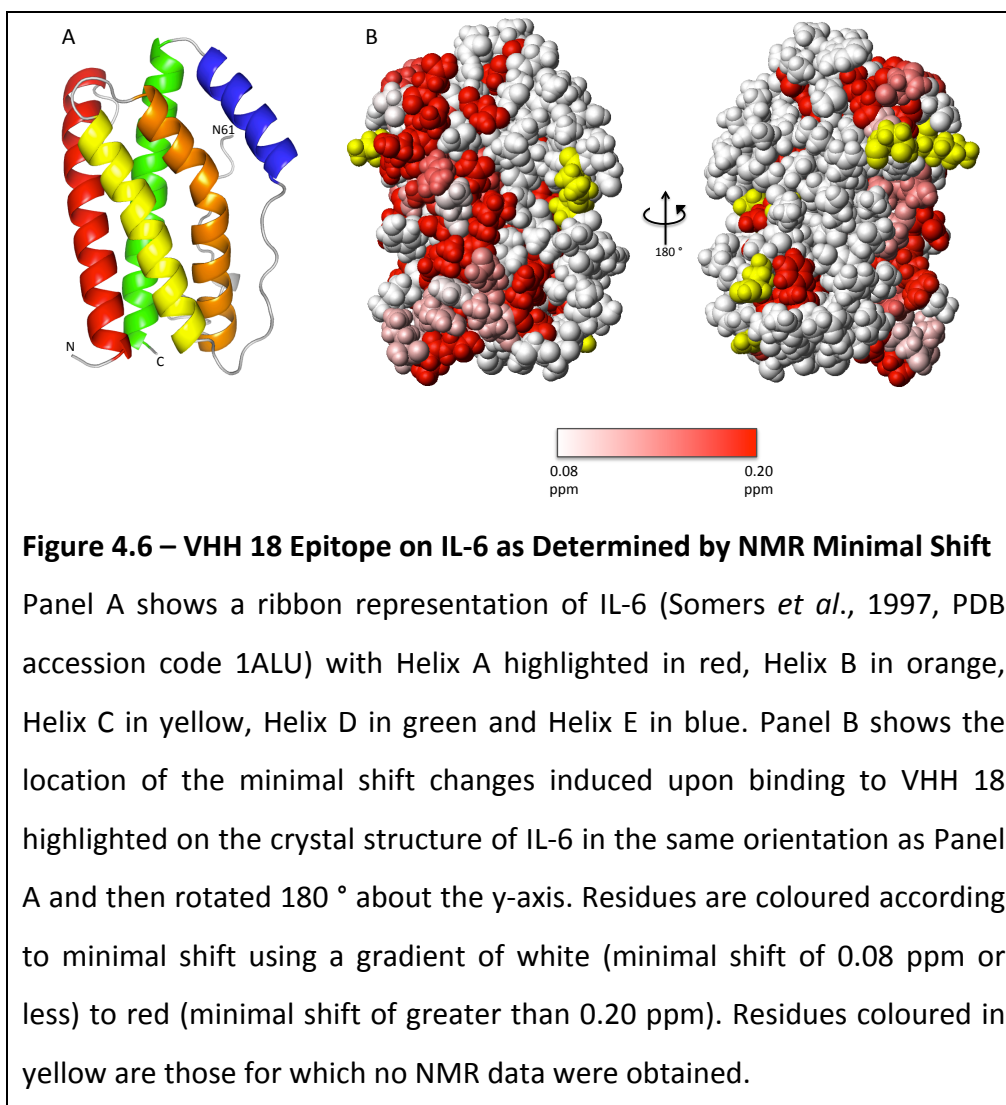
Figure 4.4 – Comparison of the Spectra of Free IL-6 and IL-6 Bound to VHH 18

Shown in blue is the ^{15}N - ^1H TROSY spectrum of $^{15}\text{N}/^{13}\text{C}/^2\text{H}$ labelled free IL-6 at a concentration of 380 μM in 25 mM sodium phosphate, 100 mM sodium chloride buffer, pH 6.4 collected at 35 $^{\circ}\text{C}$ on a 800 MHz Bruker Avance II spectrometer for 60 minutes. Overlaid in red is the equivalent spectrum for IL-6 bound to unlabelled VHH 18 at a concentration of 220 μM . Significant differences in the backbone amide chemical shifts can be seen between the free and bound forms of IL-6.

The backbone amide minimal shift values for peaks observed in the TROSY-HNCO spectra of free IL-6 compared to bound were calculated as described in Section 4.1.2 using the scaling factor values of $\alpha_{\text{HN}} = 1$, $\alpha_{\text{N}} = 0.2$ and $\alpha_{\text{C}} = 0.35$. The minimal shift values plotted against residue number and are shown in Figure 4.5.



The backbone amide minimal shift results were then mapped onto the structure of free IL-6 (Somers *et al.*, 1997, PDB accession code 1ALU) and are shown in Figure 4.6. VHH 18 was shown to be a Site II blocker in the Site II ELISA assay (as discussed in Section 1.5) and the results of the minimal shift mapping onto IL-6 agree with these results. Figure 4.7 highlights the position of gp-130 binding to Site II of IL-6 (adapted from IL-6 hexamer structure, Boulanger *et al.*, 2003, PDB accession code 1P9M) and it can be seen that this is the same epitope as calculated by the NMR minimal shift mapping.



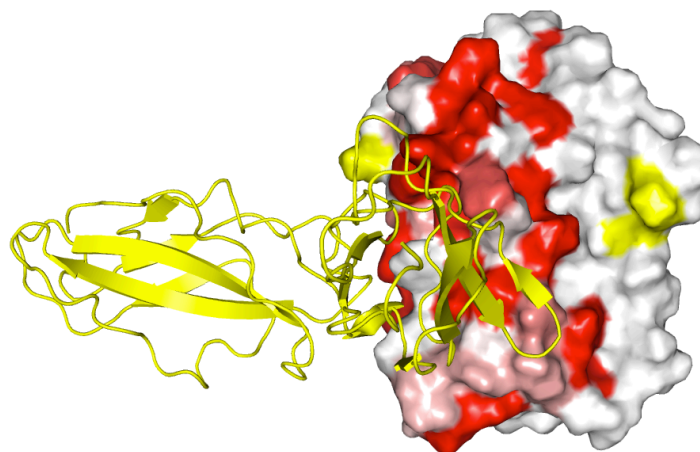


Figure 4.7 – Comparison of the VHH 18 and gp-130 Binding Sites on IL-6

The crystal structure of IL-6 (adapted from Boulanger *et al.*, 2003, PDB accession code 1P9M) is coloured, as described in Figure 4.6, according to minimal shift results for VHH 18, with the gp-130 domains 2 and 3 (gp-130 D2D3) shown as a yellow ribbon, highlighting the Site II binding site.

4.3.3 – Mapping of VHH 18 Epitope on IL-6 using HDX by MS

HDX by MS experiments were carried out for free IL-6 and for the VHH 18:IL-6 complex and Figure 4.8 shows the difference plot of the results. The plot shows two regions of Helix A and Helix C in which the deuterium uptake is much greater in the free IL-6 than when VHH 18 is bound. This suggests that these regions are no longer as accessible to solvent and are likely to be where VHH 18 binds to IL-6. When highlighted on the structure of IL-6 these regions identify the likely binding site of VHH 18 on IL-6 (shown in Figure 4.9).

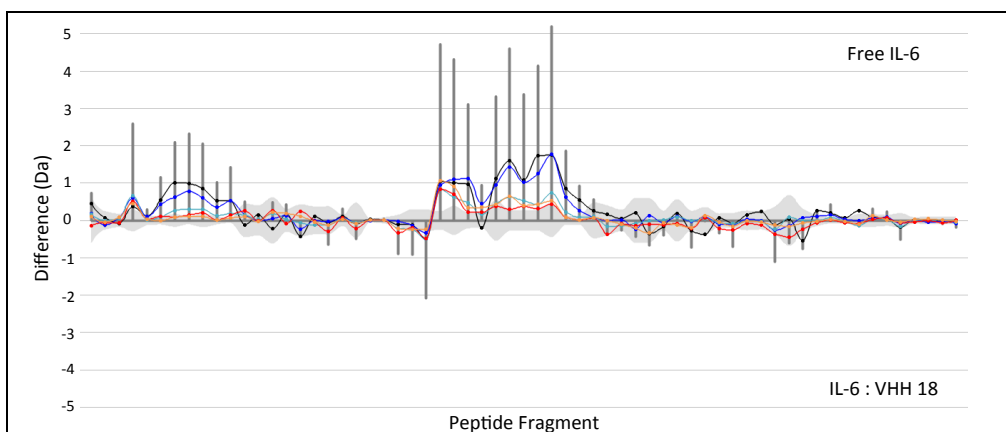
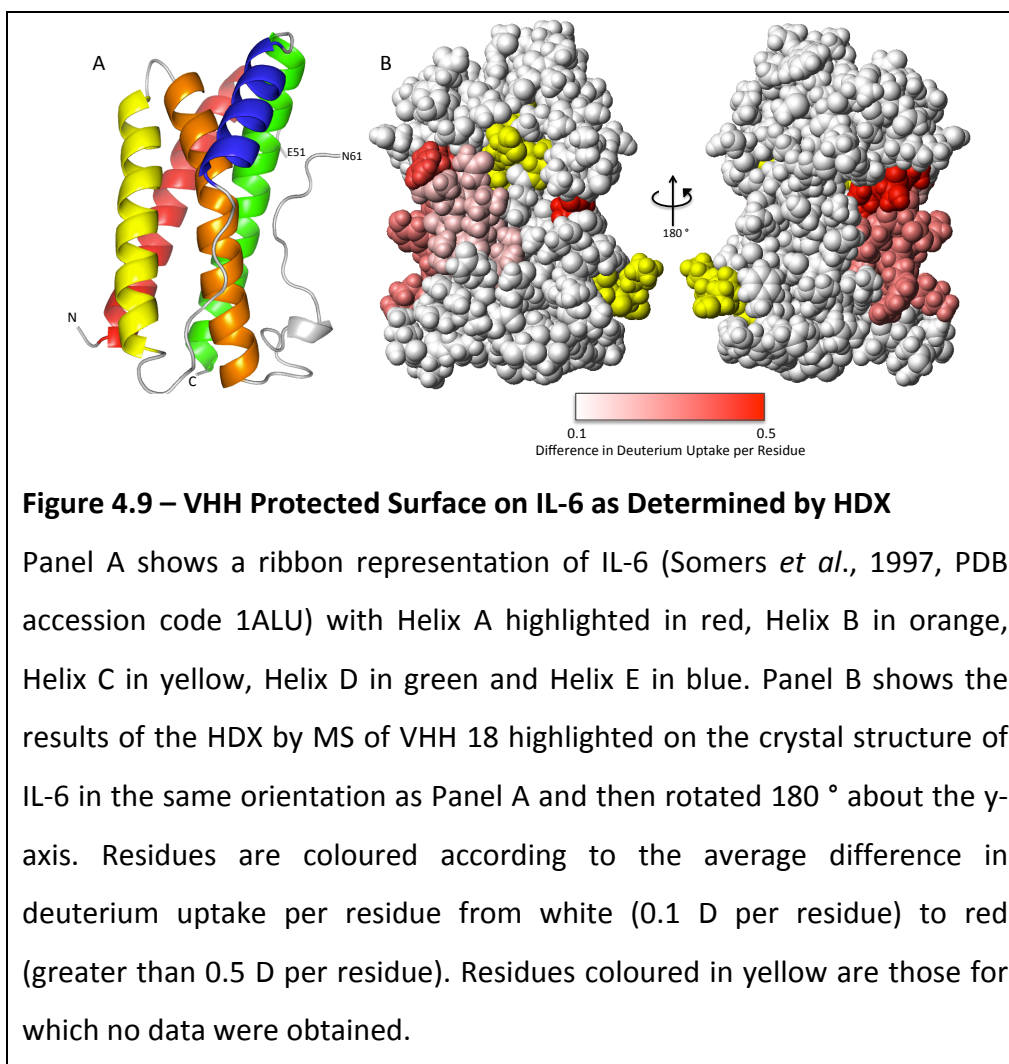


Figure 4.8 – HDX Difference Plot of VHH 18 on IL-6

The difference plot shows the difference in deuterium uptake of the unbound IL-6 compared to the IL-6 with VHH 18 bound. The coloured lines show the deuterium uptake per peptide at 1 minute (orange), 10 minutes (red), 30 minutes (light blue), 120 minutes (dark blue) and 240 minutes (black). The vertical bars represent the sum of the differences for all time points. The grey shaded area is an indicator of the experimental error associated with each measurement.



The epitope determined using HDX by MS fits well with VHH 18 being a Site II binder. Figure 4.10 highlights the position of gp-130 binding to IL-6 (adapted from IL-6 hexamer structure, Boulanger *et al.*, 2003, PDB accession code 1P9M) and is comparable to the epitope as mapped using HDX by MS.

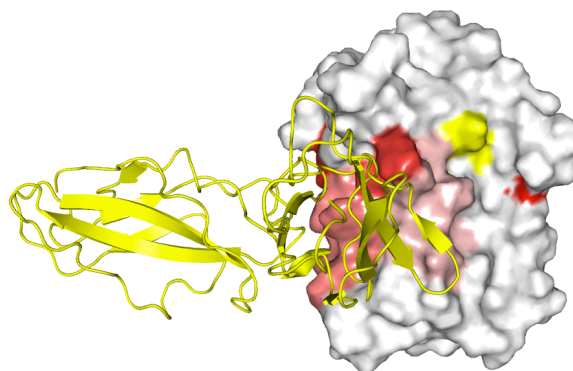


Figure 4.10 – Comparison of the VHH 18 and gp-130 Binding Sites on IL-6

The crystal structure of IL-6 (adapted from Boulanger *et al.*, 2003, PDB accession code 1P9M) is coloured, as Figure 4.9, according to the HDX by MS results for VHH 18 with gp-130 D2D3, shown as a yellow ribbon, highlighting the Site II binding site.

4.3.4 – NMR Minimal Shift Mapping of the IL-6 Paratope on VHH 67

^{15}N - ^1H HSQC and ^{15}N - ^{13}C - ^1H HNCO spectra were collected for the free VHH 67 and also for VHH 67 bound to IL-6. Over the course of the HNCO experiment, the VHH 67:IL-6 complex began to precipitate out of solution. For this reason the HSQC experiments were used instead of the HNCO experiments for the mapping of the IL-6 paratope on VHH 67. Addition of the IL-6 resulted in large chemical shift differences for many of the backbone amide groups as shown in Figure 4.11. It can be seen that some of the backbone amide signals show no significant chemical shift difference between the free and the bound forms, for example residues Arg140 and Glu191 in Figure 4.11 Panel B. This lack of movement in the backbone amide signals suggest that these residues are not affected by the binding of IL-6 and therefore unlikely to be at the binding interface. The backbone amide signals for residues such as Gly149 and Arg174 do shift between the free and bound forms of VHH 67 suggesting that they are affected by the binding of IL-6 and are more likely to be at or near the binding interface or indicate a conformational change.

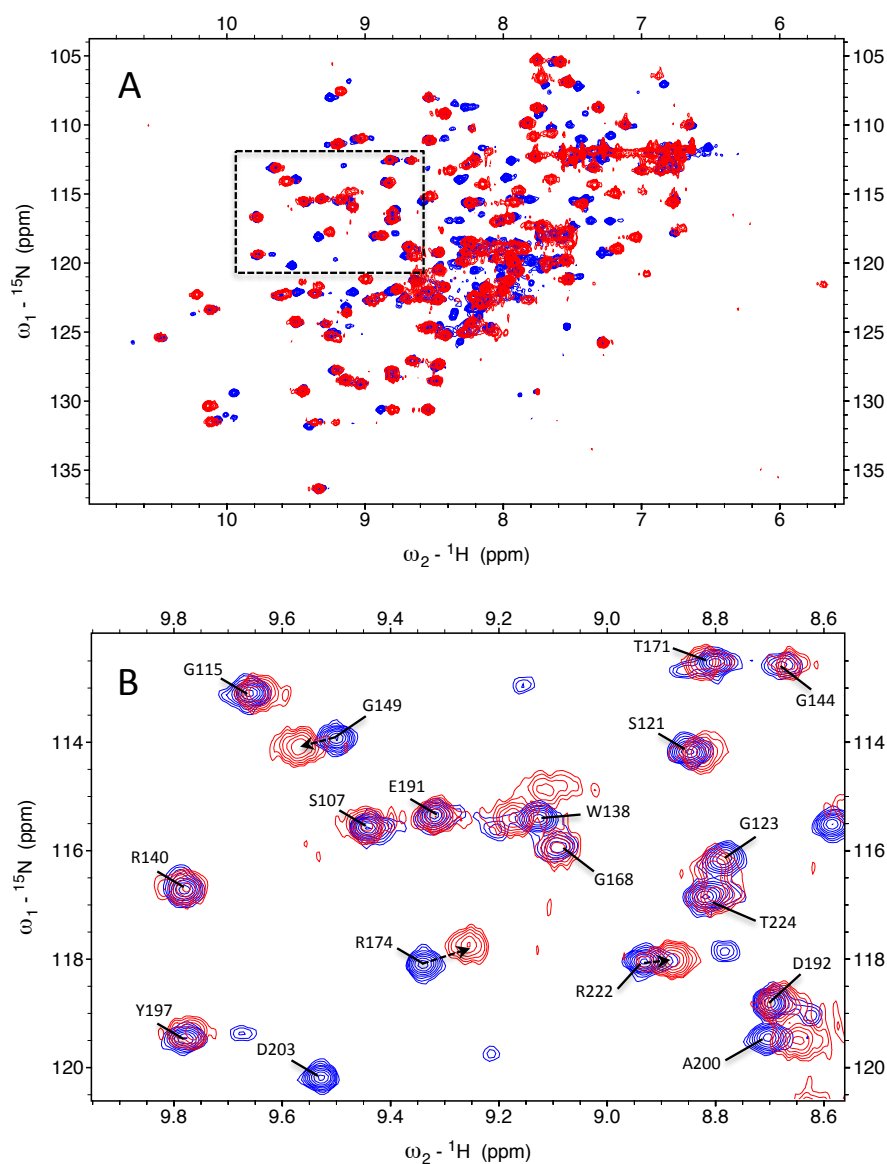


Figure 4.11 – Comparison of the Spectra for Free VHH 67 and VHH 67 Bound to IL-6

Panel A shows in blue the ^{15}N - ^1H HSQC spectrum of $^{15}\text{N}/^{13}\text{C}$ labelled free VHH 67 at a concentration of 312 μM in 25 mM sodium phosphate, 100 mM sodium chloride buffer, pH 6.4 collected at 35 $^{\circ}\text{C}$ on a 800 MHz Bruker Avance II spectrometer for 40 minutes. Overlaid in red is the equivalent spectrum for VHH 67 bound to unlabelled IL-6 at a concentration of 273 μM . The boxed area is expanded in Panel B showing that the backbone amide signals of residues such as Gly149 and Arg174 shift upon binding to IL-6.

The backbone amide minimal shift values for each free versus bound VHH 67 residue were calculated as described in Section 4.1.2 using the scaling factor values of $\alpha_{\text{HN}} = 1$ and $\alpha_{\text{N}} = 0.2$. Figure 4.12 shows these minimal shift values plotted against residue number and shows large shifts in the amide resonances of residues in CDR2 and CDR3 suggesting that these CDRs are involved in binding to IL-6. Smaller, but significant shifts, can also be seen for CDR1 and the fourth loop.

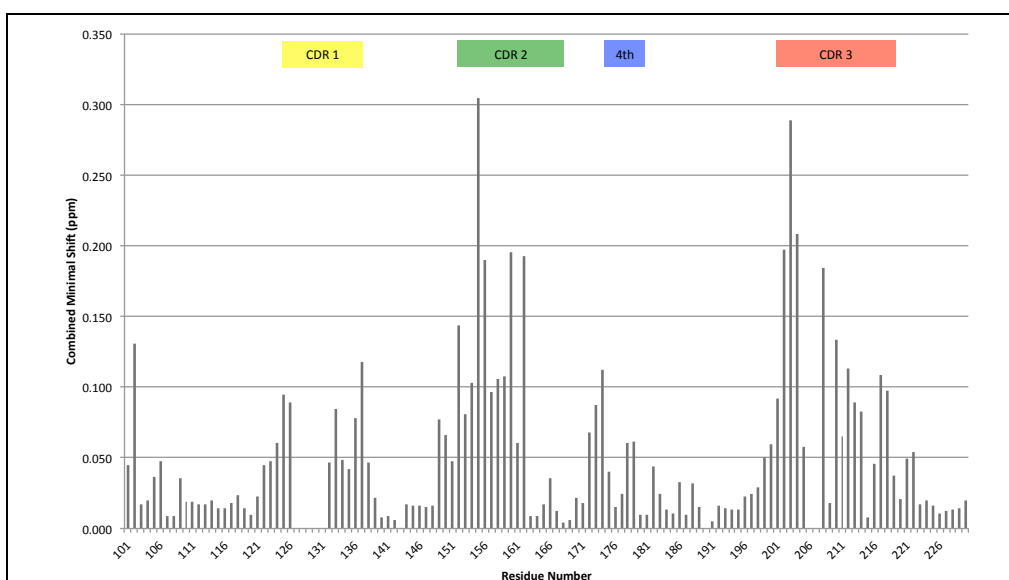
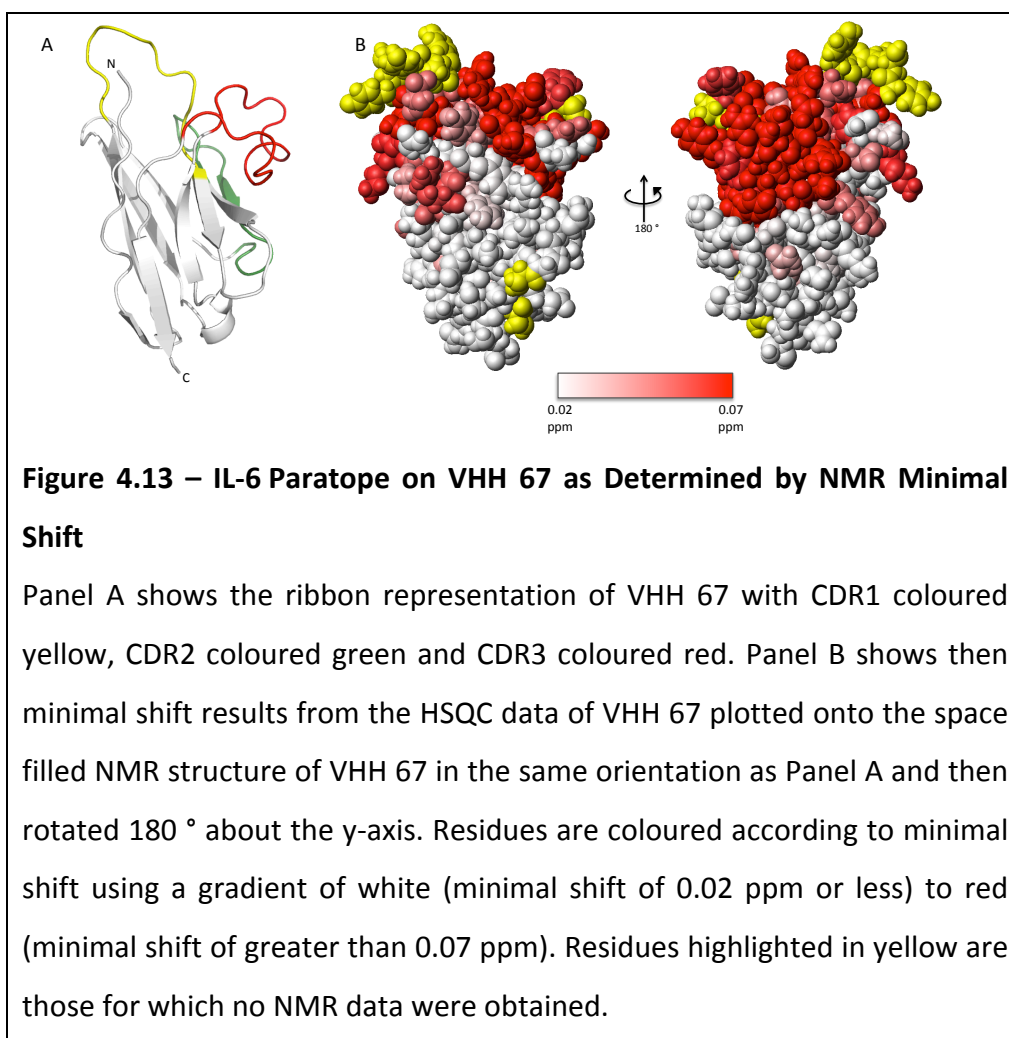


Figure 4.12 – Backbone Amide Minimal Shift Values for VHH 67 Induced upon Binding to IL-6

The graph shows the backbone amide minimal shift values calculated from the free versus bound HSQC spectra of VHH 67 plotted against residue number and highlights the regions most affected upon binding to IL-6. CDR1, CDR2 and CDR3 are highlighted in yellow, green and red respectively. The region highlighted in blue is the fourth loop which is located in the vicinity of the CDRs. The graph shows that large shifts are observed for the backbone amide groups of CDR2 and CDR3.

No additional backbone amide signals were observed in the VHH 67:IL-6 complex spectrum, which could correspond to the missing CDR1 signals in free VHH 67. This suggests that the binding of IL-6 to VHH 67 does not stabilise these residues in a single conformation. The increased backbone amide minimal shift values observed for residues Ser125 to Gly126, prior to the missing CDR1 residues, and for residues Gln133, Met136 and Gly137, after the missing signals, may suggest that this region is affected by IL-6 binding but that residues Tyr127 to Tyr131 remain in intermediate exchange in the bound form. To better visualise the residues affected by binding, Figure 4.13 shows the backbone amide minimal shift results mapped onto the NMR solution structure of VHH 67. The results show a clearly defined IL-6 paratope on VHH 67 that includes both CDR2 and CDR3 and the visible residues of CDR1. Few effects are seen in the framework regions of VHH 67 implying that no significant long range structural changes are occurring upon binding.



4.3.5 – NMR Minimal Shift Mapping of the VHH 67 Epitope on IL-6

To map the binding site of VHH 67 on IL-6, ^{15}N - ^1H TROSY and ^{15}N - ^{13}C - ^1H TROSY-HNCO spectra of free IL-6 and of the IL-6 bound to VHH 67 were collected. Shown in Figure 4.14 is an overlay of the TROSY spectra of free IL-6 and IL-6 bound to VHH 67.

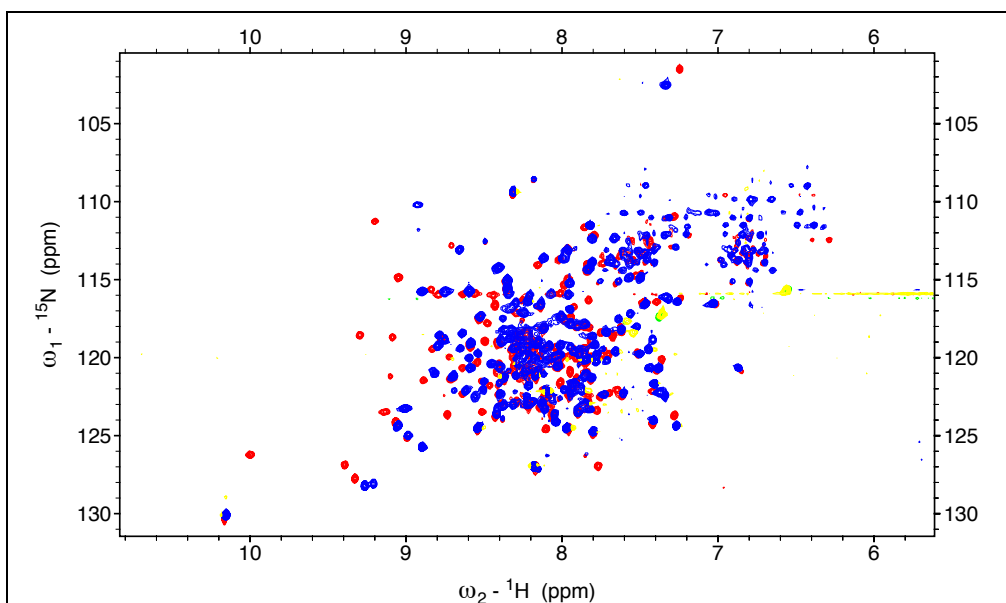


Figure 4.14 – Comparison of the Spectra of Free IL-6 and IL-6 Bound to VHH 67

Shown in blue is the ^{15}N - ^1H TROSY spectrum of $^{15}\text{N}/^{13}\text{C}/^2\text{H}$ labelled free IL-6 at a concentration of 380 μM in 25 mM sodium phosphate, 100 mM sodium chloride buffer, pH 6.4 collected at 35 $^{\circ}\text{C}$ on a 800 MHz Bruker Avance II spectrometer for 60 minutes. Overlaid in red is the equivalent spectrum for IL-6 bound to unlabelled VHH 67 at a concentration of 254 μM . Significant differences in the amide chemical shifts can be seen between the free and bound forms of IL-6.

The backbone amide minimal shift values for each residue were calculated as described in Section 4.1.2 using the scaling factor values of $\alpha_{\text{HN}} = 1$, $\alpha_{\text{N}} = 0.2$ and $\alpha_{\text{C}} = 0.35$. The minimal shift values plotted against residue number are shown in Figure 4.15

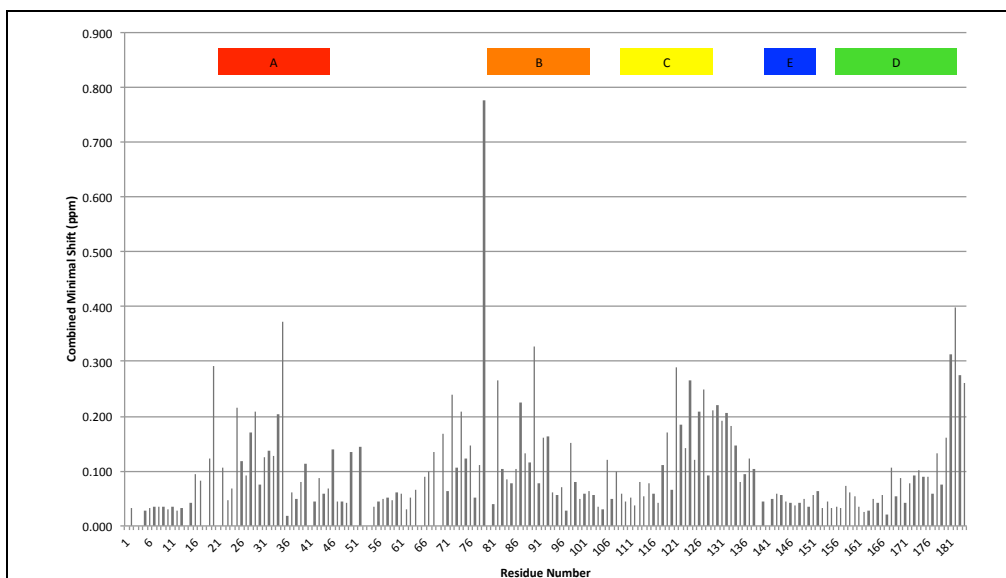
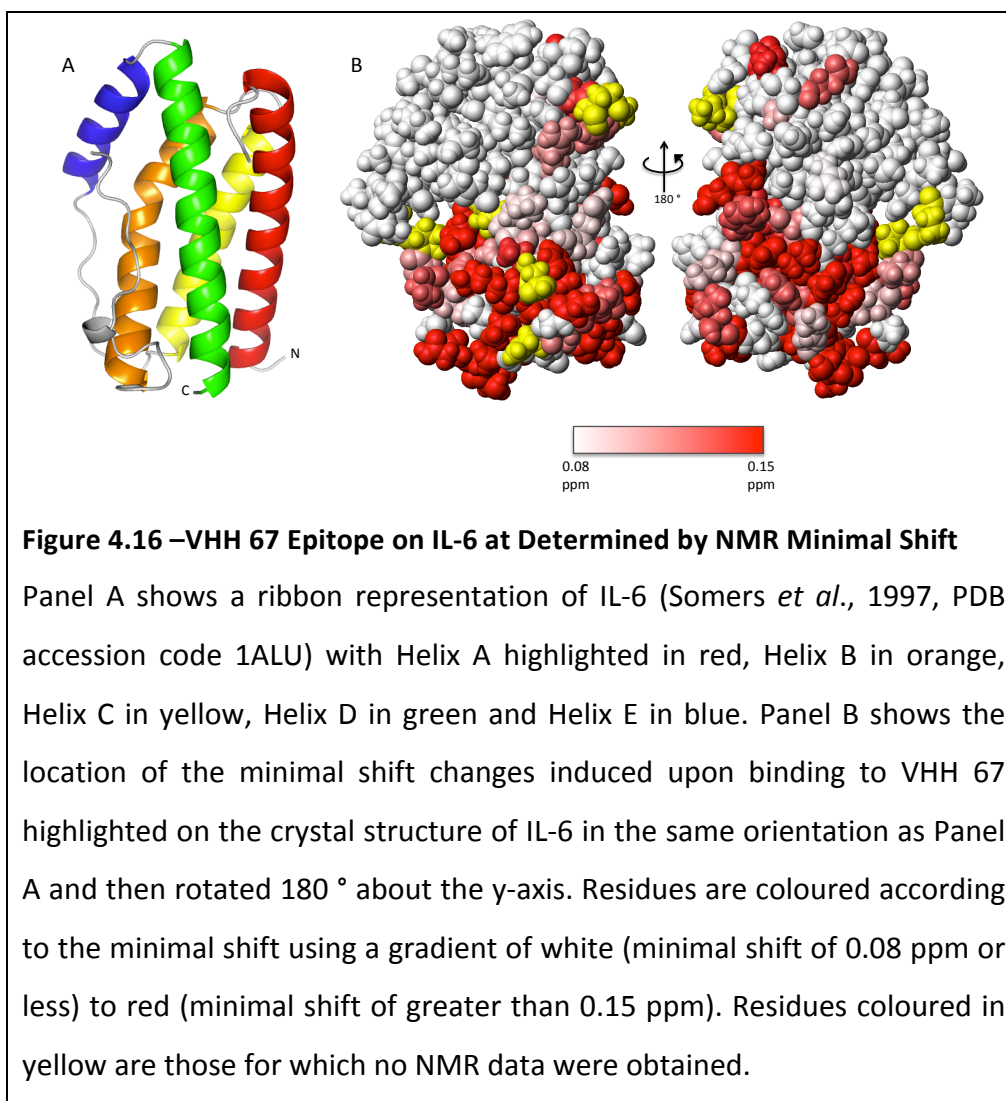


Figure 4.15 – Backbone Amide Minimal Shift Values for IL-6 Induced upon Binding to VHH 67

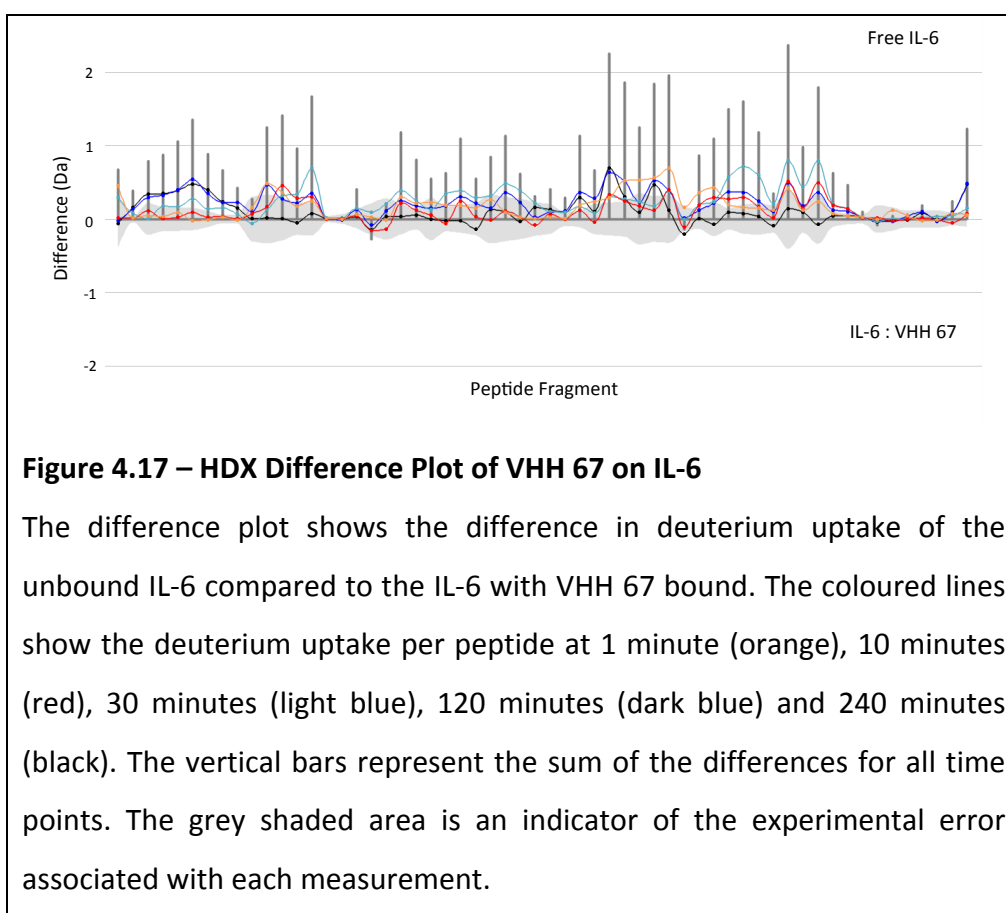
The graph shows the backbone amide minimal shift values for IL-6, induced upon binding to VHH 67, calculated from the free versus bound TROSY-HNCO spectra, plotted against residue number. The helices (A, B, C and D) of the four helix bundle are highlighted respectively with red, orange, yellow and green boxes. The additional short helix (E) lying outside the four helix bundle is highlighted with a blue box.

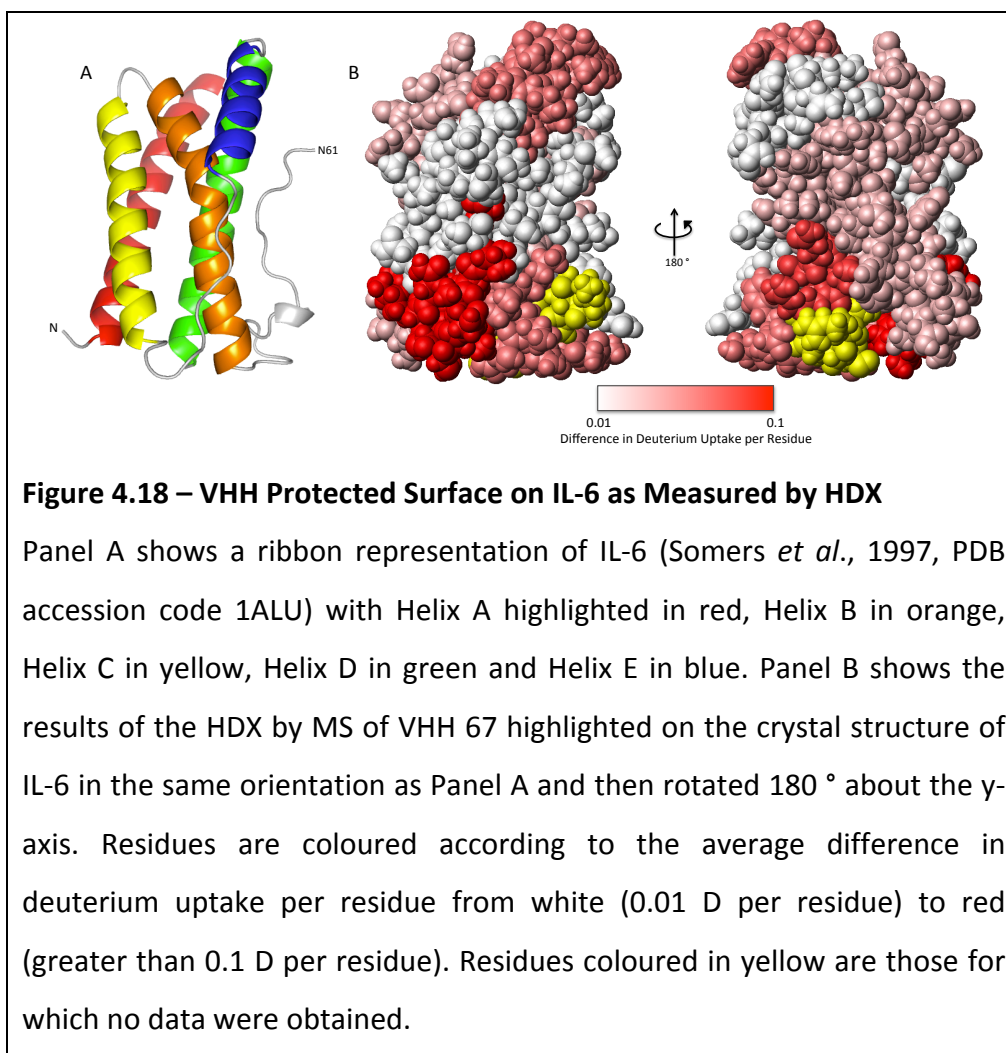
The backbone amide minimal shift results were then mapped onto the structure of IL-6 (Somers *et al.*, 1997, PDB accession code 1ALU) and are shown in Figure 4.16. The minimal shift mapping results of VHH 67 onto IL-6 show significant shifts of residues at the base of IL-6. This does not correspond to residues at Site I, Site II or Site III.



4.3.6 – Mapping of VHH 67 Epitope on IL-6 using HDX by MS

HDX by MS was carried out for free IL-6 and for IL-6 with VHH 67 bound. The difference in deuterium uptake between the two samples is shown in Figure 4.17. In general, there is little difference between the deuterium uptake of the free and the bound forms of IL-6 and, unlike VHH 18, no obvious regions of reduced uptake are observed with the VHH bound. Plotting these results onto the structure of IL-6 (Figure 4.18) does show some changes in the deuterium uptake towards the base of IL-6 (antipodal to Site III) but the data does not provide a clear epitope like that seen for the HDX by MS results of VHH 18 onto IL-6.





4.4 – Discussion

The work described in Chapter 3 highlighted significant differences in the conformational heterogeneity of the antigen-binding regions of the anti-IL-6 VHH antibodies. One of the aims of the work described in this chapter was to map the binding sites of the VHH:IL-6 complexes and in doing so, determine the role of the flexible CDRs in binding. To achieve this NMR minimal shift mapping and HDX by MS methods were used.

In the case of VHH 18, the absence of NMR signals for six residues of CDR3 suggested CDR3 may adopt multiple conformations when free in solution. When the complex was formed with IL-6, no additional backbone amide signals were observed by NMR that could be associated with the stabilisation of the flexible

loop, implying that CDR3 exists in multiple conformations even when bound to IL-6. This may mean that CDR3 of VHH 18 is not involved in the binding of IL-6, although as the residues either side of the missing signals are showing larger than average minimal shift values this seems unlikely. The more likely explanation is that the plastic nature of IL-6 (Xu *et al.*, 1996) allows continued exchange between conformations when these CDR3 residues are at the binding interface (Addis *et al.*, 2014). The paratope mapping of the VHH binding site onto IL-6 by NMR minimal shift suggested that the interaction site between VHH 18 and IL-6 is through all three CDRs with some involvement from the fourth loop located in the antigen binding region. Unfortunately, HDX by MS mapping of the paratope was not successful as digestion of the VHH did not yield suitable fragments to give a good coverage of the protein sequence. The mapping of the IL-6 binding site showed that VHH 18 binds to the same site that would be occupied by gp-130 D3 in the IL-6 signalling hexamer (Site II) which is in agreement with the results of previously reported ELISA blocking assays and cell based bioassay (Hancock, 2010 and summarised in Section 1.5). The epitope on IL-6 was fairly well defined by NMR minimal shift mapping but a more localised result was seen when using the HDX by MS method. This difference may be due to inherent differences in the way the two methods work. NMR is sensitive to any subtle changes in the chemical environment of residues upon binding and these may induce long range effects through the protein structure whereas HDX by MS is dependent on the exposure of specific amide protons to D₂O with little effect on neighbouring residues.

Minimal shift mapping of the paratope of VHH 67 identified residues of CDR2 and CDR3 as having the largest differences between the free and bound form. No additional backbone amide signals that could correspond to the missing CDR1 signals were observed. Small but significant minimal shift values were obtained for the residues either side of the missing CDR1 residues, implying that CDR1 is likely to be involved in binding but, as with VHH 18, the residues of CDR1 are still exchange broadened because CDR1 itself remains in multiple conformations when bound to IL-6 or due the dynamic nature of IL-6. The NMR epitope mapping of the interaction site on IL-6 suggested VHH 67 binds towards the base of IL-6, remote

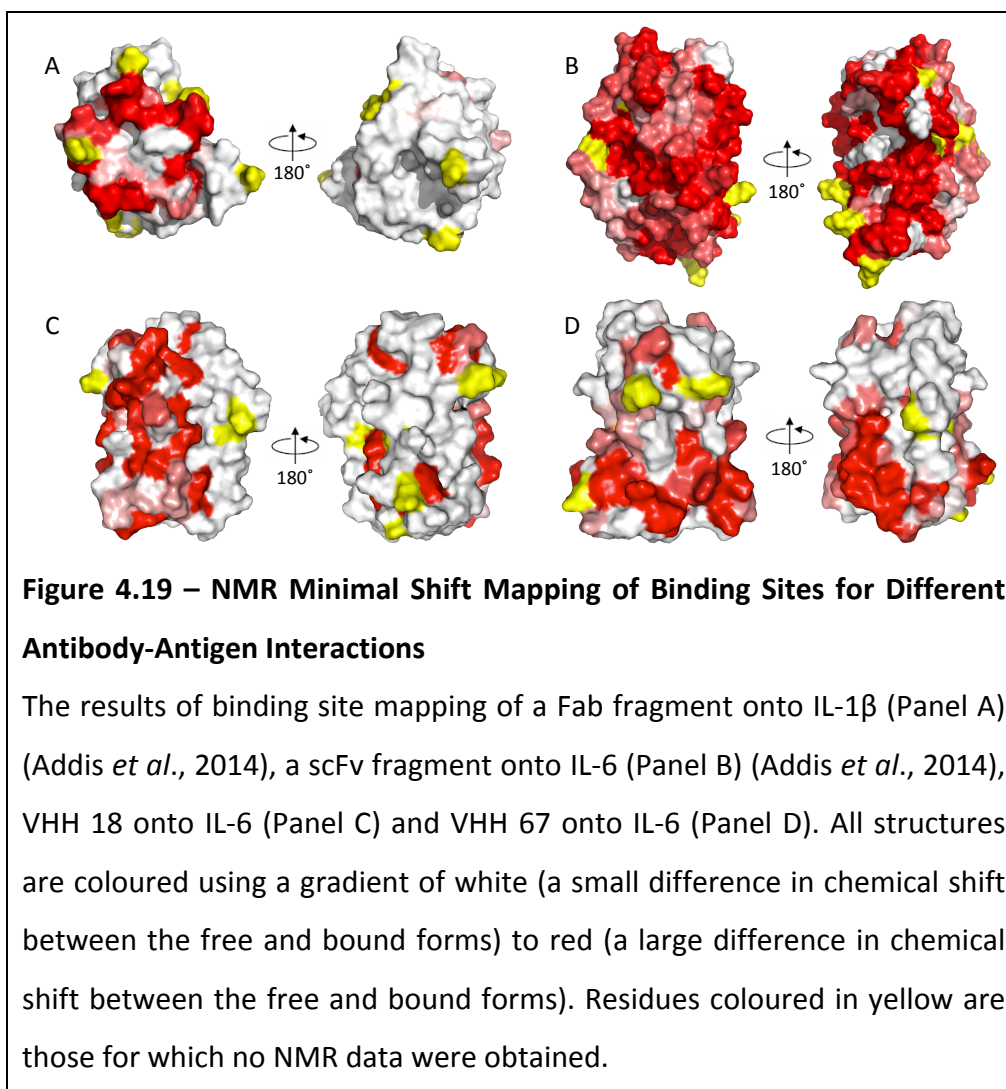
from Sites I, II and III, implying a novel binding site may have been identified. This would fit with the biological data collected for VHH 67 in which no blocking was seen at either Site II or Site III and that the formation of the signalling hexamer was not inhibited by the presence of VHH 67 (Hancock, 2010 and summarised in Section 1.5). HDX by MS showed very small differences in the deuterium exchange rates across the entire structure of IL-6 making it difficult to identify a precise binding site.

This work has shown that paratope and epitope mapping are possible using VHHs in the same way that has been reported for larger antibody fragments. The applicability of each method appears to be dependent on the binding characteristics, for example, NMR minimal shift mapping worked well for both the VHH 18:IL-6 and VHH 67:IL-6 complexes whereas using HDX by MS to map the binding sites on IL-6 was only successful for the VHH 18:IL-6 complex. The reason for the limited success with HDX by MS mapping may be due to the position of VHH binding to IL-6. The Site II interface, where VHH 18 binds, is made up of residues from Helix A and Helix C (Xu *et al.*, 1996, Somers *et al.*, 1997). By contrast, VHH 67 appears to bind at the base of IL-6 interacting with all four of the helices that make up the four helix bundle. Conformational changes brought about at the base of IL-6 by the binding of VHH 67 may result in subtle changes to the positions of all the helices, altering the solvent exposure of the amide protons across the entire protein, whereas, the effects of VHH 18 interacting with just Helix A and Helix C will be more localised.

The observation that one CDR of both VHH 18 and VHH 67 appears to be in conformational exchange in the free and bound forms agrees with work published by Addis *et al.* describing conformational heterogeneity in antibody binding (Addis *et al.*, 2014). The work describes the NMR analysis of two antibody-antigen systems: a Fab fragment binding to IL-1 β and a scFv fragment binding to IL-6. Despite very different target antigens, IL-1 β being a β -sheet protein and IL-6 being α -helical, signals corresponding to the backbone amide groups of CDR3 in the VH and VL domains were absent from the NMR spectra of the free Fab and the free

scFv. It was suggested that this conformational heterogeneity allows the initiation of binding, increasing both the likelihood of binding and the on rate. All the VHH antibodies studied in Chapter 3 show conformational heterogeneity in the free form and this remains the case in the bound form for the two VHHs analysed in this chapter. This work provides evidence of an additional type of antibody fragment exhibiting the same conformational heterogeneity. Interestingly for VHH 67, CDR1 rather than CDR3 is assumed to be adopting multiple conformations, suggesting that the soft capture mechanism is not exclusive to CDR3.

The quality of the epitope mapping results acquired with the Fab, scFv and VHH fragments does vary as illustrated in Figure 4.19. The epitope mapping of the Fab fragment onto IL-1 β highlights a discrete region to which the Fab is thought to bind (Addis *et al.*, 2014). In contrast, when mapping the epitope of the scFv onto IL-6, chemical shift differences are seen throughout the protein making it difficult to identify the region of binding (Addis *et al.*, 2014). The differences observed between the Fab and scFv results were assumed to be a result of the inherent plasticity of IL-6 rather than due to the different antibody formats. The mapping of the binding sites on IL-6 using the VHH fragments shows more defined regions of perturbed residues than was observed with the scFv, even when binding to the dynamic IL-6.



The paratope mapping by NMR for VHH 18 and VHH 67 suggests that all three of the CDR loops are involved in binding to IL-6. Analysis of the crystal structures of the VHH:IL-6 complexes described in Section 1.5 (Hancock, 2010) highlights interesting differences between the extent of CDR involvement in binding which may be related to the length of CDR3. The CDR3 loops of the five VHH:IL-6 crystal structures ranged in length from 10 residues, the same length CDR3 as VHH 18, to 20 residues, a CDR3 length one residue greater than VHH 67 (Hancock, 2010).

As described by Lo Conte *et al.*, the interface area is the “accessible surface on both partners that becomes inaccessible to solvent due the protein-protein contacts” and can be calculated by subtracting the solvent accessible surface area of the complex from the sum of the solvent accessible surfaces of the free antibody and free antigen. The interface areas calculated for the anti-IL-6 complexes are shown in Table 4.1 (Lo Conte *et al.*, 1999).

VHH	Interface Area (Å ²)
VHH 15	1767
VHH 29	1792
VHH 38	1249
VHH 56	1731
VHH 146	1173
VHH 67	1430

Table 4.1 - Interface Area of the VHH:IL-6 Complexes Calculated from the X-Ray Crystal Structures

Interface areas of the VHH:IL-6 complexes calculated from the X-ray crystal structures (Hancock, 2010) by subtracting the solvent accessible surface of the complex (using MOLMOL Koradi *et al.*, 1996) from the sum of the solvent accessible surfaces of the free VHH and free IL-6 as described by Lo Conte *et al.* (Lo Conte *et al.*, 1999). Also included in the table (highlighted in blue) is the interface area determined from the VHH67:IL-6 crystal structure as reported in Chapter 6.

The predicted interaction surface area for a conventional antibody-antigen complex is $1680 \pm 260 \text{ Å}^2$ (Lo Conte *et al.*, 1999) and the interface areas for the anti-IL-6 VHH antibodies fall within this predicted range with the exception VHH 38 and VHH 146, both of which have long CDR3 loops and bind to a cleft at Site III of IL-6. The prediction of interface area made by Lo Conte *et al.* was based predominantly on the crystal structures of conventional antibodies, with just one out of the 19 structures analysed being a VHH:antigen structure. Therefore these predictions are based mainly on antibodies where six CDR loops, three from the VH and three from the VL, can interact with the antigen. Interestingly, it appears

that VHH domains, with just three CDR loops, can form equivalent antibody-antigen interface areas.

To explore the VHH binding sites further, the VHH:IL-6 crystal structures were analysed to identify the regions of the VHH that contribute to the binding interface. The interface residues were determined by comparing the solvent accessibility of each VHH residue in the free form compared to when the VHH was bound to IL-6. Any residues for which a greater than 10 % decrease in the solvent accessible surface was seen when bound to IL-6 were considered to be at the interface. The solvent accessible surface of these interface residues were then determined to give a total interface area, the sum of the solvent accessible surface of all interface residues. From this the interface area contributed from each region of the VHH could be calculated and then the percentage contribution of each region to the interface determined. The results from this analysis are shown in Table 4.2.

		CDR Length (No. of Residues)			Total VHH Interface Area (Å ²)	VHH Interface Area (Å ²)								Percentage of Total VHH Interface Area (%)							
VHH	Site	CDR1	CDR2	CDR3		FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4		
VHH 15	III*	6	16	10	1104	0	346	0	240	279	240	0	0	31	0	22	25	22	0		
VHH 29	II	3	17	15	1075	250**	67	0	0	0	758	0	23**	6	0	0	0	71	0		
VHH 38	III	6	17	16	777	0	0	0	39	0	738	0	0	0	0	5	0	95	0		
VHH 56	III*	6	16	10	1247	0	403	0	214	250	380	0	0	32	0	17	20	30	0		
VHH 146	III	8	16	20	646	0	118	0	0	0	528	0	0	18	0	0	0	82	0		
VHH 67	-	13	17	19	917	0	15	0	295	0	606	0	0	2	0	32	0	66	0		

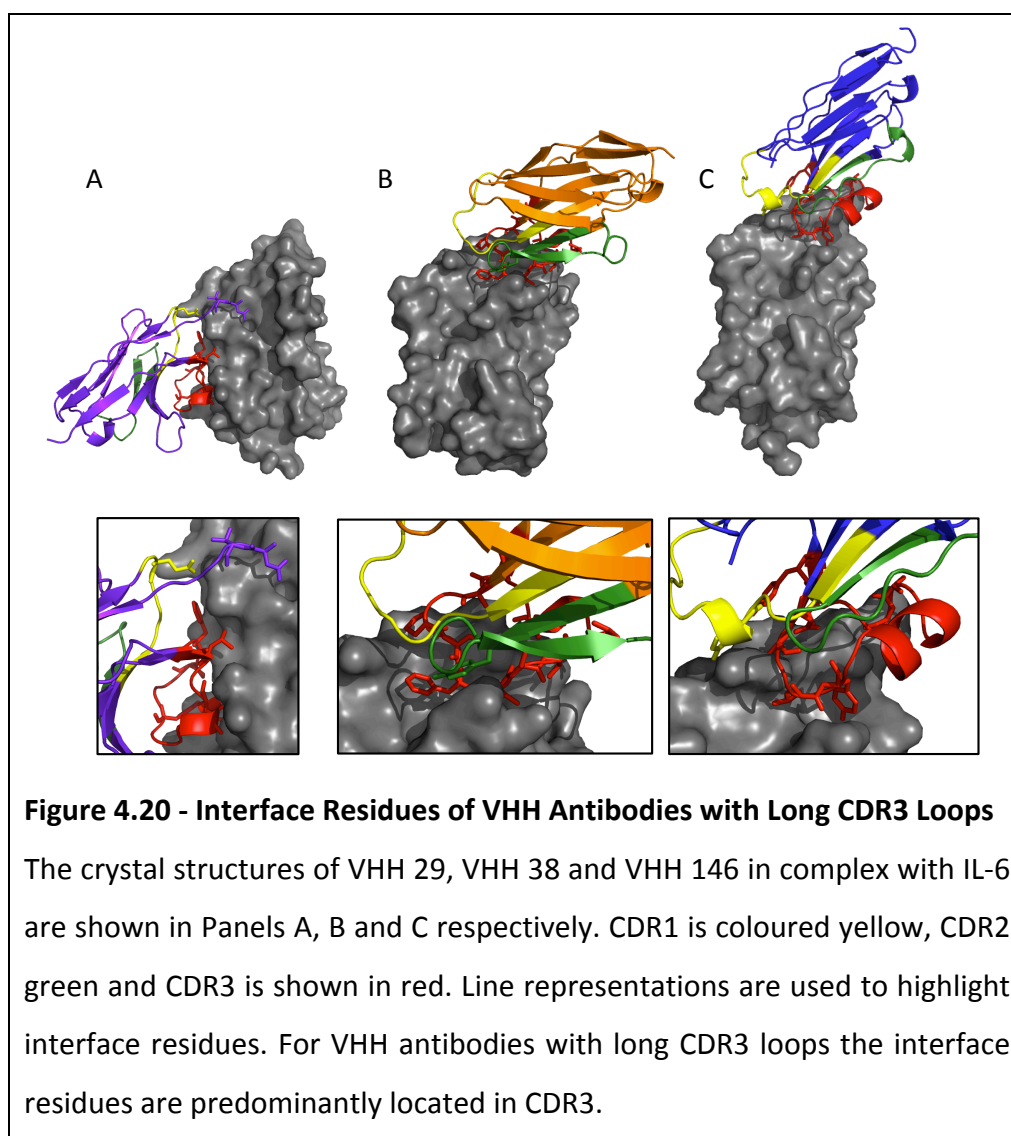
* Blocking ELISAs suggest Site III binding but crystal structure shows binding remote to Site III

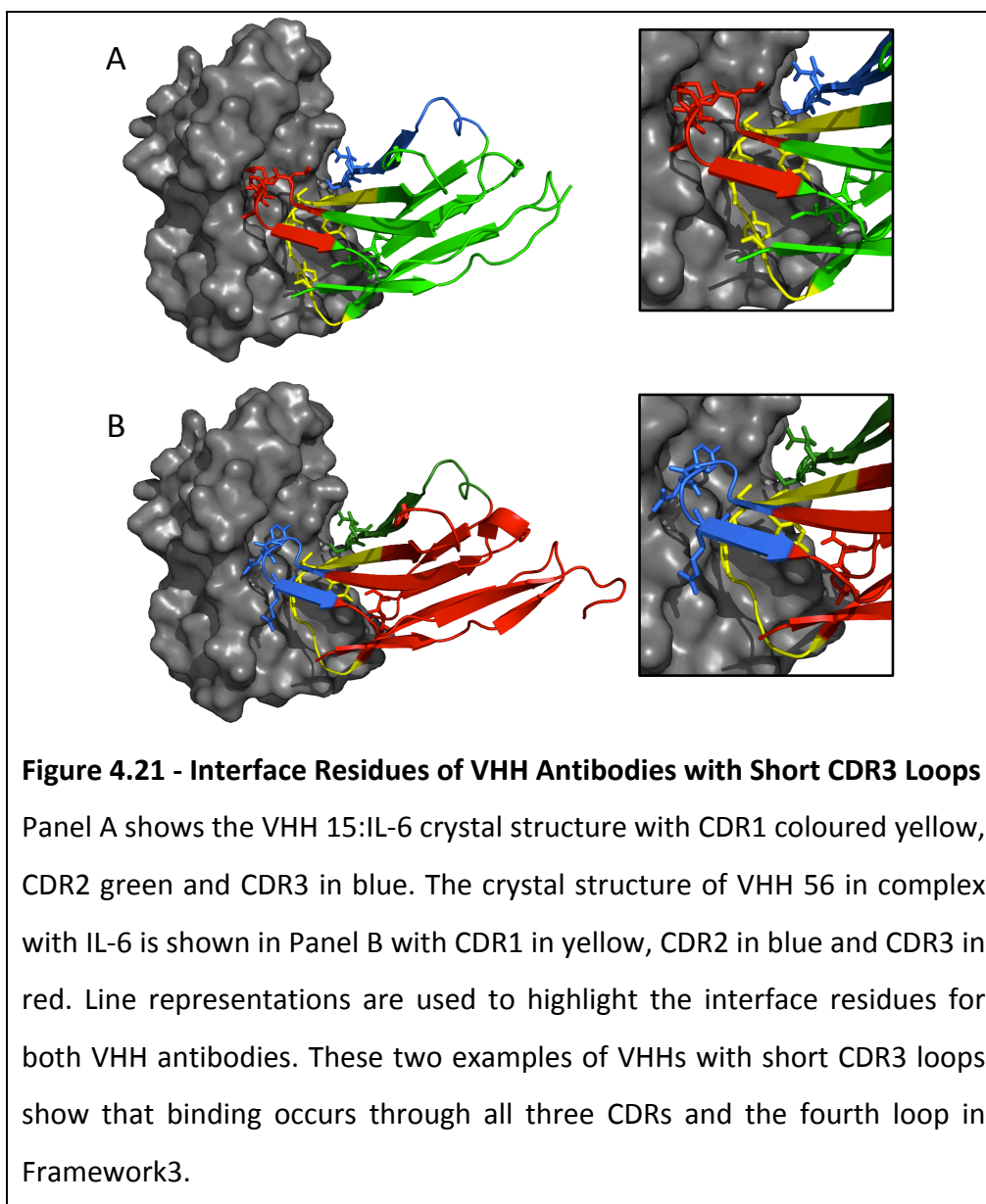
** Includes interaction through residues upstream of the mature antibody sequence

Table 4.2 – Analysis of VHH Interface Residues making up the VHH-IL-6 Binding Interface for the anti-IL-6 VHH Antibodies

Interface residues of the VHH antibodies were identified according to the difference in solvent accessible surface area in the free form compared to when bound to IL-6. The sum of the solvent accessible surface of these interface residues is termed the Total VHH Interface Area. From this, the interface area and percentage contribution for each region of the VHH were calculated. Also included in the table (highlighted in blue) are results determined from the VHH67:IL-6 crystal structure as reported in Chapter 6.

The general trend appears to be that for VHHs with elongated CDR3 loops (VHH 29, VHH 38 and VHH 146) binding occurs predominantly through CDR3 with some involvement from one of the remaining CDRs (see Figure 4.20). For those VHH antibodies with much shorter CDR3 loops, not only are all three CDRs involved in binding but the fourth loop in the Framework3 region also forms part of the VHH-IL-6 interface with all four loops contributing almost equally to the binding surface (see Figure 4.21).





The analysis of the anti-IL-6 VHH crystal structures (Hancock, 2010) has shown that for VHH domains with shorter CDR3 loops binding does not always occur predominantly through CDR3 as suggested by the literature (Muyldermans *et al.*, 2009). For the two examples of VHH domains with short CDR3 loops discussed here it appears that as the CDR3 length decreases, the contribution to binding from CDR1 and CDR2 increases along with the contribution from the framework regions, notably the fourth loop of the antigen-binding region. This implies that to compensate for the lack of the VL interactions, VHH antibodies bind through longer CDR loops or bind through framework residues in addition to the CDR loops.

Given that VHH 18 has the same length CDR3 as VHH 15 and VHH 56 we may expect residues from CDR1, CDR2 and the framework regions at the interface to compensate for the shorter CDR3. NMR minimal shift mapping identified residues in CDR1, CDR2 and Framework3 that were perturbed upon binding to IL-6. Despite signals from six of the CDR3 residues not being visible by NMR, signals corresponding to residues either side of the missing residues are perturbed. This would suggest that all three CDRs and the additional loop in Framework3 interact with IL-6. VHH 67 has a much longer CDR3 so is most comparable to VHH 146, the crystal structure of which shows that residues from CDR1 and CDR3 make up the entire interface. The NMR minimal shift mapping of VHH 67 shows a similar pattern with the most significant perturbations observed in the signals of residues in CDR2 and CDR3. The VHH 67:IL-6 crystal structure is presented in Chapter 6 and confirms that the interface is predominantly made up of residues from CDR2 and CDR3 with only one residue of CDR1 involved in binding.

The lack of published crystal structures of VHH antibodies with shorter CDR3 loops makes it difficult to test whether this relationship between CDR3 length and the involvement of framework residues in the interface holds for VHH antibodies raised against different antigens. Table 4.3 shows the same analysis as was carried out for the anti-IL-6 VHHs performed on the VHH crystal structures discussed in Section 1.2, with the exception of VHH-H14 for which only the free VHH structure was solved. For most of the VHHs (cAb-Lys3, cAb-RN05, VHH-RR6, cAb-CN05 and AMD9) binding occurs through one, two or all three CDR loops. A small number of Framework1 residues form part of the binding interface of cAb-Lys3, cAb-RN05 and AMD9 but these residues are directly adjacent to CDR1 so could be considered an extension of CDR1. AMB7 and AMD10 are interesting examples because the binding interface includes residues of CDR1, CDR2, CDR3 and framework despite having longer CDR3 loops. AMB7 and AMD10 appear to bind side on to planar sites of porcine pancreatic α -amylase (PPA) whereas AMD9 binds in a conventional manner through the antigen binding loops at the top of the VHH to a cavity of PPA. This may suggest that the involvement of framework regions is dictated by the conformation of the antigen-binding site rather than just CDR3 length.

VHH	Antigen	CDR3 Length	Percentage of Total VHH Interface Area (%)							
			FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4	
cAb-Lys3	Lysozyme	24	12*	12	0	23	0	53	0	
cAb-RN05	RNAse A	12	20*	20	0	0	0	60	0	
VHH-R6	RR6 Dye (Hapten)	17	0	27	0	46	0	27	0	
cAb-CA05	Bovine Carbonic Anhydrase	19	0	0	0	0	0	100	0	
AMB7	Porcine Pancreatic α -Amylase	19	0	8	25	17	17	33	0	
AMD9	Porcine Pancreatic α -Amylase	14	8*	16	0	38	0	38	0	
AMD10	Porcine Pancreatic α -Amylase	13	0	0	20	20	20	40	0	

* Directly adjacent to CDR1

Table 4.3 – Analysis of Residues making up the VHH-Antigen Binding Interface for Literature VHH Structures

Interface residues were identified according to the difference in solvent accessible surface area in the free form compared to when the VHH was bound to the antigen. The sum of the solvent accessible surface of these interface residues is termed the Total VHH Interface Area. From this, the interface area and percentage contribution for each region of the VHH were calculated.

4.5 - Conclusions

Studying the VHH:IL-6 complexes by NMR has identified that the dynamic CDRs reported in Chapter 3 remain dynamic when bound. This adds more evidence to the supposition by Addis *et al.*, that conformational heterogeneity may be important to efficient antibody-antigen binding.

The results of the binding site mapping for VHHs and IL-6 using minimal shift by NMR and HDX by MS showed mixed success. Paratope mapping using HDX by MS was not successful due to poor digestion of the VHH, however, paratope mapping by NMR did identify the binding site and also highlighted the continued conformational exchange of some of the CDR loops. Mapping the epitope of VHH 18 onto IL-6 was successful, with both NMR and MS identifying a defined binding area that agrees with results from the ELISA assays. The mapping of the binding site of VHH 67 onto IL-6 indicates a new binding site at the base of IL-6, antipodal to Site III.

Analysis of the VHH:IL-6 crystal structures discussed in Section 1.5 (Hancock, 2010) suggested that VHHs with elongated CDR3 loops bind predominantly through CDR3, as suggested previously (Muyldermans *et al.*, 2009), with some involvement from one other CDR. For VHHs with shorter CDR3 loops, the interface is made up of residues from all three CDRs and other framework regions, most commonly the Framework3 residues that make up the fourth loop at the antigen-binding site. VHH 18 with a short CDR3 and VHH 67 with an elongated CDR3, both fit with these findings. However, analysis of literature crystal structures suggests that this relationship may not be applicable for VHHs raised to other antigens.

In conclusion, it has been shown that VHH domains can be used in a similar manner to other antibody formats to map interaction surfaces and yield important information regarding the dynamics of the VHHs in the free and bound states. In order to help understand the impact of dynamics on the binding kinetics of VHH antibodies and antibodies in general, Chapter 5 focuses on the importance of the structured CDR3 of VHH 67.

Chapter 5 – Destabilising CDR3 of VHH 67 and the Effect on Binding to IL-6

5.1 – Introduction

The solution structure of VHH 67, presented in Chapter 3, established that the elongated CDR3 was stabilised into a single conformation in the antigen free form. The work described in Chapter 4 confirmed this stabilised CDR3 loop is likely to form part of the binding site to IL-6. Therefore, the aim of the work described in this chapter was to identify factors that may be responsible for the stabilisation of CDR3 in VHH 67 and look at the impact on binding kinetics of destabilising the structured CDR3.

As discussed in Section 3.4 (Figure 3.19), the interaction between three conserved aromatic residues, Phe139, Tyr217 and Trp220, may be responsible for stabilising CDR3. Phe139 forms part of Framework2 and is one of the characteristic VH to VHH substitutions thought to help decrease the hydrophobicity of what, in a conventional VH domain, would be the VH-VL interface. In the panel of anti-IL-6 VHH antibodies, this phenylalanine is conserved in all VHH antibodies with CDR3 lengths of 15 residues or greater and substituted with either a tyrosine, serine, histidine or valine in VHHs with shorter CDR3 loops (see Figure 5.1). This suggests that there may be a relationship between the presence of the phenylalanine and CDR3 length.

In VHH 67, Tyr217 is situated at the C-terminal of CDR3 and although the tyrosine residue is not conserved in all of the anti-IL-6 VHH antibodies, there appears to be a precedent for an aromatic residue in this position for VHHs with CDR3 lengths of 15 residues or greater (see Figure 5.1). After comparing the free NMR structure of VHH 67 with the crystal structures of the bound anti-IL-6 VHHs with long CDR3 loops, the assumption was made that Tyr217 is not directly involved in binding to IL-6. The crystal structure of VHH 67 in complex with IL-6 is presented in Chapter 6 and confirms that this assumption was correct.

Trp220 is the first residue of Framework4 and is conserved in all anti-IL-6 VHH antibodies with the exception of four VHHs in which the tryptophan is replaced by either an arginine (as in VHH 30, VHH 145 and VHH 94) or a leucine (as in VHH 26). There does not appear to be a relationship between the conservation of this tryptophan with CDR3 loop length as Trp220 is present in VHHs with CDR3 loops ranging from 7 to 20 residues (see Figure 5.1).

VHH	Framework1	CDR1	Framework2
46	QVQLVLESGGGSVQAGGSRLRLSCT-V	P G Y - - T S A S G C M A W	F R Q A P G K E R E G I A
146	DVQLVLESGGGSVQAGGSRLRLSCA-A	S G Y T D T T Y T Y C M G W	F R Q A P G K E R E A V A
2	QVQLVLESGGGSVQAGGSRLRLSCV-L	S K G - Y F V S V R C M A W	F R Q A P G K E R E G V A
67	DVQLVLESGGGSVQAGGSRLRLSCG-F	S G Y S Y T Y N Q Y Y M G W	F R Q A P G K E R E G V A
30	QVQLVLESGGGSVQAGGSRLRLSCA-A	S G Y - - T A N N Y C M G W	F R Q A P G K E R E G V A
12	QVQLVLESGGGSVQAGGSRLRLSCA-A	S G - - - E Y S S K C L G W	F R Q A P G K E R E G V A
270	EVQLVLESGGGSVQAGGSRLRLSCA-A	S G G - - L L S T Y A V G W	F R Q V P G K E C V L T T
21	DVQLVLESGGGSVQAGGSRLRLSCA-A	S G Y P Y P A N M Y C M A W	F R Q A P G K E R D W V A
63	DVQLVLESGGGSVQAGGSRLRLSCA-P	S T F - - T Y D A D C V G W	F R Q A P G K E R E G V A
103	DVQLVLESGGGSVQAGGSRLRLSCV-V	S I F - - A Y D A D C V G W	F R Q A A G L E R E G V A
145	QVQLVLESGGGSVQAGGSRLRLSCV-A	S K N - T W F S S A Y L G W	F R Q A P G K E R E G V A
3	QVQLVLESGGGSVQAGGSRLRLSCA-V	S E H - - T Y T S D C M G W	F R Q A P G K E R E R V A
40	QVQLVLESGGGSVQAGGSRLRLSCA-A	S G Y - - T Y E D C M G W	F R Q A P G K E R E A V A
66	EVQLVLESGGGSVQAGGSRLRLSCA-A	S D N - - I S S E C M G W	F R Q A P G K E R E G V A
107	QVQLVLESGGGSVQAGGSRLRLSCA-A	S G L - - T Y S T I H M G W	F R Q A P G K E R E G V A
38	QVQLVLESGGGSVQAGGSRLRLSCA-H	S G F - - T V E E N C M A W	F R Q A P G K E R E G V A
19	DVQLVLESGGGSVQAGGSRLRLSCV-A	S G L - - T V N S Y C I A W	F R Q G P G K E R E G V G
220	DVQLVLESGGGSVQAGGSRLRLSCA-V	S G Y - - Y S R Y C A G W	F R Q A P G K E R E G V A
8	QVQLVLESGGGSVQAGGSRLRLSCA-A	- E F - - T F D A D C M G W	F R Q A P G K E R E G V A
26	DVQLVLESGGGSVQAGGSRLRLSCA-V	S G F - - S F S E Y C I G W	F R Q G P G K E R E G V A
142	QVQLVLESGGGSVQAGGSRLRLSCA-A	S G Y - - T P C T Y T M S	W H R Q A P G K E R E F V S
15	QVQLVLESGGGSVQAGGSRLRLSCVAA	S G Y - - T G C T Y D M R	W Y R Q A P G K E R E F V S
22	QVQLVLESGGGSVQAGGSRLRLSCA-A	S G Y - - I G C T Y E M H	W Y R Q A P G K E R E F V S
56	QVHLVLESGGGSVQAGGSRLRLSCA-A	S G Y - - I G C T Y E T R	W Y R E A P G K D R E F V S
18	EVQLVLESGGGSVQAGGSRLRLSCA-A	S G L - - R I S E C T T G W	W Y R Q A P G K E R E L V S
94	EVQLVLESGGGSVQAGGSRLRLSCA-A	S G F - - I F S Y M A M S	W V R Q A P G K E F E W V S
97	EVQLVLESGGGSVQAGGSRLRLSCV-P	S Q Y - - T F S H C R M E	W F R Q V P G K E R E L V S

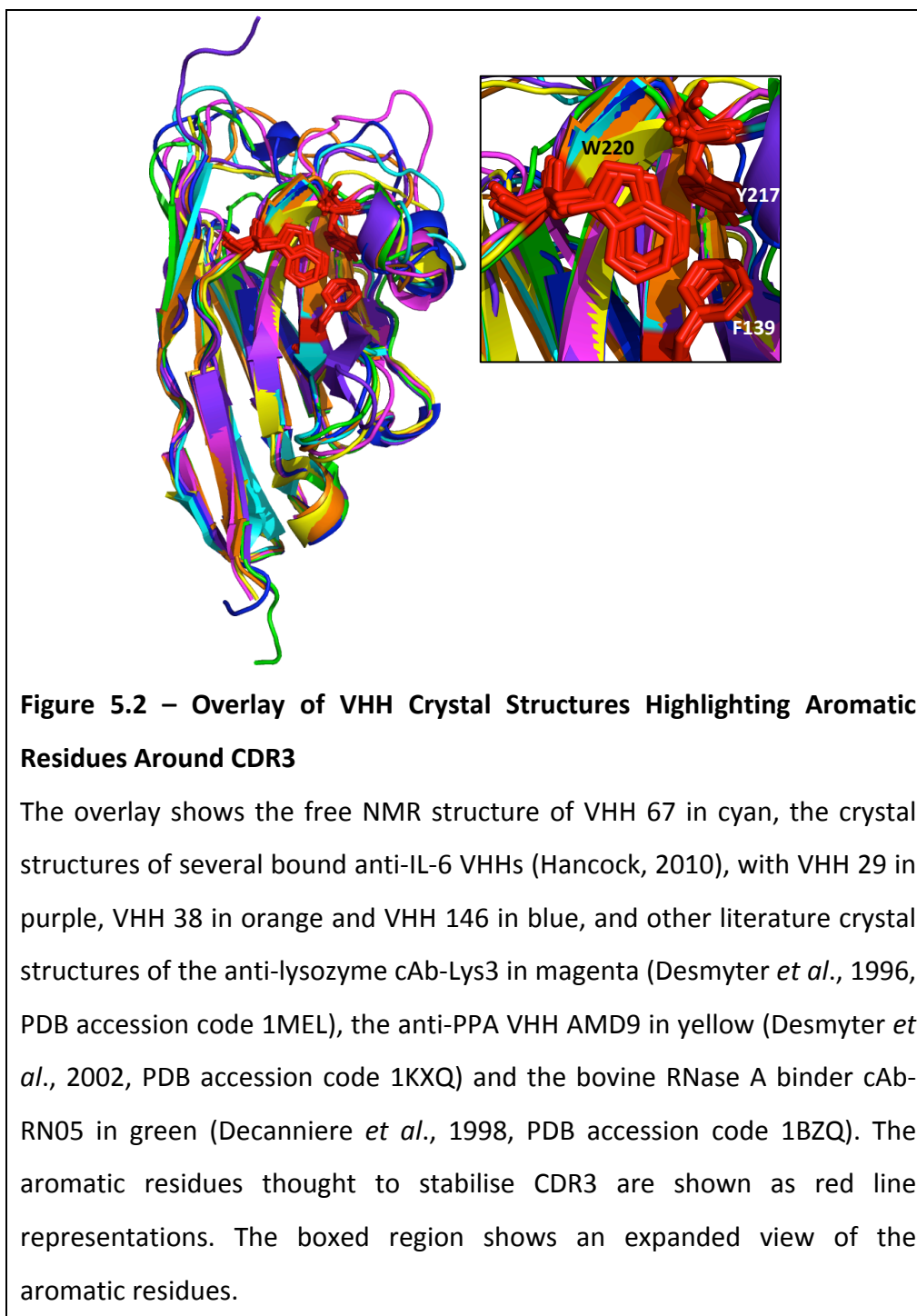
VHH	CDR2	Framework3
46	A I S S - G S F K T H Y A D S V K G	R F T I S Q D I P N D T L Y L Q M N N L E P E D T A M Y Y C A Q
146	C I D S - - G G N E L Y A D S V K G	R F T I S Q D N A K K T V Y L Q M S S L K P E D T A I Y Y C A A
2	A V S G - G G G S L Y T D S V K G	R F T I S R D N A K N T V Y L Q M S S L K P E D T A M Y Y C A A
67	S I N R - D G I R T F Y A D S V K G	R F T I S R D N A K N T V Y L Q M S S L K P E D T A I Y Y C A A
30	T I N S - G G D S T N Y A D S V K G	R F T I S R D N A K N T V Y L Q M S S L S P E D T A M Y Y C A A
12	F I N I A G G G N T Y A D S V K G	R F A I S R D N A K N M V Y L Q M N N L K P E D T A M Y Y C A E
270	S T I Q - - D G S T I Y D A S V K G	R F T I S L D N A K N M V Y L Q M S S L K P E D T A V Y Y C A A
21	N T D - - - S G G T N Y A D S V K G	R F T I S Q D R A K N T V Y L Q M N N L K P E D T A I Y Y C A A
63	H I N R - M D G T T Y A K F V K G	R F T I S R E N A K N T V Y L Q M S S L K P E D T S I Y Y C A A
103	H I N R - M D G T T Y A N F V K G	R F T I S R E N A K N T V Y L Q M S S L K P E D T A I Y Y C A A
145	S I F R - Y D D T T S Y A D S V K G	R F T I S Q V D A T N T V Y L Q M S S L K P D D T A M Y Y C A A
3	F I Y S - S G D L T Y Y D S V K G	R F T I S Q D N A K K T V Y L Q M S S L K P E D T A M Y Y C A R
40	V I H T - S S G V P Y Y A D S V K G	R F T I S Q D N A K N T V N L Q M Y S L K P E D T A M Y Y C A I
66	S I H M - - V G V E D Y A D S V K G	R F T I S Q D N V K K T V Y L Q M S S L K P E D T A I Y Y C A A
107	A F Y S - H S G G T D Y A N S V K G	R F T I S Q D L A K N T V Y L Q M S S L K V E D T A M Y Y C A A
18	R I Y T - G S G S T Y A D S V K G	R F T I S Q D N A K N T V Y L Q M S S L K P E D T A M Y Y C A A
19	F I D T - - S E T Y Y A D S V K G	R F T I S Q D N A K N T V Y L Q M S S L K P E D T A I Y Y C A A
220	V M D I - - G G D T S Y A D P V K G	R F T I S Q D N A K N M V Y L Q M S S L K P E D T A I Y Y C A A
8	V I S R - M R G T I F Y N S S V R S	R V A I S R D N A K S T V Y L Q M S S L K P E D T A M Y Y C A A
29	T I K S - G G S T W Y A D S V K G	R F D I S Q D D T K I L V Y L Q M S S L E P E D T A I Y Y C A A
26	A I G S - G G S T Y Y A D S V K G	R F T I S P D N A K N T V Y L H M N S L K P E D T A T Y Y C T T
142	S I V N - - D G T I R Y A E S V K G	R F T I S Q D N A E T T V W L Q M S S L K P E D T A M Y Y C R R
15	G I D S - - D G R A T Y A D S V K G	R F T I S Q S N A K I A V Y L Q M D S L K L E D T A M Y Y C N L
22	G I D S - - D G R T S Y V D S V K G	R F T I S Q D H A K I A V Y L Q M S S L K P E D T A M Y Y C R L
56	S I D S - - D D T T S Y A D S V K G	R F T I S Q D N A K D T V Y L Q M S A L K P E D T A M Y L C K R
18	K F S N - - L G T T W Y T G S V K G	R F T I S Q D S A K N T V Y L Q M S S L S P G D T A M Y Y C N T
94	S I G S - E S S I T S Y E D S V K G	R F T I S - D N A K N T L Y L Q M S S L K T E D T A M Y Y C T K
97	R L N N - - L G G T Y Y A D S V K G	R F T I S Q D N A K N T L Y L Q M S S L K P E D T A M Y Y C H T

VHH	CDR3	Framework4	CDR3 Length
46	N S E E I W G C G G S W Y S G Y G Y T Y W	G Q G T Q V T V S S	20
146	D S N L Y T C R F R F P A G K G A F T Y W	G Q G T Q V T V S S	20
2	A S R F D D C N S G S W S E E V A Y M S W	G R G T Q V T V S S	19
67	D R D L F P L Y - S T W S S A T R Y N Y W	G R G T Q V T V S S	19
30	A F E W S R G S - C P D L D V R D G G W	G G T Q V T V S S	18
12	R F V H F C G S G - L Y T G T D E H N W	G Q G T Q V T V S S	18
270	D S S F I L S Q - - G A S C P F P G N Y W	G Q G T Q V T V S S	18
21	V R T L F C P - - L T V T S D F K T H Y W	G Q G T Q V T V S S	18
63	R P R - - - C T N D Y T S T R N N W N Y W	G Q G T Q V T V S S	17
103	R P R - - - C D F Y Y G S S R D S W D Y W	G Q G T Q V T V S S	17
145	G L R P T W M N - - R S A N I D T G L T W	G Q G T Q V T V S S	17
3	S A R - - S N F - C T T L S V T F P W	G Q G T Q V T V S S	17
40	T G D - - Y C Y - Y P S P V K T N Y N Y W	G Q G T Q V T V S S	17
66	T T S - - T C F - Y P T L R T D G Y A Y W	G Q G T Q V T V S S	17
107	A Y G - - A D F - F T P L A P N R Y A Y W	G Q G T Q V T V S S	17
38	A T - - - P C F - V S T L I P A A Y D I W	G Q G T Q V T V S S	16
19	P R - - - R R L - C Y T L D L S N D D Y W	G Q G T Q V T V S S	16
220	N L L - - C G Y - I A T M S A H M Y N N W	G Q G T Q V T V S S	16
8	G E T C - - Y - - S D I R R A D F G W	G R G T Q V T V S S	15
29	T F T P A - C Y - - - E D S A W Y L S W	G Q G T Q V T V S S	15
26	D I A S - - - - - S C V G R K Y T L R G E T D V T V S S	G Q G T Q V T V S S	15
142	Q C V - - - - - - - Y N P G V D Y W	G Q G T Q V T V S S	10
15	Q C L - - - - - - - R Y P G E Y Y W	G Q G T Q V T V S S	10
22	T C V - - - - - - - Y N P R Q Y Y W	G Q G T Q V T V S S	10
56	Q C W - - - - - - - Y N R R Y Y W	G Q G T Q V T V S S	10
18	D L C - - - - - - - Y E N T M W	G Q G T Q V T V S S	10
94	G G K I F Y - - - - - - - D L Q Q T R	G L G T Q V T V S S	10
97	S F C - - - - - - - S - - - - - A S G W	G Q G T Q V T V S S	7

Figure 5.1 - Sequence Alignment of the anti-IL-6 VHHs

The amino acid sequences of the anti-IL-6 VHH antibodies aligned using Clustal Omega (Sievers *et al.*, 2011), is shown ordered by CDR3 length with a blue dashed line showing the cut off of 15 residues. The conserved phenylalanines in Framework2 are highlighted with an orange box; the aromatic residues in CDR3 are highlighted with black boxes; and the conserved tryptophans in Framework4 are highlighted with green boxes.

The conservation of these three aromatic residues is also seen in VHH antibodies raised to other proteins including the anti-lysozyme cAbLys3 that has a CDR3 length of 24 residues, the anti-PPA VHH AMD9 that has a CDR3 length of 14 residues and the bovine RNase A binder cAb-RN05 with a CDR3 length of 12 residues. An overlay of cAb-Lys3, AMD9, cAb-RN05 and the anti-IL-6 VHH structures is shown in Figure 5.2.



In order to test the hypothesis that the interaction between Phe139, Tyr217 and Trp220 is responsible for the stabilisation of CDR3 via the formation of an additional region of framework, a series of mutations to VHH 67, in which the aromatic residues were substituted by alanine, were prepared. The mutants were then analysed by surface plasmon resonance (SPR) to determine the effect of substituting the aromatic residues on the binding to IL-6. For the mutants displaying differences in binding kinetics over VHH 67 wild type (WT), NMR spectra were acquired to determine the structural changes resulting from the mutations.

5.2 – Material and Methods

5.2.1 – Construct Design for VHH 67 Mutants

Forward and reverse primers were designed to incorporate the single, double and triple mutations into both mammalian and *E.coli* expression vectors using either an Agilent QuikChange Lightning or QuikChange Lightning Multi Site-Directed Mutagenesis kits. The primers are shown in Figure 5.3. The forward and reverse primers of F139A, Y217A and W220A were designed to make single mutations. The forward and reverse primers Y217A W220A were designed to incorporate a double mutation and to allow a triple mutation the forward primer F139A was used in combination with the forward primer Y217A W220A.

F139A

Forward TAC TAT ATG GGG TGG **GCC** CGC CAG GCT CCA GG
 Reverse CC TGG AGC CTG GCG **GGC** CCA CCC CAT ATA GTA

Y217A

Forward TCA TCG GCC ACC CGA **GCT** AAC TAC TGG GGC CG
 Reverse CG GCC CCA GTA GTT **AGC** TCG GGT GGC CGA TGA

W220A

Forward ACC CGA TAT AAC TAC **GCG** GGC CGG GGG ACC
 Reverse GGT CCC CCG GCC **CGC** GTA GTT ATA TCG GGT

Y217A W220A

Forward GG TCA TCG GCC ACC CGA **GCT** AAC TAC **GCG** GGC CGG GG
 Reverse CC CCG GCC **CGC** GTA GTT **AGC** TCG GGT GGC CGA TGA CC

Figure 5.3 – Primers for VHH 67 Mutants

The figure shows the forward and reverse primers designed to incorporate the alanine residues into the VHH 67 mutants. Shown in yellow are the bases responsible for the aromatic to alanine substitutions.

5.2.2 – PCR Amplification of Mutant DNA for Single and Double Mutations

PCR reactions were carried out using the Agilent QuikChange Lightning Site-Directed Mutagenesis Kit and were performed using DNA from both the mammalian expression vectors, prepared by Dr. Laura Griffin (UCB), and the *E.coli* expression vectors, prepared as described in Chapter 2. The PCR reactions were performed on approximately 50 ng of DNA with 5 µl of 10 x QuikChange Lightning buffer, 0.75 µl of QuikSolution reagent, 1 µl of QuikChange Lightning enzyme, 125 ng of each oligonucleotide (forward and reverse), 1 µl of dNTP mix and made up to 50 µl in sterile dH₂O. Reactions were carried out under the following conditions:

95 °C	2 minutes	
95 °C	20 seconds	
60 °C	10 seconds	} 18 Cycles
68 °C	2.75 minutes	
68 °C	5 minutes	

5.2.3 – PCR Amplification of Mutant DNA for the Triple Mutation

PCR reactions were carried out using the Agilent QuikChange Lightning Multi Site-Directed Mutagenesis Kit and were again performed using DNA from both the mammalian and the *E.coli* expression vectors. The PCR reactions were performed on approximately 50 ng of DNA with 2.5 µl of 10 x QuikChange Lightning buffer, 0.75 µl of QuikSolution reagent, 1 µl of QuikChange Lightning Multi enzyme blend, 125 ng of the two forward oligonucleotides, 1 µl of dNTP mix and made up to 25 µl in sterile dH₂O. Reactions were carried out under the following conditions:

95 °C	2 minutes	} 30 Cycles
95 °C	20 seconds	
55 °C	30 seconds	
65 °C	2.75 minutes	
65 °C	5 minutes	

5.2.4 – Restriction Enzyme Digests

1 µl of *Dpn* I restriction enzyme was added to each PCR reaction and incubated at 37 °C for 10 minutes.

5.2.5 – Transformation of Digested DNA into Competent *E.coli* Cells

2 µl of the digested PCR products were transformed in to 40 µl of XL10-Gold ultracompetent cells as described in Section 2.2.7. The culture was then streaked onto LB-agar plates supplemented with either 50 µg ml⁻¹ kanamycin for the mammalian expression vector samples or 100 µg ml⁻¹ ampicillin for the *E.coli* expression vector samples and incubated overnight at 37 °C.

5.2.6 – Plasmid DNA Isolation for Sequencing

Single transformation colonies were picked into 3 ml of LB supplemented with either 50 µg ml⁻¹ kanamycin or 100 µg ml⁻¹ ampicillin, incubated and harvested as described in Section 2.2.3. DNA was isolated as per manufacturer's instructions using mini prep kits (Qiagen) and sequencing performed as described in Section 2.2.8.

5.2.7 – Mammalian Expression of VHH 67 Mutants

To determine the binding affinity of the mutants to IL-6, small-scale mammalian expression was performed and the supernatants analysed by SPR. Mammalian expression of VHH 67 WT was carried out alongside the mutants as a positive control. Transfections were carried out in HEK293 cells using the 293fectin lipid based transfection reagent, according to the manufacturer's instructions (Invitrogen). Transfected cells were grown at 37 °C on a shaking platform for 6 days. The supernatant was harvested by centrifugation (300 x g for 10 minutes) and were then filtered through a 0.2 µm filter and 0.01 % w/v sodium azide added to prevent microbial growth.

5.2.8 – Surface Plasmon Resonance Binding Analysis of VHH 67 Mutants to IL-6

To determine the binding affinity to IL-6 of the VHH 67 WT and mutants, SPR binding experiments were carried out on a Biacore 3000. A goat anti-llama antibody (Bethyl Laboratories) was immobilised onto the sensor of the SPR chip using amine coupling chemistry. After equilibration with the running buffer, HBS-EP (Biacore, GE Healthcare), the capture of the VHH antibodies was achieved by injecting the supernatants from the mammalian expression over the immobilised goat anti-llama antibody. The level of VHH capture was kept low, approximately 50 RU, to avoid mass transport limited binding. Affinity measurements were performed by titrating IL-6 over the captured VHH proteins at concentrations of 1.25 nM, 2.5 nM, 5 nM, 10 nM and 20 nM. Double referenced binding curves were analysed using the BIAevaluation software (Biacore AB). Kinetics parameters were determined using the Binding With Drifting Baseline fitting algorithm. To determine the affinity of weaker binders, IL-6 titrations of 100 nM and 200 nM were also included.

5.2.9 – *E.coli* Expression of Isotopically Labelled VHH 67 Mutants

For those mutants displaying a difference in affinity to IL-6 compared to VHH 67 WT, ¹⁵N/¹³C labelled protein for NMR structural analysis was expressed, refolded and purified as described in Sections 2.2.13 and 2.2.14.

5.2.10 – Circular Dichroism and Differential Scanning Calorimetry of VHH 67 Mutants

Circular dichroism (CD) analysis was carried out as described in Section 2.2.19 to confirm the VHH 67 mutants showed the same secondary structural features as VHH 67 WT and differential scanning calorimetry (DSC) analysis was carried out to determine the midpoint of thermal transition or unfolding temperature (T_m) of VHH 67 WT compared to the mutants. Samples were prepared in duplicate at a concentration of 0.5 mg ml^{-1} in the NMR buffer (see Section 3.2.2) with the reference cell containing NMR buffer only and analysed on a Microcal VP Capillary DSC instrument (GE Healthcare). The samples were heated from 10°C to 100°C at a rate of $60^\circ \text{C per hour}$ with a pre-scan of 15 minutes and a filtering period of 5 seconds. The T_m was calculated using the Origin 7.0 software (OriginLab), after blank subtraction and automatic baseline determination. This work was carried out with considerable assistance from Dr. Oliver Durrant (UCB).

5.2.11 – NMR Data Acquisition, Processing and Analysis for Sequence Specific Assignments of the VHH 67 Mutants

NMR spectra were acquired from 0.35 ml samples of 150 - 300 μM VHH in NMR buffer (see Section 3.2.2). ^{15}N - ^1H HSQC and ^{15}N - ^{13}C - ^1H HNCO spectra were collected as described in Section 3.2.2 to allow for actual shift analysis to be carried out between VHH 67 WT and the mutants. To allow backbone assignment of the VHH 67 mutants, ^{15}N - ^{13}C - ^1H HNCA (Kay et al., 1990) spectra with acquisition times of 25 ms for ^{15}N , 9 ms for ^{13}C and 50 ms for ^1H were collected using non-uniform sampling (NUS) over a period of 72 hours, with the datasets sparsed at 25 %. The non-uniform sampled data were reconstructed using istHMS (Hyberts *et al.*, 2012). Spectra were analysed using the Sparky package (T. D. Goddard and D. G. Kneller, SPARKY 3, University of California San Francisco). Assignments were made for the VHH 67 mutants by comparing the $\text{C}\alpha$ and amide chemical shifts from the ^{15}N - ^{13}C - ^1H HNCA spectra to the $\text{C}\alpha$ and amide chemical shifts assigned for the VHH 67 WT.

5.2.12 – Secondary Structure Prediction from NMR Chemical Shifts

The location of secondary structure elements were predicted by the Torsion Angle Likelihood from Shift method (TALOS+) (Cornilescu *et al.*, 1999, Shen *et al.*, 2009) as described in Section 3.2.4.

5.2.13 –Actual Shift Analysis of the VHH 67 Mutants

Following backbone assignment of the VHH 67 mutants actual shift analysis could be carried out by comparing the ^{15}N , ^{13}C and ^1H resonances of the mutants to those of the WT using the equation below:

$$\Delta\delta = \sqrt{((Mut \delta_{HN} - WT \delta_{HN}) \times \alpha_{HN})^2 + ((Mut \delta_N - WT \delta_N) \times \alpha_N)^2 + ((Mut \delta_{C'} - WT \delta_{C'}) \times \alpha_{C'})^2}$$

Scaling factors α_{HN} , α_N and $\alpha_{C'}$ are applied to each term to account for the difference in the spectral dispersion of the ^1H , ^{15}N and ^{13}C dimensions as described in Section 4.3.1. Residues most affected by the substitutions will be those with greatest actual shift values. ($\Delta\delta$).

5.3 – Results

5.3.1 – Cloning of VHH Mutants into Mammalian and *E.coli* Vectors

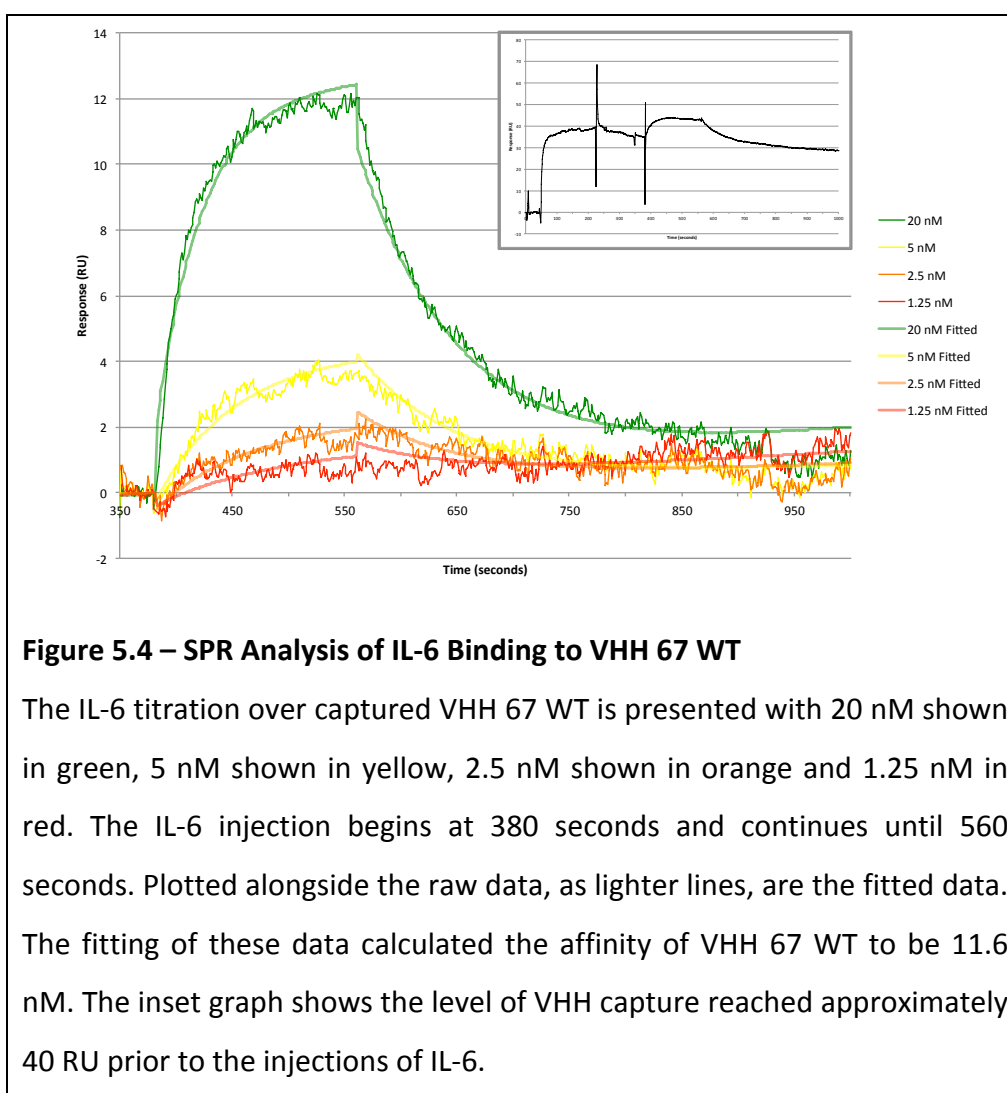
The mutant DNA was successfully cloned into both the mammalian expression vector and the *E.coli* expression vector as confirmed by DNA sequencing.

5.3.2 – Mammalian Expression of VHH 67 Mutants

Mammalian expression of the VHH 67 mutants was carried out using 7 μl of the DNA isolated as described in Section 5.2.6. Alongside the mutants, VHH 67 WT was also expressed using 1 μl of DNA as supplied by Dr. Laura Griffin (UCB). After a six-day incubation the supernatants were harvested by centrifugation and the binding to IL-6 analysed by SPR.

5.3.3 – Surface Plasmon Resonance Binding Analysis of VHH 67 Mutants to IL-6

Plotted in Figures 5.4 through to 5.7 are the results of the IL-6 titrations over the captured VHH 67 WT, VHH 67 F139A, VHH 67 Y21A and VHH 67 W220A mutants. Data has not been presented for the Y217A W220A double mutant or the F139A Y217A W220A triple mutant as no binding was observed. The results for the 10 nM samples have been removed due to poor response curves. The SPR experiments involved the capture of the VHH mutants using an anti-llama antibody. The successful capture of the mutants implies that the aromatic to alanine substitutions have not disrupted the VHH structure so much as to alter the overall VHH topology and prevent association with the anti-llama antibody.



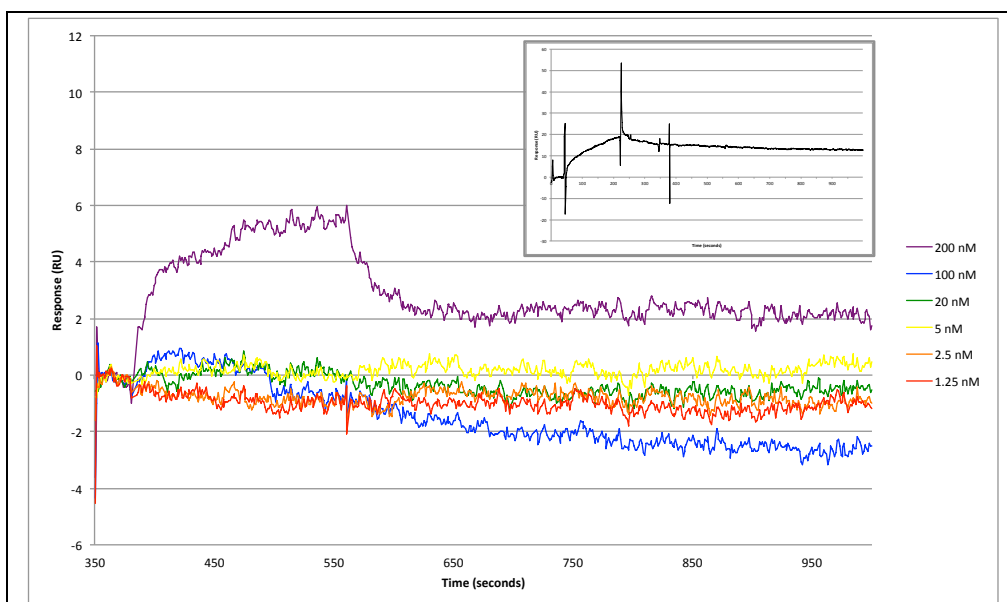


Figure 5.5 – SPR Analysis of IL-6 Binding to VHH 67 F139A

The IL-6 titration over captured VHH 67 F139A is presented with 200 nM shown in purple, 100 nM shown in blue, 20 nM shown in green, 5 nM shown in yellow, 2.5 nM shown in orange and 1.25 nM in red. The IL-6 injection begins at 380 seconds and continues until 560 seconds. The inset graph shows the level of VHH capture reached approximately 20 RU prior to the injections of IL-6. Binding was only observed at the highest concentration of IL-6.

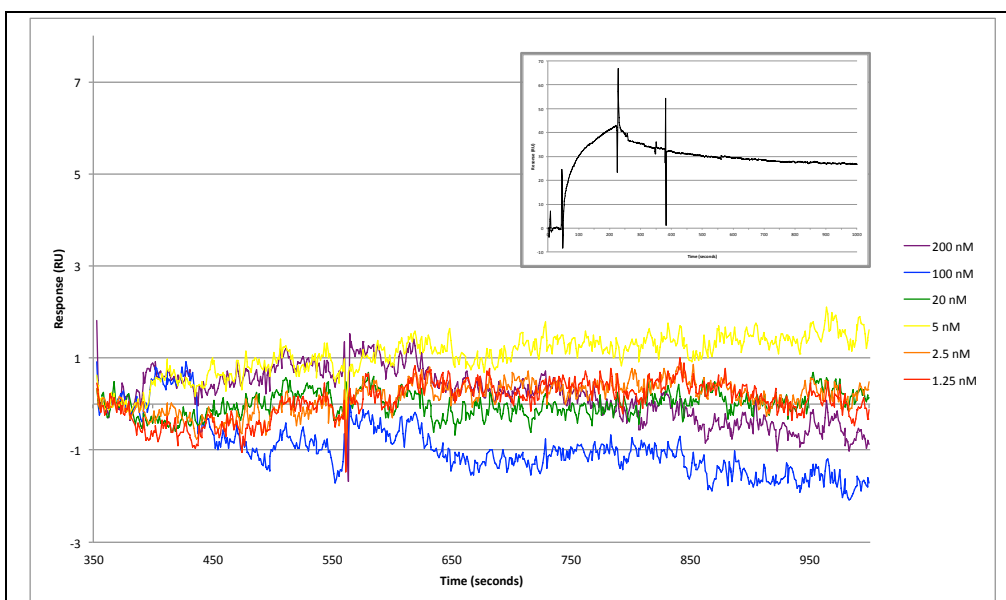


Figure 5.6 – SPR Analysis of IL-6 Binding to VHH 67 Y217A

The IL-6 titration over captured VHH 67 Y217A is presented with 200 nM shown in purple, 100 nM shown in blue, 20 nM shown in green, 5 nM shown in yellow, 2.5 nM shown in orange and 1.25 nM in red. The IL-6 injection begins at 380 seconds and continues until 560 seconds. The inset graph shows the level of VHH capture reached approximately 40 RU prior to the injections of IL-6. Binding was not observed for VHH 67 Y217A, even at the highest concentration.

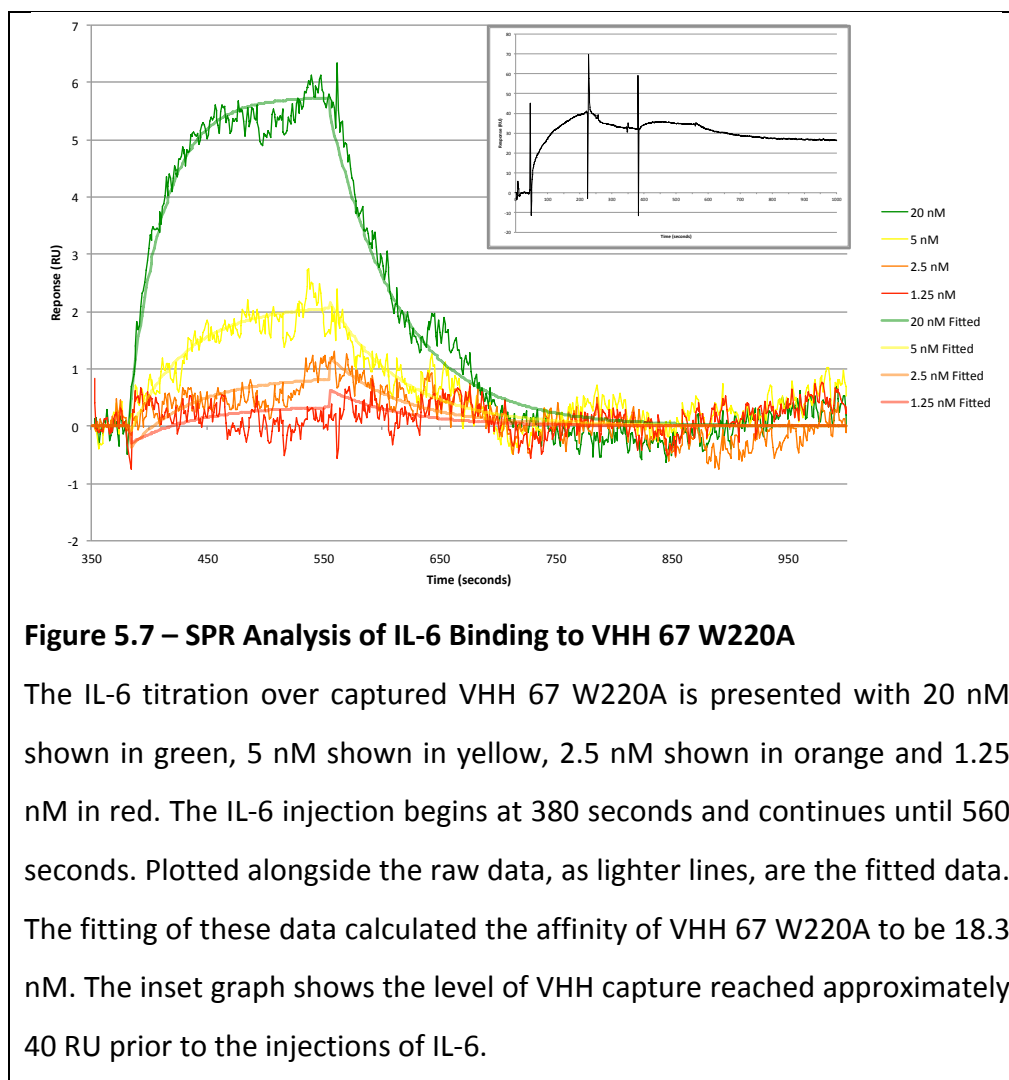


Table 5.1 shows the on rates, off rates and affinities calculated from the SPR data displayed above. The data show that the W220A substitution results in only a slight reduction in the binding of IL-6, from 12 nM to 18 nM, suggesting that this mutation does not alter the structure of VHH 67. Interestingly, analysis of the sequence alignments of the anti-IL-6 VHH antibodies suggested that the presence of this tryptophan was not related to CDR3 length, possibly indicating that it is not required for the stabilisation of the long CDR3. The F139A mutation shows a significant reduction in the affinity to IL-6 to the point that binding can only be observed with an IL-6 concentration of 200 nM. From the SPR results a K_D value of > 200 nM was estimated implying that this mutation does have a substantial impact on the structure of VHH 67. The results also suggest that the Y217A mutation has a dramatic impact on the structure of VHH 67 as no binding to IL-6 is

observed in the single mutation, double mutation or triple mutations even at the top concentration of 200 nM. Although Tyr217 forms part of CDR3, the crystal structure of VHH 67 in complex with IL-6, presented in Chapter 6, confirms that this residue does not form part of the VHH 67-IL-6 interface so the reduction in binding is not a result of the loss of a key binding residue. To investigate further the impact of these mutations on the structure of VHH 67, and in particular the conformation of CDR3, the F139A and Y217A mutants were selected for structural analysis by NMR.

	k_a ($M^{-1} s^{-1}$)		k_d (s^{-1})		K_D (nM)
	Value	Std Error	Value	Std Error	
WT	6.93×10^5	1.70×10^4	1.11×10^{-2}	1.42×10^{-4}	11.6
F139A	-	-	-	-	> 200*
Y217A	-	-	-	-	-
W220A	8.87×10^5	3.71×10^4	1.63×10^{-2}	2.73×10^{-4}	18.3
Y217A W220A	-	-	-	-	-
F139A Y217A W220A	-	-	-	-	-

* Approximate K_D value estimated from 100 nM and 200 nM IL-6 concentrations

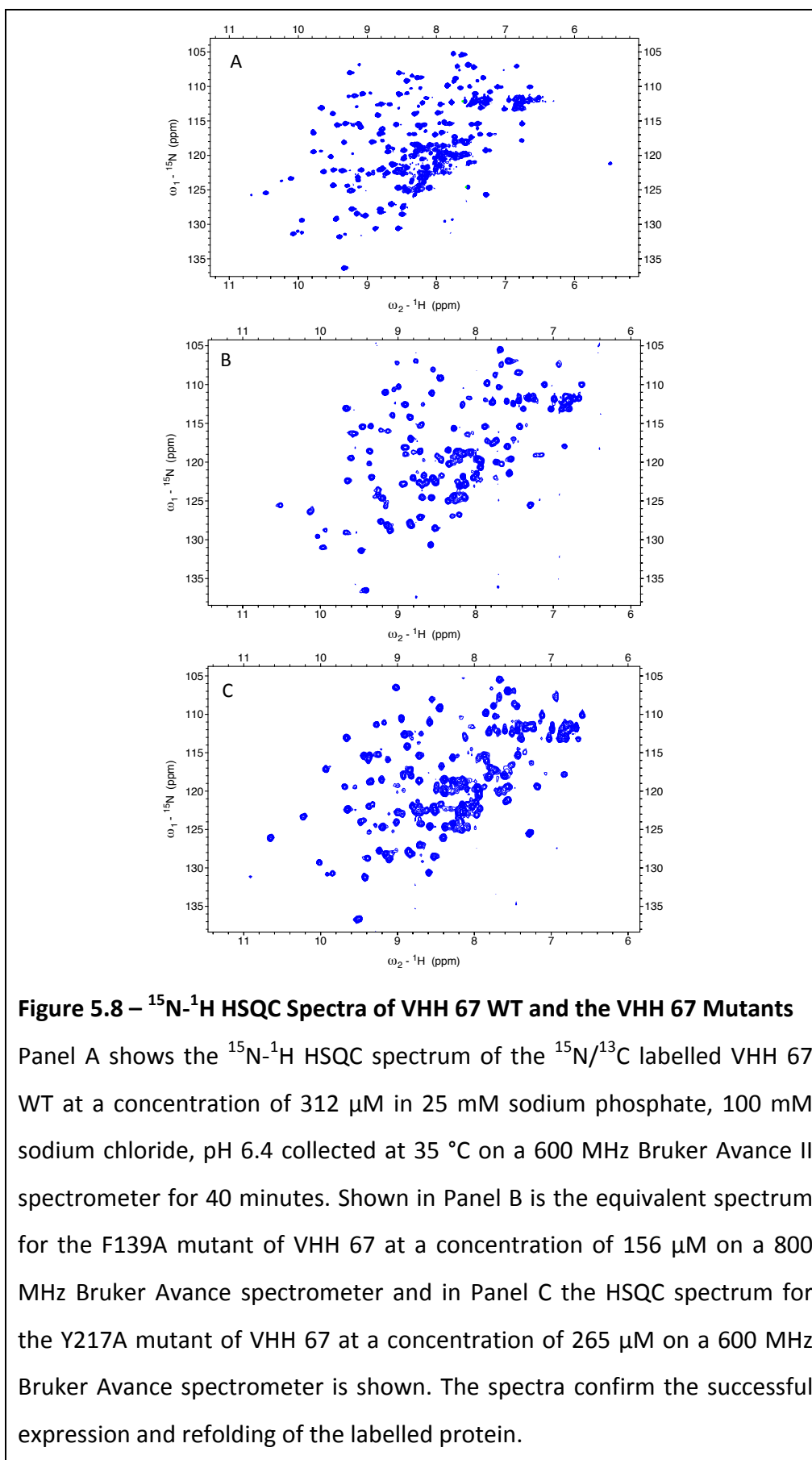
Table 5.1 – IL-6 Binding Kinetics as Measured by SPR for the VHH 67 Mutants

The results show that the W220A mutation has little impact on the binding of IL-6. However, the inclusion of the F139A or Y217A mutations in VHH 67 results in a significant drop in affinity to IL-6.

5.3.4 – *E.coli* Expression, Purification and Refolding of Isotopically Labelled VHH 67 Mutants

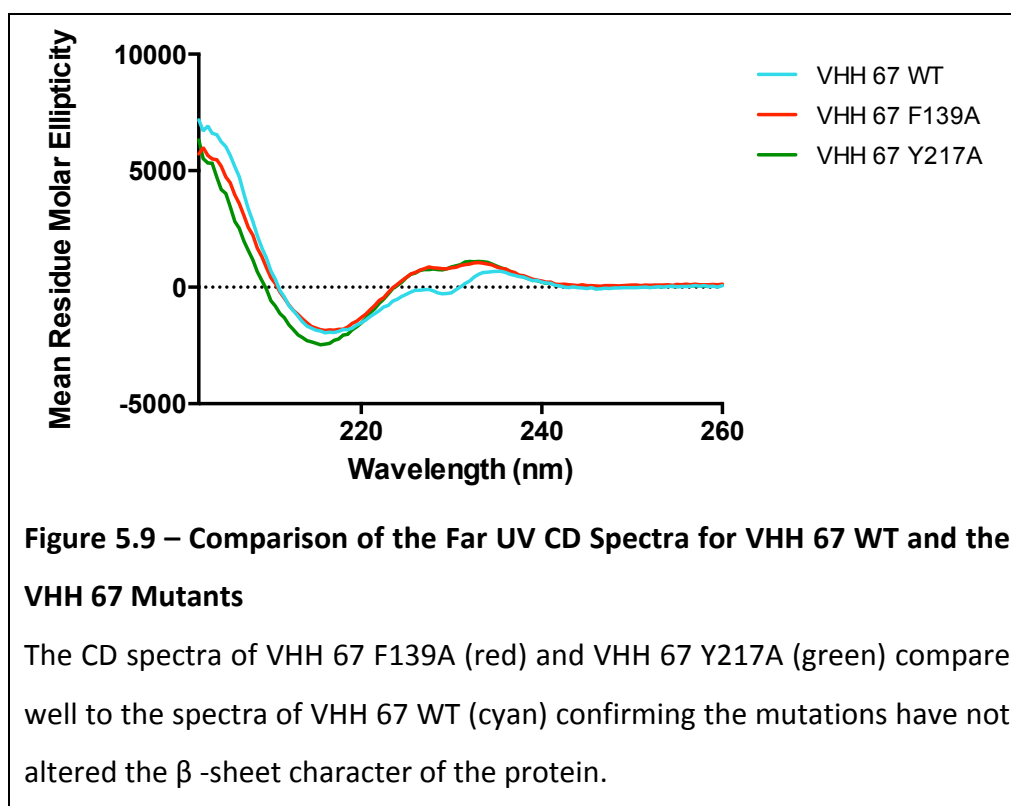
As described in Section 5.3.3, it was decided that the structures of VHH 67 F139A and VHH 67 Y217A should be investigated further by NMR. The mutants were expressed as $^{15}N/^{13}C$ labelled proteins in *E.coli*, purified and refolded as described in Sections 2.2.13 and 2.2.14. ^{15}N - 1H HSQC spectra of VHH 67 F139A (Figure 5.8 Panel B) and VHH 67 Y217A (Figure 5.8 Panel C) show many similarities to the spectra of VHH 67 WT (Figure 5.89 Panel A) suggesting that the mutants have the same overall fold as the VHH 67 WT so have been successfully expressed, purified and refolded.

Approximately 190 signals were observed in the HSQC spectrum of VHH 67 WT. Of these signals, 128 were assigned as backbone amides and 30 were assigned as side chain NH₂s or tryptophan indolic NHs. This left 30 signals unassigned which, as the majority of these signals appeared to be fairly weak, were attributed to small amounts of incorrectly folded protein. The number of peaks observed in the HSQC spectra of the VHH 67 mutants were much lower, with approximately 150 signals observed in the HSQC spectra for both VHH 67 F139A and VHH 67 Y217A.



5.3.5 – Far UV Circular Dichromism and Differential Scanning Calorimetry Analysis of VHH 67 Mutants

A comparison of the Far UV CD spectra of VHH 67 WT and the VHH 67 mutants is shown in Figure 5.9. The results confirm that the F139A and Y217A mutations have not disrupted the β -sheet character of the protein.



DSC analysis was only carried out on Y217A as insufficient purified F139A was available. Figure 5.10 displays the results of the DSC analysis confirming that, in making the tyrosine to alanine mutation, the protein remains folded. The T_m values for VHH 67 WT and VHH 67 Y217A were calculated at 69 °C and 61 °C respectively. This 8 °C reduction in the temperature of unfolding suggests that the structure of the mutant is significantly different to that of the WT protein so could indicate destabilisation of CDR3.

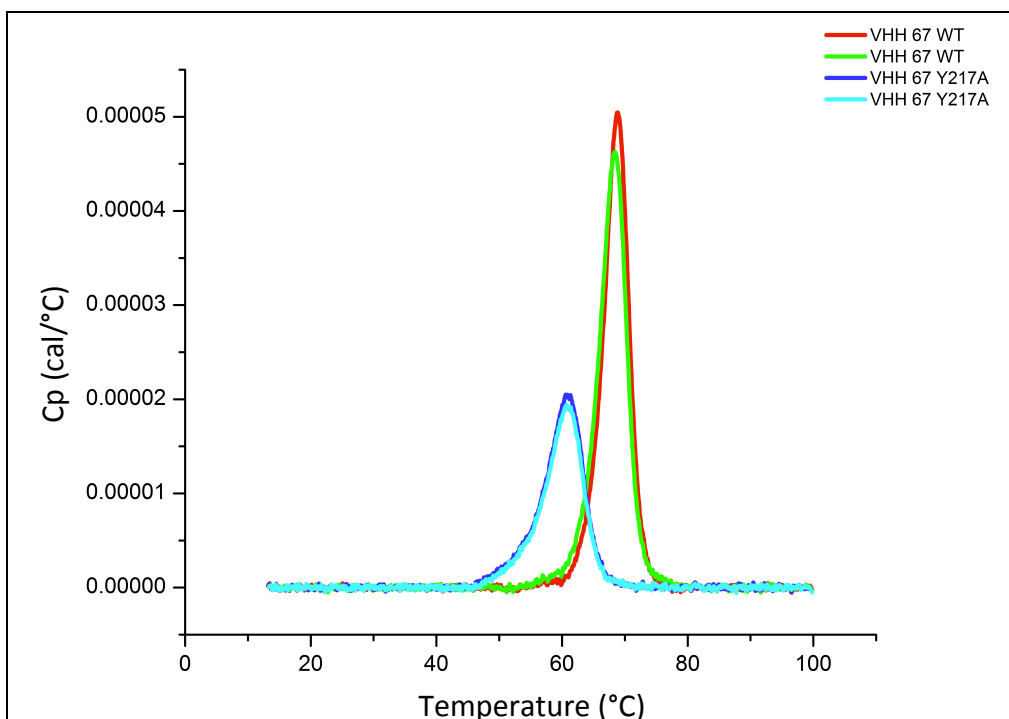


Figure 5.10 – Comparison of DSC Spectra of VHH 67 WT and VHH 67 Y217A

The graph shows the heat capacity (C_p) of VHH 67 WT (red and green) and VHH 67 Y217A (blue and cyan) plotted as a function of temperature. A reduction of 8 °C in the midpoint of unfolding (T_m) is observed between VHH 67 WT and VHH 67 Y217A suggesting the mutant protein is significantly different to the WT protein.

5.3.6 – Sequence Specific Assignments for VHH 67 F139A and Y217A

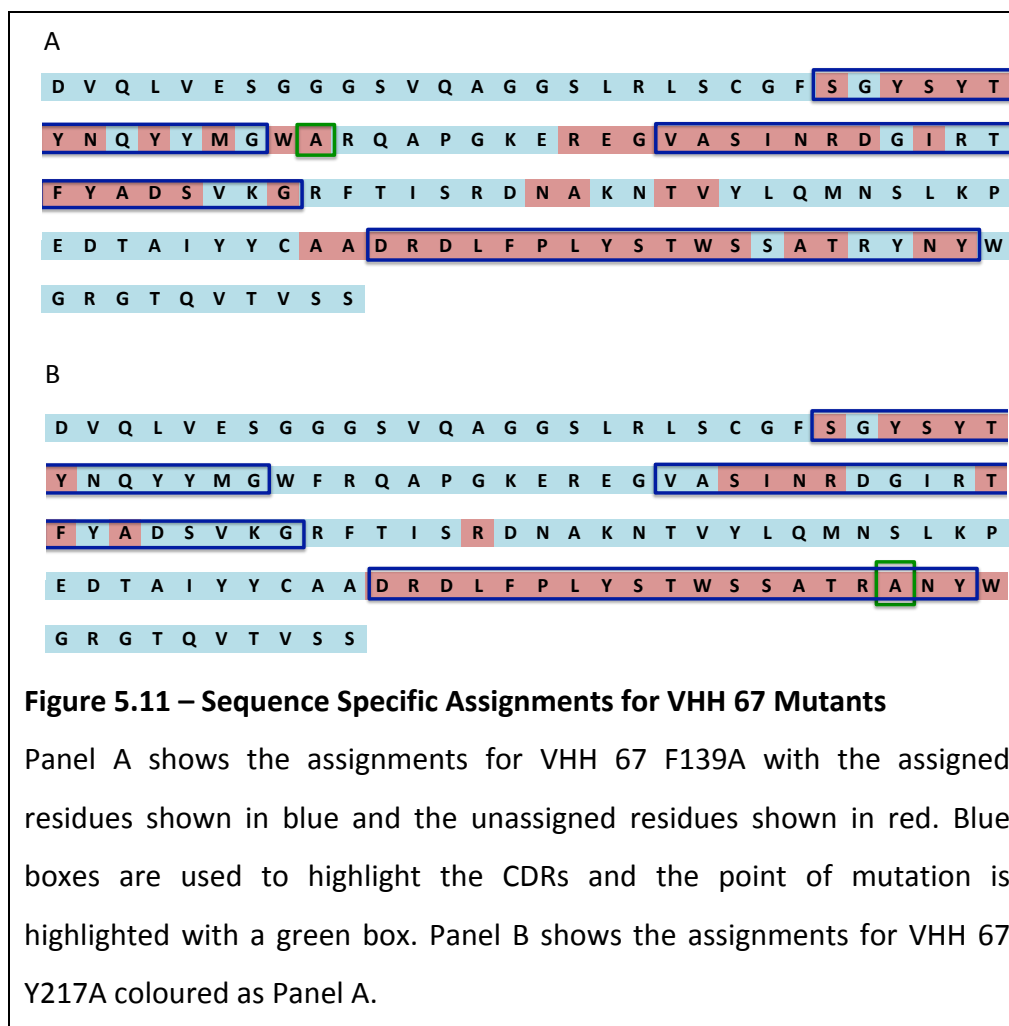
As a consequence of a significant shift in many of the backbone amide signals of the mutant proteins compared to the WT protein, a combination of ^{15}N - ^1H HSQC, ^{15}N - ^{13}C - ^1H HNCO and ^{15}N - ^{13}C - ^1H HNCA spectra were used to obtain the sequence specific backbone amide assignments of VHH 67 F139A and VHH 67 Y217A.

Sequence specific backbone amide assignments were obtained for 62 % of the residues in F139A. The backbone amide assignments for Ser125, Tyr127 to Asn132, Tyr134, Met136, Trp138 to Ala139, Arg147 to Asp156, Ile158, Phe161 to Ser165, Gly168, Asn176 to Ala177, Thr180 to Val181, Ala199 to Ser212, Ala214 to Thr215 and Asn218 to Tyr219 were not obtained. This is a substantial reduction compared

with the 96 % that were assigned in the VHH 67 WT (Section 3.3.1.2). A similar result was observed with Y217A with only 75 % of residues being assigned. The backbone amide assignments for Ser125, Tyr127 to Tyr131, Ser152 to Arg155, Thr160 to Phe161, Ala163, Arg174, Asp203 to Trp220 were not obtained. Figure 5.11 shows that, as a result of both the F139A and the Y217A mutations, the majority of signals corresponding to residues in CDR3 are no longer visible. Also missing from F139A is a large proportion of the CDR2 signals.

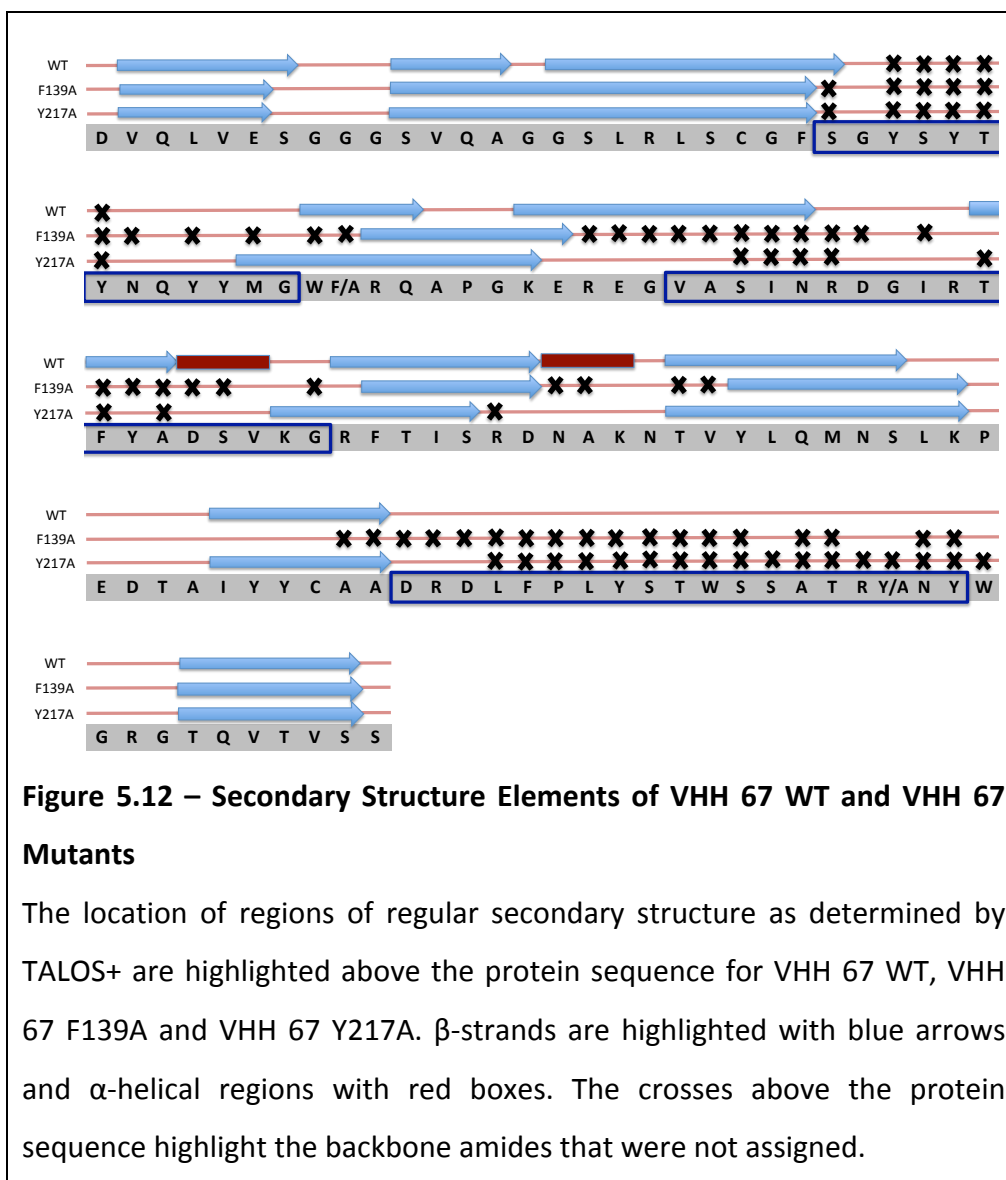
As detailed in Section 5.3.4, approximately 150 signals were observed in the HSQC spectrum of VHH 67 F139A and of these, 92 signals were assigned as backbone amides leaving approximately 60 signals unassigned. Assuming that around 30 of these signals will correspond to side chain NH₂s or tryptophan indolic NHs, as was seen with VHH 67 WT, this leaves around 30 unassigned backbone amide signals. As is highlighted in Figure 5.11 Panel A, 50 amino acids remain unassigned, therefore, around 20 backbone amide signals are absent from the HSQC spectrum.

The corresponding analysis of the HSQC spectrum of VHH 67 Y217A shows that of approximately 150 peaks observed, 104 were assigned as backbone amide signals and around 30 can be assumed to arise from side chain NH groups leaving approximately 15 unassigned backbone amide signals for the 34 amino acids left unassigned (see Figure 5.11 Panel B). Therefore, as with VHH 67 F139A, around 20 backbone amide signals are absent from the HSQC spectrum.



5.3.7 – Secondary Structure Prediction for VHH 67 F139A and Y217A

The locations of secondary structure elements for VHH 67 mutants, as determined from the chemical shift data by the program TALOS+ (Cornilescu *et al.*, 1999 and Shen *et al.*, 2009), are shown in Figure 5.12 alongside the results for VHH 67 WT. The results show that, where the signals remain visible, a similar pattern of secondary structure between the WT and the mutants is predicted. This suggests that the mutations have not disrupted the predominantly β -sheet character of the protein, a finding supported by the CD data.



5.3.8 – Comparison of the Chemical Shift Assignments for VHH 67 F139A and Y217A

Actual shift mapping was used to investigate the effects of the aromatic to alanine mutations on the structure of VHH 67. Figure 5.13 shows the HSQC spectrum of VHH 67 WT overlaid with the HSQC spectra of the two mutants and highlights the chemical shift differences observed for some amide groups between the WT and the mutants.

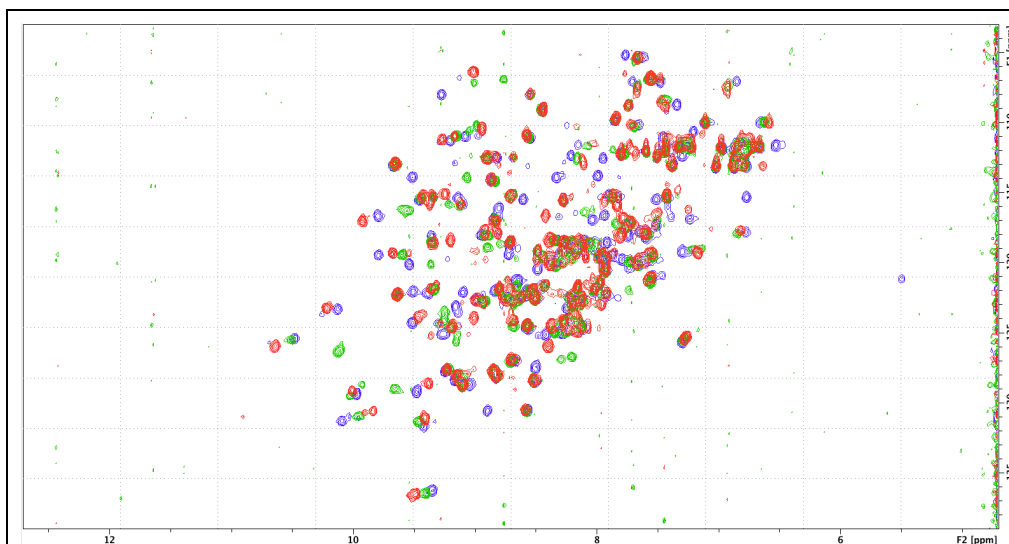
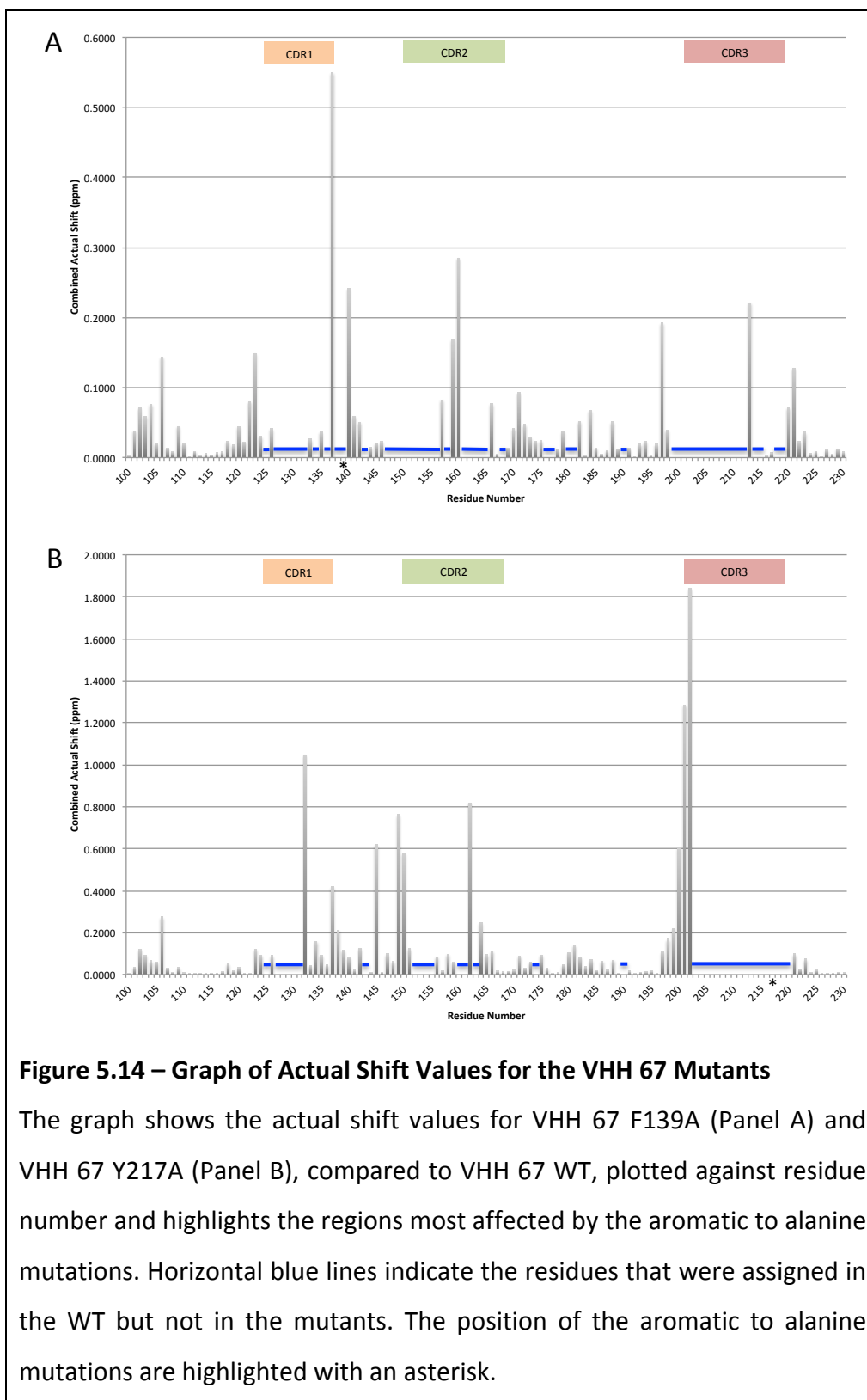


Figure 5.13 – Overlay of the ^{15}N - ^1H HSQC Spectra of VHH 67 WT and the Mutants

The figure shows the ^{15}N - ^1H HSQC spectra for $^{15}\text{N}/^{13}\text{C}$ labelled VHH 67 WT in blue, VHH 67 F139A in green and VHH 67 Y217A in red. Spectra were collected at concentrations between 156 μM and 312 μM in 25 mM sodium phosphate, 100 mM sodium chloride, pH 6.4 at 35 $^{\circ}\text{C}$ on either a 600 MHz Bruker Avance spectrometer or a 800 MHz Bruker Avance II spectrometer for between 40 and 60 minutes.

The combined actual shift values between the mutant and WT structures were calculated, using the resonances from the ^{15}N - ^{13}C - ^1H HNCO spectra, as described in Section 5.2.13 and are shown plotted against residue number in Figure 5.14. Many residues that were assigned in VHH 67 WT are no longer observable in the NMR spectra of the mutants and are highlighted in the figure.



Plotting the actual shift results onto the solution structure of VHH 67 WT highlights the regions most affected by the mutations (shown in Figure 5.15). Although chemical shift differences are observed between residues of the WT and mutant VHHs, the number of perturbed residues is less than seen in previous mapping experiments, for example the paratope mapping of VHH 67 when bound to IL-6 as reported in Section 4.3.4. More significant than the visibly perturbed residues, are the number of residues that were assigned in the WT structure but cannot be assigned in the mutant structures, 43 residues for F139A and 25 for Y217A. This is most likely due to exchange broadening of the peaks resulting in the signals disappearing into the baseline of the spectra. The residues affected (shown in green in Figure 5.15 Panel B and Panel C) are predominantly located around the position of the aromatic to alanine mutations in CDR2 and CDR3.

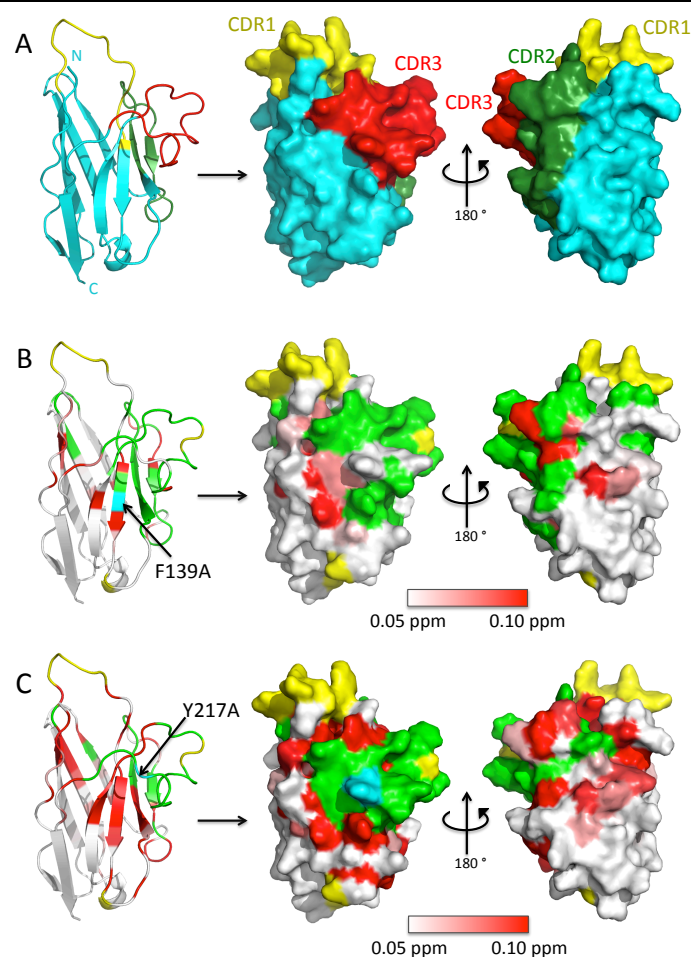


Figure 5.15 –Actual Shift Mapping of the Structural Changes Induced by the VHH 67 Mutants

Panel A shows the solution structure of VHH 67 WT coloured in cyan with CDR1, CDR2 and CDR3 highlighted in yellow, green and red respectively. The ribbon representation of VHH 67 is shown on the left, in the middle is the space filled structure in the same orientation and on the right the space filled structure rotated 180 ° around the y axis. Panel B shows the results for the actual shift mapping of VHH 67 F139A and Panel C the results for VHH 67 Y217A. The structures in Panel B and Panel C are in the same orientation as Panel A but are coloured according to the chemical shift differences using a gradient of white (shift less than 0.05 ppm) to red (shift greater than 0.1 ppm). Residues not assigned in either VHH 67 WT or the mutant are shown in yellow and residues assigned in the WT but not assigned in the mutant are shown in green. The position of the aromatic to alanine mutation is shown in cyan.

5.4 – Discussion

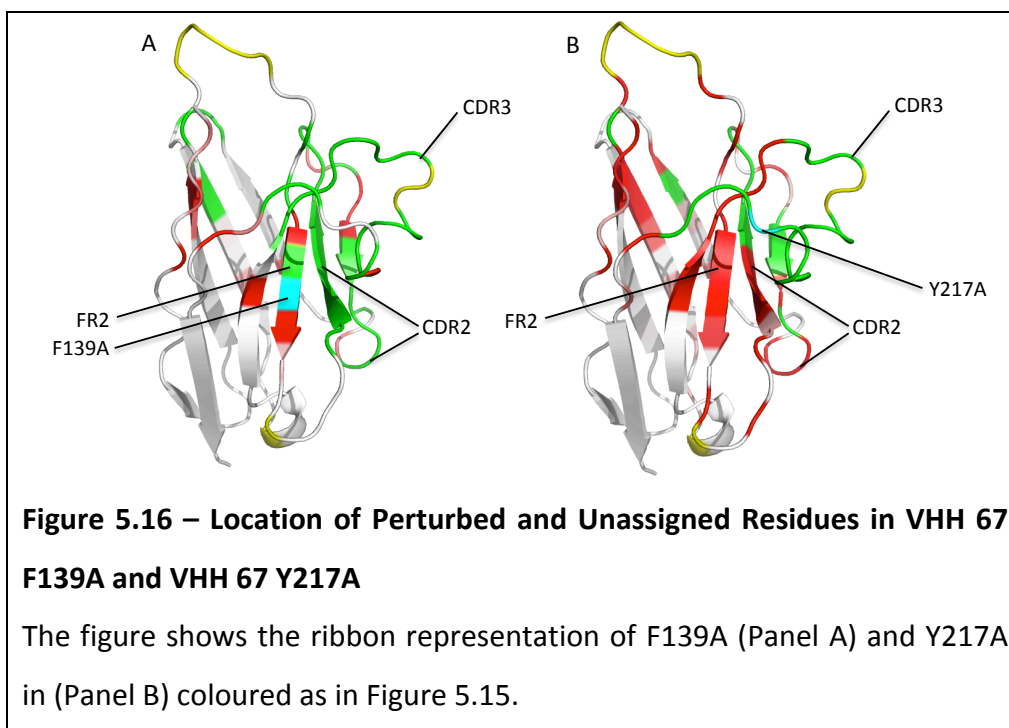
The aim of the work described in this chapter was to understand the molecular basis of the conformational stability of the CDR3 loop of VHH 67. A comparison of the solution structure of VHH 67 with the other anti-IL-6 VHH crystal structures and literature VHH structures identified an interaction between three conserved aromatic residues located in and around CDR3 that appeared to stabilise CDR3. Single, double and triple aromatic to alanine mutations were incorporated into both the mammalian and *E.coli* expression vectors to allow analysis by SPR, DSC and NMR.

The SPR experiments revealed a reduction in IL-6 affinity for VHH 67 F139A ($K_D > 200$ nM) and VHH 67 Y217A ($K_D \gg 200$ nM) compared to the WT (K_D 12 nM) but no significant change in affinity was observed with VHH 67 W220A (K_D 18 nM). DSC analysis of Y217A showed a significant reduction in the T_m compared to WT suggesting a change in the structure and the NMR analysis of F139A and Y217A indicated that the mutations did not appear to disrupt the predominantly β -sheet character of the protein but that many of the residues of Framework2, CDR2 and CDR3 that were assigned in the WT were no longer visible in the mutants.

Actual shift analysis of the mutants compared to the WT was carried out to identify the regions of the VHH in which chemical shift perturbations were observed. Combining these results with the location of the unassigned residues gives a strong indication that these mutations have destabilised the structure of CDR3 and this in turn has had an impact on residues in the vicinity of CDR3.

Although the result of both the F139A and the Y217A substitutions appears to destabilise the CDR3 loop, the affect on affinity and the nature of the perturbations observed by NMR for the two mutants do differ considerably. The proportion of assigned residues in VHH 67 F139A (62 %) was lower than that of VHH 67 Y217A (75 %). Highlighting the unassigned residues on the structure of VHH 67 (Figure 5.16) shows that many residues equivalent to those unassigned in F139A are in fact visible in Y217A but are significantly perturbed, especially in

Framework2 and CDR2. If the F139A and Y217A mutations have destabilised CDR3 and resulted in the loop being more dynamic, we would expect the residues in Framework2 and CDR2 to be perturbed as the chemical environment would have been altered and this appears to be the result observed with Y217A. The fact that in F139A the signals for these residues are not observed suggests that they are themselves now dynamic or, more likely, in contact with the dynamic CDR3 some of the time and hence the signals will be exchange broadened. Therefore, a conclusion that could be made from these results is that the Y217A substitution results in CDR3 being destabilised and in a conformation, or conformations, that do not contact Framework2 and CDR2 whereas the F139A substitution also results in the destabilisation of CDR3 but the conformations sampled by the dynamic CDR3 includes a conformation, or conformations, that make contact with Framework2 and CDR2, similar to the original conformation.



Presented in Figure 5.17 are the proposed results of each of the three mutations. When Trp220 is substituted for an alanine, the interaction between Phe139 of Framework2 and Tyr217 of CDR3 remains. The comparable affinity to IL-6 observed for VHH 67 W220A (K_D 18 nM) and for VHH 67 WT (K_D 12 nM) implies that this interaction is sufficiently strong to maintain the CDR3 loop in the structured, binding conformation.

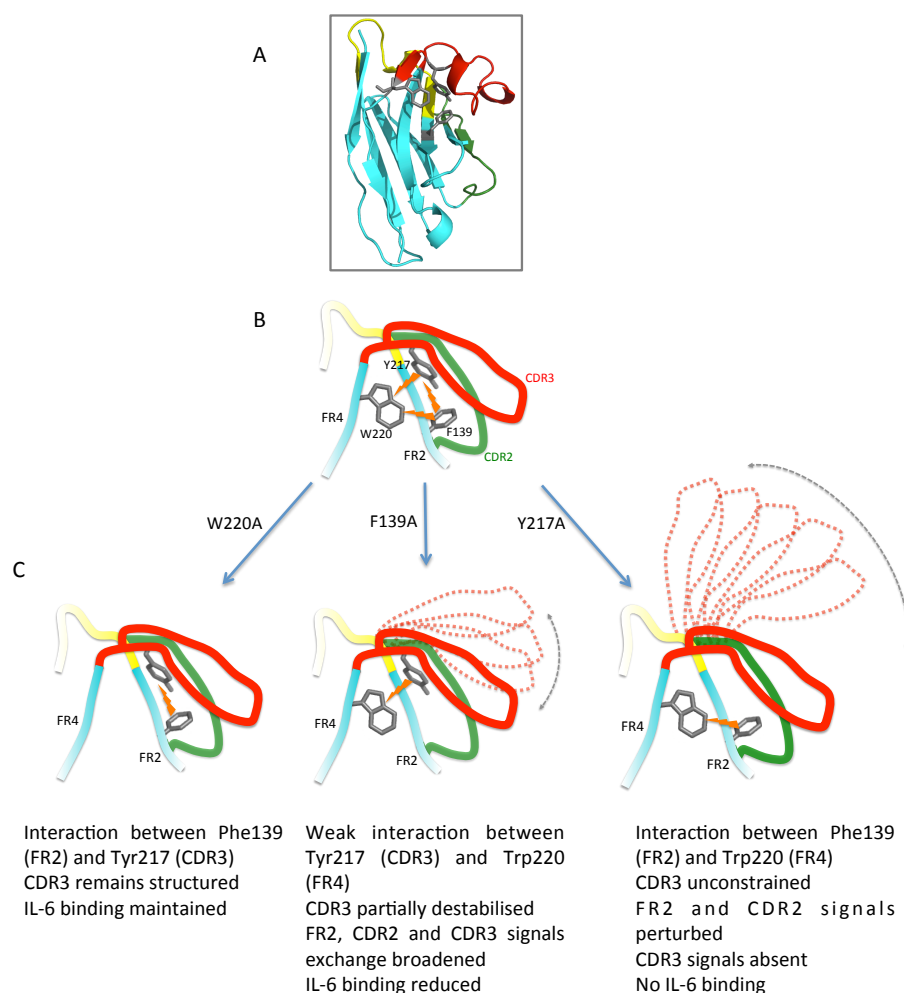


Figure 5.17 - Proposed Changes in CDR3 Structure and Dynamics Resulting from the Aromatic to Alanine Mutations

A ribbon representation of VHH 67 WT is shown in Panel A with CDR1 highlighted in yellow, CDR2 in green and CDR3 in red. The aromatic residues Phe139, Tyr217 and Trp220 are shown as grey line representations. A schematic showing the interaction between Phe139 of Framework2, Tyr217 of CDR3 and Trp220 of Framework4 is shown in Panel B, coloured as in Panel A. Panel C shows each of the mutations made to VHH 67 and the proposed changes to the structure of CDR3 as a result of the mutations.

The substitution of Phe139 in Framework2 resulted in a significant reduction in the affinity to IL-6 compared to VHH 67 WT ($K_D > 200$ nM compared to K_D 12 nM) and also resulted in the loss of backbone amide signals corresponding to residues in FR2, CDR2 and CDR3 when analysed by NMR. The proposed structural changes for the F139A mutation are shown in Figure 5.17. CDR3 may be partially destabilised due to the absence of Phe139 but if the interaction between Tyr217 and Trp220 remains, CDR3 may be somewhat restricted and only able to adopt a limited number of conformations. If one of these conformations is the conformation observed in VHH 67 WT, with CDR3 folded against Framework2 and CDR2, it is likely that backbone amide signals for residues in these regions would be exchange broadened and may even be absent from the NMR spectrum. This increased flexibility would also result in an entropic penalty having to be paid upon binding which could explain the decreased affinity to IL-6.

When Tyr217 is substituted the resulting NMR spectrum and the affinity to IL-6 show significant differences to the results obtained with VHH 67 F139A. These may be rationalised by a difference in the conformations adopted by CDR3 in VHH 67 Y217A as presented in Figure 5.17. Without the interaction between Tyr217 and either Phe139 or Trp220, the CDR3 loop is likely to be more flexible than was observed in VHH 67 F139A and VHH 67 W220A. This increase in entropy may explain the absence of binding to IL-6. In contrast to VHH 67 F139A, residues in Framework2 and CDR2 of VHH 67 Y217A were visible by NMR but were significantly perturbed. This implies that any increase in the flexibility or conformational heterogeneity in these regions does not result in exchange broadening of NMR signals but does suggest that the chemical environment of these residues has altered from VHH 67 WT, most likely due to CDR3 no longer being in contact with these regions.

5.5 – Conclusions

The mutagenesis studies undertaken in this chapter have shown that the substitution of the aromatic residues Phe139 and Tyr217 from VHH 67 has a considerable impact on the dynamics and conformational stability of the structured CDR3. Both mutations appear to destabilise CDR3 making it more dynamic and resulting in exchange broadening that ultimately leads to the loss of signals from the NMR spectra of VHH 67 F139A and VHH 67 Y217A. The conformations adopted by the newly liberated CDR3 appear to differ between the two mutants as seen by the effect on binding to IL-6 and by NMR analysis.

The structured CDR3 of VHH 67 may be a unique example of where the static character of the loop is critical to antigen binding. This structured CDR3 in a free antibody is not common either in the anti-IL-6 VHHs (as described in Section 3.4) or in other conventional antibody derived Fabs and scFvs as discussed in Section 4.4 where flexibility is thought to be critical to allow soft capture of an antibody by its antigen (Addis *et al.*, 2014). The work in Chapter 6 investigates the binding between VHH 67 and IL-6 in an attempt to gain more understanding into the mechanism of binding.

Chapter 6 – Functional Effects of VHH 67 on IL-6 Signalling

6.1 – Introduction

The work described in Chapter 3 identified that, unlike other anti-IL-6 VHHs, VHH 67 appears to have an entirely structured CDR3. Chapter 5 highlighted that in making this CDR3 more dynamic a significant reduction in binding to IL-6 is observed. The NMR minimal shift analysis and the hydrogen deuterium exchange (HDX) as monitored by (MS) analysis discussed in Chapter 4 both pointed towards VHH 67 binding to IL-6 at a novel binding site at the base of the four helix bundle. This observation fits with results from the ELISA and bioassay data, described in Section 1.5, that could not positively identify VHH 67 as a Site II or Site III binder and showed VHH 67 did not inhibit the formation of the IL-6 signalling complex (Hancock, 2010). The aim of the work described in this chapter was to carry out further experiments, utilising X-ray crystallography and surface plasmon resonance (SPR), to help understand the binding of VHH 67 to IL-6 in an attempt to explain some of the unique features displayed by VHH 67.

6.2 – Material and Methods

6.2.1 – ELISA and Cell Based Assays

As part of the work carried out by Dr Laura Griffin (UCB), a full characterisation of the purified VHH proteins was undertaken as summarised Section 1.5 (Hancock, 2010). This included Site II and Site III blocking ELISA assays to determine the site of interaction and a cell-based assay used to assess the ability of each VHH to inhibit IL-6 signalling.

6.2.2 – X-Ray Crystallography

6.2.2.1 – Complex Formation for X-Ray Crystallography

To prepare the complex sample for X-ray crystallography, a 5 % excess of VHH 67 was mixed with IL-6 and incubated at 25 °C for 2 hours. The sample was then purified by gel filtration chromatography to remove any free VHH. A Superdex 200 16/60 column (GE Healthcare) was equilibrated at 1.6 ml min⁻¹ with 50 mM sodium

chloride, 25 mM Tris, pH 7.5. The complexed sample was loaded onto the column at a flow rate of 1 ml min⁻¹ and 2 ml fractions were collected between 50 and 100 minutes. Fractions were analysed by analytical gel filtration and the appropriate fractions pooled. The concentration of the pooled fractions were determined by absorbance at 280 nm and then concentrated, using a 10,000 Da MWCO Millipore Amicon Ultra 15 ml centrifugal filter unit to a concentration of 20 mg ml⁻¹. The final purity of the complex was then confirmed by analytical gel filtration.

6.2.2.2 – Crystallisation Trials

This work was carried out with considerable assistance from Dr. Gareth Hall (University of Leicester) who kindly collected and processed the diffraction data and carried out the refinement of the structure.

Crystallisation trials were set up using the MorpheusTM, PACT, JCSG+, MIDAS, and Clear Strategy 1 screens (Molecular Dimensions), utilising the Cartesian Honeybee 8+1 micro-dispensing system. The protein to precipitant ratio was mixed at 1:1, to a final volume of 0.2 µl, using MRC crystallisation 96-well sitting drop vapour diffusion plates (Molecular Dimensions). Crystal trays were set up at 19 °C, with crystals being observed in the MorpheusTM screen after only 2 days, in a condition containing 25 % PEG 3350, 25 % PEG 1000, 25 % MPD, 0.1 M MOPS/HEPES buffer solution, pH 7.5, and 0.12 M ethylene glycol solution. Optimisation of this condition was carried out by varying the precipitant, pH and ethylene glycol concentrations, and by keeping the incubation temperature at 19 °C. Fewer, larger crystals were observed in lower precipitant concentrations, whereas increasing the salt concentration also resulted in fewer larger crystals, but these were visually more disordered. The optimisation did not show any significant improvements over the initial conditions so the final crystallisation conditions remained as 25 % PEG 3350, 25 % PEG 1000, 25 % MPD, 0.1 M MOPS/HEPES buffer solution, pH 7.5, and 0.12 M ethylene glycol solution.

6.2.2.3 – Crystal Diffraction and Data Collection

Diffraction data for the VHH 67:IL-6 crystal were collected on Beamline I03 at the Diamond Light Source synchrotron using $\lambda = 0.96863 \text{ \AA}$ and a Pilatus3 6M area detector. Fortunately, the crystallisation mother liquor did not require a cryoprotectant, allowing the crystal to be immediately flash frozen in liquid nitrogen. Initial diffraction allowed the crystal to be indexed by collecting two images separated by 90° with an exposure time of 0.2 seconds. A collection strategy was established in which 360° of data were collected, by exposing the crystal for 0.2 seconds, per 0.2° oscillation. The detector distance was set at 0.579 m, allowing the data to be collected to a maximum resolution of $1.75 \text{ \AA}$.

6.2.2.4 – Indexing, Integration and Scaling of Data

The VHH 67:IL-6 crystals that were generated under the final optimised crystallisation conditions, as detailed in Section 6.2.2.2, are shown in Figure 6.1.

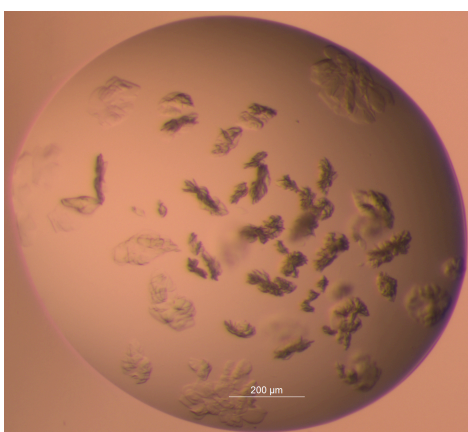


Figure 6.1 - Examples of the VHH 67:IL-6 Crystals

The figure shows the VHH 67:IL-6 crystals grown after 2 days at 19°C in 25 % PEG 3350, 25 % PEG 1000, 25 % MPD, 0.1 M MOPS/HEPES buffer solution, pH 7.5, and 0.12 M ethylene glycol solution.

XDS (Kabsch, 2010) was used to index and integrate the images collected from the VHH 67:IL-6 crystal. From the data collected a primitive orthorhombic Bravais lattice, with regularised cell dimensions, $a = 47.80 \text{ \AA}$, $b = 57.65 \text{ \AA}$ and $c = 119.83 \text{ \AA}$; and angles $\alpha = 90.0^\circ$, $\beta = 90.0^\circ$ and $\gamma = 90.0^\circ$, was determined. To compensate for fluctuations in beam intensity and the absorption of the crystal the intensities from the IL-6:VHH 67 dataset were scaled using SCALA (CCP4), using the dataset integrated in spacegroup $P2_122_1$.

6.2.2.5 – Molecular Replacement

The previously solved NMR structure for IL-6 (Xu *et al.*, 1997, PDB accession code 1IL6,) was used for molecular replacement. A homology model of VHH 67 was made using Swissmodel (Guex *et al.*, 2009) based on the solved VHH structure (Loris *et al.*, 2003, PDB accession code 1MVF). A Matthews coefficient calculation suggested that the most likely solution contained one IL-6 molecule and one VHH 67 molecule in the asymmetric unit, giving a solvent content of 44.3 %. PHASER (McCoy *et al.*, 2005) was applied to execute the molecular replacement, setting it to search for one copy of IL-6 and one copy of VHH 67, in the spacegroup $P2_122_1$. PHASER found a single molecular replacement solution in $P2_122_1$ giving a z-score of 14.1 and a log likelihood gain (LLG) score of 86.77.

6.2.2.6 – Model Building and Refinement

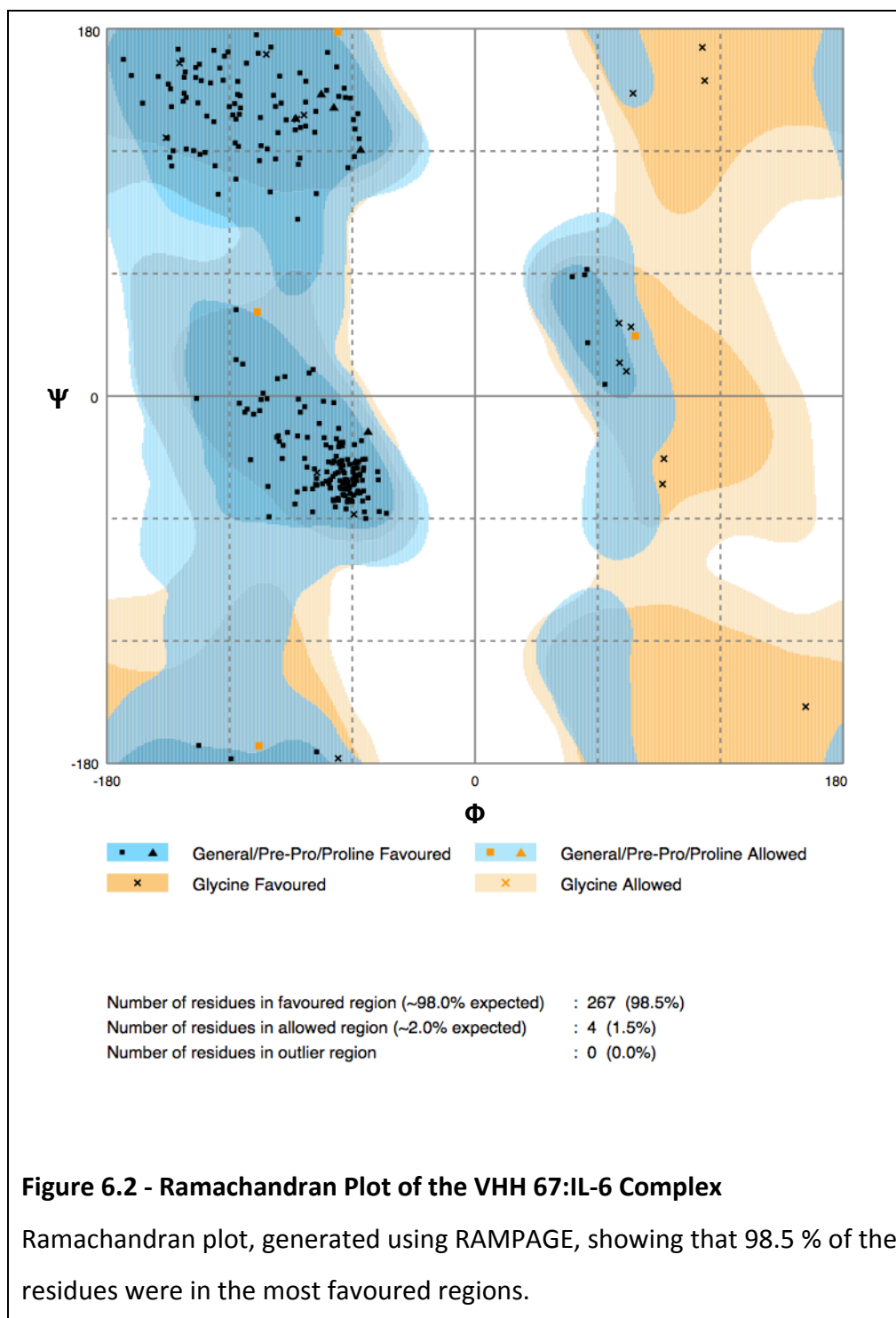
The solution from PHASER was loaded into Coot (Emsley and Cowtan, 2004) to allow manual model building to be carried out. The electron density map for the IL-6 molecule correlated well with the molecular replacement model, with the exception of residues Asn49 to Asn61 in the region between Helix A and Helix B; and Asp135 to Thr139 in the region between Helix C and Helix E; which were disordered and subsequently not modelled. Sufficient electron density was also unavailable to model fifteen N-terminal residues of the VHH 67, incorporating the His₆-tag and TEV cleavage site, and as such these residues were omitted from the structure. In addition, significant structural refinement was required for residues Tyr127 to Tyr134, Arg155 to Ile158 and Arg202 to Arg226, representing CDR1, CDR2 and CDR3, respectively, reflecting significant differences between the

homology model and the final structure of VHH 67. Refinement was carried out, using phenix_refine (Adams *et al.*, 2010), TLS refinement (translation/liberation/screw) and an appropriate weighting term. Model building and refinement ceased when the R_{free} value remained constant and the structure validation methods suggested no outliers.

6.2.2.7 – Structure Validation

The structure was validated throughout the model building and refinement process using both the Validate functions in Coot and the statistics outputted by phenix.refine. Refinement stopped when the R_{free} value ceased to reduce following further rounds of model building and refinement. The resulting R_{factor} and R_{free} values were 23.1 % and 28.2 %, respectively. The Comprehensive validation function in Phenix were applied to validate the final structure. The Ramachandran plot produced by RAMPAGE (Lovell *et al.*, 2002) showed that 98.5 % of residues were in the most favoured regions, with 1.5 % within the additional allowed regions. The final statistics and Ramachandran plot for the crystal structure of VHH 67:IL-6 are shown in Table 6.1 and Figure 6.2.

Crystal Data			
Space Group			P 2 ₁ 2 2 ₁
Unit-cell dimensions (Å)		Unit-cell angles (°)	
<i>a</i>	47.80	α	90.00
<i>b</i>	57.65	β	90.00
<i>c</i>	119.83	γ	90.00
Protein molecules per unit cell			2
Data-collection statistics			
Resolution (Å)			2.91
Number of measured reflections			46806
Number of unique reflections			7639
Average redundancy			6.1(5.8)
Average <i>I</i> / σ			19.5 (2.9)
Completeness (30.9-2.91Å) (%)			99.4 (98.2)
<i>R</i> _{merge}			0.05 (0.58)
Final refinement statistics			
Protein atoms			2247
Solvent atoms			0
<i>R</i> factor (28.83-2.91 Å) (<i>R</i> free)			0.231 (0.282)
R.m.s. deviations from ideal geometry			
Bond distances (Å)			0.006
Bond angles (°)			1.151
Average <i>B</i> Factors (Å ²)			
Wilson <i>B</i> -Factor			86.8
Main-chain atoms			98.0
Side-chain atoms			104.4
Solvent			n/a
Solvent Content (%)			44.3
† High-resolution shell, 3.07 - 2.91			
Table 6.1 - X-Ray Diffraction Analysis Statistics for VHH 67:IL-6 Complex			



6.2.3 – Surface Plasmon Resonance

To further investigate the effects of the presence of the VHH antibodies on the formation of the IL-6 signalling complex, SPR experiments were set up on a Biacore 3000 with considerable help from Dr. Terry Baker (UCB).

The following constructs were immobilised onto the sensor of the SPR chip using amine coupling chemistry as per manufacturer's instructions (GE Healthcare): gp-80 Domains 2 and 3 fused to human scFc (gp-80 D2D3 hscFc); gp-130 extra cellular domain human Fc (gp-130 ECD hFc); and gp-130 Domain 1 scFc (gp-130 D1 scFc). Passed over the sensors were the following samples: (1) IL-6 control, (2) gp-80 control, (3) IL-6:gp-80 control, (4) VHH control, (5) IL-6:VHH complex, (6) gp-80:VHH complex and (7) IL-6:gp-80:VHH complex. The running buffer was HBS-EP (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005 % (v/v) P20) (GE Healthcare) and sensors were regenerated using 40 mM HCl. All samples were made up to a concentration of 300 nM in the running buffer and then mixed at a ratio of 1:1:1 (IL-6:gp-80:VHH) to give a final concentration of 100 nM (running buffer was used in place of samples when required).

To measure the effect of the VHH on Site I binding, the response for IL-6 alone was compared to the response for the IL-6:VHH complex when passed over the immobilised gp-80 D2D3 hscFc. To measure the effect of the VHH on Site II binding the response for the IL-6:gp-80 complex was compared to response for the IL-6:gp-80:VHH complex when passed over the immobilised gp-130 ECD hFc. Finally, to measure the effect of binding at Site III, the response of the IL-6:gp-80 complex was compared to that of the IL-6:gp-80:VHH complex was passed over the immobilised gp-130 D1 scFc. Figure 6.3 shows a schematic of the three experiments.

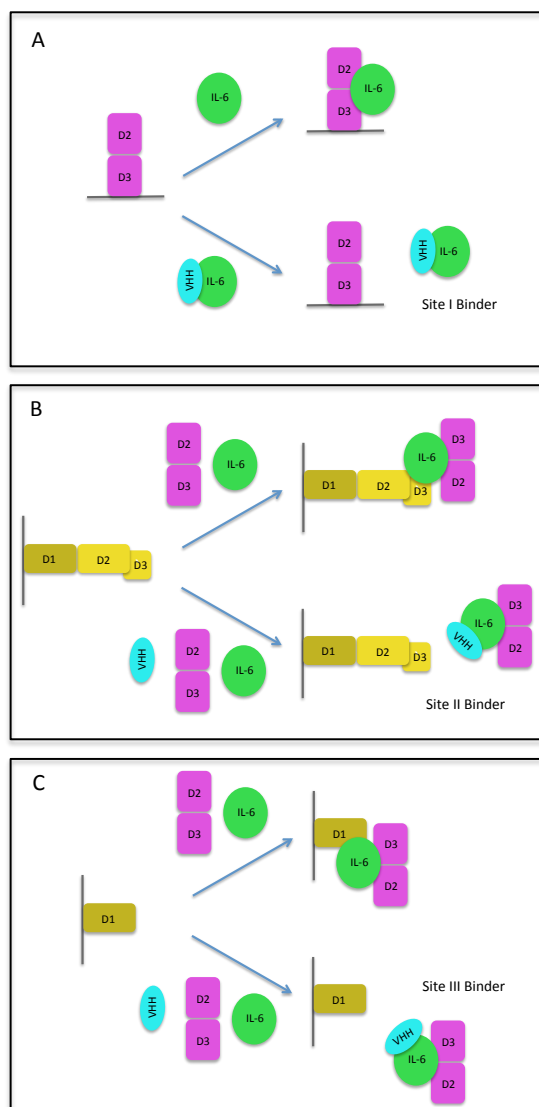


Figure 6.3 – Characterisation of the VHH Binding Sites by SPR

Panel A shows a schematic of the experiment used to determine the effect of the VHH on Site I binding, Panel B shows a schematic of the experiment measuring the effect of Site II binding and Panel C shows a schematic of the experiment used to measure the effect of binding at Site III. IL-6 is shown in green, gp-80 in magenta, gp-130 in yellow and the VHH in cyan.

Double referenced binding curves were analysed using BIAevaluation software. The response in Response Units (RU) was corrected for molecular weight and then stoichiometric ratios calculated by dividing the response in the presence of the VHH by the response for the appropriate control. The dissociation rate constants (k_d) were determined using a biphasic Langmuir dissociation algorithm.

6.2.4 – NMR Minimal Shift Mapping

Minimal shift mapping of the epitope of VHH 67 onto IL-6 has been described in Section 4.3.5. The same method (as described in Section 4.1.2) was used to determine the epitope of VHH 94 on IL-6.

6.3 - Results

6.3.1 – ELISA and Cell Based Assay

The results from the ELISA site blocking assays are shown in Figures 6.4 and 6.5 (adapted from Hancock, 2010). Data were collected for the majority of the panel of anti-IL-6 VHH antibodies but to simplify the analysis only a selection of the results are shown. Alongside the data for VHH 67 are the data for VHH 15, VHH 29, VHH 38, VHH 56 and VHH 146 as these are the VHHs for which crystal structures were obtained by Dr. Laura Griffin (UCB) and have been discussed in previous chapters. The binding of VHH 94 to IL-6 will also be discussed later in this chapter so results for VHH 94 have also been included. Unfortunately, VHH 18, for which the solution structure was solved in Chapter 3, was not tested as a purified protein in the ELISA assays or the bioassay so could not be included in the figures.

The results of the Site II blocking ELISA assay are shown in Figure 6.4. Inhibition of the binding of IL-6 to gp-130 D3 is observed in the presence of VHH 29 with a half maximal inhibitory concentration (IC_{50}) of 17.7 nM. This result is consistent with the crystal structure identifying VHH 29 binding as a Site II binder. VHH 38 and VHH 146 show no significant inhibition of IL-6 binding to gp-130 D3 binding at Site II (at the maximum concentration of 75 nM less than 25 % inhibition was observed for VHH 38 and less than 10 % inhibition was observed for VHH 146). This is consistent with the crystal structures, which show both VHH 38 and VHH 146

binding at Site III. The crystal structures identified VHH 15 and VHH 56 binding in the vicinity of Site III but are not Site III cleft binders like VHH 38 and VHH 146. The Site II blocking ELISA results for VHH 15 show approximately 50 % inhibition at the top concentration measured so although VHH 15 does not bind at Site II there may be allosteric effects leading to some inhibition of gp-130 D3 binding at Site II. VHH 56 does not inhibit the binding at Site II. The binding site of VHH 94 is at present unknown but, with an IC_{50} value of 9.8 nM calculated, VHH 94 does appear to inhibit the binding of gp-130 D3 to IL-6 at Site II. At the time of running this assay the binding site of VHH 67 was also unknown but results of epitope mapping by both NMR and HDX suggested that it may bind at the base of IL-6 (antipodal to Site III). With a maximum inhibition of 30 %, the Site II blocking ELISA results suggest that VHH 67 is unlikely to bind at Site II.

VHH 18 was not measured as a purified protein but initial screening of VHH 18 as a transient suggested an inhibition of IL-6 binding to gp-130 D3 and was therefore classed as a Site II blocker (data not shown) which is consistent with the epitope mapping results obtained by NMR and HDX.

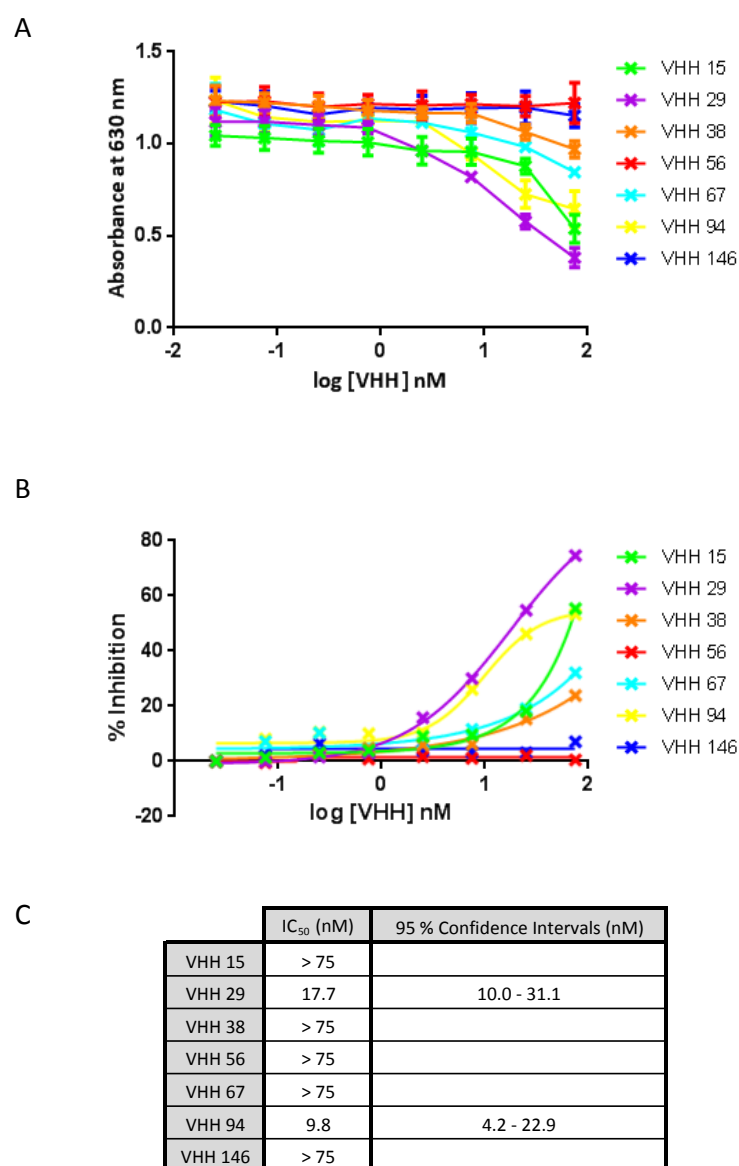


Figure 6.4 – Identification of Site II Binding VHH Antibodies by ELISA

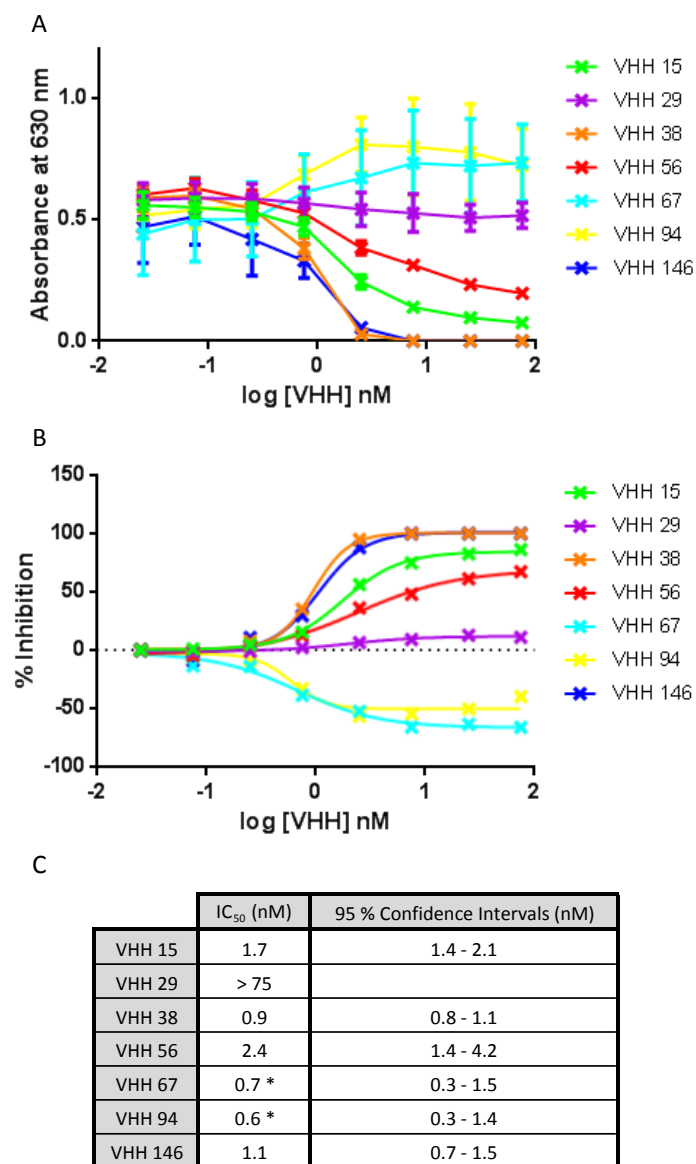
The graph in Panel A shows the results of the Site II blocking ELISA assay for VHH 15 (green), VHH 29 (purple), VHH 38 (orange), VHH 56 (red), VHH 67 (cyan), VHH 94 (yellow) and VHH 146 (blue). Triplicate data were collected and the mean values are plotted with error bars indicating one standard deviation (sd). Panel B shows the same results plotted as a percentage inhibition of gp-130 D3 binding at Site II. Panel C shows the IC₅₀ values calculated (where appropriate) alongside the 95 % confidence intervals after the data were fitted using a dose response model in GraphPad Prism (log (inhibitor) vs. variable slope - four parameters).

Shown in Figure 6.5 are the results of the Site III blocking ELISA for the same seven VHHs. The VHHs can be divided into three groups: those which show inhibition of Site III; those which show no effect on the binding of gp-130 D1 at Site III; and those which appear to enhance the binding of gp-130 D1 at Site III.

VHH 38 and VHH 146, known from the crystal structures to be Site III binders, fall as expected into the first category. IC_{50} values for VHH 38 and VHH 146 were calculated as 0.9 nM and 1.1 nM respectively and show complete inhibition of gp-130 D1 binding at concentrations of 7.5 nM and above. VHH 15 and VHH 56, known from the crystal structures to bind in the vicinity of Site III, have IC_{50} values of around 2 nM but do not completely block the binding of gp-130 D1 at the maximum concentration. This is consistent with VHH 15 and VHH 56 binding near Site III, so having some inhibitory effect, but not in the cleft blocking the binding of gp-130 D1.

VHH 29 shows no effect on the binding at Site III with an inhibition of just 10 % at the maximum concentration. This is consistent with the crystal structure and the Site II blocking ELISA results identifying VHH 29 as a Site II binder.

VHH 67 and VHH 94 are very interesting as in the presence of the antibodies an enhancement rather than inhibition of IL-6 binding to Site III is observed, with IC_{50} values calculated at 0.6 to 0.7 nM. This unexpected enhancement in binding of gp-130 D1 to IL-6, suggests that VHH 67 and VHH 94 are binding to a regulatory site away from Site III but are positively modulating the binding at Site III via an allosteric mechanism, which may point towards a new opportunity for drug development.

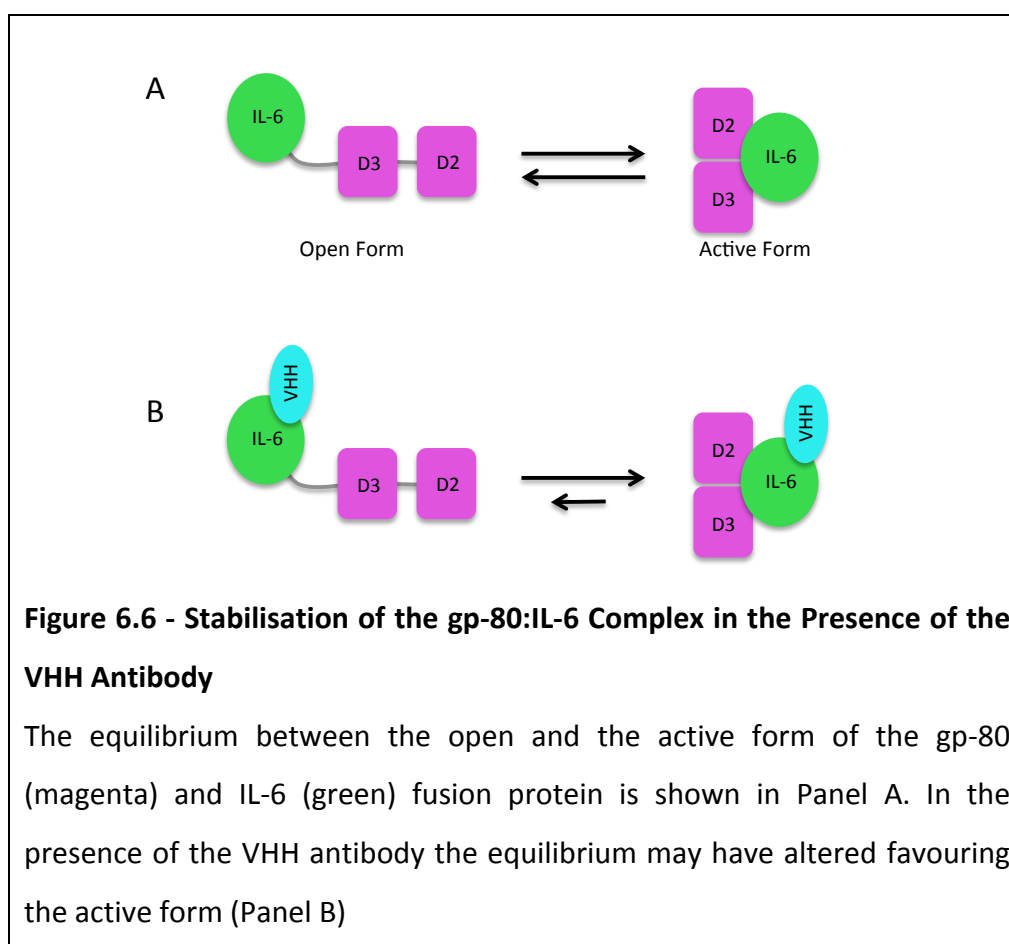


* IC₅₀ value corresponds to a negative % inhibition
(enhancement in the binding of gp-130 D1)

Figure 6.5 – Identification of Site III Binding VHH Antibodies by ELISA

The graph in Panel A shows the results of the Site III blocking ELISA assay for VHH 15 (green), VHH 29 (purple), VHH 38 (orange), VHH 56 (red), VHH 67 (cyan), VHH 94 (yellow) and VHH 146 (blue). Triplicate data were collected and the mean values are plotted with error bars indicating one standard deviation (sd). Panel B shows the same results plotted as a percentage inhibition of gp-130 D1 binding at Site III. Panel C shows the IC₅₀ values calculated (where appropriate) alongside the 95 % confidence intervals after the data were fitted using a dose response model in GraphPad Prism (log (inhibitor) vs. variable slope - four parameters).

There are two possible scenarios that could rationalise the enhanced binding of IL-6 to gp-130 in the presence of VHH 67 and VHH 94 as observed in the Site III blocking ELISA. Firstly, the VHH antibody binds to IL-6 remote from Site III but induces conformational changes in IL-6 that result in enhanced binding to gp-130 at Site III. Secondly, the apparent enhancement in binding is the result of the VHH antibody stabilising the IL-6:gp-80 complex. The IL-6:gp-80 complex used in the ELISA assays was a fusion of IL-6 and gp-80 with the three domains (IL-6, gp-80 D2 and gp-80 D3) fused via the flexible N- and C-termini of IL-6 and gp-80 respectively. The format of this fusion protein is shown in Figure 6.6 and is designed to allow self-configuration into the active form that binds to gp-130 D1. It is likely that the fusion protein is in equilibrium between an open and an active form so if the VHH antibody stabilises the active form, the equilibrium is shifted towards this form and an enhancement in binding to gp-130 would be observed.



The inhibition of IL-6 signalling results for the anti-IL-6 VHHs are shown in Figure 6.7. A reduction of the STAT3 response is seen in the presence of VHH 15, VHH 29, VHH 38, VHH 94 and VHH 146 suggesting these five VHHs disrupt IL-6 signalling. VHH 56 and VHH 67 show no significant reduction in STAT3 response suggesting that binding to IL-6 occurs at a site that does not inhibit the formation of the IL-6 signalling complex and, therefore, does not disrupt signalling. This is consistent with the results from the ELISA blocking assays for VHH 56 and VHH 67 that show no significant inhibition of either gp-130 D3 at Site II or gp-130 D1 at Site III.

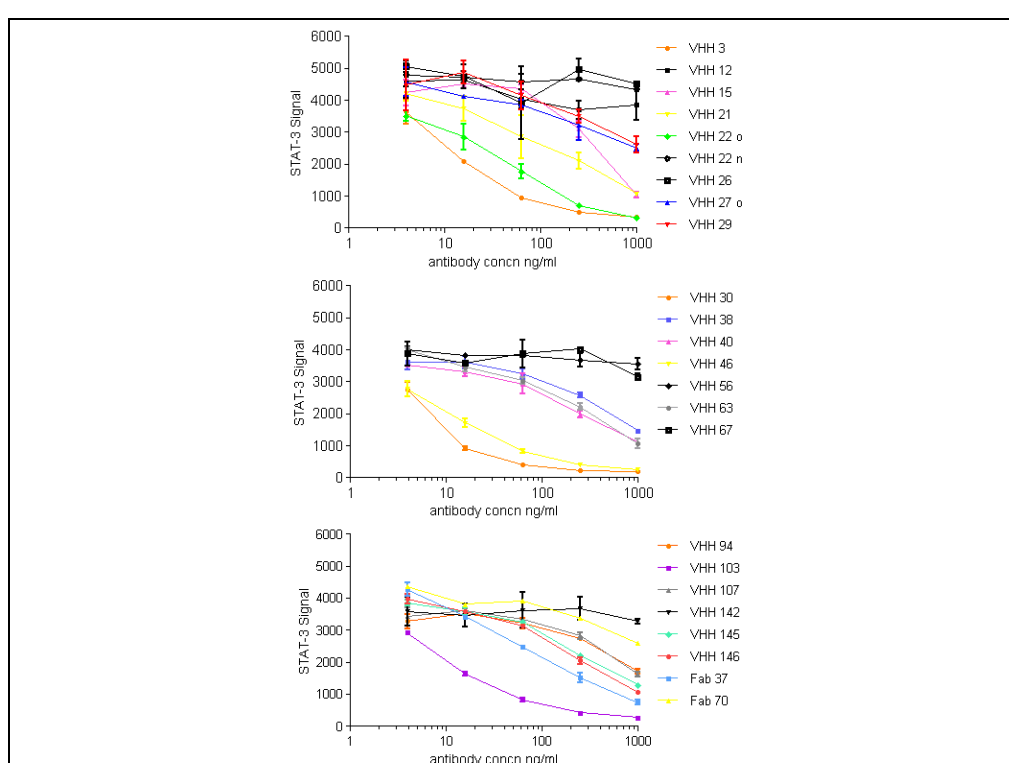
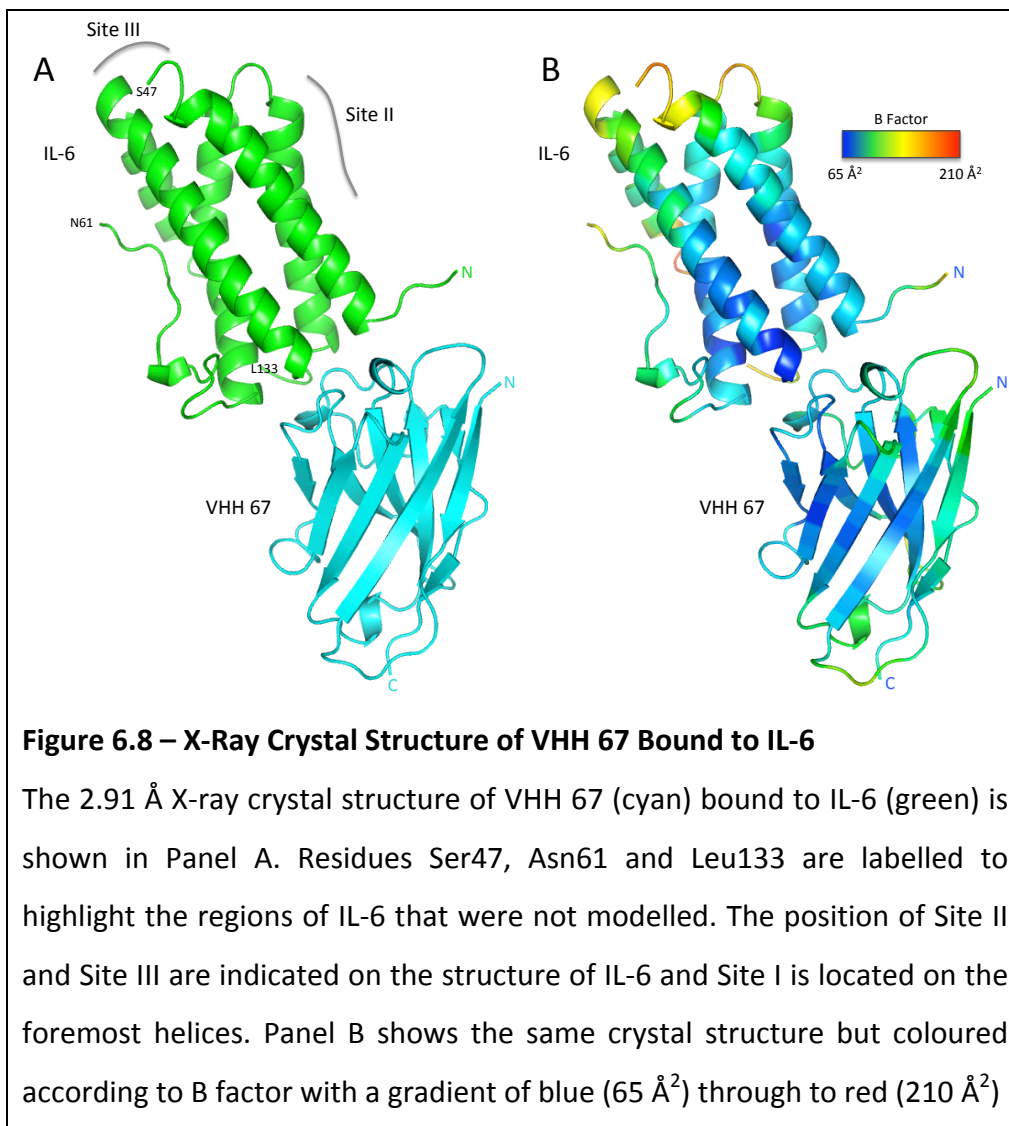


Figure 6.7 - Inhibition of IL-6 Signalling for all anti-IL-6 VHH Antibodies

Figure reproduced with the permission of Dr. Laura Griffin (Hancock, 2010). The STAT3 response in the presence of the VHH antibodies is shown. A reduction in the STAT3 response due to inhibition of the IL-6 signalling complex can be seen for VHH 15 (pink triangles), VHH 29 (red triangles), VHH 38 (blue squares), VHH 94 (orange circles) and VHH 46 (red circles). The STAT3 response in the presence of VHH 56 (black diamonds) and VHH 67 (black squares) does not reduce suggesting that these VHHs do not bind through Site II or Site III.

6.3.2 – X-Ray Crystallography of VHH 67 : IL-6

The crystal structure obtained for VHH 67 bound to IL-6 is shown in Figure 6.8, which reveals that VHH 67 binds to the base of IL-6 (antipodal to Site III).



6.3.2.1 – Residues and CDR Dynamics of VHH 67 as Revealed by the VHH 67:IL-6 Crystal Structure

The solvent accessible surface for each residue was calculated for free VHH 67 and for VHH 67 in complex with IL-6 using MOLMOL (Koradi *et al.*, 1996). Figure 6.9 Panel A shows the difference in the percentage of the solvent accessible surface (free - bound) plotted against residue number. The residues for which the solvent accessible surface is greater, by more than 10 %, in the free VHH 67 than in the complex were considered to be at the VHH 67-IL-6 interface. These residues are highlighted on the structure in Figure 6.9 Panel B.

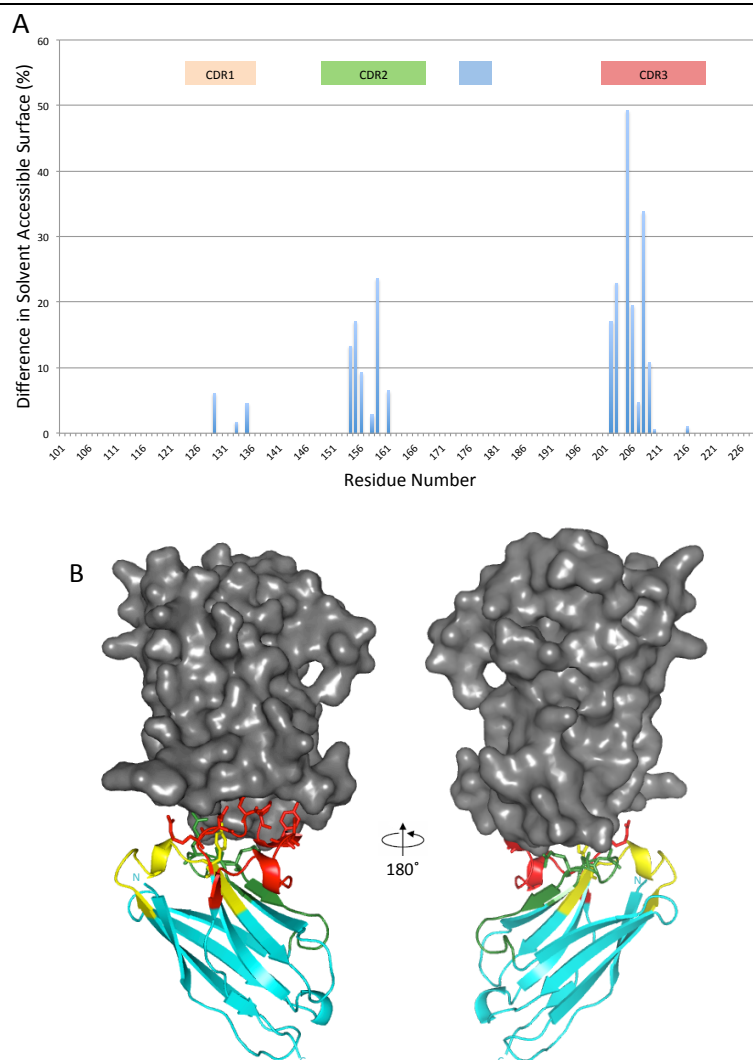
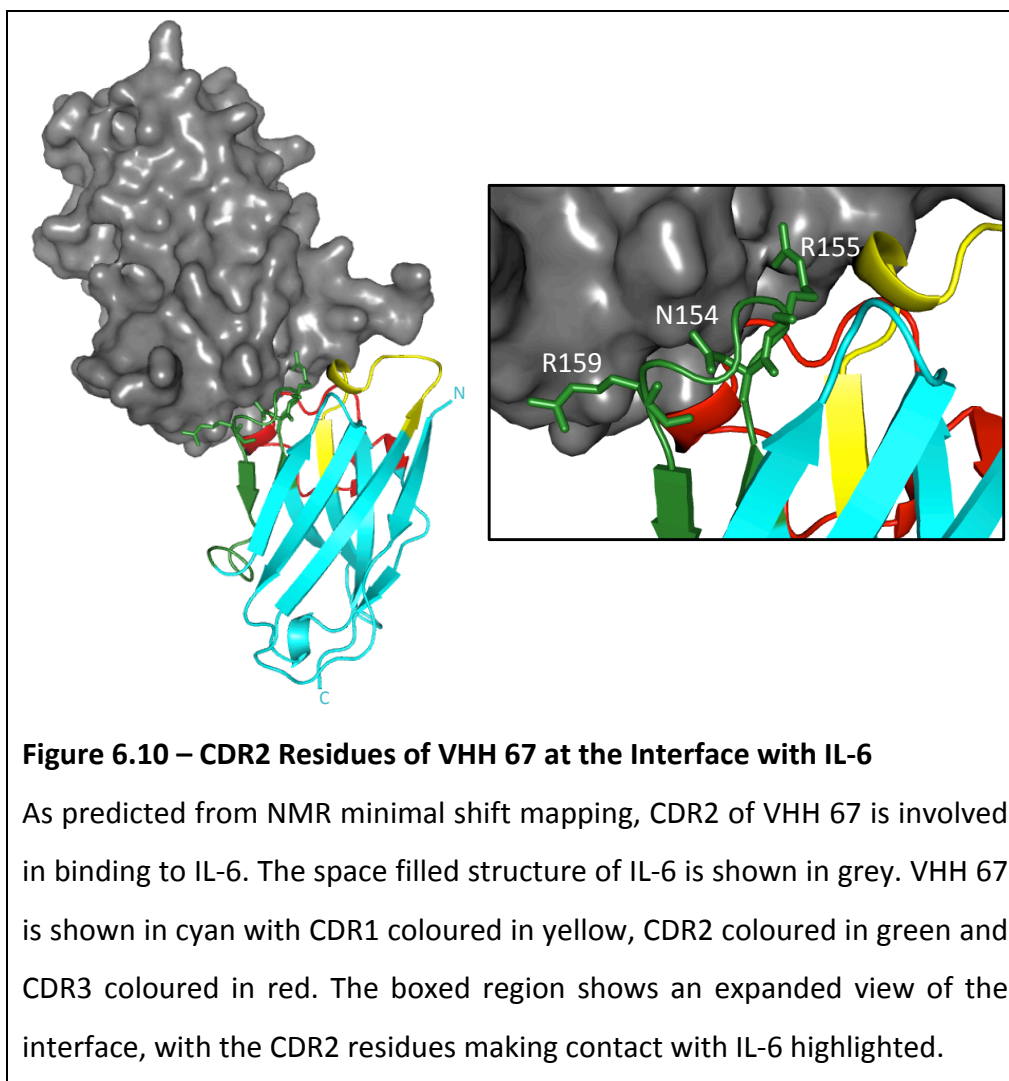


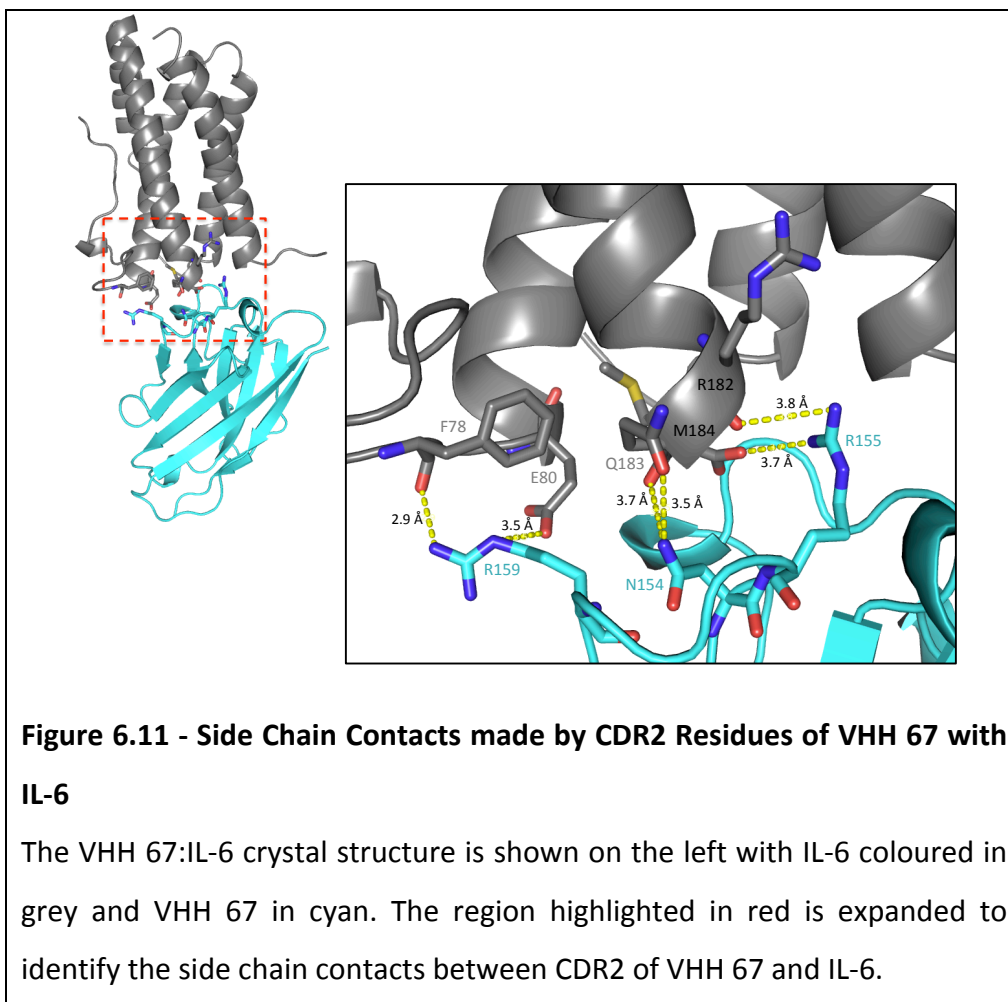
Figure 6.9 - VHH 67 Interface with IL-6

The graph in Panel A shows the difference in solvent accessible surface for free VHH 67 compared to VHH 67 in complex with IL-6. Panel B shows the results of these calculations plotted onto the crystal structure of VHH 67 in complex with IL-6. IL-6 is shown in grey and VHH 67 shown in cyan with CDR1, CDR2 and CDR3 coloured in yellow, dark green and red respectively. The residues for which the solvent accessible surface is greater, by more than 10 %, in the free VHH 67 than when in complex with IL-6 are shown as line representations. The results show residues Tyr135 of CDR1, Asn154, Arg155 and Arg159 of CDR2 and Arg202, Asp203, Phe205, Pro206, Leu207, Tyr208 and Ser209 of CDR3 make up the majority of the VHH 67-IL-6 interface.

The minimal shift analysis by NMR suggested that both CDR2 and CDR3 were involved in the binding of VHH 67 to IL-6 and this is confirmed by the crystal structure. Figure 6.10 shows selected residues of CDR2 forming part of the interface with IL-6.



The key interactions between the side chains the CDR2 residues of VHH 67 and IL-6 are shown in Figure 6.11. The N δ 2 atom in the side chain of Asn154 forms two hydrogen bonds with Gln183 of IL-6; the NH₂ group of Arg155 forms a hydrogen bond to Met184 and a salt bridge to Arg182 of IL-6; and the side chain of Arg159 forms a hydrogen bond with Phe78 and a salt bridge to Glu80.



The side chains of two residues of CDR3 (Phe205 and Tyr208) are seen extending from VHH 67 into a cleft at the base of IL-6 (see Figure 6.12). As with previous examples of VHH crystal structures including cAb-Lys3 (Desmyter *et al.*, 1996) and VHH 146:IL-6 (Hancock, 2010), these two residues are in the N-terminal portion of CDR3 and the C-terminal portion of CDR3 appears to act as a region of extra framework and is not involved in binding to IL-6. In addition to these hydrophobic interactions are two hydrogen bonds, between Asp203 of VHH 67 and Ser22 of IL-6 and Leu207 of VHH 67 and Met184 of IL-6 (see Figure 6.13).

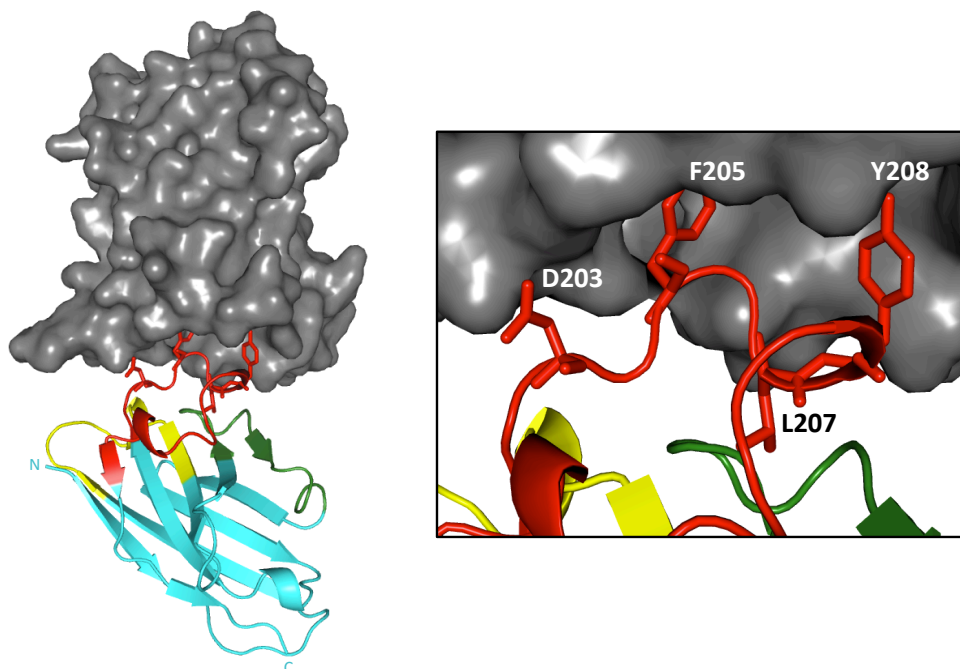


Figure 6.12 – CDR3 Residues of VHH 67 at the Interface with IL-6

As predicted from NMR minimal shift mapping, CDR3 of VHH 67 is involved in binding to IL-6. The space filled structure of IL-6 is shown in grey. VHH 67 is shown in cyan with CDR1 highlighted in yellow, CDR2 highlighted in green and CDR3 highlighted in red. The boxed region shows an expanded view of the interface, with Phe205 and Tyr208 (shown as line representations) extending from VHH 67 into a cleft at the base of IL-6.

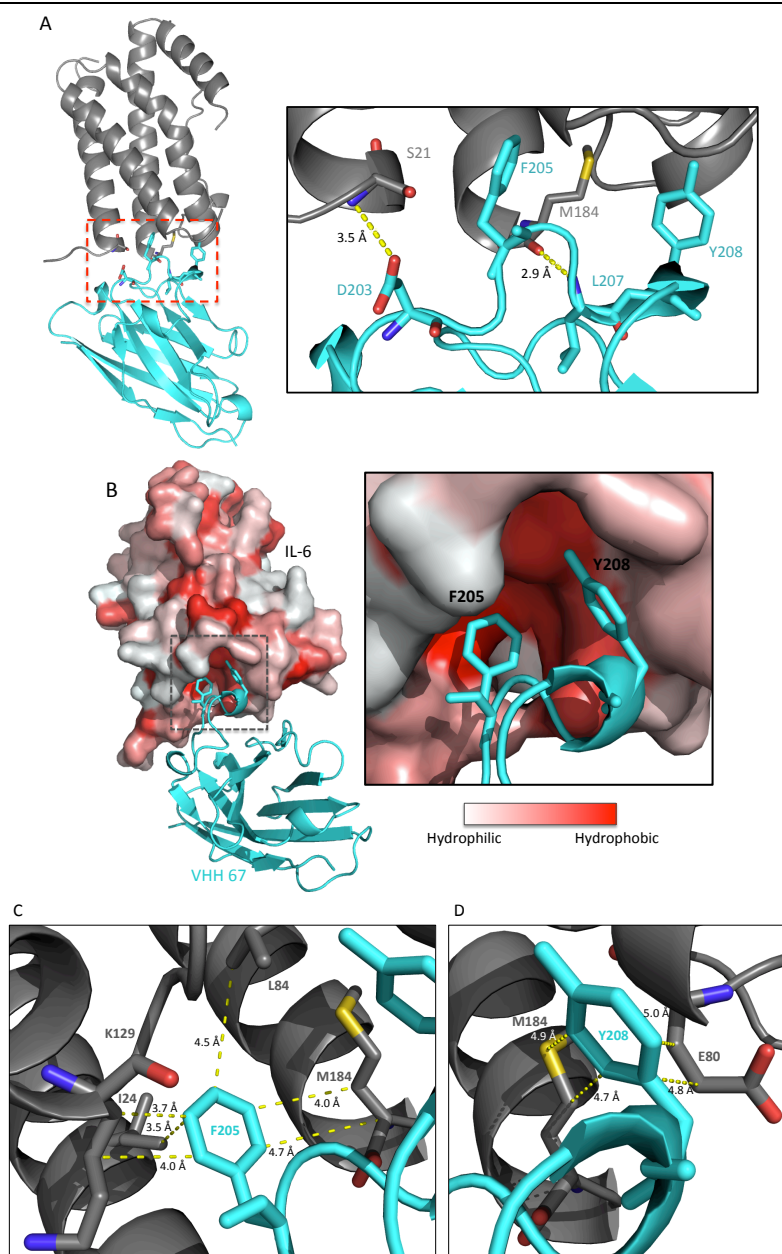
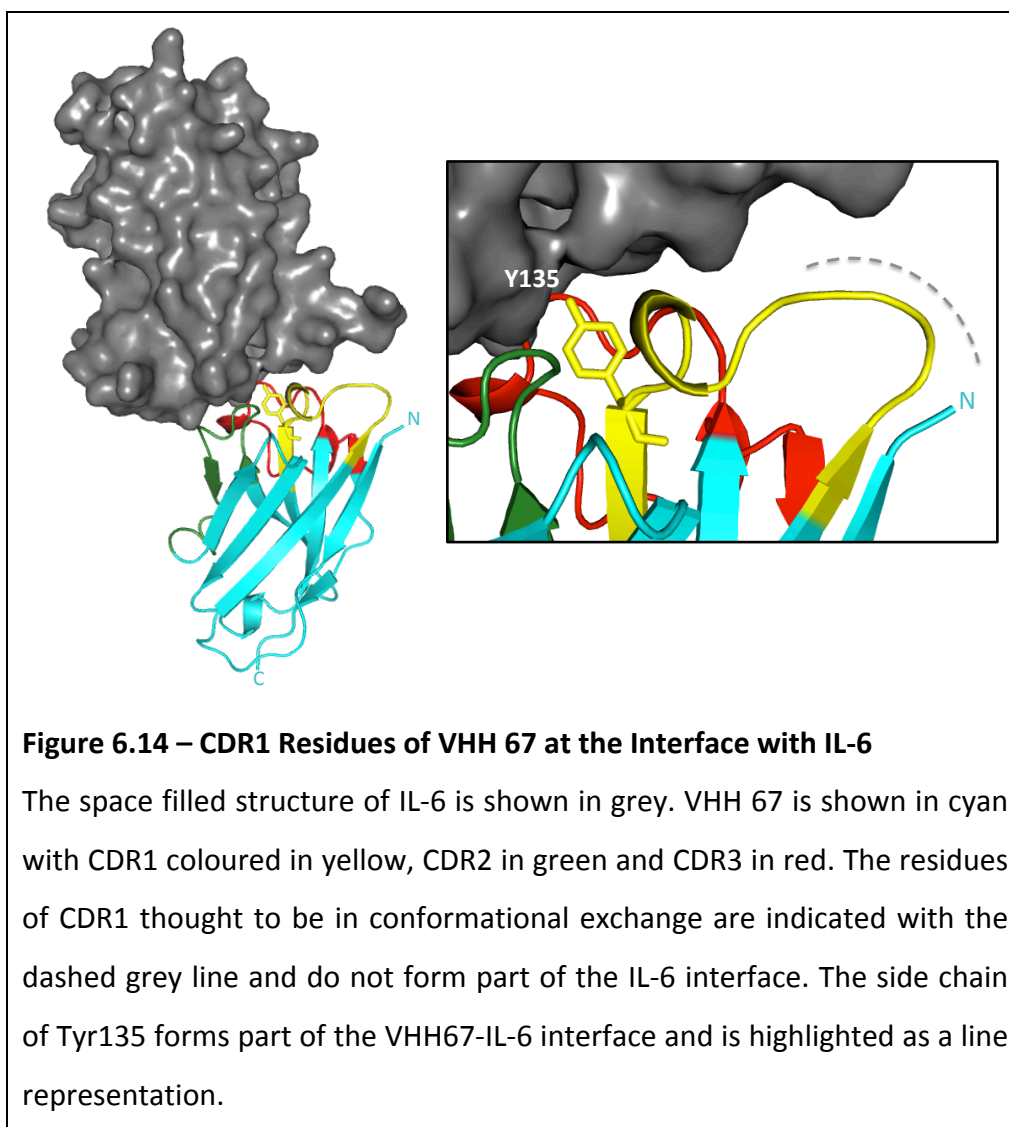


Figure 6.13 - Side Chain Contacts made by CDR3 Residues of VHH 67 with IL-6

In Panel A the VHH 67:IL-6 crystal structure is shown on the left with IL-6 coloured in grey and VHH 67 in cyan. The region highlighted in red is expanded to identify the side chain contacts between the CDR3 residues Asp203 and Leu207 of VHH 67 and IL-6. In Panel B IL-6 is coloured according to hydrophobicity (Eisenberg *et al.*, 1984) with the most hydrophobic residues coloured in red. The expanded view on the right shows that Phe205 and Tyr208 protrude into a hydrophobic pocket at the base of IL-6. Panel C and Panel D show the hydrophobic interactions made between Phe205 and Tyr208 of VHH 67 and IL-6

In the free solution structure of VHH 67 five residues of CDR1 were believed to be in conformational exchange. The continued absence of backbone signals corresponding to these residues from the VHH bound to IL-6 suggested that these residues were not stabilised upon binding to IL-6. The crystal structure confirms that these residues do not form part of the interface with IL-6 (shown in Figure 6.14) and may still be dynamic when in complex with IL-6. The B factors in this region are also higher than seen for the framework regions of VHH 67 and the other two CDRs (see Figure 6.16) supporting the fact that these residues may be in a more dynamic region of the protein. Tyr135, located towards the C-terminus of CDR1 does form part of the interface with IL-6 as shown in Figure 6.14. Figure 6.15 highlights the hydrogen bonds formed between the OH group of the tyrosine side chain and Ser22 and Met184 of IL-6.



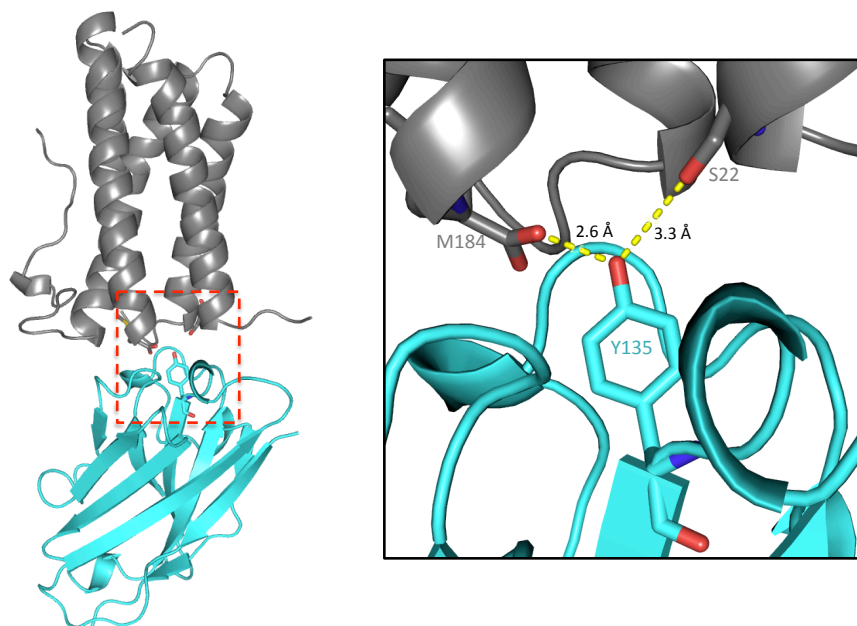
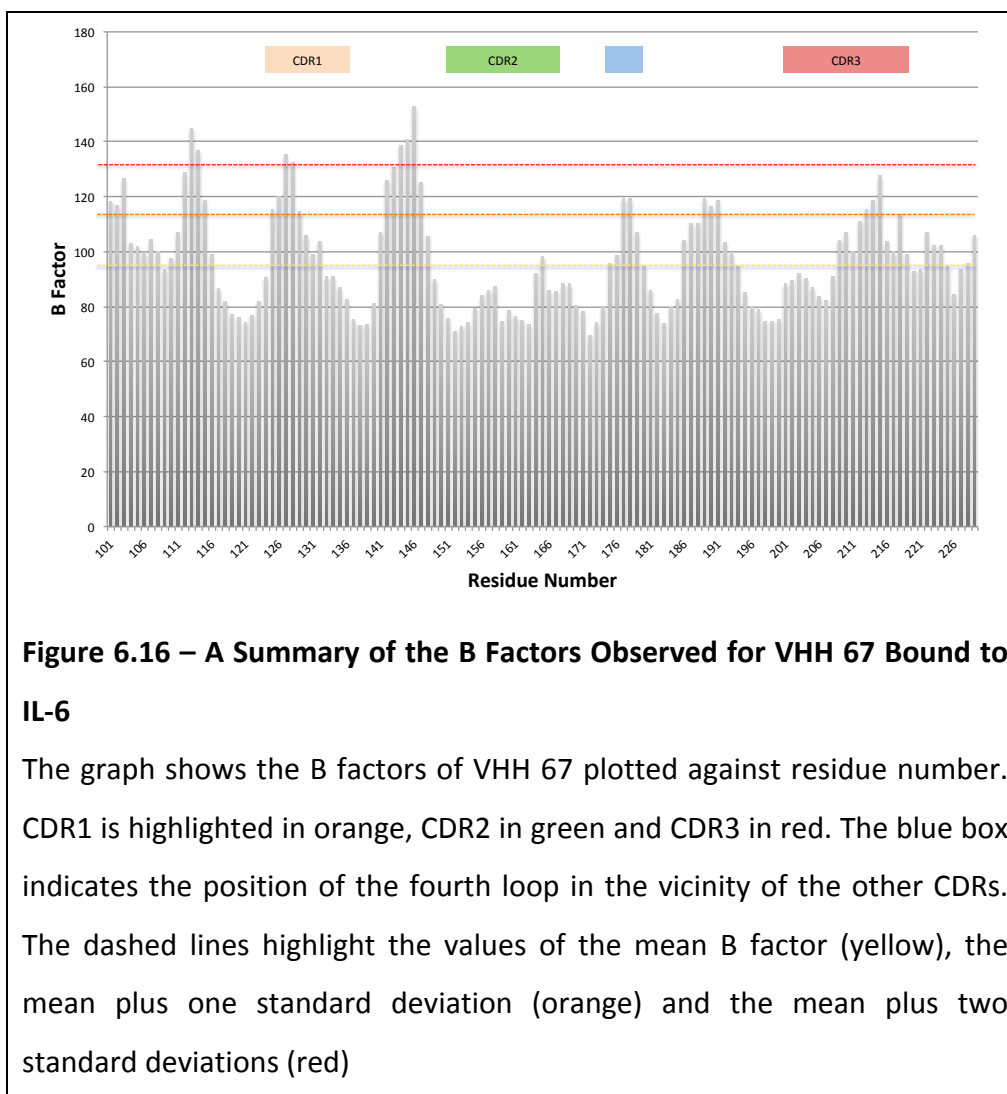


Figure 6.15 - Side Chain Contacts made by Tyr135 of CDR1 with IL-6

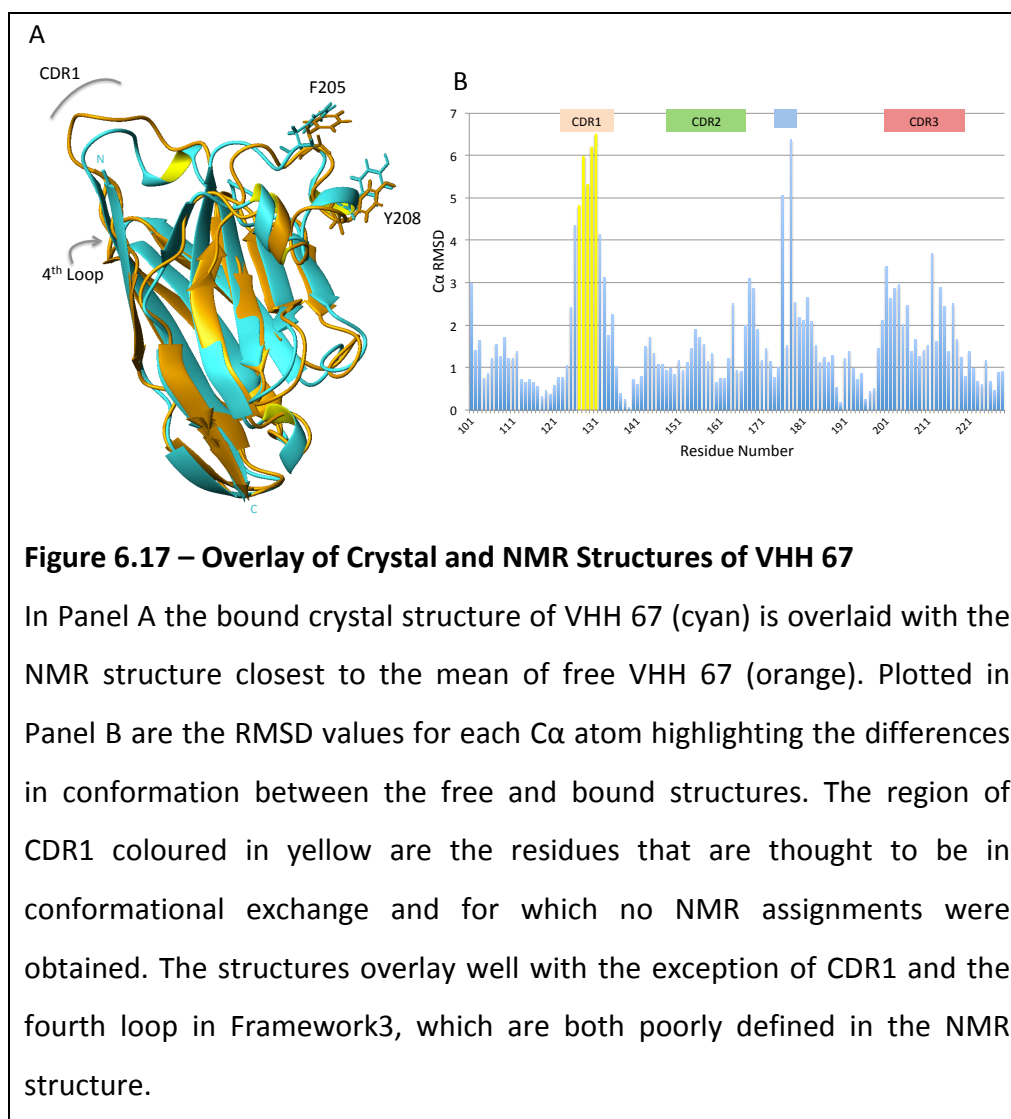
The VHH 67:IL-6 crystal structure is shown on the left with IL-6 coloured in grey and VHH 67 in cyan. The region highlighted in red is expanded to show the side chain contacts of Tyr135 of CDR1.

The graph shown in Figure 6.16 plots the B factors for the C α atoms against residue number for VHH 67. B factors for CDR1 and CDR3 are greater than the average along with the fourth loop at the antigen-binding site comprising of residues Asp175 to Thr180. The other regions with higher than average B factors are the turns between the β sheets: residues Ser111 to Gly115, Gln141 to Glu148 and Asn186 to Ala194.



Of the four free solution structures that were studied in Chapter 3, VHH 67 appeared to be the least dynamic. Unlike VHH 18, VHH 29 and VHH 146, the entire length of the long CDR3 was visible by NMR along with NOEs between the residues in CDR3 and Framework 2. Overlaying the free solution structure of VHH 67 with the bound crystal structure (Figure 6.17 Panel A) shows very little difference in the conformation of the two structures. Backbone superimposition of the NMR structure closest to the mean and the crystal structure (residues Asp101 to Ser229) gives an overall RMSD of 1.59 Å. However, calculating the RMSD values for each Cα atom highlights two regions in which the conformation of the free and bound structures do differ (see Figure 6.17 Panel B), these are CDR1 and the fourth loop in Framework3. CDR1 was poorly defined in the free NMR structures and shows high B factors in the crystal structure so it is unsurprising that there is poor

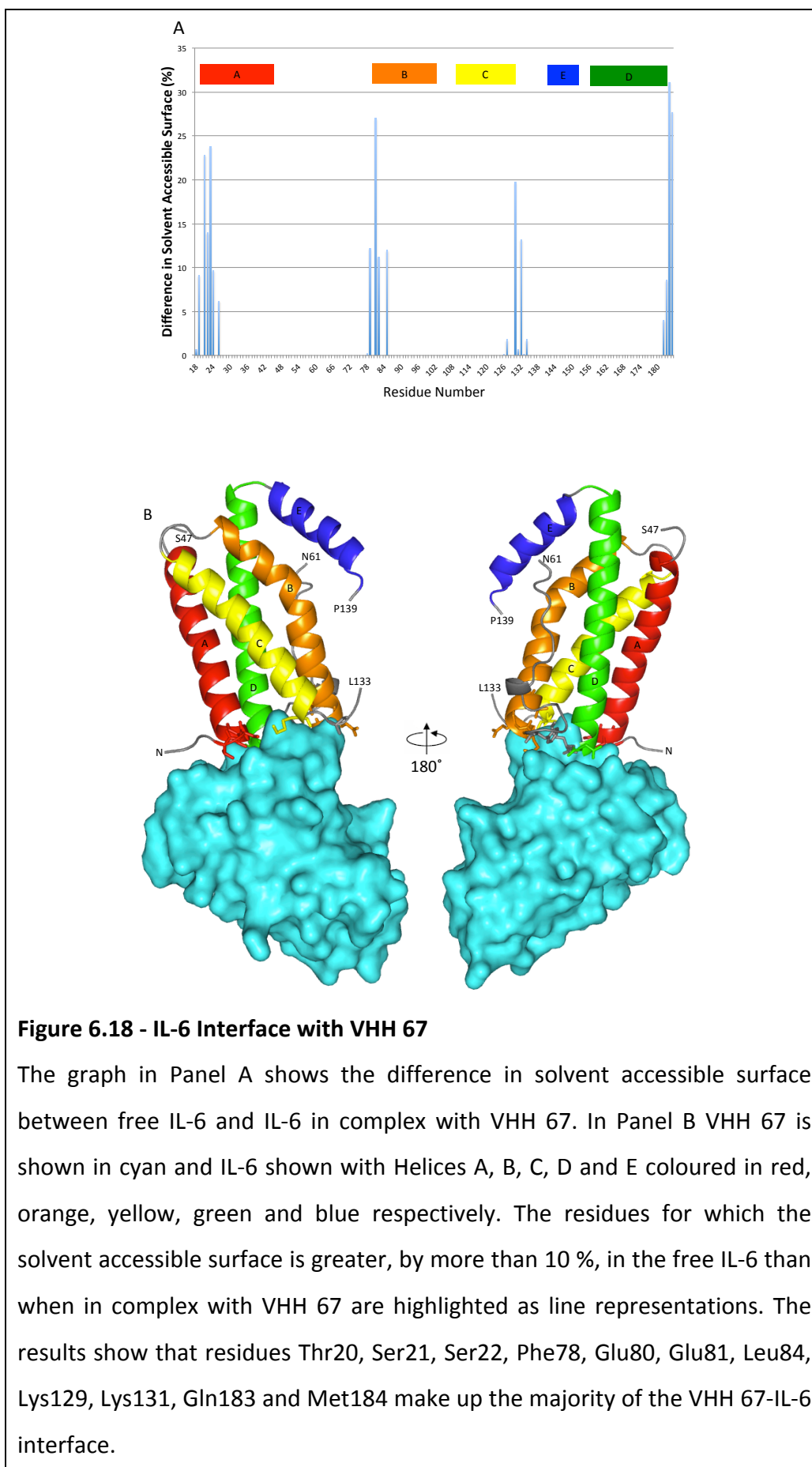
agreement between the two structures for this region. Closer examination of the family of NMR structures of VHH 67 (Figure 3.15) shows that the region of Framework3 that makes up the fourth loop of the antigen binding site is less well defined than other areas of framework so again it is not surprising that the conformation of the free NMR structure and the bound crystal structure may vary more in this region.



Not only do the backbone atoms of the free NMR structure and the bound crystal structure overlay well but also the side chain groups of the two residues that reach into the cleft at the base of IL-6 are positioned almost identically between the two structures (shown in Figure 6.17). This suggests that Phe205 and Tyr208 are pre-positioned into the correct orientation for binding thus no entropic penalty is paid upon binding.

6.3.2.2 – Interface Residues of IL-6 as Revealed by the VHH 67:IL-6 Crystal Structure

The residues of IL-6 at the interface with the VHH were calculated from the VHH 67:IL-6 crystal structure as described in Section 6.3.2.1 and the results are presented in Figure 6.18. The interface includes residues from the N-termini of Helix A and Helix B, the C-terminus of Helix C, the turn between Helices C and D and the N terminus of Helix D. In Figure 6.19 the results of the binding site mapping by NMR minimal shift and HDX are shown alongside the interface residues determined from the crystal structure. Both the NMR and HDX mapping predict the IL-6 epitope to include the N-termini of Helix A and Helix B and the C-termini of Helix C and Helix D but the predicted binding site also includes a number of residues extending along the helices and in the loop regions between the helices. This may, as described in Section 4.4, be a result of VHH 67 binding end on to the four helix bundle resulting in a ripple effect through IL-6 making the binding surface appear larger than just the interface residues.



	NMR	HDX	X-Ray		NMR	HDX	X-Ray		NMR	HDX	X-Ray		NMR	HDX	X-Ray
P1				S52				N103		✓		Q154		✓	
V2				S53				R104	✓	✓		N155	✓		
P3				K54				F105		✓		Q156		✓	
P4				E55				E106	✓	✓		W157		✓	
G5				A56				S107		✓		L158		✓	
E6				L57				S108		✓		Q159		✓	
D7				A58				E109		✓		D160			
S8				E59				E110		✓		M161			
K9				N60				Q111		✓		T162		✓	
D10				N61	✓			A112		✓		T163		✓	
V11				L62	✓			R113		✓		H164		✓	
A12				N63				A114		✓		L165		✓	
A13				L64				V115				I166	✓	✓	
P14				P65	-			Q116	✓			L167		✓	
H15				K66	✓			M117	✓			R168	✓	✓	
R16				M67	✓✓			S118				S169		✓	
Q17				A68				T119	✓✓			F170		✓	
P18				E69	-			K120	✓✓			K171		✓	
L19	✓✓	✓		K70	✓✓	-		V121	✓			E172	✓	✓	
T20		✓	★	D71	✓	-		L122	✓✓			F173		✓	
S21		✓	★	G72	✓✓	-		I123	✓			L174		✓✓	
S22		✓	★	C73		-		Q124	✓✓			Q175		✓✓	
E23	✓✓	✓		F74	✓✓	✓		F125	✓✓	✓✓		S176	✓✓	✓✓	
R24	✓	✓		Q75		✓		L126		✓✓		S177		✓✓	
I25				S76	✓	✓		Q127	✓✓	✓✓		L178	✓	✓✓	
D26	✓✓	✓		G77	✓✓	✓		K128	✓✓	✓✓		R179	✓✓	✓✓	
K27	✓✓	✓		F78	-	✓	★	K129	✓✓	✓✓	★	A180	✓✓	✓✓	
Q28		✓		N79		✓		A130	✓✓	✓✓		L181	✓✓	-	
I29	✓✓	✓		E80		✓	★	K131	✓✓	✓✓	★	R182	✓✓	-	
R30	✓✓	✓		E81	✓✓	✓	★	N132		✓✓		Q183		-	★
Y31	✓✓	✓		T82		✓		L133		✓✓		M184		-	★
I32	✓✓	✓		C83		✓		D134	✓	✓✓					
L33	✓✓	✓		L84	✓	✓	★	A135	✓✓	✓✓					
D34		✓		V85	✓✓	✓		I136		✓✓					
G35				K86	✓	✓		T137		✓✓					
I36		✓		I87	✓	✓		T138							
S37		✓		I88	✓✓	✓		P139	-	✓✓					
A38	✓	✓		T89				D140							
L39		✓		G90	✓✓			P141	-						
R40		✓		L91	✓✓			T142							
K41	-	✓		L92				T143							
E42				E93				N144							
T43				F94				A145							
C44	✓✓			E95				S146							
N45				V96				L147		✓					
K46				Y97				L148		✓					
S47				L98				T149		✓					
N48	✓			E99				K150		✓					
M49	✓			Y100				L151		✓					
C50				L101		✓		Q152		✓					
E51	-			Q102		✓		A153		✓					

Epitope Mapping
✓✓ Large Differences between Free and Bound
✓ Some Difference between Free and Bound
- Data Not Available

X-Ray Crystal Structure
★ Interface Residues

Figure 6.19 - A Comparison of the VHH 67 Binding Site Mapping on IL-6 by NMR and HDX with the Interface Residues Identified from the IL-6:VHH67 Crystal Structure

The sequence of IL-6 is shown with the helices A, B, C, D and E highlighted in red, orange, yellow, green and blue respectively. Residues at the interface with VHH 67, as determined by the difference in solvent accessible surface, are highlighted with a ★. The binding site as predicted by NMR minimal shift mapping and HDX by MS are highlighted with either a ✓ or a ✓✓ depending on the differences seen in the chemical shift or rate of hydrogen deuterium exchange between free IL-6 and IL-6 bound to VHH 67.

Figure 6.20 shows the B factors for IL-6 bound to VHH 67 plotted against residue number. As expected, the helical regions of IL-6 are well modelled and show low B factors. Higher B factors are observed in the inter-helix regions, which prevented the modelling of Asn49 to Asn61 and Asp135 to Thr139. Residues Asn49 to Asn61 are often disordered in the free form (Somers *et al.*, 1997) but are stabilised upon binding of gp-130 D1 (Boulanger *et al.*, 2003) forming a loop between Helix A and Helix B.

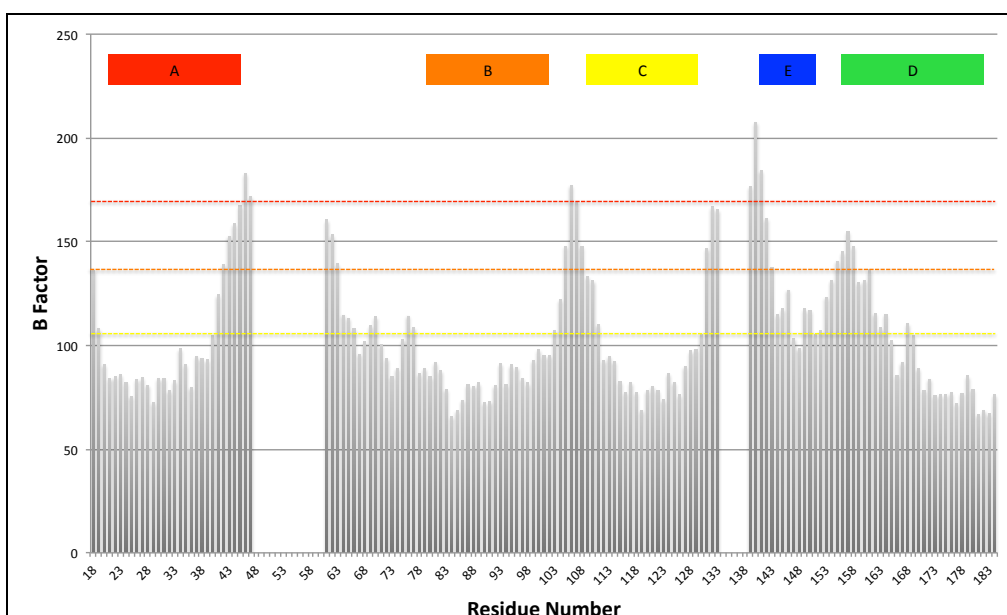
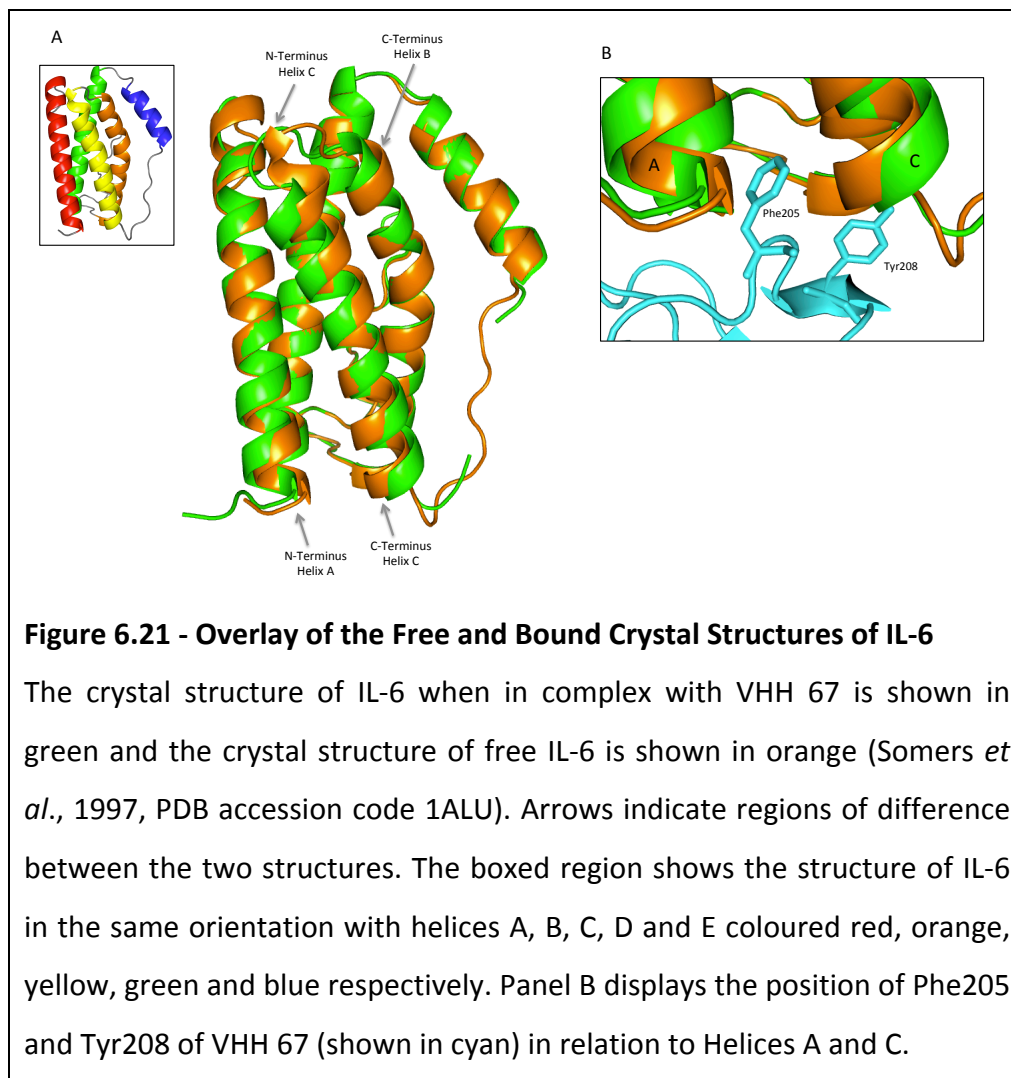


Figure 6.20 – A Summary of the B Factors Observed for IL-6 Bound to VHH 67

The graph shows the B factors obtained for IL-6 bound to VHH 67 plotted against residue number. The four helices (A, B, C and D) of the four helix bundle are highlighted by red boxes. The additional short helix (E) lying outside the four helix bundle is highlighted with a grey box. Residues Asn48 to Asn61 and Asp134 to Thr138 were not modelled as poor electron density was observed. The dashed lines highlight the values of the mean B factor (yellow), the mean plus one standard deviation (orange) and the mean plus two standard deviations (red).

An overlay of the free crystal structure of human IL-6 (Somers *et al.*, 1997, PDB accession code 1ALU) and the crystal structure of IL-6 when bound to VHH 67 is shown in Figure 6.21. As detailed above, the region between Helix A and Helix B (Asn48 to Asn61) is not modelled in either the free or the bound structure and a region between Helix C and Helix D (Asp134 to Thr138) is modelled in the free structure but could not be modelled in the bound structure. The main differences in the structures were observed in the N-terminus of Helix A and the C-terminus of Helix C where the residues Phe205 and Tyr208 are shown to interact with IL-6 in the crystal structure (see Figure 6.21 Panel B). The interaction of Helix C with VHH 67 appears to result in a slight change in conformation of the entire helix which then alters the structure of the C-terminus of Helix B along with the inter helix loop linking Helix B and Helix C. This may explain the differences in the chemical shift and the rate of hydrogen deuterium exchange observed for these regions despite not being part of the VHH 67-IL-6 interface.



6.3.2.3 – VHH 67 Binding to a New Site on IL-6

The crystal structure shows that VHH 67 binds to a site distinct from the three sites known to be involved in the formation of the IL-6 signalling complex (described in Section 1.4). Figure 6.22 shows the crystal structure of VHH 67 bound to IL-6 with the proteins that form the IL-6 signalling complex aligned to IL-6 and superimposed onto it: gp-80 domains 2 and 3 (gp-80 D2D3), binding at Site I; gp-130 domains 2 and 3 (gp-130 D2D3), binding at Site II; and gp-130 domain 1 (gp-130 D1), binding at Site III.

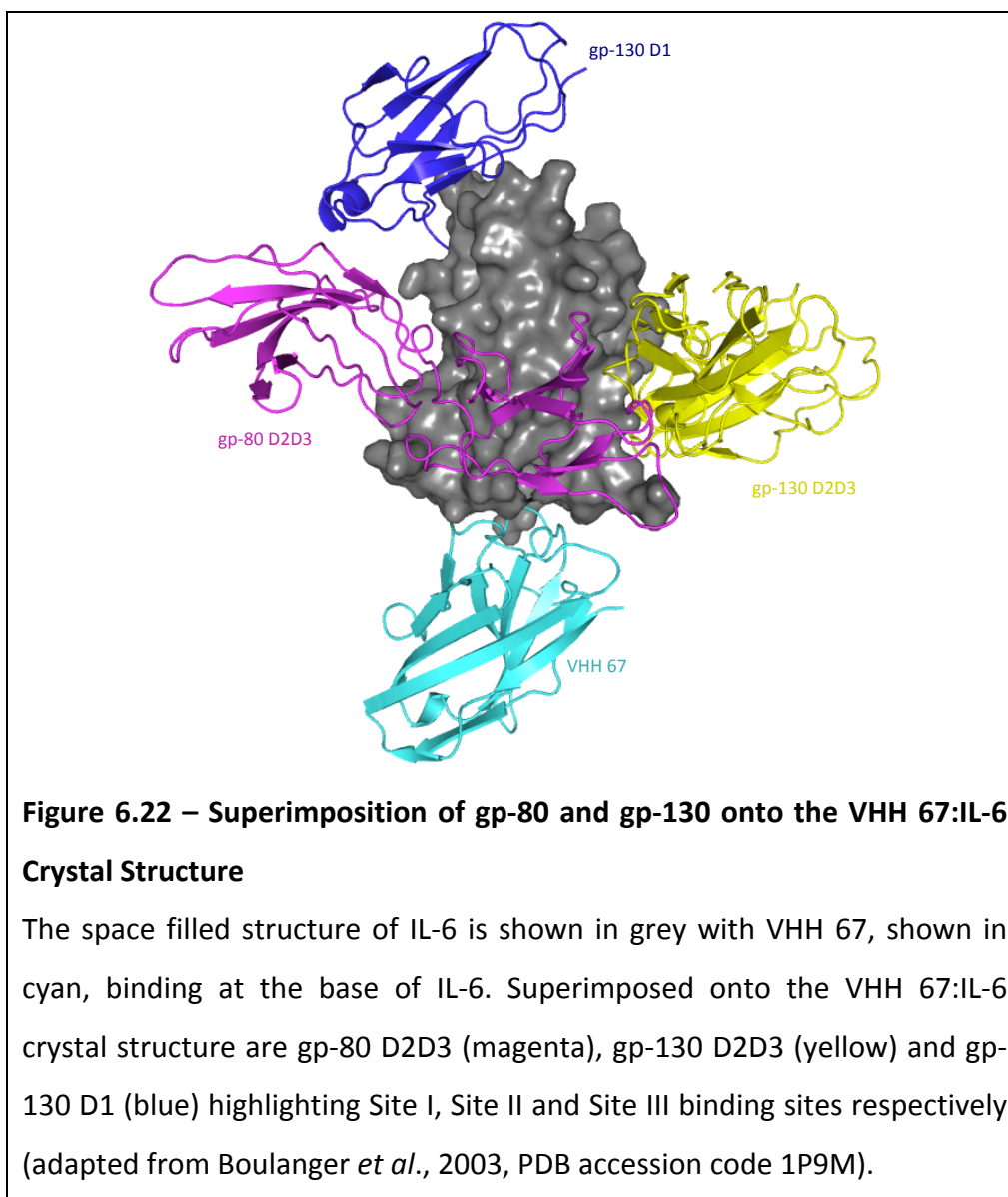
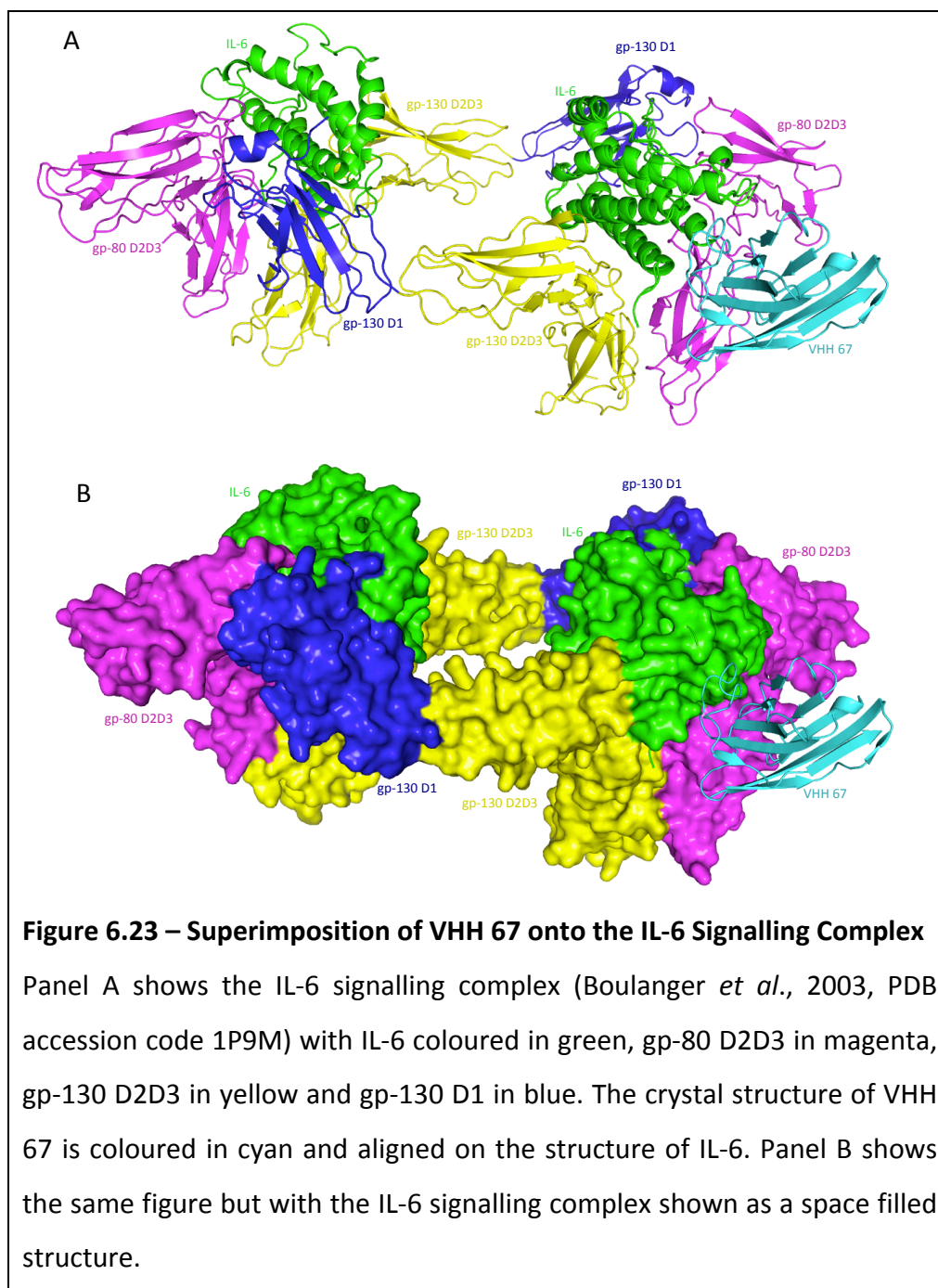


Figure 6.23 shows the crystal structure of VHH 67 aligned with one of the IL-6 molecules in the crystal structure of the IL-6 signalling complex (Boulanger *et al.*, 2003, PDB accession code 1P9M). VHH 67 does not appear to bind in a position that would inhibit the formation of the IL-6 signalling complex, which is consistent with the cell assays showing no inhibition of IL-6 signalling in the presence of VHH 67 (Section 6.3.1).



6.3.3 – Characterisation of VHH Binding Sites on IL-6 by SPR

As discussed in Section 6.3.1, the cell based bioassays showed no inhibition of IL-6 signalling in the presence of VHH 67 and as discussed in Section 6.3.2.3 this can now be explained by the new binding site identified from the VHH 67:IL-6 crystal structure. An enhanced response was observed in the Site III blocking ELISA in the presence of VHH 67, as described in Section 6.3.1, and the two potential explanations for this enhancement in IL-6 binding to gp-130 D1 were that conformational changes to IL-6 occur upon binding to VHH 67, or that VHH 67 stabilises the gp-80:IL-6 complex (see Figure 6.6). To investigate the functional effect of VHH 67 on the formation of the IL-6 signalling complex SPR experiments were performed as described in Section 6.2.3. A selection of three, previously studied, VHHs were analysed by SPR, these were: VHH 67 and VHH 94, both showing an enhanced response in the Site III blocking ELISA assay and VHH 29, a known Site II binder. VHH 29 was included as a negative control for the Site I and Site III SPR experiments but should show inhibition of binding in the Site II experiment. The presence of VHH 67 and VHH 94 in the Site III binding SPR experiment should show an increased affinity to gp-130 D1 over the control as this was the result observed in the Site III blocking ELISA. If the reason for this enhancement in gp-130 D1 binding is due to stabilisation of the gp-80:IL-6 complex then an increased affinity in the presence of the VHH should also be observed in the Site I binding SPR experiment.

Table 6.2 lists the stoichiometric ratios, calculated by dividing the response in the presence of each VHH by the response for the appropriate control, in the Site I, Site II and Site III binding site SPR experiments. The Site I experiment compares IL-6 alone and IL-6:VHH binding to immobilised gp-80, the Site II experiment compares IL-6:gp-80 and IL-6:gp-80:VHH binding to immobilised gp-130 ECD and the Site III experiment compares IL-6:gp-80 and IL-6:gp-80:VHH binding to gp-130 D1 (see Section 6.2.3 for a more detailed description). Presented in Figures 6.24, 6.25 and 6.26 are the SPR sensorgrams for each of the binding experiments and tables detailing the calculated dissociation rate constants.

	Stoichiometric Ratio		
	VHH 67	VHH 94	VHH 29
gp-80 D2D3 Site I	2.06	1.97	0.82
gp-130 ECD Site II	0.93	0.85	0.45
gp-130 D1 Site III	1.04	0.88	0.65

Table 6.2 – Stoichiometric Ratios Calculated from SPR Binding Experiments

The table shows the stoichiometric ratios calculated from the SPR binding experiments used to determine the effect on the binding of IL-6 at Site I, Site II and Site III in the presence of VHH 67, VHH 94 and VHH 29. The presence of VHH 67 and VHH 94 both showed a two fold increase in the binding of IL-6 to gp-80 at Site I. The presence of VHH 29 showed an inhibition of binding of IL-6:gp-80 to gp-130 ECD. The results quoted are from a single experiment but are consistent with data collected previously (Dr. Terry Baker (UCB), unpublished data).

Enhanced binding of both the VHH 67:IL-6 complex and the VHH 94:IL-6 complex to the immobilised gp-80 D2D3 are observed in the Site I SPR binding assay and, as predicted, little effect was observed in the presence of the Site II binder VHH 29. The enhancement seen for both VHH 67 and VHH 94 was around two fold higher than seen in the absence of VHH (see Table 6.2 and Figure 6.24) and suggest some allosteric effects at Site I modulated by the binding of VHH 67 or VHH 94 to IL-6. The dissociation curves in the presence of all VHs can be fitted using a biphasic function (see Figure 6.24). The IL-6 control and VHH 29:IL-6 show similar off rates with approximately 75 % contribution from a faster rate constant (average value of $1.9 \times 10^{-2} \text{ s}^{-1}$) and the remainder from a slower rate constant (average value of $2.6 \times 10^{-3} \text{ s}^{-1}$). In the presence of VHH 67 and VHH 94 the dissociation profile of IL-6 is very different. Although both dissociation curves were fitted to a biphasic model, the dissociation occurs predominantly (> 90 %) with a rate constant of around $1.1 \times 10^{-5} \text{ s}^{-1}$, two to three orders of magnitude slower than was observed for IL-6 alone and in the presence of the Site II binder.

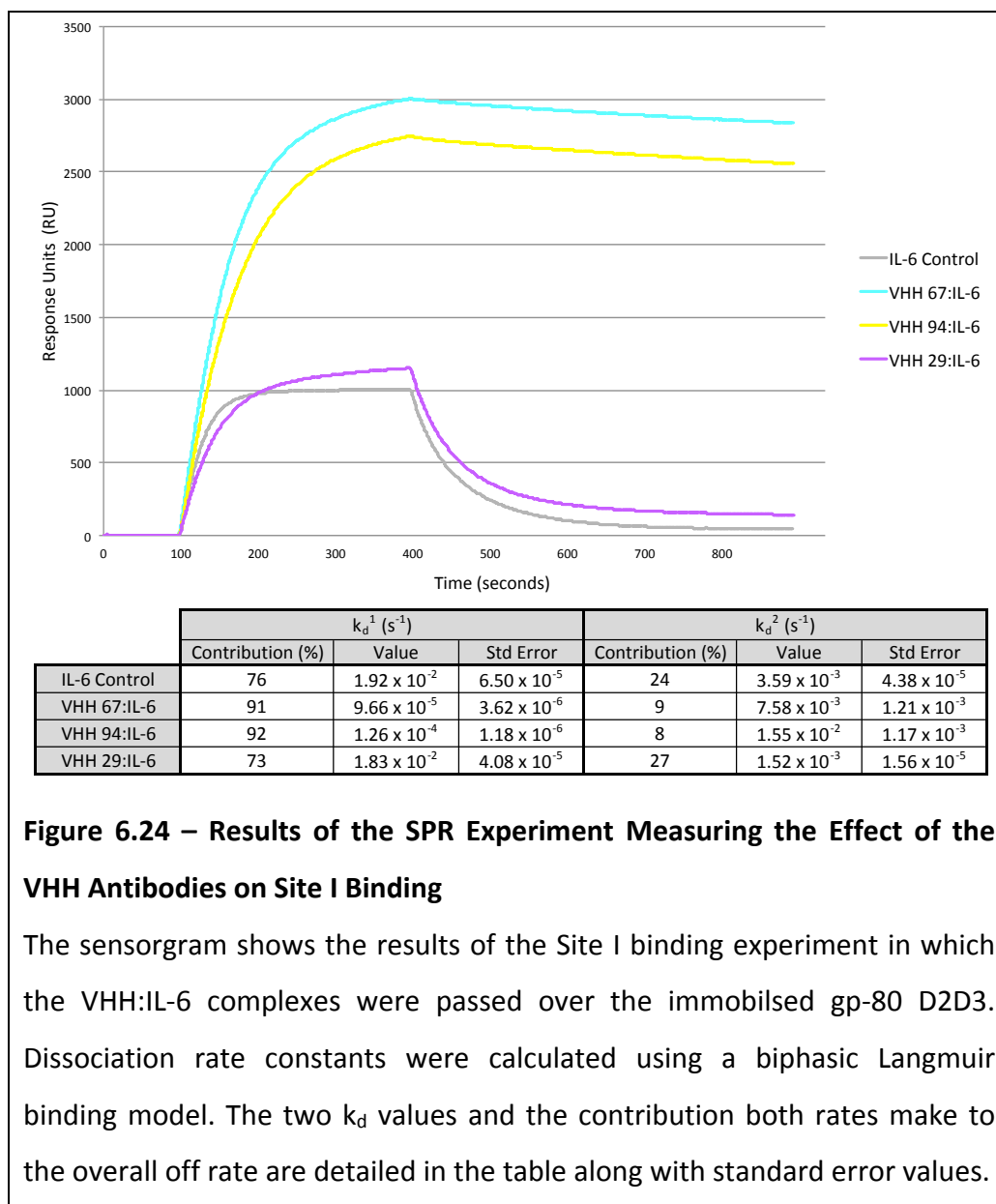


Figure 6.25 shows the effect of the presence of the VHHs on the binding of the IL-6:gp-80 complex to the immobilised gp-130 ECD. No change in response was seen over the control when either VHH 67 or VHH 94 were present (see Table 6.2). As expected, a reduction in binding is observed in the presence of VHH 29 which has been shown to bind to Site II and therefore should inhibit the interaction between the IL-6:gp-80 complex and the immobilised gp-130 (see Table 6.2). The off rates of the IL-6:gp-80 control, the VHH 67:IL-6:gp-80 complex and the VHH 94:IL-6:gp-80 complex can be predominantly fitted (97 %) to a single rate constant with a value of approximately $3.9 \times 10^{-5} s^{-1}$. The off rate in the presence of VHH 29 is

approximately 20-fold slower than is seen for IL-6:gp-80 control but it can be seen in Figure 6.25 that it is the on rate that has altered compared to the control. This slower on rate accounts for the reduction in the binding of the IL-6:gp-80 complex to gp-130 ECD (a stoichiometric ratio of 0.45 in the presence of VHH 29 compared to a stoichiometric ratio of 1 for the control, as shown in Table 6.2).

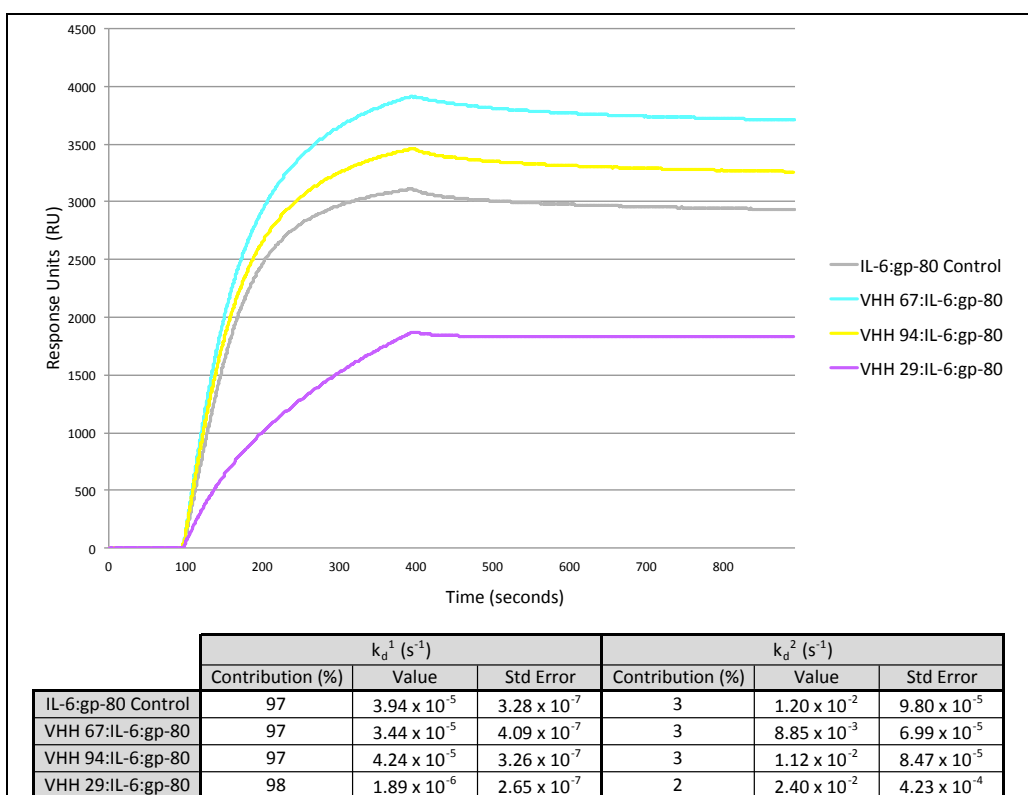


Figure 6.25 – Results of the SPR Experiment Measuring the Effect of the VHH Antibodies on Site II Binding

The sensorgram shows the results of the Site II binding experiment in which the VHH:IL-6:gp-80 complexes were passed over the immobilised gp-130 ECD. Dissociation rate constants were calculated using a biphasic Langmuir binding model. The two k_d values and the contribution both rates make to the overall off rate are detailed in the table along with standard error values.

The final sensorgram in Figure 6.26 shows VHH 67, VHH 94 and VHH 29 have little effect on binding at Site III (see Table 6.2). The dissociation profiles of the control and in the presence of each of the VHHs are very similar with all data being fitted using a biphasic function with k_d values falling in the range of $1.11 \times 10^{-2} \text{ s}^{-1}$ to $8.81 \times 10^{-3} \text{ s}^{-1}$.

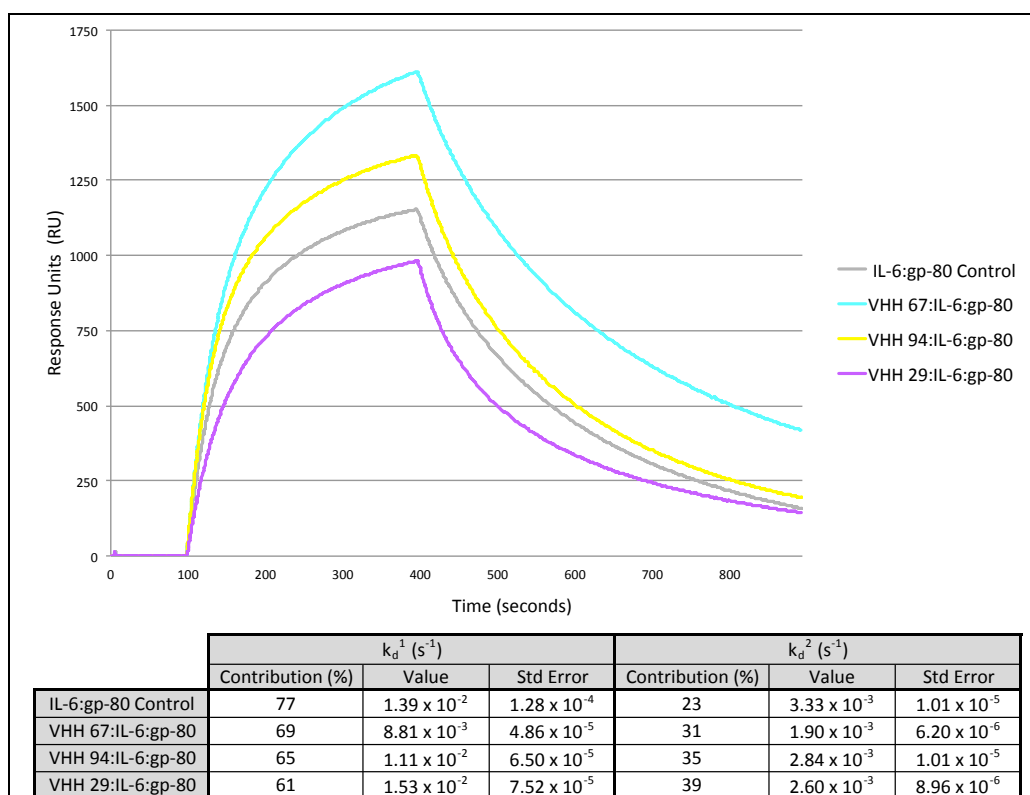


Figure 6.26 – Results of the SPR Experiment Measuring the Effect of the VHH Antibodies on Site III Binding

The sensorgram shows the results of the Site III binding experiment in which the VHH:IL-6:gp-80 complexes were passed over the immobilised gp-130 D1. Dissociation rate constants were calculated using a biphasic Langmuir binding model. The two k_d values and the contribution both rates make to the overall off rate are detailed in the table along with standard error values.

6.3.4 – NMR Minimal Shift Mapping of VHH 94 Epitope on IL-6

From the Site III blocking ELISA assay, the presence of both VHH 67 and VHH 94 appeared to increase the binding of hyper IL-6 to gp-130 D1. NMR minimal shift mapping of VHH 94 was undertaken to understand whether the binding mode of VHH 94 is similar to that of VHH 67. As with VHH 67, ^{15}N - ^1H TROSY and ^{15}N - ^{13}C - ^1H TROSY-HNCO spectra of free IL-6 and of the IL-6 bound to VHH 94 were collected. The results of the minimal shift mapping are highlighted on the structure of IL-6 (Somers *et al.*, 1997, PDB accession code 1ALU) in Figure 6.27. The results suggest a binding site similar to that seen with VHH 67, with the perturbed residues predominantly located at the base of IL-6, suggesting that VHH 94 may bind in the same region as VHH 67.

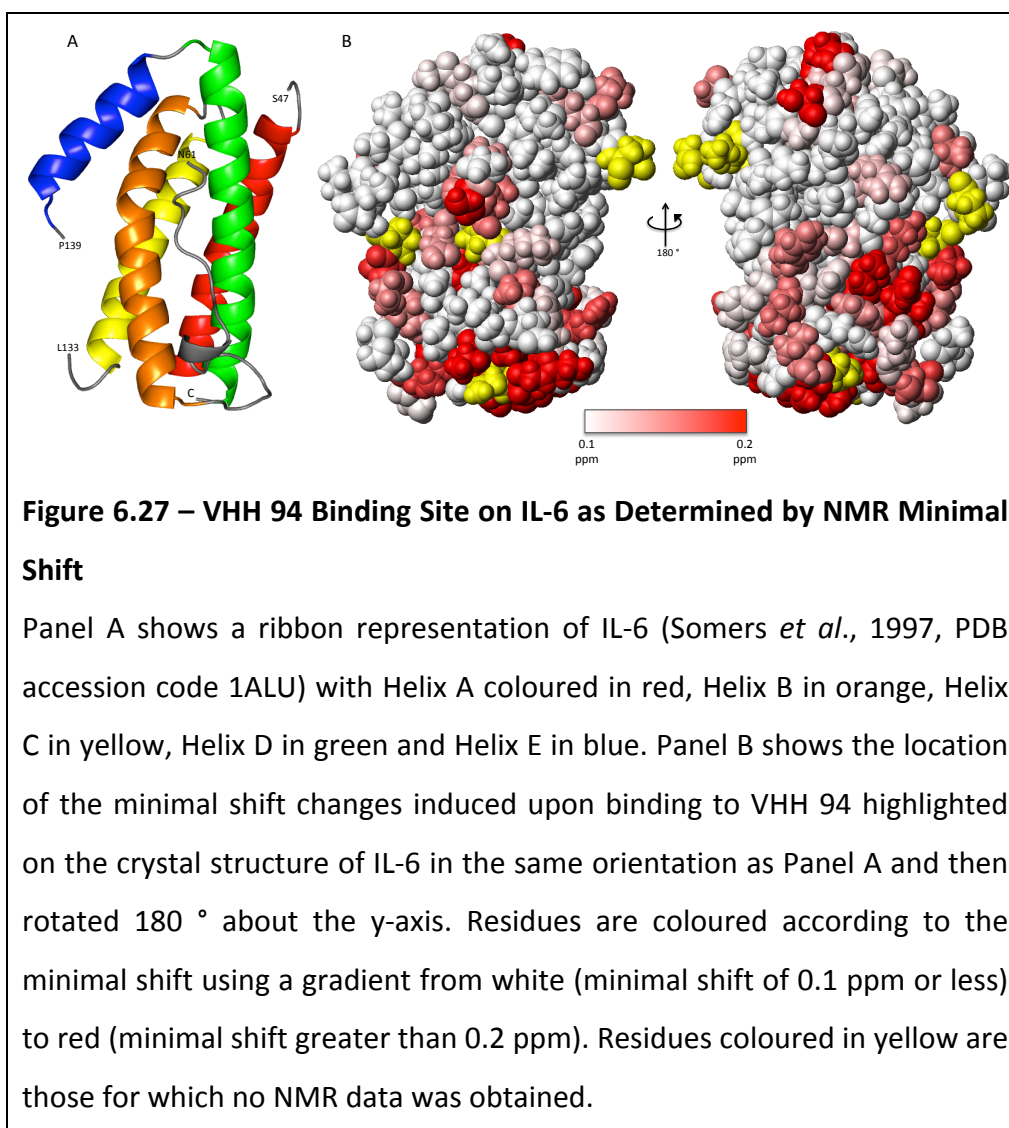
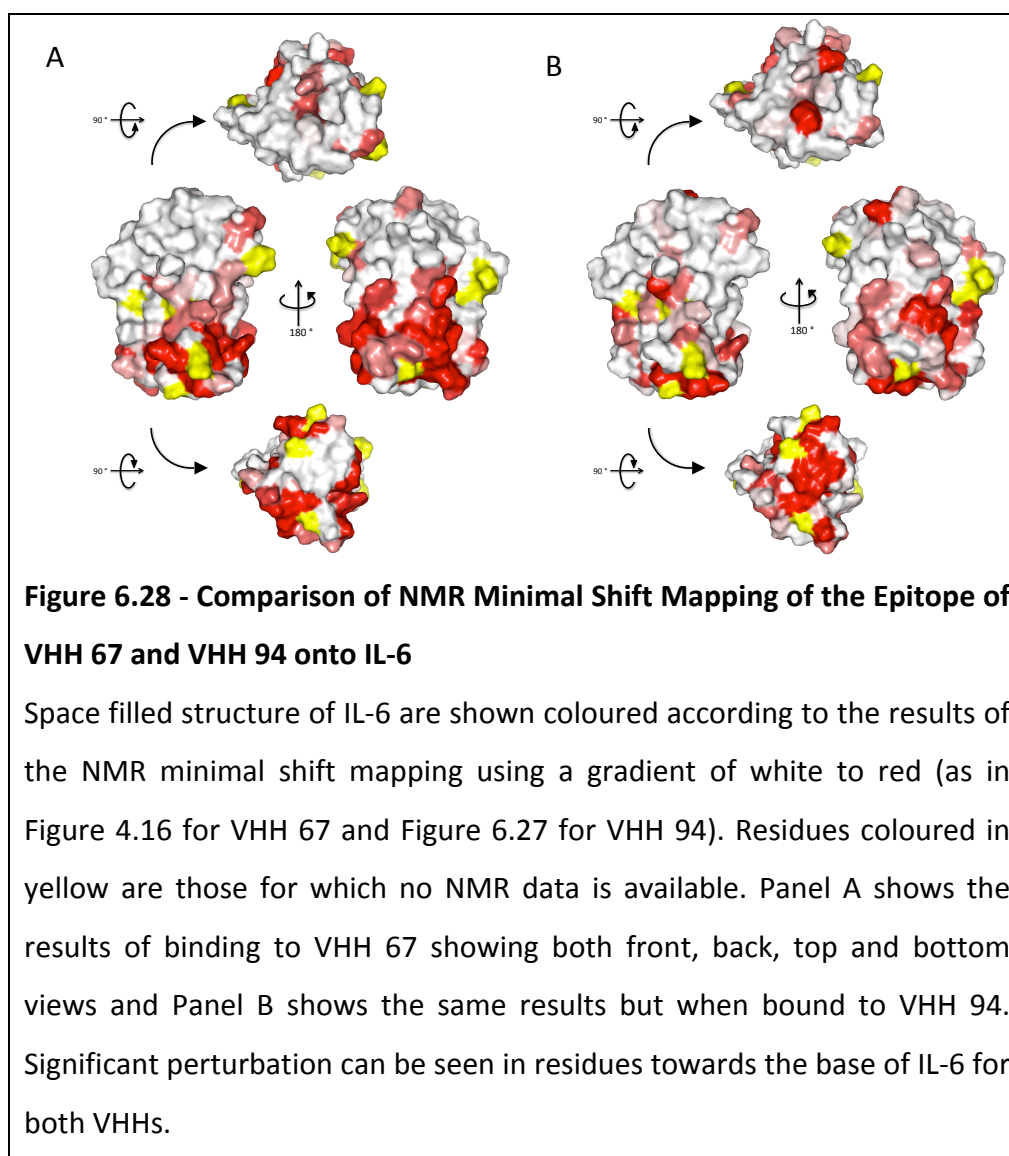


Figure 6.28 shows a comparison between the perturbed residues when IL-6 is bound to VHH 67 (Panel A) and when bound to VHH 94 (Panel B).



6.4 – Discussion

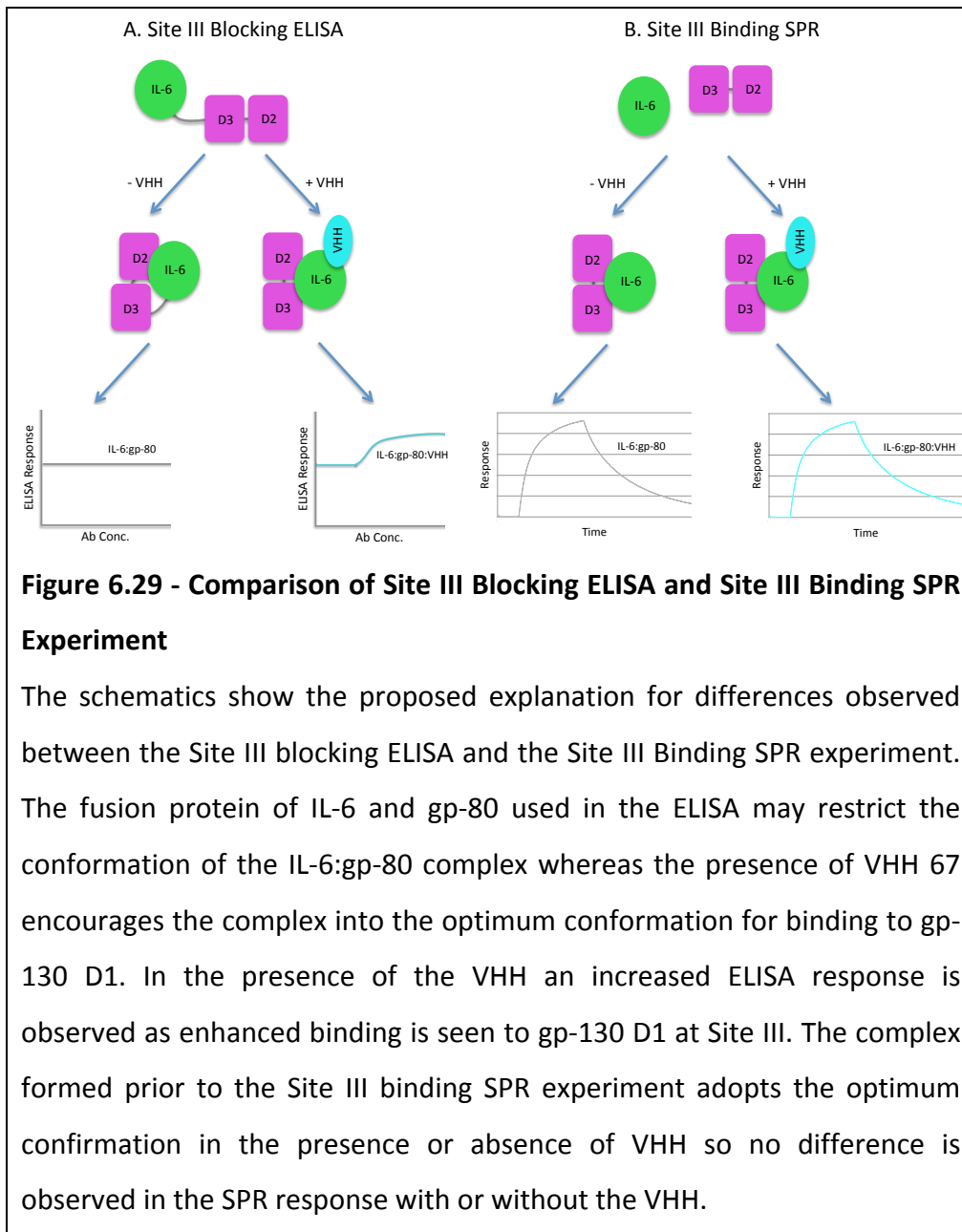
The assumption that VHH 67 was binding to a site distinct from Site I, Site II and Site III on IL-6 was confirmed by the X-ray crystal structure. A comparison of the bound crystal structure with the free NMR structure shows very little difference in the structure of the antigen-binding region implying that the free structure is pre-orientated into its bound conformation and therefore loses no entropy in binding. Observations made in Chapter 5 suggest that making CDR3 more flexible has a detrimental effect on antigen binding, this could be due to the entropic penalty of

a disordered CDR3 becoming ordered upon binding. Having the crystal structure of VHH 67 bound to IL-6 confirms that none of the mutated residues (as shown in Section 5.1) have any direct contact with IL-6, therefore, the changes in affinity seen with the F139A and Y217A mutations are most likely the result of changes in dynamics rather than removal of key binding residues.

SPR experiments were performed in an attempt to understand the biological impact of the new binding site identified by the VHH 67:IL-6 crystal structure. The Site I binding SPR experiments revealed an increase in the binding of IL-6 to gp-80 when VHH 67 and VHH 94 were present suggesting that these two VHs may enhance the interaction between IL-6 and gp-80, stabilising the IL-6:gp-80 complex. This offers an explanation for the enhancement in binding in the Site III blocking ELISA as described in Section 6.3.1 and Figure 6.6. However, given the enhanced response for VHH 67 and VHH 94 in the Site III blocking ELISA, an increased response was also expected in the Site III SPR experiment but this is not the case. It is proposed that this could be due to either differences in the reagents used or differences in the concentrations of reagents used in the two experiments.

The interaction between IL-6 and gp-80 is relatively weak with a K_D of approximately 1 nM (Yamasaki *et al.*, 1988). In the Site III blocking ELISA the concentration of IL-6-gp-80 fusion protein used was 100 ng ml⁻¹ or, given that IL-6:gp-80 fusion is approximately 42 kDa in size, 2 nM. With a K_D of 1 nM, at this concentration it is likely that IL-6:gp-80 fusion is in equilibrium between the open and active form (as shown in Figure 6.6 Panel A), therefore, if the VHH does enhance the formation of the complex the effect will be observed by an enhancement in the binding to gp-130 D1 in the presence of the VHH over IL-6:gp-80 alone (as discussed in Section 6.3.1). The Site III SPR binding experiment was carried out with an IL-6:gp-80 concentration of 100 nM, significantly greater than the K_D . Therefore, it is likely that the equilibrium would be favoured towards the active form so any enhancement in binding between IL-6 and gp-80 that may be achieved in the presence of the VHH may not be apparent by enhanced binding of the IL-6:gp-80:VHH complex to gp-130 D1.

Alternatively, as described in Section 6.3.1, a fusion of IL-6 and gp-80 was used in the blocking ELISAs whereas for the SPR experiments a complex of IL-6 and gp-80 was formed prior to titration over gp-130 D1. The reason for using the fusion protein was to encourage, by the close proximity of the IL-6 and gp-80 domains, the relatively weak Site I interaction between IL-6 and gp-80 (Yamasaki *et al.*, 1988). Original fusion constructs included a flexible (Gly₄Ser)₂ linker between the IL-6 and gp-80 D3 (Fischer *et al.*, 1997) but this was optimised by removing the (Gly₄Ser)₂ linker in favour of using the flexible N- and C-termini of IL-6 and gp-80 to join the domains (Hancock, 2010). This may have introduced steric hindrance into the fusion protein resulting in a suboptimal, but still active, conformation being formed. The results from the Site I SPR experiment confirms an enhancement in the binding of IL-6 to gp-80 in the presence of VHH 67 and VHH 94 and it may be that this interaction between the VHH and the IL-6 of the fusion protein encourages the formation of the optimum IL-6:gp-80 complex and overcomes the steric hindrance. This would then explain the enhanced binding to gp-130 D1 in the ELISA as the interaction at Site III is reliant of the formation of the IL-6:gp-80 complex (Boulanger *et al.*, 2003). The complex formed between IL-6 and gp-80 for the Site III SPR experiment is not restricted as the domains are not covalently linked, therefore, the conformation of the IL-6:gp-80 complex remains the same in the absence or presence of the VHH. Figure 6.29 illustrates the this proposed explanation of the differences between the Site III blocking ELISA and the Site III binding SPR experiment.



Revisiting the NMR minimal shift mapping shows large perturbations in the region of IL-6 that interacts with gp-80 D2D3 when VHH 67 is bound (Figure 6.30). These chemical shift differences may be indicative of structural changes that result in the enhanced binding to gp-80 D2D3.

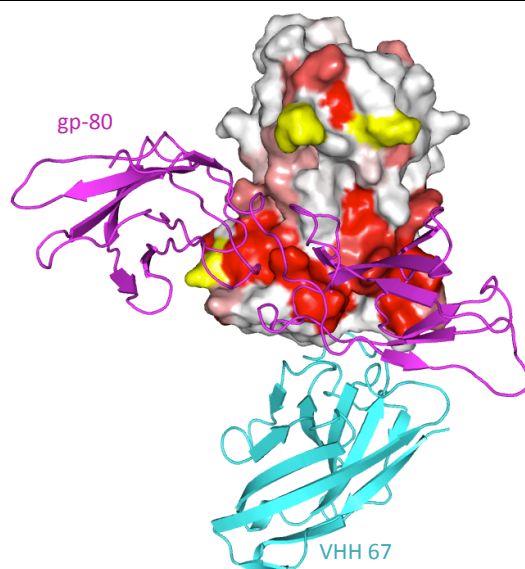


Figure 6.30 - NMR Minimal Shift Perturbations Induced upon the Binding of VHH 67 to IL-6 at the gp-80 Binding Site

The crystal structure of VHH 67:IL-6 is shown with VHH 67 as a cyan ribbon and IL-6 as a space filled structure coloured according to the NMR minimal shift results using a gradient from white (small shifts) to red (large shifts). The magenta ribbon highlights the position of gp-80 D2D3 in the IL-6 signalling complex (adapted from Boulanger *et al.*, 2003, PDB accession code 1P9M and aligned with the IL-6 crystal structure). Large chemical shift perturbations in the structure of IL-6 can be seen in residues that would form the interface between IL-6 and gp-80.

6.5 – Conclusions

The experiments described in this chapter have confirmed that VHH 67 binds to a novel binding site at the base of IL-6 consistent with results from the NMR binding site mapping, ELISA and cell based assays. Another interesting finding is that the binding of VHH 67 at this novel site appears to cause allosteric changes in IL-6 that result in the enhanced binding of gp-80 D2D3. A second example of a VHH exhibiting the signs of allosteric modulation at the gp-80 D2D3 binding site was also identified.

Chapter 7 – Using VHH Antibody Fragments as a Tool for Drug Discovery

7.1 – Introduction

The final objective of this project was to evaluate the use of VHH antibodies as a tool for small molecule drug discovery. This chapter aims to collate the findings from the previous chapters in order to discuss the application of using VHHs to aid the drug discovery process.

7.2 – Discussion

With over 25 antibodies now approved for use by the FDA (Reichert, 2010) and a further 350 currently in clinical trials (Reichert, 2013) no one can deny the interest in, and success of, using antibodies as therapeutics. However, disadvantages, such as limited tissue penetration and the expense that can be associated with manufacturing such complex molecules, means that the search for small molecule alternatives to antibody treatments are also actively pursued, with the ideal scenario being to use the high specificity of antibodies to guide small molecule drug design. Not only can antibodies be used to help design small molecules but the risk associated with small molecule drug discovery can be reduced by using antibodies to validate biological pathways and define the region of interaction (Lawson, 2012).

The availability of structural information, from established techniques such as X-ray crystallography and NMR, can offer advantages when designing small molecules to modulate protein-protein interactions. Identification of residues making up the protein-protein interface can act as a template from which peptidic or small molecule mimics can be designed. Typically for antibody-antigen interactions, structural information from antibody fragments such as Fabs and scFvs would be used to gain an insight into the characteristics of the binding site. The work described in this project not only supports the use of VHH antibodies as

alternatives to these larger antibody fragments, but also offers significant benefits over current strategies.

The methods used for the expression and purification of the VHH antibodies studied led to the generation of high yields of pure VHH proteins (up to approximately 50 mg l⁻¹ for unlabelled protein and around 20 mg l⁻¹ for labelled protein) which provided high quality NMR, crystallography and SPR data. Unfortunately, for the panel of VHH antibodies studied, conditions to express soluble VHHs in *E.coli* were not identified, however, there are many examples of soluble VHH expression described in the literature (Desmyter *et al.*, 1996; Ghahroudi *et al.*, 1997; Lauwereys *et al.*, 1998; Muyldermans *et al.*, 2001; Vranken *et al.*, 2002 and Vuchelen *et al.*, 2009).

The physical characteristics of VHH antibodies, such as their small size compared to Fabs and enhanced stability compared to scFvs, offer an obvious advantage compared to conventional antibody fragments. For a small number of VHHs, signs of aggregation were observed but overall the samples were easier to handle than scFvs due to the reduced size and because they consist of a single domain. The stability of the VHH antibodies allowed collection of NMR data at 35 °C over periods of several days without degradation of the sample.

VHH fragments have the advantage that all antigen binding affinity and specificity is encoded in a single domain, therefore, if using the structural features of the antibody to guide small molecule design, the same information would be gained from a 15 kDa VHH as from a 28 kDa scFv or a 50 kDa Fab.

The work described in Chapter 3 shows that high quality NMR spectra can be obtained from VHH proteins, with very little signal overlap observed in the 2D and 3D spectra. This allowed the solution structures of two of the unbound anti-IL-6 VHHs to be determined in a much quicker timeframe than would be possible for larger antibody fragments. The solution structures gave an interesting insight into the dynamics of the VHH CDR loops with both structured and flexible loops being

identified. The work presented in Chapter 5 revealed that deconstructing CDR3 resulted in significant conformational changes and reduced affinity, which emphasises the importance of the structured CDR3 loop to the interaction between VHH 67 and IL-6. In this case the more dynamic CDR1 may help to guide the VHH antibody into position, but it is the structured CDR3 loop that is responsible for binding so the interactions between CDR3 and the antigen would be used as a template from which to design a small molecule. This highlights that when looking to design small molecules to mimic the binding of an antibody to a particular antigen, an understanding of the dynamics of the CDRs at the binding interface and the possible conformations sampled by the CDRs could be very valuable. NMR offers an insight into the dynamics not necessarily obtained from crystallography where protein dynamics may be affected by the crystal packing.

X-ray crystallography is one method that can be used to identify the binding interfaces within a complex, but if suitable crystals of the complex cannot be produced, as was the case with the VHH 18:IL-6 complex, other methods such as minimal shift mapping by NMR and HDX by MS can be used as an alternative. The work described in Chapter 4 confirmed that the mapping of the binding interfaces of both the VHH and the antigen, in this case IL-6, could be achieved using NMR minimal shift and HDX by MS methods. Paratope mapping by NMR implied that all three CDRs of the VHHs were likely to form the VHH-IL-6 interface and in the case of VHH 67 this was later confirmed with the VHH 67:IL-6 crystal structure. The implication that all three CDR loops of the VHHs were involved in binding led to further analysis of the anti-IL-6 VHH crystal structures (Hancock, 2010) to determine the interface area. This showed that VHH antibodies are capable of forming interface areas comparable to that of conventional antibody fragments through either the elongated CDR3 loop or through the framework regions in addition to the CDR loops. This large interface area may also explain how VHH antibodies can form high affinity interactions with an antigen despite having fewer CDR loops.

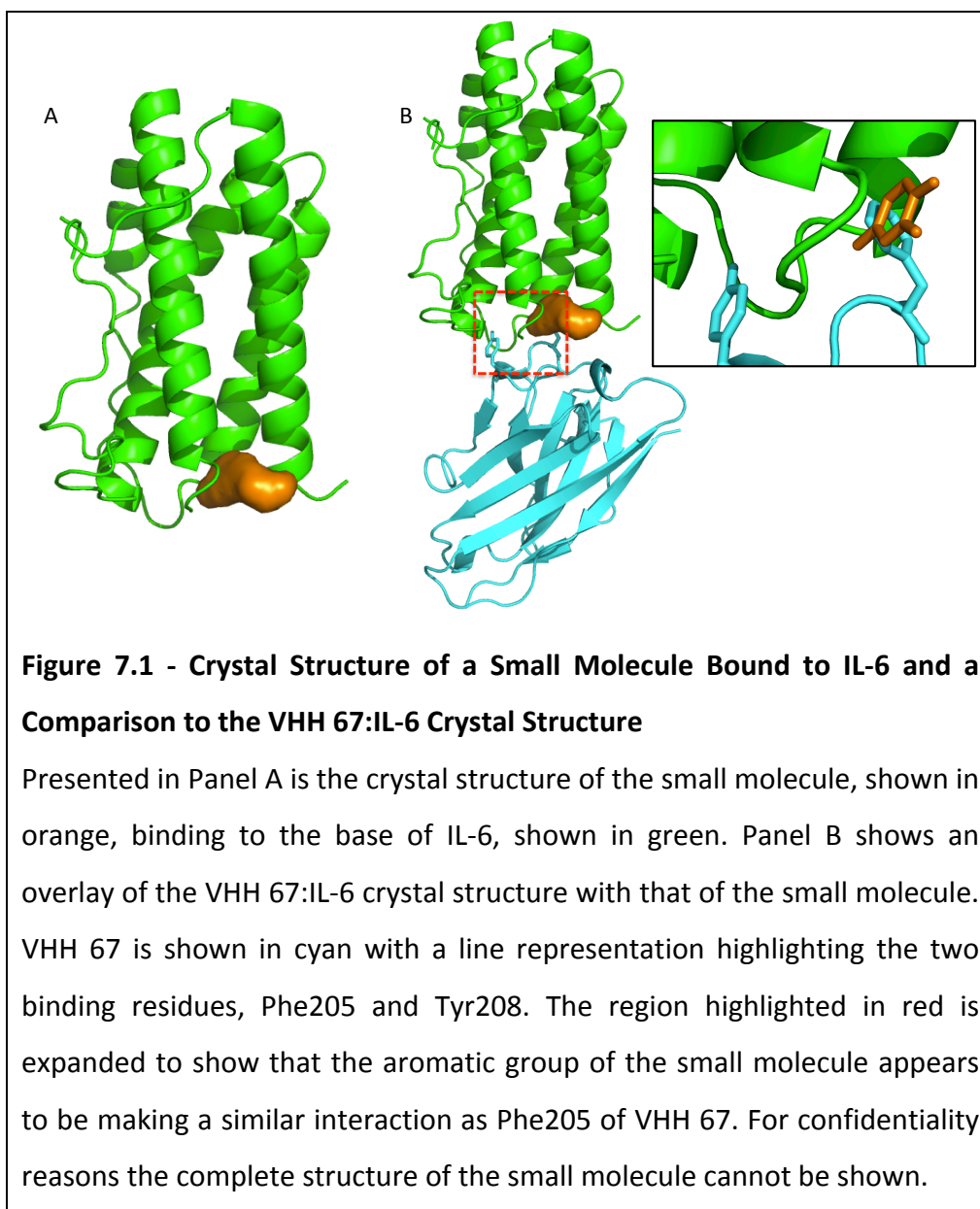
Of the examples discussed in this thesis, the epitope mapping of IL-6 using the VHH antibodies was more successful than previous attempts using scFvs (Addis *et al.*, 2014), with a better defined epitope observed using the smaller antibody fragment. The successful epitope mapping results obtained using VHH antibodies suggest that, as with conventional antibodies, it will be possible to screen a target antigen for potential binding sites but with the added benefit of the improved physical properties of the VHH antibodies. Another advantage of using VHH fragments over scFvs and Fabs for epitope mapping is that the overall size of the antibody:antigen complex will be smaller and therefore less likely to result in shorter NMR relaxation times and the associated loss in sensitivity.

A unique characteristic of the VHH antibodies is the elongated CDR3 loop and this feature could offer advantages over the CDR loops of conventional antibody fragments. For example, the elongated CDR3 loops associated with VHH antibodies are perceived to have the ability to access alternative binding sites (Muyldermans, 2001). Additional residues in the N-terminal portion of CDR3 of VHH antibodies such as cAb-Lys3 (Desmyter *et al.*, 1996) have been shown to extend from the antigen binding site into a cleft of the antigen and may provide starting points for the design of small molecule binders that may not have been discovered with conventional antibodies.

An interesting outcome of the structural work with VHH 67 is its potential application to the design of phage display libraries. The objective of a phage display campaign is to obtain a suitable antibody scaffold from a naïve immunisation library and synthetically optimise the scaffold to improve the affinity and specificity of the antibody for the chosen target (Pande *et al.*, 2010). From the work described in this project and from previously reported literature examples, a set of recommendations could be made to allow a library to be designed using a VHH as a scaffold. The NMR and crystal structures of VHH 67 (Chapter 3 and Chapter 6) and the cAb-Lys3 crystal structure (Desmyter *et al.*, 1996) highlight that binding occurs through the N-terminal region of CDR3 whereas the C-terminal region of the long CDR3 folds back over the VHH creating an extra section of

framework. The work described in Chapter 5 revealed that destabilising this additional region of framework had a dramatic and negative effect on the affinity of the VHH to IL-6. This suggests that if VHH 67 was to be used as scaffold for a library, residues in the C-terminal region of CDR3 should not be altered as the stability of the VHH could be compromised with little chance of any enhancement in affinity. Instead, the optimisation should be focussed on the N-terminal region of CDR3 as these are the residues more likely to form the antigen-binding site. In addition to this, if the strategy was to extend the CDR3 loop into a cleft of the antigen to gain greater affinity and specificity, it is again the N-terminal region in to which the extra residues should be incorporated.

The example described below illustrates how using VHH antibody fragments to design small molecule binders may be possible. As a result of screening a fragment library for activity towards IL-6, a small molecule binder was identified. The X-ray crystal structure of the small molecule bound to IL-6 was solved and shows the small molecule binding to IL-6 in the same location as was observed with VHH 67 (see Figure 7.1 Panel A). An overlay of the small molecule crystal structure with the VHH 67 crystal structure (as discussed in Chapter 6) shows the aromatic group of the small molecule making the same interaction with IL-6 as Phe205 of VHH 67 (Figure 7.1 Panel B). The next step would be to elaborate the initial small molecule hit to enhance the affinity of the molecule to IL-6. Using the VHH 67:IL-6 complex structure as a guide, a modification to the small molecule could be made to incorporate an additional aromatic group that, through the design on an appropriate linker, would mimic the interaction between Tyr208 of VHH 67 and IL-6.



The identification of this small molecule binding to IL-6 in the same location as VHH 67 was a serendipitous discovery made independently to the work carried out in this project but clearly illustrates the great potential of antibody guided small molecule drug design and discovery.

7.3 – Conclusions

In conclusion, the work described in this thesis clearly indicates that VHH antibody fragments are a good alternative to conventional antibody fragments for the structural characterisation of antibody-antigen interactions and the subsequent design of small molecule binders. The information gained from VHH antibodies offer equal value to that acquired using Fabs and scFvs with respect to binding site mapping, the determination of solution structures and insights into conformational dynamics but the small size, relative simplicity and the enhanced stability of VHH antibodies offer savings in the time and resources required to obtain detailed structural information. Characteristics such as the binding affinity originating from a single domain and the elongated CDR3 loops could potentially offer more simplistic small molecule design strategies and access to binding sites that are unique to VHH antibodies.

Appendices

A.1 - Minimal Media

$(\text{NH}_4)_2\text{SO}_4$	1.0 g l^{-1}	$(^{15}\text{N} \text{ labelled if required})$
Na_2HPO_4	6.8 g l^{-1}	
KH_2PO_4	3.0 g l^{-1}	
NaCl	0.5 g l^{-1}	
Na_2SO_4	0.3 mM	
EDTA	50.0 mg l^{-1}	
MnCl_2	16.0 mg l^{-1}	
FeCl_3	5.0 mg l^{-1}	
ZnCl_2	0.5 mg l^{-1}	
CuCl_2	0.1 mg l^{-1}	
CoCl_2	0.1 mg l^{-1}	
H_3BO_3	0.1 mg l^{-1}	
MgSO_4	1.0 mM	
CaCl_2	0.3 mM	
d-Biotin	1.0 mg l^{-1}	
Thiamine	1.0 mg l^{-1}	
Glucose	2.0 g l^{-1}	$(^{13}\text{C} \text{ labelled if required})$

Made up in dH_2O and the pH adjusted to pH 7.4 with NaOH.

A.2 - TALOS Based Torsion Angle Constraints used for Structural Calculations

A.2.1 - VHH 18 Torsion Angle Constraints

Residue	Angle	Constraint Range	
102 VAL	PHI	-143	-3
	PSI	56	184
103 GLN	PHI	-168	-96
	PSI	122	170
104 LEU	PHI	-167	-83
	PSI	99	175
105 VAL	PHI	-173	-97
	PSI	107	151
106 GLU	PHI	-146	-34
	PSI	104	164
107 SER	PHI	-170	-66
	PSI	142	182
111 SER	PHI	-137	-73
	PSI	116	172
112 VAL	PHI	-152	-112
	PSI	132	176
113 GLN	PHI	-175	-35
	PSI	108	172
114 ALA	PHI	-75	-35
	PSI	115	155
115 GLY	PHI	70	114
	PSI	-38	14
116 GLY	PHI	-145	-37
	PSI	89	205
117 SER	PHI	-175	-95
	PSI	130	174
118 LEU	PHI	-162	-118
	PSI	105	173
119 ARG	PHI	-172	-96
	PSI	111	159
120 LEU	PHI	-148	-96
	PSI	110	166
121 SER	PHI	-152	-92
	PSI	103	163
122 CYS	PHI	-152	-104
	PSI	117	157
123 ALA	PHI	-152	-88
	PSI	105	149
124 ALA	PHI	-162	-86
	PSI	126	166

Residue	Angle	Constraint Range	
125 SER	PHI	-163	-47
	PSI	106	178
128 ARG	PHI	-104	-56
	PSI	57	181
129 ILE	PHI	-77	-37
	PSI	-68	-12
130 SER	PHI	-88	-48
	PSI	-51	9
131 GLU	PHI	-130	-66
	PSI	-44	20
132 CYS	PHI	-165	-89
	PSI	127	175
133 THR	PHI	-159	-55
	PSI	101	145
134 THR	PHI	-155	-67
	PSI	111	151
135 GLY	PHI	-180	-64
	PSI	136	188
136 TRP	PHI	-168	-92
	PSI	119	163
137 TYR	PHI	-168	-100
	PSI	143	183
138 ARG	PHI	-168	-92
	PSI	121	169
139 GLN	PHI	-180	-68
	PSI	109	149
140 ALA	PHI	-125	-41
	PSI	87	171
141 PRO	PSI	118	158
143 LYS	PHI	-154	-62
	PSI	65	205
144 GLU	PHI	-150	-26
	PSI	116	176
145 ARG	PHI	-125	-53
	PSI	93	157
146 GLU	PHI	-146	-86
	PSI	115	155
147 LEU	PHI	-99	-55
	PSI	104	152
148 VAL	PHI	-145	-69
	PSI	-46	-6
149 SER	PHI	-193	-105
	PSI	134	174
150 LYS	PHI	-159	-107
	PSI	106	162
151 PHE	PHI	-165	-85
	PSI	106	146

Residue	Angle	Constraint Range	
152 SER	PHI	-177	-53
	PSI	115	187
153 ASN	PHI	30	70
	PSI	20	64
154 LEU	PHI	-116	-60
	PSI	-28	24
155 GLY	PHI	53	101
	PSI	-5	51
156 THR	PHI	-128	-68
	PSI	127	183
157 THR	PHI	-172	-92
	PSI	130	182
158 TRP	PHI	-161	-109
	PSI	109	153
159 TYR	PHI	-155	-99
	PSI	123	171
160 THR	PHI	-88	-48
	PSI	97	177
161 GLY	PHI	-110	-22
	PSI	-69	15
162 SER	PHI	-87	-47
	PSI	-42	2
163 VAL	PHI	-136	-72
	PSI	-22	34
164 LYS	PHI	-139	1
	PSI	61	181
165 GLY	PHI	67	111
	PSI	-44	28
166 ARG	PHI	-112	-52
	PSI	118	182
167 PHE	PHI	-188	-68
	PSI	124	180
168 THR	PHI	-165	-89
	PSI	85	165
169 ILE	PHI	-139	-95
	PSI	110	150
170 SER	PHI	-166	-82
	PSI	107	175
171 GLN	PHI	-149	-109
	PSI	118	174
172 ASP	PHI	-154	-62
	PSI	96	172
173 SER	PHI	-107	-23
	PSI	-65	11
174 ALA	PHI	-89	-45
	PSI	-53	-9
175 LYS	PHI	-115	-71

Residue	Angle	Constraint Range	
176 ASN	PHI	-143	-31
	PSI	91	195
177 THR	PHI	-171	-87
	PSI	127	175
178 VAL	PHI	-156	-108
	PSI	112	152
179 TYR	PHI	-145	-105
	PSI	121	161
180 LEU	PHI	-151	-91
	PSI	98	146
181 GLN	PHI	-138	-66
	PSI	97	161
182 MET	PHI	-169	-77
	PSI	113	173
183 ASN	PHI	-150	-82
	PSI	139	187
184 SER	PHI	34	74
	PSI	20	60
185 LEU	PHI	-136	4
	PSI	96	164
186 SER	PHI	-170	-42
	PSI	70	190
187 PRO	PSI	-50	-10
190 THR	PHI	-96	-52
	PSI	110	150
191 ALA	PHI	-127	-83
	PSI	-65	15
192 MET	PHI	-155	-39
	PSI	114	154
193 TYR	PHI	-125	-85
	PSI	114	170
194 TYR	PHI	-157	-113
	PSI	131	179
195 CYS	PHI	-161	-121
	PSI	120	176
196 ASN	PHI	-170	-118
	PSI	111	191
197 THR	PHI	-156	-96
	PSI	130	170
198 ASP	PHI	-163	-95
	PSI	143	183
199 LEU	PHI	-167	-27
	PSI	67	207
200 CYS	PHI	-175	-91
	PSI	77	217
208 TRP	PHI	-219	-79
	PSI	111	191

Residue	Angle	Constraint Range	
210 GLN	PHI	-151	-59
	PSI	-45	43
212 THR	PHI	-158	-58
	PSI	100	144
213 GLN	PHI	-132	-64
	PSI	105	149
214 VAL	PHI	-140	-84
	PSI	104	144
215 THR	PHI	-161	-73
	PSI	100	140
216 VAL	PHI	-144	-80
	PSI	98	150
217 SER	PHI	-138	-62
	PSI	63	187
218 SER	PHI	-104	-40
	PSI	-58	-6

A.2.2 - VHH 67 Torsion Angle Constraints

Residue	Angle	Constraint Range	
96 LEU	PHI	-143	-3
	PSI	99	187
97 TYR	PHI	-194	-54
	PSI	69	201
98 PHE	PHI	-130	10
	PSI	97	153
101 ASP	PHI	-119	-63
	PSI	-56	20
102 VAL	PHI	-183	-59
	PSI	108	160
103 GLN	PHI	-155	-95
	PSI	114	154
104 LEU	PHI	-161	-81
	PSI	102	174
105 VAL	PHI	-156	-100
	PSI	98	158
106 GLU	PHI	-132	-32
	PSI	106	166
107 SER	PHI	-159	-79
	PSI	103	243
109 GLY	PHI	57	105
	PSI	-2	38
111 SER	PHI	-149	-77
	PSI	109	177
112 VAL	PHI	-161	-89
	PSI	115	179
113 GLN	PHI	-184	-48
	PSI	103	179
114 ALA	PHI	-76	-36
	PSI	114	154
115 GLY	PHI	70	110
	PSI	-28	16
116 GLY	PHI	-148	-40
	PSI	89	209
117 SER	PHI	-177	-93
	PSI	125	177
118 LEU	PHI	-166	-122
	PSI	105	173

Residue	Angle	Constraint Range	
119 ARG	PHI	-164	-92
	PSI	111	151
120 LEU	PHI	-149	-85
	PSI	109	169
121 SER	PHI	-157	-89
	PSI	112	164
122 CYS	PHI	-137	-81
	PSI	78	146
124 PHE	PHI	-153	-105
	PSI	103	163
125 SER	PHI	-159	-51
	PSI	122	182
133 GLN	PHI	-122	-50
	PSI	-42	18
135 TYR	PHI	-110	-58
	PSI	-60	-16
136 MET	PHI	-185	-73
	PSI	132	180
138 TRP	PHI	-161	-109
	PSI	129	169
139 PHE	PHI	-174	-106
	PSI	131	183
140 ARG	PHI	-166	-94
	PSI	114	166
141 GLN	PHI	-172	-72
	PSI	105	149
146 GLU	PHI	-159	-39
	PSI	120	180
147 ARG	PHI	-119	-43
	PSI	114	154
148 GLU	PHI	-159	-83
	PSI	112	160
150 VAL	PHI	-133	-57
	PSI	-54	-10
151 ALA	PHI	-189	-97
	PSI	116	184
152 SER	PHI	-152	-112
	PSI	106	146
153 ILE	PHI	-151	-111
	PSI	119	175
154 ASN	PHI	-132	-52
	PSI	151	203
155 ARG	PHI	-80	-40
	PSI	-55	-3
156 ASP	PHI	-118	-78
	PSI	-13	27

Residue	Angle	Constraint Range	
157 GLY	PHI	48	104
	PSI	-8	44
158 ILE	PHI	-118	-62
	PSI	-58	14
160 THR	PHI	-159	-103
	PSI	142	182
161 PHE	PHI	-189	-61
	PSI	134	178
162 TYR	PHI	-174	-102
	PSI	133	173
163 ALA	PHI	-122	-42
	PSI	110	166
164 ASP	PHI	-76	-36
	PSI	-61	-9
165 SER	PHI	-101	-45
	PSI	-55	-7
166 VAL	PHI	-131	-87
	PSI	-26	34
167 LYS	PHI	-79	-39
	PSI	111	151
168 GLY	PHI	65	109
	PSI	-35	25
169 ARG	PHI	-104	-64
	PSI	110	186
170 PHE	PHI	-194	-62
	PSI	132	180
171 THR	PHI	-148	-92
	PSI	102	142
172 ILE	PHI	-141	-97
	PSI	107	167
173 SER	PHI	-158	-78
	PSI	110	174
174 ARG	PHI	-171	-111
	PSI	129	173
175 ASP	PHI	-184	-52
	PSI	97	161
176 ASN	PHI	-106	-22
	PSI	-61	7
177 ALA	PHI	-90	-50
	PSI	-48	-8
178 LYS	PHI	-113	-73
	PSI	-17	23
179 ASN	PHI	36	76
	PSI	29	69
180 THR	PHI	-156	-84
	PSI	114	178

Residue	Angle	Constraint Range	
181 VAL	PHI	-149	-109
	PSI	107	147
182 TYR	PHI	-152	-100
	PSI	117	169
183 LEU	PHI	-163	-75
	PSI	93	153
184 GLN	PHI	-127	-67
	PSI	86	162
185 MET	PHI	-167	-75
	PSI	113	173
186 ASN	PHI	-154	-78
	PSI	124	188
187 SER	PHI	-175	-27
	PSI	114	214
188 LEU	PHI	-145	-25
	PSI	-78	80
189 LYS	PHI	-190	-50
	PSI	100	170
191 GLU	PHI	-83	-43
	PSI	-51	-7
192 ASP	PHI	-109	-69
	PSI	-39	17
193 THR	PHI	-94	-54
	PSI	116	160
195 ILE	PHI	-129	-49
	PSI	113	157
196 TYR	PHI	-124	-76
	PSI	115	167
197 TYR	PHI	-147	-107
	PSI	120	164
198 CYS	PHI	-136	-96
	PSI	94	150
199 ALA	PHI	-136	-92
	PSI	125	165
200 ALA	PHI	-166	-114
	PSI	130	194
202 ARG	PHI	-95	-43
	PSI	-61	-5
203 ASP	PHI	-105	-53
	PSI	-54	10
208 TYR	PHI	-96	-44
	PSI	-51	1
209 SER	PHI	-147	-43
	PSI	-69	27

Residue	Angle	Constraint Range	
210 THR	PHI	-136	-44
	PSI	-50	26
212 SER	PHI	-114	-54
	PSI	-37	27
213 SER	PHI	-146	-54
	PSI	61	153
214 ALA	PHI	-80	-36
	PSI	-58	-10
215 THR	PHI	-89	-49
	PSI	-42	6
216 ARG	PHI	-130	-58
	PSI	-41	35
218 ASN	PHI	-101	-61
	PSI	-58	-6
220 TRP	PHI	-161	-109
	PSI	131	171
224 THR	PHI	-154	-70
	PSI	105	149
225 GLN	PHI	-133	-65
	PSI	100	152
226 VAL	PHI	-161	-65
	PSI	106	146
227 THR	PHI	-152	-68
	PSI	102	142
228 VAL	PHI	-147	-87
	PSI	112	152
229 SER	PHI	-154	-66
	PSI	99	167

A.3 - Chemical Shift Index Data

A.3.1 - Sequence Specific Assignments Obtained for VHH 18

E101	C'	176.3	L104	H α	4.966
	N	119.8		H β 2	1.314
	H	8.102		H β 3	1.152
	C α	56.37		H γ	0.906
	C β	30.89		Q δ 1	0.701
	C γ	-		Q δ 2	0.657
	H α	4.329	V105	C'	176.5
	H β 2	-		N	120.3
	H β 3	-		H	8.585
	H γ 2	-		C α	62.21
	H γ 3	-		C β	35.35
V102	C'	176.1		C γ 1	21.07
	N	120.7		C γ 2	21.28
	H	8.101		H α	4.383
	C α	61.74		H β	2.021
	C β	33.33		Q γ 1	0.959
	C γ 1	20.83		Q γ 2	0.959
	C γ 2	22.09	E106	C'	177.2
	H α	4.638		N	131.9
	H β	2.042		H	10.040
	Q γ 1	1.031		C α	56.51
	Q γ 2	0.970		C β	30.81
Q103	C'	173.3		C γ	37.65
	N	123.4		H α	5.960
	H	8.474		H β 2	2.211
	C α	55.57		H β 3	2.119
	C β	33.30		H γ 2	2.777
	C γ	34.19		H γ 3	2.516
	H α	4.639	S107	C'	173.5
	H β 2	2.019		N	115.1
	H β 3	1.936		H	9.245
	H γ 2	2.254		C α	57.74
	H γ 3	2.254		C β	65.55
	H ϵ 21	-		H α	4.866
	H ϵ 22	-		H β 2	3.944
	N ϵ 2	-		H β 3	3.818
L104	C'	176.1	G108	C'	174.4
	N	122.6		N	107.8
	H	8.366		H	8.651
	C α	53.66		C α	45.08
	C β	44.81		H α 2	4.711
	C γ	28.15		H α 3	3.754
	C δ 1	23.32			
	C δ 2	26.64			

G109	C'	172.9	A114	C'	178.2
	N	105.4		N	124.3
	H	7.541		H	8.263
	C α	45.32		C α	53.83
	H α 2	3.950		C β	18.04
	H α 3	3.639		H α	3.701
G110	C'	171.5	G115	Q β	1.404
	N	107.0		C'	175.5
	H	7.592		N	112.8
	C α	44.94		H	9.606
	H α 2	4.235		C α	44.86
	H α 3	3.974		H α 2	4.467
S111	C'	174.4	G116	H α 3	3.574
	N	115.7		C'	170.6
	H	8.295		N	109.1
	C α	56.56		H	8.439
	C β	64.23		C α	44.63
	H α	5.694		H α 2	4.345
	H β 2	3.837		H α 3	3.777
V112	H β 3	3.702	S117	C'	173.1
	C'	173.7		N	110.0
	N	121.7		H	7.869
	H	8.766		C α	56.57
	C α	59.42		C β	67.21
	C β	35.89		H α	5.594
	C γ 1	20.53		H β 2	3.768
	C γ 2	21.55	L118	H β 3	3.684
	H α	4.606		C'	173.7
	H β	2.073		N	121.5
	Q γ 1	0.823		H	8.488
	Q γ 2	0.822		C α	54.46
Q113	C'	175.9		C β	48.29
	N	122.8		C γ	26.70
	H	8.226		C δ 1	24.11
	C α	55.05		C δ 2	26.55
	C β	30.20		H α	4.405
	C γ	34.15		H β 2	1.344
	H α	4.614		H β 3	1.274
	H β 2	2.045		H γ	1.282
	H β 3	1.981	R119	Q δ 1	0.782
	H γ 2	2.416		Q δ 2	0.666
	H γ 3	2.416		C'	175.3
	H ϵ 21	7.607		N	122.1
	H ϵ 22	6.787		H	8.229
	N ϵ 2	112.1		C α	54.79
				C β	32.45
				C γ	27.71
				C δ	43.55

R119	H α	4.883	A124	C β	22.95
	H β 2	1.728		H α	5.376
	H β 3	1.600		Q β	1.243
	H γ 2	1.295	S125	C'	174.0
	H γ 3	1.229		N	116.1
	H δ 2	2.878		H	8.893
	H δ 3	2.868		C α	57.73
L120	C'	177.1		C β	65.33
	N	127.1		H α	4.823
	H	8.758		H β 2	-
	C α	53.25		H β 3	-
	C β	43.64	G126	C'	171.9
	C γ	26.47		N	111.9
	C δ 1	21.43		H	8.845
	C δ 2	26.22		C α	46.09
	H α	4.990		H α 2	4.253
	H β 2	1.176		H α 3	3.537
	H β 3	0.915	L127	C'	-
	H γ	1.178		N	113.9
	Q δ 1	0.247		H	7.459
	Q δ 2	-0.068		C α	54.02
S121	C'	172.7		C β	43.88
	N	114.6		C γ	-
	H	8.951		C δ 1	28.19
	C α	57.03		C δ 2	26.68
	C β	66.10		H α	4.562
	H α	5.088		H β 2	1.770
	H β 2	3.789		H β 3	1.516
C122	H β 3	3.701		H γ	-
	C'	171.5		Q δ 1	0.900
	N	124.8		Q δ 2	0.656
	H	8.992	R128	C'	176.3
	C α	54.78		N	-
	C β	40.71		H	-
	H α	4.845		C α	54.74
	H β 2	3.278		C β	29.64
	H β 3	2.622		C γ	-
A123	C'	177.0		C δ	-
	N	128.8		H α	-
	H	8.456		H β 2	-
	C α	51.29		H β 3	-
	C β	20.99		H γ 2	-
	H α	4.869		H γ 3	-
	Q β	1.363		H δ 2	-
A124	C'	177.3		H δ 3	-
	N	125.9	I129	C'	176.4
	H	8.336		N	129.7
	C α	50.86		H	8.945

I129	C α	65.84	T134	C'	173.7
	C β	39.24		N	126.7
	C γ 1	30.65		H	8.876
	C γ 2	16.27		C α	61.93
	C δ 1	14.55		C β	69.31
	H α	3.515		C γ 2	21.73
	H β	1.227		H α	5.127
	H γ 12	1.191		H β	3.809
	H γ 13	1.090		Q γ 2	0.888
S130	Q γ 2	0.621	G135	C'	171.9
	Q δ 1	0.543		N	114.8
	C'	175.6		H	9.123
	N	115.0		C α	43.60
	H	8.170		H α 2	5.118
	C α	60.12		H α 3	3.417
	C β	63.05	T136	C'	175.3
	H α	4.296		N	119.0
	H β 2	4.054		H	8.901
	H β 3	4.054		C α	56.07
E131	C'	175.4		C β	32.59
	N	118.6		H α	5.723
	H	7.554		H β 2	3.149
	C α	55.74		H β 3	2.933
	C β	30.11		H δ 1	6.990
	C γ	36.97		H ϵ 1	7.524
	H α	4.544		H ϵ 3	7.752
	H β 2	2.318		H η 2	7.043
	H β 3	1.912		H ζ 2	-
	H γ 2	2.282		H ζ 3	6.925
C132	H γ 3	2.173		N ϵ 1	120.2
	C'	173.8	Y137	C'	175.2
	N	116.8		N	123.7
	H	7.409		H	10.450
	C α	55.51		C α	56.21
	C β	49.46		C β	43.70
	H α	4.783		H α	5.686
	H β 2	2.996		H β 2	3.136
	H β 3	2.996		H β 3	2.907
T133	C'	174.6		Q δ	6.874
	N	116.7		Q ϵ	6.629
	H	8.950	R138	C'	174.2
	C α	62.95		N	116.8
	C β	69.97		H	9.691
	C γ 2	21.17		C α	53.62
	H α	4.933		C β	33.01
	H β	3.865		C γ	24.67
	Q γ 2	0.945		C δ	45.27
				H α	5.781
				H β 2	0.923

R138	H β 3	0.874	K143	C β	35.54
	H γ 2	1.486		C γ	25.02
	H γ 3	1.486		C δ	28.79
	H δ 2	2.845		C ϵ	42.22
	H δ 3	2.743		H α	4.771
Q139				H β 2	2.043
	C'	174.4		H β 3	1.960
	N	122.9		H γ 2	1.452
	H	9.442		H γ 3	1.359
	C α	55.25		H δ 2	1.696
	C β	33.20		H δ 3	1.696
	C γ	-		H ϵ 2	3.021
	H α	4.878		H ϵ 3	3.021
	H β 2	2.351	E144	C'	176.9
	H β 3	2.170		N	118.9
	H γ 2	2.350		H	8.322
	H γ 3	2.377		C α	55.86
	H ϵ 21	7.770		C β	30.71
	H ϵ 22	6.793		C γ	36.52
	N ϵ 2	112.1		H α	4.339
A140	C'	-		H β 2	2.109
	N	136.6		H β 3	1.960
	H	9.379		H γ 2	2.386
	C α	50.02		H γ 3	2.340
	C β	18.43	R145	C'	175.6
	H α	4.860		N	122.7
	Q β	1.489		H	8.790
P141	C'	178.3		C α	57.34
	C α	64.44		C β	30.74
	C β	31.68		C γ	27.50
	C γ	28.15		C δ	43.43
	C δ	50.47		H α	4.575
	H α	4.332		H β 2	1.728
	H β 2	2.305		H β 3	1.641
	H β 3	1.929		H γ 2	1.585
	H γ 2	2.174		H γ 3	1.503
	H γ 3	2.020		H δ 2	2.453
	H δ 2	3.880		H δ 3	2.366
G142	H δ 3	3.660	E146	C'	175.1
				N	125.2
	C'	174.0		H	9.416
	N	112.6		C α	54.33
	H	8.716		C β	33.52
	C α	45.87		C γ	35.66
	H α 2	4.133		H α	5.009
K143	H α 3	3.761		H β 2	2.183
	C'	176.1		H β 3	2.086
	N	119.9		H γ 2	2.314
	H	8.004		H γ 3	2.302
	C α	54.32			

L147	C'	176.6	F151	C'	175.7
	N	127.6		N	124.9
	H	8.891		H	9.246
	C α	55.89		C α	56.96
	C β	41.91		C β	41.96
	C γ	26.95		H α	4.956
	C δ 1	27.00		H β 2	3.286
	C δ 2	21.86		H β 3	2.656
	H α	3.733		Q δ	7.121
	H β 2	1.712		Q ϵ	7.347
	H β 3	1.204		H ζ	-
	H γ	1.323	S152	C'	174.9
	Q δ 1	0.363		N	122.5
	Q δ 2	0.272		H	8.863
V148	C'	176.3		C α	57.97
	N	122.0		C β	66.03
	H	9.425		H α	4.925
	C α	62.13		H β 2	4.157
	C β	33.85		H β 3	4.142
	C γ 1	21.01	N153	C'	175.1
	C γ 2	22.50		N	119.3
	H α	4.627		H	8.362
	H β	2.242		C α	55.67
	Q γ 1	1.134		C β	36.78
	Q γ 2	1.124		H α	4.330
S149	C'	175.1		H β 2	2.782
	N	112.4		H β 3	2.543
	H	7.888		H δ 21	7.446
	C α	57.05		H δ 22	6.834
	C β	66.19		N δ 2	113.7
	H α	5.573	L154	C'	177.7
	H β 2	4.061		N	117.1
	H β 3	3.590		H	7.645
K150	C'	173.4		C α	54.32
	N	122.0		C β	42.20
	H	8.734		C γ	26.92
	C α	55.79		C δ 1	25.25
	C β	37.97		C δ 2	22.69
	C γ	25.26		H α	4.472
	C δ	29.66		H β 2	1.704
	C ϵ	41.44		H β 3	1.503
	H α	5.556		H γ	1.577
	H β 2	1.722		Q δ 1	0.867
	H β 3	1.614		Q δ 2	0.816
	H γ 2	1.317			
	H γ 3	1.317			
	H δ 2	1.309			
	H δ 3	1.309			
	H ϵ 2	2.506			
	H ϵ 3	2.256			

G155	C'	174.0	T160	C'	175.5
	N	108.2		N	115.8
	H	7.853		H	7.378
	C α	45.74		C α	61.61
	H α 2	4.053		C β	68.33
T156	H α 3	3.422	G161	C γ 2	22.23
	C'	174.1		H α	3.855
	N	111.8		H β	4.396
	H	7.296		Q γ 2	1.353
	C α	60.61		C'	-
	C β	70.06		N	114.0
	C γ 2	22.08		H	8.480
	H α	4.479		C α	48.55
	H β	4.193	S162	H α 2	4.019
	Q γ 2	1.130		H α 3	3.582
T157	C'	173.7		C'	175.8
	N	113.9		N	-
	H	8.447		H	-
	C α	59.93		C α	60.35
	C β	72.34		C β	63.03
	C γ 2	23.13		H α	-
	H α	5.189		H β 2	-
	H β	3.957		H β 3	-
	Q γ 2	1.191	V163	C'	175.3
W158	C'	173.7		N	112.4
	N	121.8		H	7.354
	H	7.971		C α	59.96
	C α	57.97		C β	31.26
	C β	33.13		C γ 1	20.37
	H α	4.571		C γ 2	20.32
	H β 2	3.195		H α	4.155
	H β 3	2.754		H β	0.901
	H δ 1	7.400		Q γ 1	0.148
	H ϵ 1	10.110		Q γ 2	0.184
	H ϵ 3	6.952	K164	C'	177.4
	H η 2	7.137		N	125.4
	H ζ 2	7.508		H	7.118
	H ζ 3	6.546		C α	58.52
	N ϵ 1	130.1		C β	32.26
Y159	C'	173.1		C γ	25.17
	N	123.9		C δ	29.38
	H	7.441		C ϵ	42.02
	C α	56.49		H α	3.575
	C β	42.55		H β 2	1.697
	H α	4.617		H β 3	1.605
	H β 2	2.721		H γ 2	1.304
	H β 3	2.125		H γ 3	1.311
	Q δ	6.972		H δ 2	1.636
	Q ϵ	6.762			

K164	Hδ3	1.636	I169	Cγ1	29.36
	Hε2	2.943		Cγ2	29.36
	Hε3	2.901		Cδ1	15.78
G165				Hα	5.734
	C'	174.0		Hβ	1.964
	N	116.0		Hγ12	1.315
	H	9.119		Hγ13	1.354
	Cα	45.47		Qγ2	1.326
	Hα2	4.271		Qδ1	1.042
R166	Hα3	3.511	S170		
	C'	175.8		C'	171.5
	N	117.2		N	118.7
	H	7.713		H	8.724
	Cα	57.25		Cα	57.85
	Cβ	31.19		Cβ	65.64
	Cγ	28.55		Hα	4.771
	Cδ	43.42		Hβ2	3.910
	Hα	4.616		Hβ3	3.910
	Hβ2	2.128	Q171	C'	173.8
	Hβ3	1.670		N	120.1
	Hγ2	1.459		H	8.712
	Hγ3	1.368		Cα	54.81
	Hδ2	3.290		Cβ	34.55
	Hδ3	3.290		Cγ	-
F167				Hα	4.733
	C'	175.0		Hβ2	1.591
	N	120.1		Hβ3	1.513
	H	7.561		Hγ2	-
	Cα	52.66		Hγ3	-
	Cβ	39.61		Hε21	-
	Hα	6.129		Hε22	-
	Hβ2	2.965		Nε2	-
	Hβ3	2.970	D172	C'	176.8
	Qδ	7.055		N	124.9
T168	Qε	7.272		H	8.689
	Hζ	7.136		Cα	52.53
	C'	173.8		Cβ	42.76
	N	112.9		Hα	5.018
	H	8.847		Hβ2	2.989
	Cα	61.55	S173	Hβ3	2.619
	Cβ	72.34		C'	177.4
	Cγ2	21.76		N	121.0
	Hα	5.088		H	9.114
	Hβ	3.853		Cα	61.59
	Qγ2	1.213		Cβ	62.70
I169				Hα	-
	C'	172.5		Hβ2	-
	N	133.5		Hβ3	-
	H	9.485			
	Cα	58.77			
	Cβ	41.62			

A174	C'	179.7	V178	C γ 1	22.52
	N	125.3		C γ 2	22.53
	H	8.375		H α	4.606
	C α	54.98		H β	1.712
	C β	18.32		Q γ 1	1.057
	H α	4.310		Q γ 2	0.859
	Q β	1.537	Y179	C'	172.1
K175	C'	177.3		N	126.8
	N	114.9		H	9.076
	H	7.490		C α	56.79
	C α	55.35		C β	41.85
	C β	32.82		H α	5.590
	C γ	25.80		H β 2	2.769
	C δ	29.02		H β 3	2.769
	C ϵ	42.12		Q δ	6.880
	H α	4.400		Q ϵ	6.708
	H β 2	2.046	L180	C'	174.4
	H β 3	2.038		N	122.0
	H γ 2	1.528		H	8.785
	H γ 3	1.403		C α	53.37
	H δ 2	1.692		C β	43.31
	H δ 3	1.692		C γ	-
	H ϵ 2	3.018		C δ 1	23.20
	H ϵ 3	3.018		C δ 2	25.15
N176	C'	173.1		H α	4.738
	N	126.6		H β 2	0.811
	H	8.369		H β 3	-0.385
	C α	53.03		H γ	-
	C β	39.19		Q δ 1	0.784
	H α	4.407		Q δ 2	0.224
	H β 2	3.691	Q181	C'	174.4
	H β 3	2.432		N	128.8
	H δ 21	8.114		H	9.077
	H δ 22	7.228		C α	55.11
	N δ 2	112.4		C β	29.30
T177	C'	171.7		C γ	33.51
	N	110.0		H α	4.221
	H	7.501		H β 2	2.046
	C α	61.44		H β 3	1.948
	C β	74.28		H γ 2	2.378
	C γ 2	20.94		H γ 3	2.378
	H α	5.313		H ϵ 21	-
	H β	3.309		H ϵ 22	-
	Q γ 2	0.354		N ϵ 2	-
V178	C'	174.1	M182	C'	173.1
	N	125.6		N	127.4
	H	8.390		H	8.878
	C α	60.51		C α	54.87
	C β	35.36		C β	35.38

M182	C γ	29.67	P187	C'	-
	C ϵ	-		C α	66.17
	H α	4.085		C β	31.98
	H β 2	1.724		C γ	28.56
	H β 3	1.114		C δ	51.21
	H γ 2	1.519		H α	4.426
	H γ 3	1.519		H β 2	2.508
	Q ϵ	-		H β 3	1.964
N183	C'	173.5	G188	H γ 2	2.246
	N	119.8		H γ 3	1.800
	H	7.754		H δ 2	3.883
	C α	51.20		H δ 3	3.670
	C β	40.94		C'	174.5
	H α	5.144		N	-
	H β 2	3.140		H	-
	H β 3	2.801		C α	46.17
	H δ 21	7.374		H α 2	-
	H δ 22	6.783		H α 3	-
	N δ 2	113.1	D189	C'	176.7
S184	C'	175.9		N	118.3
	N	111.2		H	8.722
	H	8.614		C α	54.90
	C α	57.77		C β	42.74
	C β	61.95		H α	4.846
	H α	3.687		H β 2	3.016
	H β 2	4.043		H β 3	2.858
	H β 3	3.687	T190	C'	174.5
L185	C'	176.8		N	120.5
	N	119.3		H	7.727
	H	8.305		C α	64.71
	C α	56.44		C β	70.00
	C β	42.90		C γ 2	20.04
	C γ	27.96		H α	4.398
	C δ 1	24.37		H β	4.394
	C δ 2	27.12		Q γ 2	1.558
	H α	4.034	A191	C'	175.8
	H β 2	1.401		N	128.6
	H β 3	1.313		H	9.282
	H γ	1.407		C α	52.98
	Q δ 1	0.901		C β	21.79
	Q δ 2	0.561		H α	4.517
S186	C'	-		Q β	1.258
	N	119.6	M192	C'	175.7
	H	9.753		N	118.0
	C α	55.57		H	7.601
	C β	65.80		C α	54.54
	H α	5.106		C β	33.37
	H β 2	4.107			
	H β 3	3.797			

M192	C γ	32.23	T197	C'	172.4
	C ϵ	-		N	112.0
	H α	4.940		H	8.392
	H β 2	2.093		C α	58.68
	H β 3	1.907		C β	70.87
	H γ 2	2.526		C γ 2	24.66
	H γ 3	2.526		H α	5.007
	Q ϵ	-		H β	4.235
Y193	C'	175.0	D198	Q γ 2	1.032
	N	126.1		C'	174.5
	H	9.335		N	120.1
	C α	57.78		H	8.631
	C β	40.92		C α	51.61
	H α	5.478		C β	45.12
	H β 2	3.061		H α	4.839
	H β 3	2.903		H β 2	2.409
	Q δ	6.913		H β 3	2.222
Y194	Q ϵ	-	L199	C'	177.1
	C'	-		N	115.0
	N	118.6		H	8.383
	H	9.634		C α	55.91
	C α	56.04		C β	38.97
	C β	44.10		C γ	27.16
	H α	5.323		C δ 1	25.87
	H β 2	3.297		C δ 2	23.11
	H β 3	2.850		H α	3.950
	Q δ	6.886		H β 2	1.937
C195	Q ϵ	6.623		H β 3	1.638
	C'	171.9		H γ	1.396
	N	-		Q δ 1	0.824
	H	-		Q δ 2	0.791
	C α	52.95	C200	C'	-
	C β	46.24		N	117.8
	H α	-		H	8.869
	H β 2	-		C α	55.22
	H β 3	-		C β	40.47
N196				H α	4.798
	C'	172.8		H β 2	3.566
	N	120.7		H β 3	2.613
	H	8.459	P201	C'	-
	C α	51.10		C α	64.53
	C β	43.42		C β	31.73
	H α	4.735		C γ	28.34
	H β 2	1.269		C δ	50.28
	H β 3	1.276		H α	4.318
	H δ 21	-		H β 2	2.305
	H δ 22	-		H β 3	1.906
	N δ 2	-		H γ 2	2.153
				H γ 3	1.966

P201	Hδ2	3.849	N206	C'	-
	Hδ3	3.849		N	-
				H	-
W202	C'	-		Cα	-
	N	-		Cβ	-
	H	-		Hα	-
	Cα	-		Hβ2	-
	Cβ	-		Hβ3	-
	Hα	-		Hδ21	-
	Hβ2	-		Hδ22	-
	Hβ3	-		Nδ2	-
	Hδ1	7.162			
	Hε1	9.930	T207	C'	171.2
	Hε3	-		N	-
	Hη2	-		H	-
	Hζ2	7.420		Cα	60.40
	Hζ3	-		Cβ	69.94
	Nε1	128.3		Cγ2	-
				Hα	-
Y203	C'	-		Hβ	-
	N	-		Qγ2	-
	H	-			
	Cα	-	W208	C'	178.2
	Cβ	-		N	120.0
	Hα	-		H	8.246
	Hβ2	-		Cα	56.96
	Hβ3	-		Cβ	33.83
	Qδ	-		Hα	5.164
	Qε	-		Hβ2	3.544
				Hβ3	2.946
Y204	C'	-		Hδ1	7.220
	N	-		Hε1	9.990
	H	-		Hε3	7.240
	Cα	-		Hη2	5.112
	Cβ	-		Hζ2	6.888
	Hα	-		Hζ3	6.085
	Hβ2	-		Nε1	130.2
	Hβ3	-			
	Qδ	-	G209	C'	174.2
	Qε	-		N	110.4
				H	8.824
E205	C'	-		Cα	45.28
	N	-		Hα2	4.982
	H	-		Hα3	4.615
	Cα	-			
	Cβ	-	Q210	C'	177.5
	Cγ	-		N	117.4
	Hα	-		H	8.906
	Hβ2	-		Cα	56.80
	Hβ3	-		Cβ	30.60
	Hγ2	-		Cγ	34.41
	Hγ3	-		Hα	4.611
				Hβ2	2.434

Q210	Hβ3	2.292	T215	C'	172.2
	Hγ2	2.690		N	124.8
	Hγ3	2.690		H	8.621
	Hε21	7.770		Cα	61.98
	Hε22	6.988		Cβ	70.59
	Nε2	112.6		Cγ2	21.98
G211				Hα	4.661
	C'	-		Hβ	3.976
	N	111.8		Qγ2	1.173
	H	9.481	V216	C'	175.8
	Cα	44.56		N	128.7
	Hα2	-		H	8.479
Hα3	3.772	Cα		60.03	
T212	C'	171.8		Cβ	32.48
	N	117.3		Cγ1	21.04
	H	8.943	Cγ2	20.20	
	Cα	60.71	Hα	4.835	
	Cβ	70.85	Hβ	1.880	
	Cγ2	20.17	Qγ1	0.655	
	Hα	4.702	Qγ2	0.470	
	Hβ	3.868	S217	C'	173.2
	Qγ2	1.140		N	122.9
Q213	C'	175.6		H	8.544
	N	131.1		Cα	57.69
	H	8.499		Cβ	65.01
	Cα	56.92		Hα	4.634
	Cβ	29.84		Hβ2	3.899
	Cγ	-		Hβ3	3.833
	Hα	4.595		S218	C'
	Hβ2	2.188	N		121.8
	Hβ3	2.056	H		8.153
Hγ2	-	Cα	60.68		
Hγ3	-	Cβ	64.91		
Hε21	6.853	Hα	4.312		
Hε22	6.740	Hβ2	-		
Nε2	111.7	Hβ3	3.890		
V214	C'	174.9			
	N	128.4			
	H	8.827			
	Cα	62.05			
	Cβ	34.21			
	Cγ1	20.16			
	Cγ2	22.88			
	Hα	4.608			
	Hβ	2.453			
	Qγ1	0.647			
	Qγ2	0.605			

A.3.2 - Sequence Specific Assignments Obtained for VHH 67

D101	C'	175.2	V105	C'	176.0
	N	119.3		N	120.0
	H	8.291		H	8.317
	C α	55.11		C α	61.87
	C β	41.83		C β	35.06
	H α	4.574		C γ 1	20.88
	H β 2	2.694		C γ 2	-
	H β 3	2.694		H α	4.349
V102	C'	175.0	E106	H β	1.936
	N	119.3		Q γ 1	0.915
	H	7.284		Q γ 2	0.874
	C α	61.41		C'	176.5
	C β	34.61		N	131.4
	C γ 1	21.64		H	10.070
	C γ 2	21.19		C α	57.10
	H α	4.057		C β	31.07
	H β	1.046		C γ	37.82
	Q γ 1	0.268		H α	5.517
Q103	Q γ 2	0.062		H β 2	2.229
	C'	173.8		H β 3	2.229
	N	125.1		H γ 2	2.862
	H	8.268		H γ 3	2.367
	C α	54.46	S107	C'	173.5
	C β	31.76		N	115.6
	C γ	33.84		H	9.440
	H α	4.472		C α	57.85
	H β 2	1.763		C β	65.57
	H β 3	1.611		H α	4.817
	H γ 2	2.122		H β 2	3.869
	H γ 3	2.067		H β 3	3.869
L104	H ϵ 21	7.302	G108	C'	174.3
	H ϵ 22	6.651		N	108.1
	N ϵ 2	111.7		H	8.542
	C'	176.3		C α	44.86
	N	125.2	G109	H α 2	4.658
	H	8.406		H α 3	3.749
	C α	52.85		C'	172.9
	C β	43.78		N	105.4
	C γ	26.73		H	7.622
	C δ 1	24.15		C α	45.20
	C δ 2	25.77		H α 2	4.020
	H α	5.187		H α 3	3.539
	H β 2	1.362	G110	C'	171.5
	H β 3	1.022		N	106.9
	H γ	1.248		H	7.548
	Q δ 1	0.571		C α	44.83
	Q δ 2	0.497			

G110	H α 2	4.238	G116	C'	170.5
	H α 3	3.939		N	109.2
S111	C'	174.3	G116	H	8.430
	N	115.6		C α	44.48
	H	8.262		H α 2	4.321
	C α	56.40		H α 3	3.756
	C β	64.19	S117	C'	173.0
	H α	5.649		N	109.9
	H β 2	3.762		H	7.841
V112	H β 3	3.679		C α	56.44
	C'	173.6		C β	67.20
	N	121.9		H α	5.587
	H	8.784		H β 2	3.759
	C α	59.46		H β 3	3.664
	C β	35.76	L118	C'	173.5
	C γ 1	20.63		N	122.1
	C γ 2	20.64		H	8.560
	H α	4.551		C α	54.44
	H β	2.028		C β	47.97
	Q γ 1	0.804		C γ	26.83
	Q γ 2	0.804		C δ 1	-
Q113	C'	175.9		C δ 2	25.80
	N	122.9		H α	4.349
	H	8.186		H β 2	1.260
	C α	54.89		H β 3	1.266
	C β	30.14		H γ	1.270
	C γ	33.98		Q δ 1	0.700
	H α	4.600		Q δ 2	0.672
	H β 2	2.060	R119	C'	175.5
	H β 3	2.020		N	122.6
	H γ 2	2.400		H	8.126
	H γ 3	2.399		C α	54.46
	H ϵ 21	7.587		C β	32.33
	H ϵ 22	6.760		C γ	27.92
	N ϵ 2	112.2		C δ	43.44
A114	C'	178.1		H α	4.916
	N	124.4		H β 2	1.721
	H	8.251		H β 3	1.621
	C α	53.70		H γ 2	1.258
	C β	17.86		H γ 3	1.208
	H α	3.639		H δ 2	2.944
	Q β	1.362		H δ 3	2.944
G115	C'	175.4	L120	C'	177.0
	N	113.1		N	127.1
	H	9.657		H	8.659
	C α	44.72		C α	53.15
	H α 2	4.445		C β	43.76
	H α 3	3.554		C γ	26.28

L120	C δ 1	-	S125	H β 3	3.484
	C δ 2	26.14			
	H α	4.900			
	H β 2	1.147			
	H β 3	0.880			
	H γ	1.212			
	Q δ 1	0.282			
	Q δ 2	-0.033			
S121	C'	173.0	Y127	C'	-
	N	114.2		N	-
	H	8.845		H	-
	C α	56.84		C α	-
	C β	66.64		C β	-
	H α	5.452		H α	-
	H β 2	3.752		H β 2	-
	H β 3	3.666		H β 3	-
C122	C'	172.7	S128	Q δ	-
	N	122.7		Q ϵ	-
	H	8.976			
	C α	53.28		C'	-
	C β	41.72		N	-
	H α	5.366		H	-
	H β 2	3.083		C α	-
	H β 3	2.980		C β	-
G123			Y129	H α	-
	C'	171.4		H β 2	-
	N	116.2		H β 3	-
	H	8.784			
	C α	44.55			
	H α 2	4.633			
F124	H α 3	3.581	T130	C'	-
				N	-
				H	-
				C α	61.42
				C β	70.28
				C γ 2	22.45
	C'	174.8	Y131	H α	4.510
	N	124.8		H β	4.588
	H	8.103		Q γ 2	1.291
	C α	56.07			
	C β	42.71			
	H α	5.006			
S125	H β 2	2.509			
	H β 3	2.384			
	Q δ	6.600			
	Q ϵ	6.303			
	H ζ	-			

Y131	C α	59.94	Y135	H β 3	2.910
	C β	39.06		Q δ	7.270
	H α	3.412		Q ϵ	7.050
	H β 2	2.812	M136	C'	175.5
	H β 3	2.635		N	115.5
	Q δ	-		H	7.494
	Q ϵ	-		C α	52.16
N132	C'	174.6		C β	34.74
	N	108.2		C γ	30.66
	H	8.202		C ϵ	16.83
	C α	60.27		H α	6.077
	C β	36.45		H β 2	1.832
	H α	3.897		H β 3	1.732
	H β 2	2.924		H γ 2	2.543
	H β 3	2.605		H γ 3	2.017
	H δ 21	7.455		Q ϵ	1.774
	H δ 22	6.857	G137	C'	170.4
Q133	N δ 2	111.8		N	108.0
	C'	175.6		H	9.258
	N	115.2		C α	44.72
	H	7.890		H α 2	5.070
	C α	55.43		H α 3	3.707
	C β	28.45	W138	C'	175.5
	C γ	34.18		N	115.4
	H α	4.023		H	9.125
	H β 2	1.937		C α	55.52
	H β 3	1.875		C β	33.29
	H γ 2	2.392		H α	5.875
	H γ 3	2.276		H β 2	3.169
	H ϵ 21	7.244		H β 3	2.880
	H ϵ 22	6.795		H δ 1	6.924
	N ϵ 2	111.9		H ϵ 1	7.525
Y134	C'	176.8		H ϵ 3	6.914
	N	116.6		H η 2	6.997
	H	7.922		H ζ 2	6.978
	C α	58.60		H ζ 3	7.692
	C β	38.57		N ϵ 1	121.0
	H α	4.313	F139	C'	174.6
	H β 2	2.616		N	123.4
	H β 3	2.554		H	10.110
	Q δ	6.911		C α	56.19
	Q ϵ	6.645		C β	45.36
Y135	C'	174.6		H α	5.431
	N	125.2		H β 2	3.175
	H	9.260		H β 3	2.914
	C α	59.13		Q δ	6.717
	C β	39.97		Q ϵ	7.022
	H α	3.906		H ζ	-
	H β 2	2.918			

R140	C'	173.9	G144	H	8.675
	N	116.7		C α	45.68
	H	9.779		H α 2	4.108
	C α	53.62		H α 3	3.735
	C β	32.65	K145	C'	176.1
	C γ	24.20		N	120.0
	C δ	44.93		H	7.933
	H α	5.950		C α	54.30
	H β 2	0.834		C β	35.44
	H β 3	0.781		C γ	24.73
	H γ 2	1.470		C δ	28.60
	H γ 3	1.431		C ϵ	41.95
	H δ 2	2.707		H α	4.767
	H δ 3	2.660		H β 2	2.050
Q141	C'	174.3		H β 3	1.942
	N	122.3		H γ 2	1.422
	H	9.364		H γ 3	1.303
	C α	55.15		H δ 2	1.679
	C β	33.01		H δ 3	1.680
	C γ	29.46		He2	3.016
	H α	4.863		He3	3.010
	H β 2	1.662	E146	C'	176.8
	H β 3	1.658		N	118.7
	H γ 2	2.356		H	8.248
	H γ 3	2.320		C α	55.54
	He21	7.781		C β	30.73
	He22	6.790		C γ	36.33
	Ne2	112.3		H α	4.361
A142	C'	-		H β 2	2.094
	N	136.3		H β 3	1.925
	H	9.339		H γ 2	2.365
	C α	49.92		H γ 3	2.302
	C β	18.33	R147	C'	175.6
	H α	4.824		N	121.4
	Q β	1.453		H	8.634
P143	C'	178.3		C α	57.26
	C α	64.36		C β	30.54
	C β	31.72		C γ	27.38
	C γ	27.96		C δ	42.86
	C δ	50.35		H α	4.500
	H α	4.285		H β 2	1.632
	H β 2	2.271		H β 3	1.616
	H β 3	1.892		H γ 2	1.516
	H γ 2	2.139		H γ 3	1.421
	H γ 3	1.984		H δ 2	2.248
	H δ 2	3.836		H δ 3	2.247
	H δ 3	3.630	E148	C'	176.1
G144	C'	174.0		N	124.4
	N	112.6		H	9.496

E148	C α	54.06	I153	H α	5.319
	C β	33.48		H β	1.539
	C γ	35.60		H γ 12	1.693
	H α	5.019		H γ 13	-
	H β 2	2.096		Q γ 2	1.046
	H β 3	2.096		Q δ 1	0.871
	H γ 2	2.280	N154	C'	175.8
	H γ 3	2.280		N	123.3
G149	C'	172.9		H	8.235
	N	113.9		C α	51.64
	H	9.497		C β	37.90
	C α	46.88		H α	4.775
	H α 2	2.956		H β 2	2.907
	H α 3	2.654		H β 3	2.901
V150	C'	174.0		H δ 21	-
	N	122.1		H δ 22	-
	H	8.822		N δ 2	-
	C α	63.56	R155	C'	175.7
	C β	33.96		N	117.0
	C γ 1	21.24		H	7.224
	C γ 2	21.29		C α	60.37
	H α	4.436		C β	28.77
	H β	2.197		C γ	26.76
	Q γ 1	1.195		C δ	43.41
	Q γ 2	1.054		H α	3.325
A151	C'	174.7		H β 2	1.904
	N	118.6		H β 3	1.390
	H	7.902		H γ 2	0.516
	C α	51.15		H γ 3	0.238
	C β	23.96		H δ 2	2.767
	H α	4.996		H δ 3	2.685
	Q β	1.362	D156	C'	177.6
S152	C'	171.5		N	110.7
	N	114.0		H	7.208
	H	8.314		C α	53.17
	C α	57.38		C β	40.76
	C β	66.56		H α	4.390
	H α	5.302		H β 2	2.848
	H β 2	4.089		H β 3	2.551
	H β 3	3.377	G157	C'	173.4
I153	C'	171.8		N	107.2
	N	123.2		H	7.459
	H	9.141		C α	45.50
	C α	58.82		H α 2	4.194
	C β	43.30		H α 3	3.482
	C γ 1	28.52	I158	C'	176.1
	C γ 2	16.10		N	118.5
	C δ 1	15.21		H	8.094

I158	C α	62.12	Y162	C β	43.72
	C β	38.42		H α	4.846
	C γ 1	27.82		H β 2	3.019
	C γ 2	17.46		H β 3	2.340
	C δ 1	12.42		Q δ	6.979
	H α	4.145		Q ϵ	6.625
	H β	2.048	A163	C'	179.2
	H γ 12	1.341		N	124.9
	H γ 13	1.251		H	8.274
	Q γ 2	0.882		C α	52.11
	Q δ 1	0.826		C β	19.21
R159	C'	173.8		H α	4.705
	N	119.9		Q β	1.849
	H	7.785	D164	C'	178.3
	C α	54.97		N	123.7
	C β	32.04		H	9.132
	C γ	27.42		C α	58.12
	C δ	43.14		C β	40.47
	H α	4.604		H α	4.351
	H β 2	1.632		H β 2	2.732
	H β 3	1.651		H β 3	2.732
	H γ 2	1.618	S165	C'	175.8
	H γ 3	1.612		N	110.3
	H δ 2	3.328		H	8.232
	H δ 3	3.296		C α	59.80
T160	C'	172.6		C β	62.89
	N	111.1		H α	4.281
	H	7.439		H β 2	3.877
	C α	58.04		H β 3	3.877
	C β	72.49	V166	C'	175.2
	C γ 2	23.09		N	112.0
	H α	4.939		H	7.375
	H β	3.591		C α	60.09
	Q γ 2	1.038		C β	31.66
F161	C'	172.6		C γ 1	20.48
	N	118.1		C γ 2	20.50
	H	7.730		H α	4.156
	C α	56.95		H β	1.062
	C β	42.55		Q γ 1	0.290
	H α	4.159		Q γ 2	0.294
	H β 2	1.484	K167	C'	177.4
	H β 3	0.842		N	125.7
	Q δ	6.807		H	7.279
	Q ϵ	7.186		C α	58.21
	H ζ	7.274		C β	32.28
				C γ	24.83
Y162	C'	176.0		C δ	29.49
	N	113.8		C ϵ	41.97
	H	7.977			
	C α	56.72			

K167	H α	3.673	I172	C'	171.6
	H β 2	1.818		N	131.8
	H β 3	1.781		H	9.405
	H γ 2	1.443		C α	58.02
	H γ 3	1.400		C β	41.92
	H δ 2	1.789		C γ 1	28.62
	H δ 3	1.789		C γ 2	15.14
	H ϵ 2	3.068		C δ 1	-
	H ϵ 3	3.032		H α	5.671
G168	C'	174.0		H β	1.601
	N	115.9		H γ 12	2.288
	H	9.088		H γ 13	1.084
	C α	45.35		Q γ 2	0.934
	H α 2	4.260		Q δ 1	0.894
	H α 3	3.514			
R169	C'	175.8	S173	C'	171.8
	N	117.3		N	118.4
	H	7.734		H	8.309
	C α	57.36		C α	57.70
	C β	30.88		C β	65.54
	C γ	-		H α	4.611
	C δ	-		H β 2	-
	H α	4.583		H β 3	-
	H β 2	-	R174	C'	172.8
	H β 3	-		N	118.1
	H γ 2	-		H	9.340
	H γ 3	-		C α	54.24
	H δ 2	-		C β	36.04
	H δ 3	-		C γ	24.63
				C δ	44.45
				H α	5.448
F170	C'	174.9		H β 2	2.264
	N	119.8		H β 3	1.485
	H	7.565		H γ 2	1.730
	C α	52.48		H γ 3	1.734
	C β	39.50		H δ 2	3.028
	H α	6.124		H δ 3	2.824
	H β 2	2.931	D175	C'	177.0
	H β 3	2.927		N	121.2
	Q δ	7.035		H	8.651
	Q ϵ	7.299		C α	52.50
	H ζ	7.096		C β	42.72
T171	C'	174.1		H α	5.012
	N	112.5		H β 2	3.133
	H	8.799		H β 3	2.571
	C α	61.25	N176	C'	176.5
	C β	72.19		N	124.4
	C γ 2	21.61		H	9.293
	H α	5.114		C α	54.75
	H β	3.794			
	Q γ 2	1.137			

N176	C β	36.93	T180	H β	3.382
	H α	5.042		Q γ 2	0.658
	H β 2	3.121	V181	C'	171.7
	H β 3	2.947		N	124.8
	H δ 21	7.698		H	8.545
	H δ 22	6.837		C α	59.16
	N δ 2	107.1		C β	34.43
				C γ 1	23.03
A177	C'	179.0		C γ 2	22.40
	N	122.4		H α	5.044
	H	8.593		H β	2.151
	C α	53.80		Q γ 1	1.185
	C β	18.97		Q γ 2	0.979
	H α	4.505	Y182	C'	175.5
	Q β	1.542		N	125.1
K178	C'	176.3		H	9.230
	N	115.4		C α	56.59
	H	7.407		C β	42.19
	C α	55.21		H α	5.691
	C β	33.67		H β 2	2.866
	C γ	25.36		H β 3	2.802
	C δ	29.19		Q δ	6.985
	C ϵ	41.80		Q ϵ	6.751
	H α	4.355	L183	C'	174.3
	H β 2	2.016		N	122.6
	H β 3	1.431		H	8.715
	H γ 2	1.451		C α	53.32
	H γ 3	1.321		C β	42.70
	H δ 2	1.647		C γ	26.78
	H δ 3	1.647		C δ 1	22.82
	H ϵ 2	2.985		C δ 2	25.53
	H ϵ 3	2.985		H α	4.729
				H β 2	0.761
N179	C'	173.3		H β 3	-0.641
	N	117.9		H γ	0.535
	H	7.734		Q δ 1	0.658
	C α	53.46		Q δ 2	0.246
	C β	36.40	Q184	C'	174.3
	H α	3.277		N	128.8
	H β 2	2.985		H	9.025
	H β 3	2.633		C α	55.03
	H δ 21	7.113		C β	29.15
	H δ 22	6.650		C γ	33.47
	N δ 2	110.0		H α	4.179
				H β 2	1.993
				H β 3	1.911
T180	C'	173.9		H γ 2	2.376
	N	108.8		H γ 3	1.918
	H	7.322		H ϵ 21	7.016
	C α	60.75			
	C β	73.39			
	C γ 2	22.01			
	H α	5.263			

Q184	He22	6.866	K189	C'	-
	Ne2	113.3		N	122.3
M185				H	9.627
	C'	172.9		C α	53.38
	N	127.8		C β	34.32
	H	8.814		C γ	25.23
	C α	54.73		C δ	29.33
	C β	34.97		C ϵ	41.93
	C γ	29.86		H α	4.868
	C ϵ	17.74		H β 2	2.144
	H α	4.007		H β 3	2.131
	H β 2	1.747		H γ 2	1.601
	H β 3	1.026		H γ 3	1.490
	H γ 2	1.491		H δ 2	1.670
	H γ 3	0.048		H δ 3	1.653
N186	Q ϵ	1.065		He2	2.894
				He3	2.892
	C'	173.4	P190	C'	179.0
	N	120.0		C α	66.20
	H	7.718		C β	31.75
	C α	51.06		C γ	28.38
	C β	40.81		C δ	50.36
	H α	5.124		H α	4.303
	H β 2	3.136		H β 2	2.447
	H β 3	2.782		H β 3	1.938
	H δ 21	7.357		H γ 2	2.290
	H δ 22	6.760		H γ 3	1.781
	N δ 2	113.0		H δ 2	3.851
				H δ 3	3.628
S187	C'	176.1	E191	C'	176.2
	N	111.1		N	115.4
	H	8.538		H	9.321
	C α	57.60		C α	58.63
	C β	61.83		C β	28.44
	H α	3.650		C γ	37.13
	H β 2	4.033		H α	4.334
	H β 3	4.045		H β 2	2.085
L188				H β 3	2.085
	C'	176.9		H γ 2	2.236
	N	119.1		H γ 3	2.236
	H	8.187	D192	C'	176.7
	C α	56.56		N	118.8
	C β	42.84		H	8.701
	C γ	28.20		C α	55.16
	C δ 1	26.94		C β	42.14
	C δ 2	24.52		H α	4.789
	H α	3.948		H β 2	3.096
	H β 2	1.402		H β 3	2.833
	H β 3	1.322			
	H γ	1.340			
	Q δ 1	0.524			
	Q δ 2	0.922			

T193	C'	174.3	Y197	Qε	6.632	
	N	120.6				
	H	7.906		C198	C'	170.9
	Cα	65.09			N	125.4
	Cβ	69.90			H	10.470
	Cγ2	21.56			Cα	53.20
	Hα	4.369			Cβ	42.95
	Hβ	4.349			Hα	4.176
	Qγ2	1.540			Hβ2	2.378
		Hβ3	2.232			
A194	C'	175.6	A199		C'	174.8
	N	128.5		N	127.5	
	H	9.154		H	8.486	
	Cα	52.73		Cα	50.54	
	Cβ	21.74		Cβ	21.42	
	Hα	4.477		Hα	4.572	
	Qβ	1.188		Qβ	-0.408	
I195	C'	175.6	A200	C'	176.6	
	N	118.0		N	119.5	
	H	7.578		H	8.705	
	Cα	61.67		Cα	50.53	
	Cβ	38.37		Cβ	26.55	
	Cγ1	28.50		Hα	5.896	
	Cγ2	17.81		Qβ	1.479	
	Cδ1	12.92	D201	C'	175.0	
	Hα	4.406		N	115.5	
	Hβ	1.700		H	8.587	
	Hγ12	0.874		Cα	56.63	
	Hγ13	-		Cβ	49.23	
	Qγ2	0.335		Hα	5.097	
	Qδ1	0.866		Hβ2	2.814	
Y196	C'	174.8	R202	Hβ3	1.971	
	N	127.8		C'	176.8	
	H	9.226		N	122.1	
	Cα	57.83		H	9.084	
	Cβ	40.82		Cα	57.36	
	Hα	5.188		Cβ	31.88	
	Hβ2	2.982		Cγ	28.26	
	Hβ3	2.775		Cδ	42.90	
	Qδ	6.914		Hα	4.698	
Qε	6.639					
Y197	C'	174.6	D203	Hβ2	1.588	
	N	119.5		Hβ3	1.516	
	H	9.777		Hγ2	1.096	
	Cα	56.55		Hγ3	0.331	
	Cβ	43.22		Hδ2	2.448	
	Hα	5.225		Hδ3	2.387	
	Hβ2	3.277				
	Hβ3	2.875		C'	174.4	
	Qδ	6.910		N	120.2	

D203	H	9.525	L207	Cδ1	25.84
	Cα	54.29		Cδ2	24.16
	Cβ	41.75		Hα	3.894
	Hα	5.104		Hβ2	1.929
	Hβ2	2.843		Hβ3	1.373
	Hβ3	2.420		Hγ	1.425
L204			Y208	Qδ1	1.019
	C'	175.1		Qδ2	0.786
	N	121.2			
	H	5.481		C'	175.9
	Cα	52.90		N	112.6
	Cβ	41.27		H	7.940
	Cγ	26.10		Cα	59.47
	Cδ1	25.99		Cβ	37.29
	Cδ2	25.94		Hα	3.244
	Hα	3.986		Hβ2	3.085
	Hβ2	1.138		Hβ3	2.970
	Hβ3	-0.182		Qδ	7.093
	Hγ	-0.005		Qε	6.796
	Qδ1	-	S209	C'	175.5
	Qδ2	-		N	112.0
F205	C'	-		H	6.824
	N	120.1		Cα	57.78
	H	7.627		Cβ	64.27
	Cα	56.49		Hα	4.346
	Cβ	39.82		Hβ2	3.498
	Hα	4.383		Hβ3	3.247
	Hβ2	3.227	T210	C'	178.0
	Hβ3	2.862		N	112.5
	Qδ	7.309		H	7.282
	Qε	7.401		Cα	62.64
	Hζ	7.350		Cβ	70.46
P206				Cγ2	21.16
	C'	-		Hα	4.030
	Cα	61.81		Hβ	3.758
	Cβ	32.38	W211	Qγ2	0.762
	Cγ	23.83		C'	177.0
	Cδ	49.08		N	117.8
	Hα	3.942		H	7.634
	Hβ2	1.434		Cα	59.78
	Hβ3	1.174		Cβ	27.66
	Hγ2	1.587		Hα	3.991
	Hγ3	1.430		Hβ2	3.314
	Hδ2	3.108		Hβ3	3.052
	Hδ3	3.058		Hδ1	6.861
L207	C'	177.7		Hε1	9.948
	N	-		Hε3	7.115
	H	-		Hη2	6.571
	Cα	57.34		Hζ2	7.188
	Cβ	42.19		Hζ3	6.204
	Cγ	26.75			

W211	Nε1	129.4	Y217	C'	176.9
				N	117.4
S212	C'	174.2		H	7.498
	N	111.7		Cα	57.33
	H	6.521		Cβ	38.12
	Cα	57.22		Hα	4.509
	Cβ	61.79		Hβ2	2.772
	Hα	3.578		Hβ3	2.115
	Hβ2	3.162		Qδ	-
	Hβ3	3.162		Qε	-
S213	C'	174.6	N218	C'	174.8
	N	119.5		N	122.1
	H	7.856		H	9.487
	Cα	56.96		Cα	55.43
	Cβ	62.69		Cβ	40.13
	Hα	4.604		Hα	4.553
	Hβ2	3.859		Hβ2	2.599
	Hβ3	3.822		Hβ3	1.960
				Hδ21	7.815
A214	C'	179.6		Hδ22	6.761
	N	130.6		Nδ2	115.4
	H	8.885	Y219	C'	173.4
	Cα	55.46		N	117.0
	Cβ	18.08		H	8.019
	Hα	3.681		Cα	57.73
	Qβ	1.137		Cβ	40.27
T215	C'	175.6		Hα	5.100
	N	105.2		Hβ2	2.869
	H	7.755		Hβ3	2.792
	Cα	63.32		Qδ	-
	Cβ	68.56		Qε	-
	Cγ2	21.89	W220	C'	178.1
	Hα	4.044		N	120.5
	Hβ	4.208		H	8.474
	Qγ2	1.185		Cα	56.83
R216	C'	174.9		Cβ	34.06
	N	118.2		Hα	4.901
	H	7.584		Hβ2	3.454
	Cα	54.36		Hβ3	2.800
	Cβ	28.99		Hδ1	6.832
	Cγ	27.09		Hε1	9.457
	Cδ	40.81		Hε3	7.098
	Hα	4.145		Hη2	4.906
	Hβ2	1.867		Hζ2	7.428
	Hβ3	1.856		Hζ3	6.168
	Hγ2	1.503		Nε1	129.3
	Hγ3	1.503	G221	C'	173.9
	Hδ2	3.161		N	111.1
	Hδ3	3.023		H	9.064

G221	C α	44.70	V226	H	8.810
	H α 2	4.722		C α	61.98
	H α 3	4.549		C β	34.27
R222	C'	177.9		C γ 1	19.90
	N	118.1		C γ 2	19.89
	H	8.932		H α	4.651
	C α	56.88		H β	2.381
	C β	31.70		Q γ 1	0.613
	C γ	27.22		Q γ 2	0.614
	C δ	43.94	T227	C'	172.1
	H α	4.583		N	124.7
	H β 2	2.103		H	8.561
	H β 3	2.103		C α	61.90
	H γ 2	1.999		C β	70.47
	H γ 3	1.911		C γ 2	21.82
	H δ 2	3.355		H α	4.623
	H δ 3	3.355		H β	3.949
G223	C'	173.4		Q γ 2	1.146
	N	111.3	V228	C'	175.7
	H	9.187		N	128.6
	C α	44.19		H	8.496
	H α 2	3.747		C α	59.94
	H α 3	3.576		C β	-
T224	C'	172.0		C γ 1	20.87
	N	116.9		C γ 2	20.23
	H	8.819		H α	4.822
	C α	59.98		H β	1.918
	C β	70.71		Q γ 1	0.655
	C γ 2	19.94		Q γ 2	0.445
	H α	4.763	S229	C'	173.2
	H β	3.884		N	122.6
	Q γ 2	1.173		H	8.485
Q225	C'	175.2		C α	57.62
	N	130.6		C β	65.11
	H	8.552		H α	4.614
	C α	56.86		H β 2	3.900
	C β	29.52		H β 3	3.818
	C γ	32.92	S230	C'	-
	H α	4.427		N	121.9
	H β 2	2.061		H	8.148
	H β 3	2.056		C α	60.57
	H γ 2	2.262		C β	64.70
	H γ 3	2.081		H α	4.292
	H ϵ 21	6.952		H β 2	3.858
	H ϵ 22	6.749		H β 3	3.858
	N ϵ 2	112.0			
V226	C'	174.9			
	N	128.3			

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Published Papers

^{15}N , ^{13}C and ^1H Resonance Assignments and Secondary Structure Determination of a Variable Heavy Domain of a Heavy Chain Antibody

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^{15}N , ^{13}C and ^1H resonance assignments and secondary structure determination of a variable heavy domain of a heavy chain antibody

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Abstract Heavy chain antibodies differ in structure to conventional antibodies lacking both the light chain and the first heavy chain constant domain (CH1). Characteristics of the antigen-binding variable heavy domain of the heavy chain antibody (VHH) including the smaller size, high solubility and stability make them an attractive alternative to more traditional antibody fragments for detailed NMR-based structural analysis. Here we report essentially complete backbone and side chain ^{15}N , ^{13}C and ^1H assignments for a free VHH. Analysis of the backbone chemical shift data obtained indicates that the VHH is comprised predominantly of β -sheets corresponding to nearly 60 % of the protein backbone.

Keywords Heavy chain antibody · VHH · NMR resonance assignments · Secondary structure

Biological context

We have previously shown that antibody fragments, such as antigen binding fragments (Fabs) and single chain

variable domain constructs (scFvs), can be used as alternatives to full length antibodies to investigate the structural characteristics of antibody-antigen binding (Wilkinson et al. 2009). Reducing the size of fragments further to just the heavy chain variable domain (VH) has proved less successful as removal of the light chain variable domain (VL) exposes a large hydrophobic surface that can cause VH fragments to aggregate (Ward et al. 1989). The possibility of producing stable VH domains, without the need for substitution of selected hydrophobic residues at the VH-VL interface has become more likely with the discovery of a class of antibodies that have evolved without light chains (Hamers-Casterman et al. 1993).

Heavy chain antibodies (HCAs) are produced alongside conventional antibodies in Camelidae, but lack both the light chain and the first heavy chain constant domain (CH1). The antigen-binding variable heavy domains of heavy chain antibodies (VHH) are less prone to aggregation as the residues situated at what would be the VH-VL interface in conventional antibodies are less hydrophobic (Muyldermans et al. 1994). Another interesting characteristic of VHHs are elongated complementarity determining regions (CDR). These longer CDRs may explain why HCAs exhibit similar affinity and specificity to conventional antibodies despite having fewer CDRs to interact with antigens (Muyldermans et al. 1994). HCAs may also be able to access alternative binding sites, such as clefts and cavities on protein targets not accessible by the relatively flat paratopes of conventional antibodies (Sheriff and Constantine 1996).

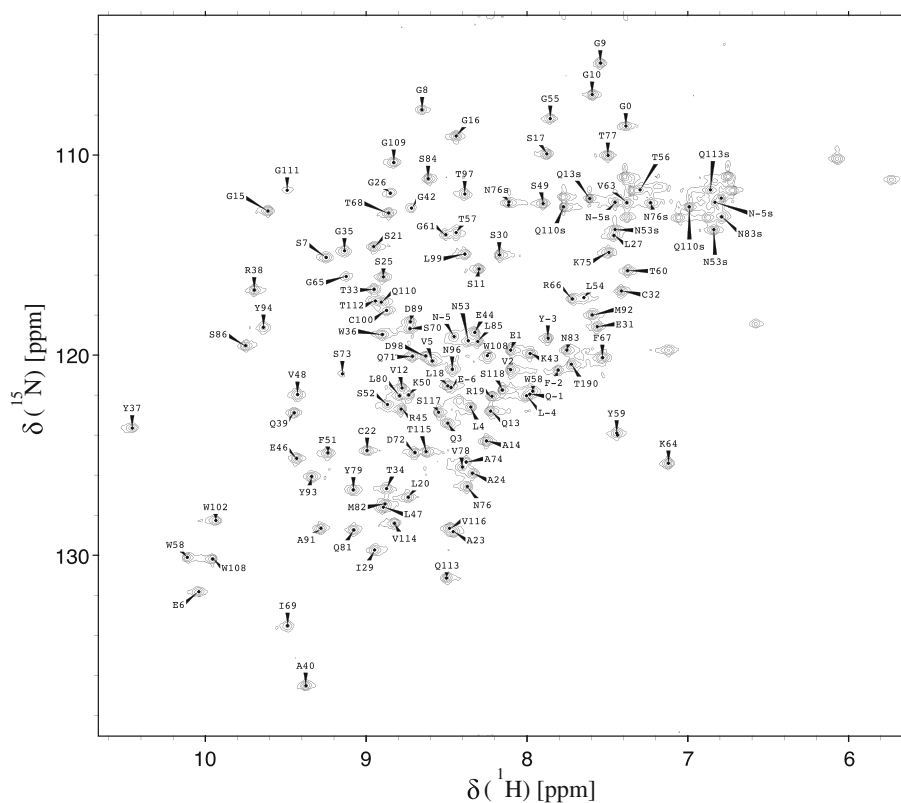
The smaller size, high solubility and stability of VHH domains make them an attractive alternative to more traditional antibody fragments (such as Fabs and scFvs) for detailed NMR-based structural analysis. In particular, to probe the conformational properties and features of the

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Fig. 1 $^{15}\text{N}/^1\text{H}$ HSQC spectrum of VHH 18. Sequence-specific assignments of signals from backbone amide groups are indicated by residue type and number. In addition, side chain amide group assignments are labelled 's'. Signals labelled with residue numbers <1 are associated with the histidine tag and TEV cleavage site



CDR loops in both the free and antigen bound states, to improve our understanding of antibody recognition of target proteins and the molecular processes involved. In this communication we report essentially complete backbone and side chain resonance assignments for an unbound VHH domain, referred to as VHH 18, as well as the positions of elements of regular secondary structure. VHH 18 is a 118 residue protein isolated from a heavy chain camelid antibody raised against the cytokine interleukin-6.

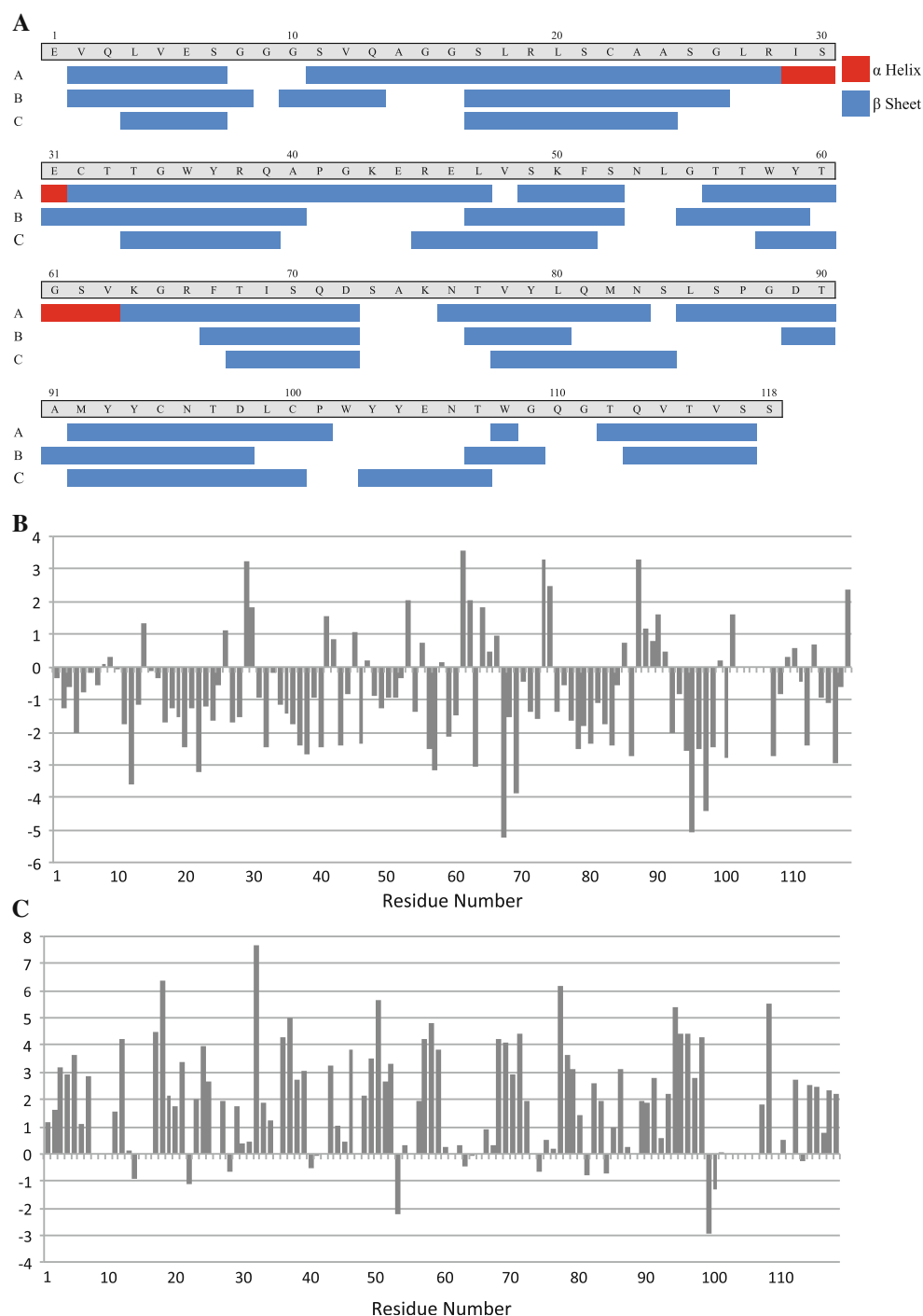
Methods and experiments

Uniformly ^{15}N and $^{15}\text{N}/^{13}\text{C}$ labelled samples of VHH 18 (with an N terminal hexahistidine tag and TEV cleavage site) were prepared from a pET21-based *Escherichia coli* expression vector. Samples were prepared from cells grown in minimal medium containing 1 g/l > 99 % ^{15}N ammonium sulphate and/or 2 g/l > 99 % ^{13}C glucose as the sole sources of nitrogen and carbon. A ^{13}C labelled sample for the $^{13}\text{C}/^1\text{H}$ NOESY-HSQC experiment was prepared as described above, but with the addition of non-isotopically labelled aromatic amino acids to the minimal medium (His, Phe, Trp and Tyr at 50 mg/l). VHH 18 was expressed as insoluble inclusion bodies, which were isolated, resolubilised in 6 M guanidine hydrochloride and purified under denaturing conditions on a Ni-NTA column according to the manufacturer's instructions (QIAGEN).

The VHH 18 containing fractions were pooled, diluted to 2 mg/ml and the protein refolded by dilution into 50 mM TRIS, 1 M guanidine hydrochloride, 1 mM reduced glutathione and 0.2 mM oxidised glutathione buffer at pH 8.5 (method adapted from Williamson et al. 1996). The refolded VHH 18 was concentrated by ultrafiltration on a Vivaflow 200 5,000 Da molecular weight cut off tangential flow concentrator according to the manufacturer's instructions (Sartorius).

NMR spectra were acquired from 0.35 ml samples of 300–400 μM VHH 18 in a 25 mM sodium phosphate, 100 mM sodium chloride, 10 μM EDTA and 0.02 % (w/v) sodium azide buffer at pH 6.4 containing 10 % $\text{D}_2\text{O}/90$ % H_2O or 100 % D_2O as appropriate. NMR data were acquired at 35 °C on 600 MHz Bruker Avance, 600 MHz Bruker DRX or 800 MHz Bruker Avance II spectrometers. The 2D and 3D spectra recorded to make sequence-specific ^{15}N , ^{13}C and ^1H resonance assignments were: $^1\text{H}-^1\text{H}$ TOCSY with mixing times of 30 and 60 ms; $^1\text{H}-^1\text{H}$ NOESY with an NOE mixing time of 125 ms; $^{15}\text{N}/^1\text{H}$ HSQC; TOCSY-HSQC with a mixing time of 55 ms; $^{15}\text{N}/^1\text{H}$ NOESY-HSQC and $^{13}\text{C}/^1\text{H}$ HSQC-NOESY with NOE mixing times of 125 ms; $^{13}\text{C}/^1\text{H}$ HSQC; HCCH-TOCSY with a mixing time of 19.5 ms and $^{15}\text{N}/^{13}\text{C}/^1\text{H}$ HNCACB, CBCA(CO)NH and HNCO (reviewed in Cavanagh et al. 2006). Typical acquisition times in F_1 and F_2 for the 3D experiments were 16.8–20 ms for ^{15}N , 6.8–8 ms for ^{13}C (20 ms in the HNCO) and 15–19 ms for ^1H , with an

Fig. 2 Secondary structure of VHH 18. Regions of regular secondary structure, as determined by the programmes TALOS (row A) and CSI (row B), are highlighted below the protein sequence in panel a. For comparison, the secondary structure reported for an unrelated free VHH domain is also shown (row C, Renisio et al. 2002). β sheets are shown in blue and α helical regions in red. Panel b shows the deviation of the observed $\text{C}\alpha$ chemical shifts from the random coil values (Wishart and Sykes 1994). Residues within β sheet regions are characterised by negative secondary shifts of >0.7 ppm and the helical regions by positive shifts of >0.7 ppm (Wishart and Sykes 1994). An equivalent plot showing the $\text{C}\beta$ secondary shift is shown in panel c. Residues within β sheet regions are characterised by positive secondary shifts of >0.7 ppm and the helical regions by negative shifts of >0.7 ppm (Wishart and Sykes 1994)



acquisition time of 50 ms in F_3 (^1H). Typical acquisition times in 2D experiments were 40 ms (^{15}N), 9 ms (^{13}C) or 35–40 ms (^1H) in F_1 and 100–200 ms in F_2 (^1H). 3D experiments were collected over approximately 72 h, 2D ^1H experiments over approximately 24 h, and 2D $^{15}\text{N}/^1\text{H}$ or $^{13}\text{C}/^1\text{H}$ spectra over 40 min. The WATERGATE method was used for water suppression when necessary. 2D and 3D NMR data were processed using NMRPipe (Delaglio et al. 1995), with linear prediction used, when required, to

extend the effective acquisition times by up to two fold in indirect dimensions. Spectra were analysed using the Sparky package (T. D. Goddard and D. G. Kneller, SPARKY 3, University of California, San Francisco).

VHH 18 gives rise to well-resolved spectra, as illustrated by the $^{15}\text{N}/^1\text{H}$ HSQC spectrum shown in Fig. 1. This allowed for almost complete backbone resonance assignments to be made. The locations of secondary structure elements, as determined from the chemical shift data by the

programmes TALOS and CSI, are shown in Fig. 2. As expected, this analysis revealed that the VHH 18 structure is comprised predominantly of β -sheets, with TALOS suggesting that the domain is composed of 10 separate β -sheets corresponding to over 80 % of the protein backbone. CSI predicts a similar pattern of 11 β -sheets corresponding to nearly 60 % of the protein backbone, however, it is noticeable that in a few cases the β -sheets identified by CSI are significantly shorter than those predicted by TALOS. Comparison of our results for VHH 18 to a previously published NMR structure of an unrelated free VHH (Renisio et al. 2002), suggests that the CSI approach more reliably identifies the secondary structure elements of VHH 18.

Extent of assignments and data deposition

Sequence-specific backbone amide assignments were obtained for all residues in VHH 18 with the exception of R28, S62, G88, C95 and W102 to T107 (91 %), and for C α and C β signals apart from W102 to N106 (95 %). All H α and H β signals were assigned with the exception of E1 (H β 2 and H β 3), C22 (H α), S25 (H β 2 and H β 3), R28, S62, S73, G88, C95 and W102 to T107 (89 %). For the remaining non-exchangeable side chain signals (^{15}N , ^{13}C and ^1H) assignments were obtained apart from E1, Q3, L27 (C γ and H γ), R28, Q39 (C γ), F51 (H ζ), Q71, L80 (C γ and H γ), Q81 (H ϵ 22 and N ϵ 2), Y93 (Q ϵ), N96 (H δ 21 and H δ 22), Q113 (C γ , H γ 2 and H γ 3) and W102 (H ϵ 3, H η 2 and H ζ 3). In addition, no side chain assignments were obtained for Y103 to T107. The comprehensive ^{15}N , ^{13}C and ^1H resonance assignments obtained for VHH 18 have been deposited in the BioMagResBank database (accession number 18837).

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Conformational Heterogeneity in Antibody-Protein Antigen Recognition:

Implications for High Affinity Protein Complex Formation

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Conformational Heterogeneity in Antibody-Protein Antigen Recognition

IMPLICATIONS FOR HIGH AFFINITY PROTEIN COMPLEX FORMATION

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Background: Antibodies are essential components of the immune system which recognize specific antigens with high affinity.

Results: Protein antigen binding sites on antibodies show conformational exchange on a millisecond to second timescale.

Conclusion: Conformational heterogeneity at high affinity protein-protein interaction sites may be common and facilitate efficient protein complex formation.

Significance: High affinity protein-protein interactions are critical for many biological processes.

Specific, high affinity protein-protein interactions lie at the heart of many essential biological processes, including the recognition of an apparently limitless range of foreign proteins by natural antibodies, which has been exploited to develop therapeutic antibodies. To mediate biological processes, high affinity protein complexes need to form on appropriate, relatively rapid timescales, which presents a challenge for the productive engagement of complexes with large and complex contact surfaces (~ 600 – 1800 Å²). We have obtained comprehensive backbone NMR assignments for two distinct, high affinity antibody fragments (single chain variable and antigen-binding (Fab) fragments), which recognize the structurally diverse cytokines interleukin-1 β (IL-1 β , β -sheet) and interleukin-6 (IL-6, α -helical). NMR studies have revealed that the hearts of the antigen binding sites in both free anti-IL-1 β Fab and anti-IL-6 single chain variable exist in multiple conformations, which interconvert on a timescale comparable with the rates of antibody-antigen complex formation. In addition, we have identified a conserved antigen binding-induced change in the orientation of the two variable domains. The observed conformational heterogeneity and slow dynamics at protein antigen binding sites appears to be a conserved feature of many high affinity protein-protein interfaces structurally characterized by NMR, suggesting an essential role in protein complex formation. We propose that this behavior may reflect a soft capture, protein-protein docking mechanism, facilitating formation of

high affinity protein complexes on a timescale consistent with biological processes.

Antibodies are both an integral component of the adaptive immune system and a highly important class of protein therapeutic. Their highly specific and modular nature, coupled with the potential to bind to a huge range of target molecules, makes them one of the most important types of therapeutic available today. Since monoclonal antibodies were first produced in the 1970s (1) there have been significant advances in the ability to engineer antibodies. For example, the successful humanization of mouse antibodies, together with the ability to now produce completely human monoclonal antibodies, has allowed the development of highly specific and potent therapeutics (2). Due to the modular nature of these proteins a number of antigen-binding derivatives have been developed as potential therapeutics, including the fragment antigen-binding (Fab)⁶ and single chain variable fragment (scFv). In addition, a variety of more elaborate antibody-based therapeutics is under evaluation, which provide further functionality required for specific applications (3).

To date, a number of Fab fragments have been licensed as therapeutics, with several other antibody fragments in various stages of clinical development. There are currently >30 United States Food and Drug Administration approved, antibody-derived therapeutics available, with at least nine of these generating revenues of >1 billion dollars per year (4). Antibody-based therapeutics now constitute a large proportion of global therapeutic sales, and with development times comparable with small molecule drugs and higher success rates, will remain a major part of the pharmaceutical industry.

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⁶ The abbreviations used are: Fab, fragment antigen binding; AEBSF, 4-(2-aminoethyl) benzenesulfonylfluoride; CDR, complementarity determining region; HSQC, heteronuclear single quantum coherence; PDB, Protein Data Bank; scFv, single chain variable fragment; TROSY, transverse relaxation optimized spectroscopy; V_H, variable heavy; V_L, variable light.

The high specificity and affinity of antigen recognition lie at the heart of both therapeutic antibody development and the adaptive immune response. This is primarily mediated by the hypervariable loops formed by the complementarity determining regions (CDRs), however, the conformational properties of the CDR loops, together with potential conformational changes induced by antigen binding, remain poorly characterized. The lack of information relating to the antigen binding site also includes very limited knowledge of potential conformational heterogeneity and dynamics on a slow or fast timescale (seconds to picoseconds).

Almost all of the high resolution structures currently available for antibodies have been determined by x-ray crystallography, which limits the ability to gain insights into functionally important conformational dynamics. In addition, there are a relatively limited number of cases where structures have been obtained for both the free antibody and the complex formed with its target protein (5). Consequently, no clear picture has emerged concerning the potential conformational changes induced by antigen binding, and the structural nature of the CDR loops in both the presence and absence of antigen. NMR spectroscopy-based structural biology has now advanced to the point at which detailed structural information can be obtained for proteins and complexes of at least 100 kDa in size (6). This enables the use of NMR spectroscopy to probe the conformational features and properties of both free and antigen-bound antibody fragments, such as scFvs and Fabs.

In this paper, we report the acquisition and analysis of high quality three-dimensional $^{15}\text{N}/^1\text{H}$ and $^{15}\text{N}/^{13}\text{C}/^1\text{H}$ NMR spectra for both free and antigen-bound scFvs and Fabs. This has allowed the determination of comprehensive sequence-specific backbone resonance assignments for the antibody fragments, which have revealed conserved structural changes within distinct antibody fragments upon binding to structurally diverse protein antigens. Furthermore, the NMR data show that the core of the antigen binding sites, in particular the CDR3 loops, exist in multiple conformational states that interconvert on a relatively slow timescale (milliseconds to seconds). Interestingly, this behavior appears to be a conserved feature of many high affinity protein-protein interaction sites (7–13), suggesting a key role in the formation of tight protein complexes.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—The humanized anti-IL-6 scFv and anti-IL-1 β Fab antibody fragments, human IL-1 β and human IL-6 were all produced as soluble proteins in *Escherichia coli*-based expression systems. Tandem pTTod-based expression vectors encoding the heavy and light chain of the anti-IL-6 Fab and anti-IL-1 β Fab were provided by UCB. To produce the anti-IL-6 scFv, the parent Fab vector was modified by restriction digestion and PCR to produce a single expressible gene encoding the variable light domain (V_L), (Gly $_4$ Ser) $_4$ linker, variable heavy domain (V_H) and His $_6$ tag, as described previously for an anti-IL-1 β scFv (14). pET-21-based vectors encoding IL-1 β or IL-6 with an N-terminal His $_6$ tag containing a tobacco etch virus protease site in the linker were also provided by UCB (14, 15).

The Fab and scFv expression vectors were transformed into W3110 *E. coli*-competent cells for the production of ^{15}N , $^{15}\text{N}/^2\text{H}$, $^{15}\text{N}/^{13}\text{C}/^2\text{H}$, or unlabeled protein. Cells containing the anti-IL-1 β Fab vector were grown in either Luria-Bertani (LB)-rich medium, or a modified Spizizen's minimal medium (16, 17) and expression induced at 37 °C (14). Uniformly ^{15}N labeled and unlabeled anti-IL-6 scFv were similarly expressed; however, $^{15}\text{N}/^2\text{H}$ - and $^{15}\text{N}/^{13}\text{C}/^2\text{H}$ -labeled proteins were produced using a small volume, high cell density expression procedure (18) to obtain high yields of deuterated protein from 50-ml cultures.

IL-1 β was expressed in Tuner (DE3) pLysS *E. coli* cells at 25 °C, as described previously (14). For the production of IL-6, the expression vector was transformed into Origami B DE3 pLysS *E. coli* cells, which were grown in either LB, modified Spizizen's minimal medium, or high cell density minimal medium (18), containing 100 $\mu\text{g}/\text{ml}$ carbenicillin, 12.5 $\mu\text{g}/\text{ml}$ tetracycline, 15 $\mu\text{g}/\text{ml}$ kanamycin, and 34 $\mu\text{g}/\text{ml}$ chloramphenicol. Cultures were grown to an A_{600} of 0.3–0.6 at 37 °C, cooled to 17 °C, IL-6 expression induced by the addition of 100 μM IPTG, and cells harvested after approximately a 16-h induction.

The anti-IL-6 scFv, IL-1 β , and IL-6 were purified as described previously (14, 15) using a combination of nickel affinity (Ni-nitrilotriacetic acid) and size exclusion chromatography (Superdex 75). The anti-IL-1 β Fab was also purified as reported previously (14) using a combination of protein G affinity (protein G-Sepharose) and size exclusion chromatography (Superdex 75). During the purification of deuterated samples of the anti-IL-6 scFv, the protein was fully denatured and refolded after nickel affinity chromatography to ensure complete exchange of any buried backbone amide groups. The scFv was denatured by the addition of 5 M guanidine hydrochloride and refolded by dialysis into a 100 mM sodium chloride and 25 mM Tris buffer at pH 7.5. Purified samples of the deuterated anti-IL-1 β Fab were similarly denatured (6 M guanidine hydrochloride) and refolded by dialysis (100 mM sodium chloride and 25 mM sodium phosphate buffer at pH 6.5) to obtain complete exchange of backbone amide groups.

Surface Plasmon Resonance—On and off rate constants for antigen binding to immobilized antibody fragments were determined by surface plasmon resonance experiments carried out essentially as described previously (15).

NMR Spectroscopy—NMR spectra were acquired from 350- μl samples of the free and antigen-bound antibody fragments (170–500 μM) and of free and antibody-bound IL-1 β and IL-6 (200–400 μM). The IL-1 β , anti-IL-1 β Fab, and IL-1 β -Fab complex were in a 100 mM sodium chloride, 25 mM sodium phosphate, 10 μM EDTA, 100 μM AEBSE, and 0.02% (w/v) sodium azide buffer at pH 6.5 (90% $\text{H}_2\text{O}/10\%$ D_2O). Backbone resonance assignments were initially obtained for IL-6 in a 100 mM sodium chloride and 20 mM sodium phosphate buffer at pH 6.4 (95% $\text{H}_2\text{O}/5\%$ D_2O). The anti-IL-6 scFv, IL-6-scFv complex and comparable free IL-6 samples were in a similar buffer containing 100 mM sodium chloride, 25 mM sodium acetate, 10 μM EDTA, 200 μM PMSF, and 0.02% sodium azide buffer at pH 5.5 (90% $\text{H}_2\text{O}/10\%$ D_2O). NMR data were collected at 35, 40, and 45 °C for free IL-1 β , 25, 35, and 40 °C for free IL-6, 40 °C for the free and antigen-bound anti-IL-6 scFv, and at 45 °C for the free and IL-1 β -bound Fab, using either 600 MHz Bruker DRX or

800 MHz Bruker Avance spectrometers equipped with triple resonance ($^{15}\text{N}/^{13}\text{C}/^1\text{H}$) cryoprobes.

A series of two- and three-dimensional TROSY-based spectra (19) were acquired to obtain sequence specific backbone resonance assignments for the anti-IL-6 scFv, anti-IL-1 β Fab, and antigen-bound complexes, including $^{15}\text{N}/^1\text{H}$ TROSY (19), $^{15}\text{N}/^{13}\text{C}/^1\text{H}$ HNCO, HNCA, HN(CO)CA (20), HNCACB (21) and HN(CO)CACB (22). Typical acquisition times for the two-dimensional experiments were 30 ms in F_1 (^{15}N) and 60 ms in F_2 (^1H), with a total experimental time of 30 to 90 min. Typical acquisition times for the triple-resonance three-dimensional experiments were 6–9 ms in indirect ^{13}C apart from HNCO which were 18–22 ms, 20–25 ms in indirect ^{15}N and 60–90 ms in direct ^1H , with an overall experimental time of 60 to 96 h. Long range backbone amide NOE data were also obtained from $^{15}\text{N}/^1\text{H}$ NOESY-TROSY and NOESY-HSQC spectra (23), which were recorded with acquisition times of 12–15 ms in indirect ^1H , 9–12 ms in indirect ^{15}N , and 60–90 ms in direct ^1H , with an NOE mixing time of 500 ms for the anti-IL-1 β Fab, 450 ms for the anti-IL-6 scFv-IL6 complex, and 700 ms for the free scFv. Total acquisition times for the NOE experiments were 87–92 h. A series of double- and triple-resonance spectra were also recorded to obtain essentially complete sequence-specific backbone resonance assignments for IL-6 and IL-1 β , as described previously (14, 15).

Partially aligned samples of the free and IL-1 β -bound anti-IL-1 β Fab were produced by the addition of Pf1 phage (Asla Biotech) at 3–4 mg/ml. Backbone amide residual dipolar coupling values were derived from measured differences in the $^{15}\text{N}/^1\text{H}$ scalar couplings seen in spectra acquired from isotropic and partially aligned samples, with the separation between equivalent peaks in $^{15}\text{N}/^1\text{H}$ HSQC and temperature-compensated TROSY spectra used to determine the couplings (24). Individual two-dimensional spectra were collected for 5–12 h, with acquisition times of 60 ms in direct ^1H and 50 ms in indirect ^{15}N .

All NMR data were processed using Topspin 2.1 (Bruker Biospin) and analyzed using the SPARKY package (Goddard and Kneller, SPARKY 3, University of California, San Francisco).

Chemical Shift-based Mapping of Interaction Sites—Backbone chemical shift (^{15}N , ^{13}C , and ^1H) comparisons between free and bound antibody fragments and antigens were made using the minimal shift method (7–9) and by direct comparison of assigned backbone signals for the free and bound proteins where available. For the anti-IL-1 β Fab, peaks from the comprehensively assigned HNCO spectrum of the free protein were compared with the unassigned HNCO spectrum of the Fab-IL-1 β complex. In the case of the anti-IL-6 scFv, this approach was extended to combine an assigned free *versus* bound comparison with minimal shift analysis for residues that were not assigned in both states, which provides the fullest overall picture of the backbone chemical shift changes induced by complex formation. Minimal shift analysis was used to map the affects of antibody binding on IL-1 β and IL-6.

Modeling of the scFv and Fab Structures—Homology models of the anti-IL-1 β Fab and anti-IL-6 scFv were produced as described previously, with the residual dipolar coupling refined model of the anti-IL-1 β scFv (PDB accession code 2KH2) used

as a template for the anti-IL-6 scFv (14). The homology model obtained for the anti-IL-1 β Fab was further refined using HADDOCK (25), with a combination of backbone amide residual dipolar coupling data, chemical shift-derived backbone dihedral angles, and backbone H_N - H_N NOEs included as experimental restraints (14). Analysis of the scFv and Fab structural models produced, including the mapping of antigen binding-induced chemical shift changes, was carried out using the PyMOL molecular graphics package.

RESULTS AND DISCUSSION

NMR Spectroscopy of Antigen-binding Antibody Fragments—NMR samples of the anti-IL-6 scFv and anti-IL-1 β Fab were found to be stable for many days at 40 and 45 °C, respectively, which allowed the acquisition of a range of high quality two-dimensional and three-dimensional NMR spectra, as illustrated in Figs. 1 and 2. The spectra obtained show excellent dispersion of backbone signals (^{15}N , ^{13}C , and ^1H) and good signal to noise ratios. The line widths observed for resonances in highly deuterated samples of the antibody fragments allowed the recording of an extensive set of triple resonance spectra for both the free and antigen-bound anti-IL-6 scFv and for the free anti-IL-1 β Fab. The correlations detected between backbone signals in these spectra, together with backbone amide NOEs identified in three-dimensional ^{15}N -edited NOESY experiments, enabled the determination of comprehensive sequence-specific backbone resonance assignments (H_N , N, C_α , C_β , and C') for both the anti-IL-6 scFv (free and bound to IL-6) and the free anti-IL-1 β Fab using well established procedures (7, 8, 14, 26, 27).

The majority of the detectable backbone amide signals (^{15}N and ^1H) were assigned for the free anti-IL-6 scFv, with 192 of the 218 possible backbone amide groups identified (88% excluding the linker (20), His₆ tag (6), prolines (9), and N-terminal residue). Backbone amide signals were not identified for Ser⁹, Leu⁴⁷, Asp⁵⁰, Ser⁷⁷, Ser⁹¹-Tyr⁹⁴, Trp⁹⁶, Gly¹⁴⁵, Asp¹⁶², Met¹⁶³, Tyr²²⁴, Cys²²⁵, Arg²²⁷-Glu²³⁶, Gly²⁴¹, and Thr²⁴² (Fig. 3). The extent of the backbone assignments obtained for the scFv bound to IL-6 was slightly higher, with 198 possible backbone amide groups identified (91%). Backbone amide resonances were not assigned for Leu⁴⁷, Asp⁵⁰, Asp⁵⁶, Ser⁷⁷, Ser⁹¹-Tyr⁹⁴, Trp⁹⁶, Thr⁹⁷, Gly¹³⁸, Gly¹⁴⁵, Tyr²²⁴, Cys²²⁵, His²²⁸-Thr²³¹, Asp²³³, Tyr²³⁴, and Thr²⁴². For both the free and IL-6-bound scFv nearly all of the backbone amide-associated spin systems observed in spectra were assigned to specific residues, with only a few very weak (3–4) or incomplete systems (5–6) remaining unidentified.

For the free anti-IL-1 β Fab, ^{15}N and ^1H resonances from 338 of the 419 assignable backbone amide groups were identified (81% excluding prolines (23) and the two N-terminal residues). Backbone amide signals were not assigned for Ile², Gln⁶-Ser⁷, Ser⁹, Asn²⁸, Trp³⁵, Gly⁴¹, Gln⁴⁵, Asn⁵⁰, Gly⁶⁶, His⁹⁰-Leu⁹⁴, Phe⁹⁶, Ser¹⁵⁶, Gly¹⁵⁷, Ser¹⁶⁸, Ser²⁰², Ser²⁰³, Gly²¹², Glu²¹³, Glu²¹⁵, Val²¹⁶, Gly²²², Asp²⁴², Asp²⁴⁷, Leu²⁵⁹, Val²⁶², Ala²⁶³, Gly²⁶⁸, Gly²⁷⁰, Tyr²⁷³, Phe²⁷⁴, Asp²⁷⁶, Thr²⁷⁷, Gly²⁸⁰, Tyr³⁰⁸-Phe³²⁰, Ser³³³, Ser³⁴⁷-Thr³⁵⁵, Val³⁶²-Phe³⁶⁵, Thr³⁸⁰-Gly³⁸², Ser³⁹², Ser³⁹³, Ser⁴⁰⁶, Ser⁴⁰⁷, Lys⁴³⁴-Lys⁴³⁸, and His⁴⁴⁰-Ala⁴⁴³ (Fig. 3). More than 94% of the backbone amide-associated spin

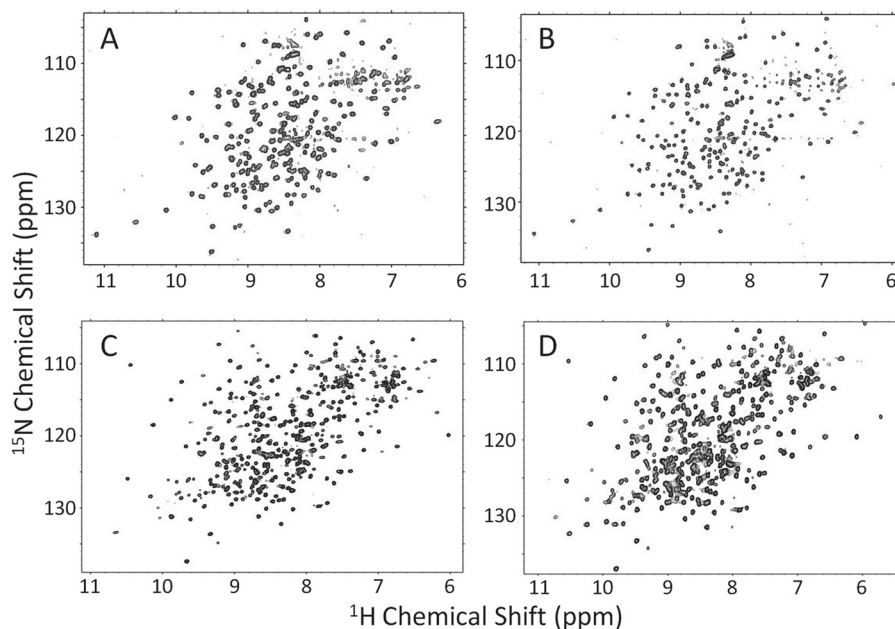


FIGURE 1. **Comparison of $^{15}\text{N}/^1\text{H}$ TROSY spectra for free and antigen-bound anti-IL-6 scFv and anti-IL-1 β Fab.** A and B, $^{15}\text{N}/^1\text{H}$ TROSY spectra of ^{15}N -labeled anti-IL-6 when free (A) and bound to unlabeled IL-6 (B). C and D, similarly, ^{15}N -labeled anti-IL-1 β Fab spectra when free (C) and bound to unlabeled IL-1 β (D).

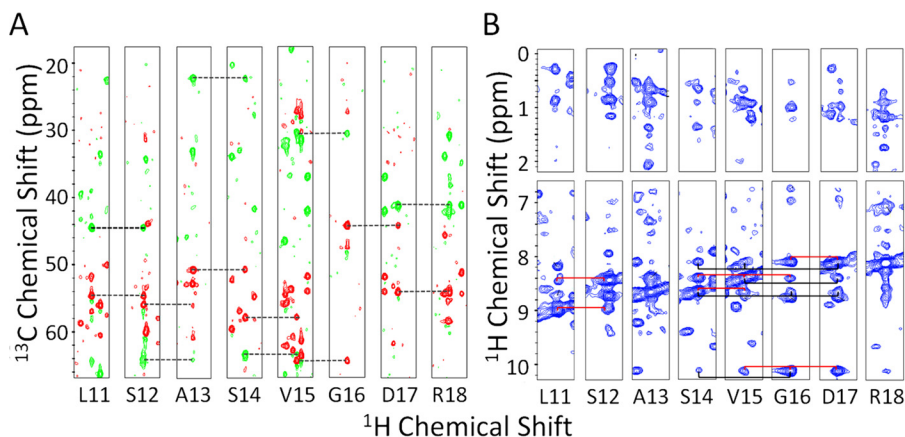


FIGURE 2. **Strip plots representative of three-dimensional NMR spectra collected for the free anti-IL-1 β Fab.** A, series of ^{15}N strips from an HNCACB experiment, illustrating the sequential connections used to assist the assignment of backbone signals for Leu¹¹-Arg¹⁸. B, equivalent ^{15}N strips from a $^{15}\text{N}/^1\text{H}$ NOESY-HSQC spectrum, with sequential H_N - H_N NOEs involving Leu¹¹-Arg¹⁸ highlighted.

systems detected for the Fab were assigned to specific residues, with the assignment of only approximately 20 backbone amide signals remaining unclear. We have previously reported essentially complete backbone resonance assignments for the equivalent scFv bound to IL-1 β (14). Spectra acquired for the scFv and Fab bound to IL-1 β show strikingly similar shifts for backbone signals (H_N , N, and C') arising from the shared antigen binding variable domains (V_H and V_L), which clearly indicates an essentially identical structure for the two variable domains and the interface formed with IL-1 β . This is also reflected in the near perfect overlay of $^{15}\text{N}/^1\text{H}$ HSQC spectra acquired for IL-1 β bound to the scFv and Fab (14).

Analysis of HNCB spectra collected for the free and antigen-bound anti-IL-1 β Fab revealed the appearance of 17 new peaks upon binding to IL-1 β . Comparison with the fully assigned spectrum available for the equivalent scFv bound to IL-1 β (14) clearly showed that these new peaks arose from the backbone

amides of residues in the antigen binding CDR loops, in particular, Asn³¹, Phe⁹¹, Trp⁹², Ser⁹³, Leu⁹⁴, and Phe⁹⁶ in the V_L domain and Asp²⁴⁷, Gly²⁶⁸, Gly²⁷⁰, Gln³¹³, Asn³¹⁴, Lys³¹⁵, Lys³¹⁶, Leu³¹⁷, Thr³¹⁸, Trp³¹⁹, and Phe³²⁰ in the V_H domain (Figs. 3 and 4). Overall, by reference to the assigned spectra of the free anti-IL-1 β Fab and equivalent scFv bound to IL-1 β , 355 of the 419 (85%) assignable backbone amide signals (^{15}N and ^1H) were identified for the IL-1 β -bound Fab.

The completeness of the backbone assignments obtained for the antigen binding variable domains of the anti-IL-6 scFv and anti-IL-1 β Fab are shown in Fig. 3, which highlights many similarities. The overall assignment level for both proteins is high; however, the assignments are slightly more complete for the anti-IL-6 scFv than for the anti-IL-1 β Fab, with better coverage for both the CDR loops and the framework residues. Interestingly, for both the IL-6- and IL-1 β -targeted antibodies the assignment of the CDRs is incomplete in the absence of bound

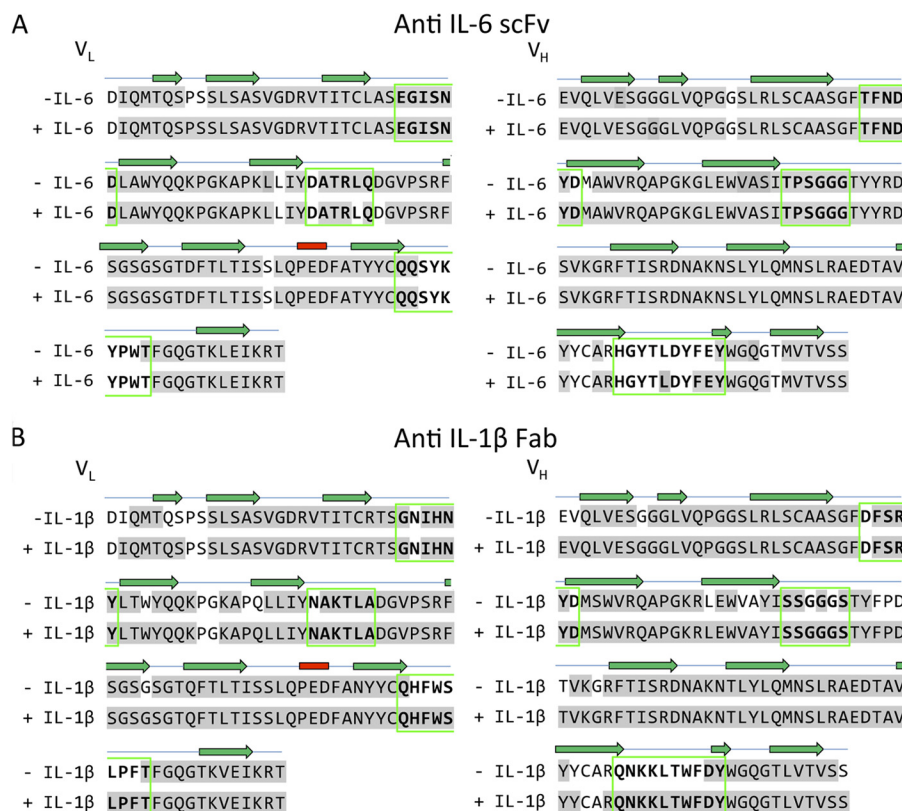


FIGURE 3. Comparison of the extent of the backbone NMR assignments obtained for the variable domains (V_L and V_H) of the free and antigen-bound forms of the anti-IL-6 scFv (A) and anti-IL-1β Fab (B). Residues for which backbone resonance assignments were made are highlighted in gray for both the free and antigen-bound antibody fragments. The positions of elements of regular secondary structure and CDR loops are also indicated, with α-helices shown as red bars, β-sheets as green arrows, and CDRs as green boxes. The overall assignment level for the free scFv is slightly higher than for the Fab, but for both antibodies backbone signals from residues in CDR3 of V_L and V_H remain largely unassigned due to the absence of detectable backbone amide resonances.

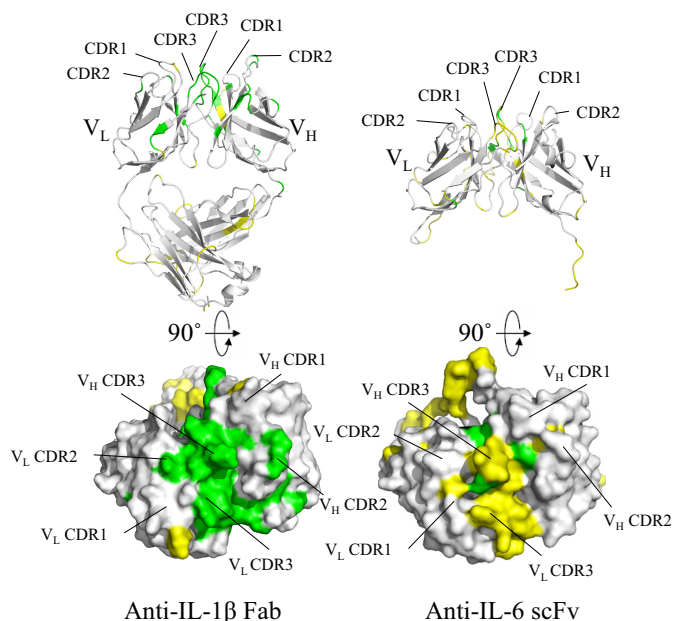


FIGURE 4. Localization of antibody residues with nonobservable backbone amide NMR signals in the free state and appearance upon antigen binding. Antibody residues with backbone amide resonances that were observable only after antigen binding (green) or were missing in both the free and bound states (yellow) are highlighted on the structural models of the anti-IL-1β Fab and anti-IL-6 scFv. Backbone amide signals were not observed for the heart of the antigen binding site in both free antibody fragments. The majority of these signals appeared for the anti-IL-1β Fab on binding to IL-1β, but remained unobservable for the anti-IL-6 scFv bound to IL-6.

antigen. This lack of assignments for the CDR loops reflects the absence of backbone amide signals from many residues present in these regions, in particular, for CDR3 of both the V_L and V_H domains, which indicates the presence of a number of discreet structural states interconverting on a relatively slow timescale (milliseconds to seconds) (28). The striking similarity in the behavior of the CDRs in both the anti-IL-1β Fab and anti-IL-6 scFv suggests that conformational heterogeneity is a common feature of antibody CDR loops, particularly toward the core of the antigen binding surface.

Comparison of the extent of the backbone assignments obtained for the antigen-bound forms of the anti-IL-6 scFv and anti-IL-1β Fab reveals a marked difference between the two antibody/antigen systems (Figs. 3 and 4). In both cases, a number of backbone amide signals were recovered upon antigen binding, which were assigned to CDR loop residues and are highlighted on the structures shown in Fig. 4. This appearance of missing backbone amide NMR signals suggests a stabilization of the CDR loops through interactions with the target proteins. However, whereas the majority of the CDR signals are visible for the anti-IL-1β Fab bound to IL-1β, only a relatively small number of new signals are seen for the anti-IL-6 scFv bound to IL-6, with a large portion of CDR3 in both V_H and V_L remaining unobservable. This difference is most likely due to the distinct structural nature of the two antigens, with IL-1β being structurally quite stable compared with a more dynamic IL-6 (14, 15). Interestingly, in spectra of the anti-IL-6 scFv

bound to IL-6 a number of residues in V_H CDR2 (Ser¹⁷⁹, Thr¹⁸¹, Ser¹⁸³, Gly¹⁸⁴, Thr¹⁸⁷, Tyr¹⁸⁸, Arg¹⁹⁰, Ser¹⁹², and Val¹⁹³) and either in, or adjacent to V_H CDR3 (Leu²³², Phe²³⁵, Tyr²³⁷, Gln²⁴⁰, and Gly²⁴¹), show significantly broader amide proton signals (>30 Hz) than observed for the majority of the protein (overall average of 26.3 ± 4.2 Hz), as shown in Fig. 5. This is consistent with some remaining slow conformational exchange behavior at the antigen binding site of the V_H domain, which is

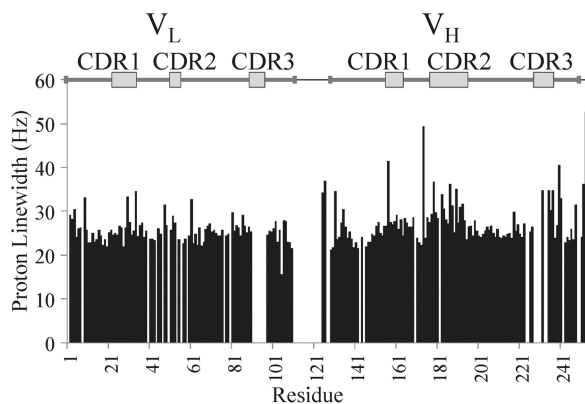


FIGURE 5. Linewidths of backbone amide proton NMR signals in a typical TROSY-HNCO spectrum of the anti-IL-6 scFv bound to IL-6. The positions of the variable domains (V_L and V_H) and antigen binding CDR loops are indicated above the histogram. Significantly broader backbone amide signals were observed for a number of residues in V_H CDR2 and either in, or adjacent to V_H CDR3, which is consistent with slow conformational exchange in the antigen binding region of the V_H domain.

presumably possible due to the relatively flexible nature of the IL-6 structure (15).

Mapping of Antigen Binding Sites—The effects of antigen binding on the backbone signals (H_N, N, and C') of the anti-IL-6 scFv and anti-IL-1β Fab were determined by a combination of minimal and actual shift analyses of HNCO spectra obtained for the free and bound antibody fragments. The results from the chemical shift analyses were mapped on to a homology model of the anti-IL-6 scFv (Fig. 6) and a refined homology model of the anti-IL-1β Fab (Fig. 7). The locations of residues affected by antigen binding further highlight the absence of NMR signals from a significant number of CDR residues, particularly in the CDR3s of both the V_H and V_L domains (Figs. 6 & 7). The binding-induced chemical shift changes also clearly show that the visible CDR signals are highly perturbed by antigen binding. An antigen binding site can be clearly identified on both the anti-IL-6 scFv and on the anti-IL-1β Fab, which is further confirmed by NMR data obtained for the corresponding IL-1β scFv (14).

Interestingly, for both antibody fragments further antigen-induced structural changes were revealed by chemical shift changes that lie beyond the CDR loops. These include changes in the framework residues supporting the CDR loops and in residues at the interface between the V_L and V_H domains. There are striking similarities in the regions affected for both antibody fragments despite binding to structurally diverse antigens, which strongly suggests that conserved conforma-

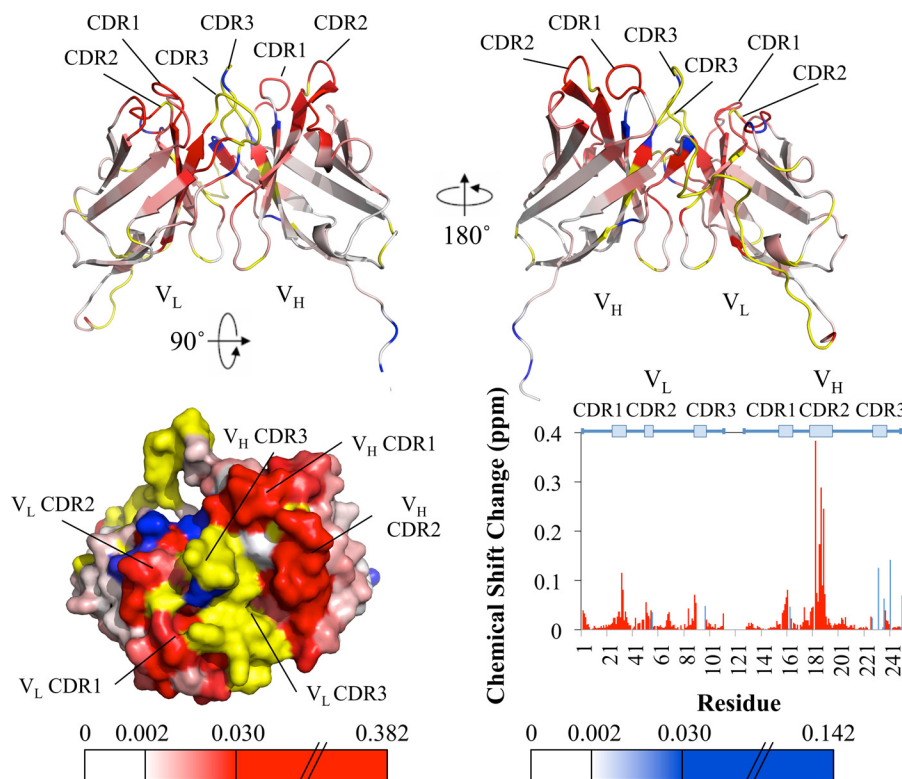


FIGURE 6. Mapping of the IL-6 interaction site on the anti-IL-6 scFv. The combined H_N, N, and C' chemical shift changes for residues between free and IL-6-bound anti-IL-6 scFv were assigned a color depending on their magnitude, with shifts of <0.002 ppm colored white, >0.03 ppm colored red, and those in between colored on a linear gradient from white to red. Similarly, for residues with backbone signals that remained unassigned in the free or bound state antigen-induced changes were determined by the minimal shift approach, with shifts of <0.002 ppm colored white, >0.03 ppm colored blue, and those in between colored on a linear gradient from white to blue. Residues for which no chemical shift data could be obtained are colored yellow. The chemical shift changes observed are mapped onto both a ribbon representation of the scFv and a surface view of the antigen binding site. The most substantial changes are seen in the CDR loops and the interface between the two variable domains.

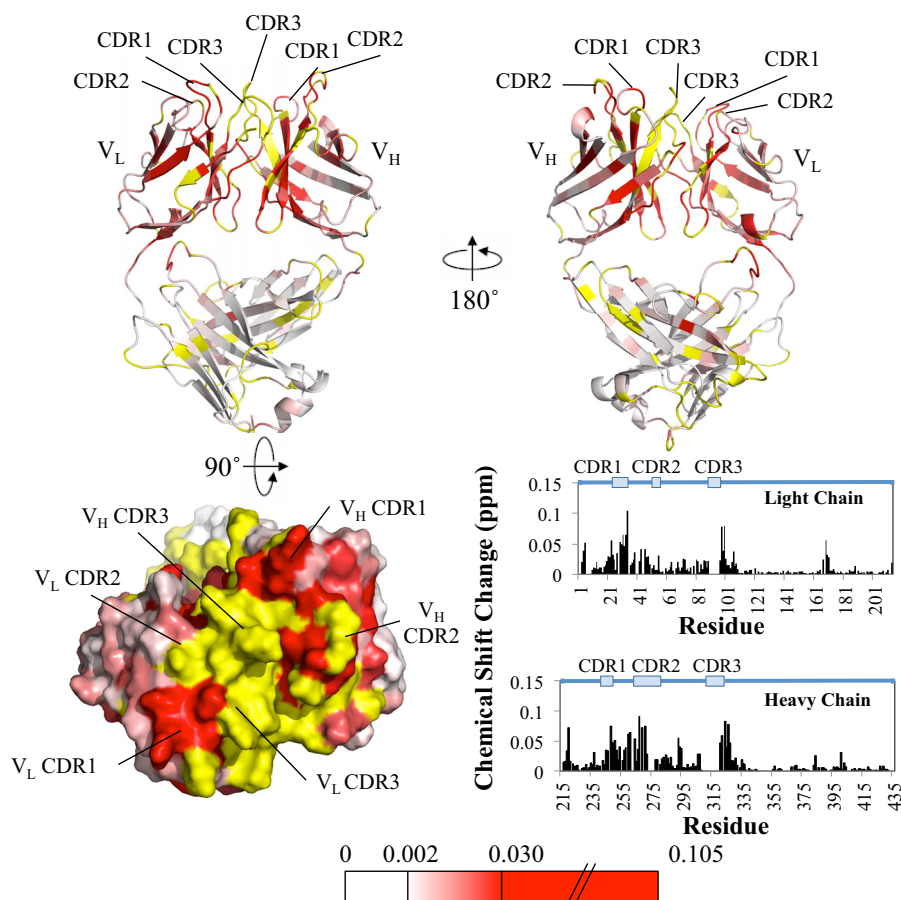


FIGURE 7. **Mapping of the IL-1 β interaction site on the anti-IL-1 β Fab.** The combined backbone minimal shift changes (H_N , N , and C') for antibody residues were determined for the free compared with IL-1 β -bound Fab. Residues with shifts of <0.002 ppm are shown in white, >0.03 in red, and in between on a linear gradient from white to red. Residues for which no minimal shift data could be obtained are shown in yellow. The chemical shift changes induced by IL-1 β binding are mapped on to both a ribbon representation of the Fab and a surface view of the antigen binding site. The most substantial changes observed are in the CDR loops, regions immediately adjacent to the CDRs, and in the interface between the two variable domains.

tional changes beyond the CDR loops are important for effective antigen binding. In contrast to the variable domains, the constant domains present in the anti-IL-1 β Fab show almost no signs of chemical shift perturbation except at the V_L/C_L interface. This most likely reflects movements of the V_L domain relative to the rest of the protein rather than structural changes within the constant domain.

Mapping of Antibody Binding Sites—Nearly complete backbone and side chain assignments have been reported previously for IL-1 β and IL-6 (29, 30), but under solution conditions different from those required for this study. It proved relatively straightforward to obtain essentially complete backbone resonance assignments for both cytokines under the new conditions, with backbone amide signals identified for all nonproline residues apart from Glu⁵¹, Lys⁵⁴, and Glu⁶⁹ in IL-6 (98%), and Ala¹, Arg⁴, Asn⁵³, and Glu⁶⁴-Asn⁶⁶ in IL-1 β (96%). The backbone assignments obtained for IL-6 and IL-1 β allowed further assessment of the nature of the interaction between the antibody fragments and their target antigens (14, 15), with minimal shift analysis providing information on the location of the antibody binding surfaces and of any structural changes induced in the bound proteins.

IL-1 β shows a discreet antibody binding site, with substantial and highly localized chemical shift perturbations (Fig. 8A), con-

sistent with a stable structure that is not significantly affected by antibody binding. In stark contrast, IL-6 shows significant perturbation of NMR signals throughout the protein as a result of scFv binding (Fig. 8B). This probably reflects the somewhat plastic and dynamic nature of the IL-6 structure, which has been described previously (15, 29), and may be related to the biological function of IL-6 and conformational changes associated with the formation of the IL-6-IL-6R α -gp130 signaling complex (15, 31). This in turn may explain the continued lack of observable backbone amide signals for residues in both CDR3 loops of the anti-IL-6 scFv bound to IL-6, with the inherent flexibility of IL-6 allowing continued exchange between distinct structural states for the contacting CDR loops. This property of the IL-6-scFv complex is in contrast to the IL-1 β -Fab, or equivalent IL-1 β -scFv complex, in which the majority of the backbone amide CDR signals are seen (Fig. 4 and Ref. 14).

The exchange between multiple conformational states on a relatively slow timescale, particularly for the CDR3s, is a conserved feature of the two distinct antibodies that have been characterized. The lack of backbone amide signals for these regions implies that a number of discreet structural states are being sampled on a millisecond to second timescale. This behavior is conserved in two antibodies with very different antigen binding sites and has recently been seen in another heavy

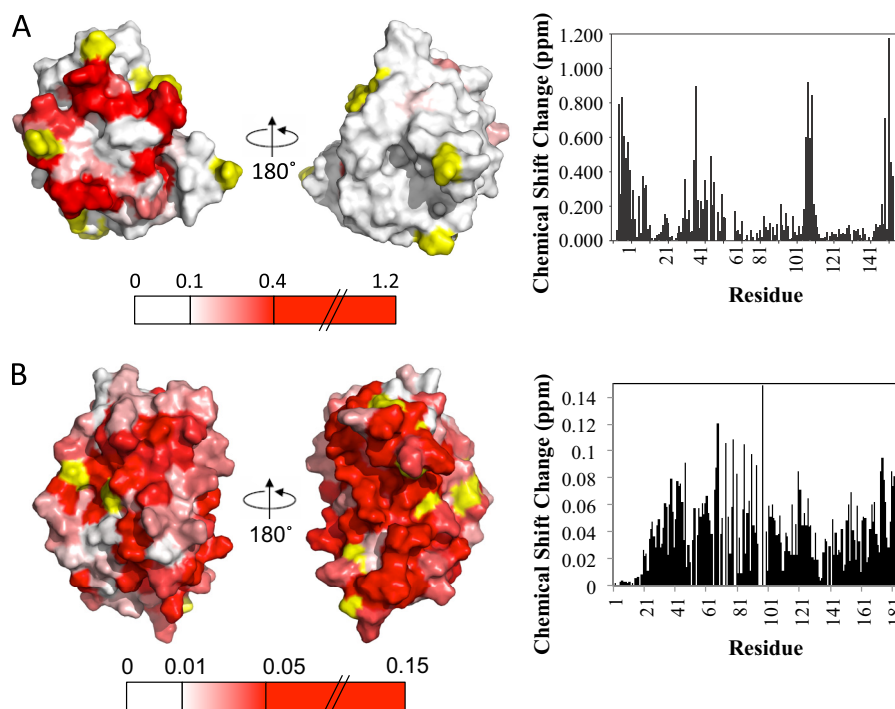


FIGURE 8. Comparison of the effects of antibody binding to IL-1 β and IL-6. Combined backbone minimal shift data were obtained for the assigned free versus antibody-bound proteins and mapped on to reported structures for the two cytokines (PDB ID 2I1B and 2IL6 for IL-1 β and IL-6, respectively). A, two-dimensional minimal shift data (H_N and N) were used for IL-1 β , with signals from residues shifted by <0.1 ppm shown in white, >0.4 ppm shown in red, and those between 0.1 and 0.4 ppm on a linear gradient from white to red. B, triple resonance minimal shift data (H_N , N, and C') were used for IL-6, with signals from residues shifted by <0.01 ppm shown in white, >0.05 ppm in red, and those between 0.01 and 0.05 ppm shown on a linear gradient from white to red. Residues for which no minimal shift data could be obtained are colored yellow for both proteins. A discreet antibody binding surface is clearly observed for IL-1 β . In contrast, for IL-6 the majority of residues show significant chemical shift changes on antibody binding, which is consistent with induced conformational changes throughout the protein.

chain antibody (32), which strongly suggests that conformational heterogeneity at the heart of the antigen binding site may be a critical feature to ensure effective binding of target proteins. This may simply reflect the need to accommodate flexible surfaces on protein antigens, but also has the potential to play a key role in facilitating productive binding to an extensive contact surface (typically $1700 \pm 260 \text{ \AA}^2$ (33)).

The slow dynamics of CDR loops at the heart of the antigen binding site and associated sampling of multiple conformations may allow antibodies to recognize and bind protein antigens when approaching at a less than optimal orientation. If this conformational heterogeneity in the CDRs allows the initiation of binding at some significant variance from the ideal orientation for interaction, it could greatly improve the chances of binding and therefore the on rate (k_a) for antibody-antigen complex formation (Fig. 9, anti-IL-1 β $k_a = 1.4 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$, $K_D = 80 \text{ pM}$ and anti-IL-6 $k_a = 2.36 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$, $K_D = 41 \text{ pM}$). This soft capture type mechanism would also allow initial intermolecular interactions at nonoptimal orientations, facilitating final docking of the proteins. The slow exchange between multiple conformations and relatively high affinity of interactions involving CDR3s appears well suited to the soft capture of protein antigens, perhaps assisted by movement of the variable domains relative to each other. This process could also serve to guide the target protein into the optimal orientation for interactions with residues in the apparently less dynamic CDR1 and CDR2 loops, which make important contributions to overall affinity and specificity (Fig. 10).

Conformational heterogeneity and associated slow motions appear to be a feature of many high affinity protein-protein interaction sites, involved in a diverse range of processes from receptor-mediated signaling to control of gene expression (7–13). This perhaps suggests that a soft capture type mechanism may be a key feature of many high affinity protein-protein interactions, facilitating a rate of complex formation consistent with that required by a range of biological processes.

Changes in the conformations and dynamics of the CDR3 loops induced by antigen binding appear to be accompanied by reorientations of the antibody variable domains relative to each other, with significant chemical changes seen for residues at the V_H/V_L interface in both the anti-IL-1 β Fab and anti IL-6 scFv (Figs. 6 and 7). A number of previous studies have reported antigen-induced changes in the orientation of the variable domains; however, no consistent picture has emerged (34–37). This may reflect the difficulty in detecting significant but small structural changes in proteins, which can now be reliably identified for relatively large proteins and complexes through the high sensitivity of backbone and side chain NMR chemical shifts to changes in conformation (6–9, 14, 15, 38).

CONCLUSIONS

The work reported here describes the results of an NMR-based study of the structural characteristics of the antigen binding regions of antibodies selected for their ability to recognize target proteins with very high specificity and affinity. This has revealed conformational heterogeneity and slow dynamics at

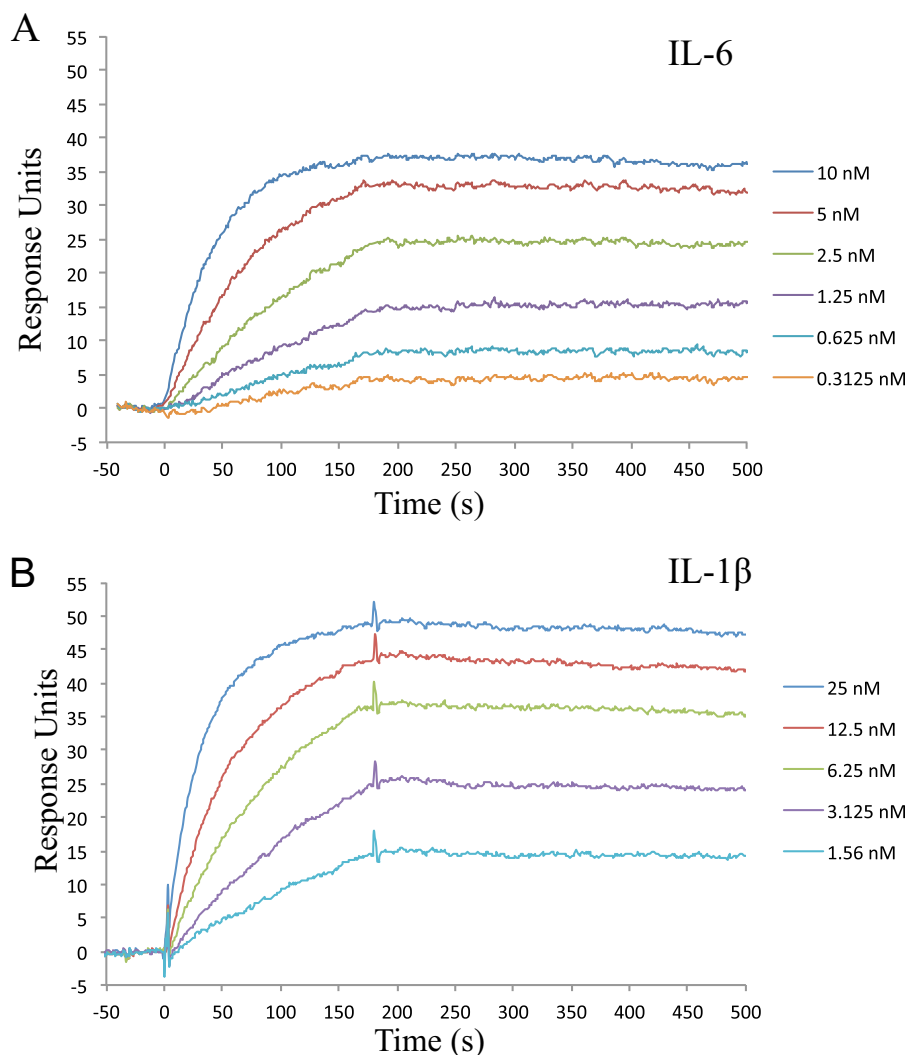


FIGURE 9. Binding of protein antigens to captured antibody fragments. Typical surface plasmon resonance sensorgrams are shown for the binding of IL-6 and IL-1 β to captured anti-IL-6 scFv (A) and anti-IL-1 β Fab (B), respectively. The responses obtained indicate very high affinity antigen binding for both antibody fragments, with a K_D of 41 pM determined for the anti-IL-6 scFv and 80 pM for the anti-IL-1 β Fab.

the heart of the antigen interaction sites, together with structural changes beyond the contact surfaces induced by antigen binding. The characterization of antibody fragments against two structurally distinct proteins, with IL-6 typical of a relatively flexible α -helical structure and IL-1 β representative of a fairly rigid β -sheet topology, has allowed the identification of antibody features and properties associated with antigen recognition that are independent of the structure of the target protein.

A number of structural features and properties have been identified that are common to both the anti-IL-1 β Fab and anti-IL-6 scFv. Perhaps the most interesting is the finding that the two distinct binding surfaces demonstrate similar conformational heterogeneity and slow dynamics for residues within the CDR3 regions of both variable domains prior to antigen binding. This sampling of a number of distinct conformations, leading to missing backbone NMR signals, appears to be an important feature of the heart of the antigen binding site, which is likely to play a key role in the recognition and/or binding of protein antigens. This inherent flexibility, sampling conforma-

tions on a millisecond to second timescale, may mediate an initial soft capture of the antigen over a range of suboptimal orientations of the two proteins, facilitating correct engagement of the antigen with other less flexible and potentially lower affinity CDR residues. This type of mechanism may help to guide initial antibody-antigen contacts down a productive binding pathway, resulting in an increased ability to bind at less than perfect orientations, which may significantly improve the rate of complex formation compared with rigid protein interaction surfaces.

Further antibody structural changes also take place following antigen binding, with an apparent change in the orientation of the two variable domains in both the anti-IL-1 β Fab and anti-IL-6 scFv. Flexibility at the V_H/V_L interface may further facilitate the soft capture mechanism by allowing an additional level of adaptability. However, changes in V_H/V_L orientation could also be linked to processes beyond antigen recognition, such as the initiation of B cell receptor signaling, which remains poorly understood. The conformational heterogeneity and slow dynamics reported here appears to be shared by many high

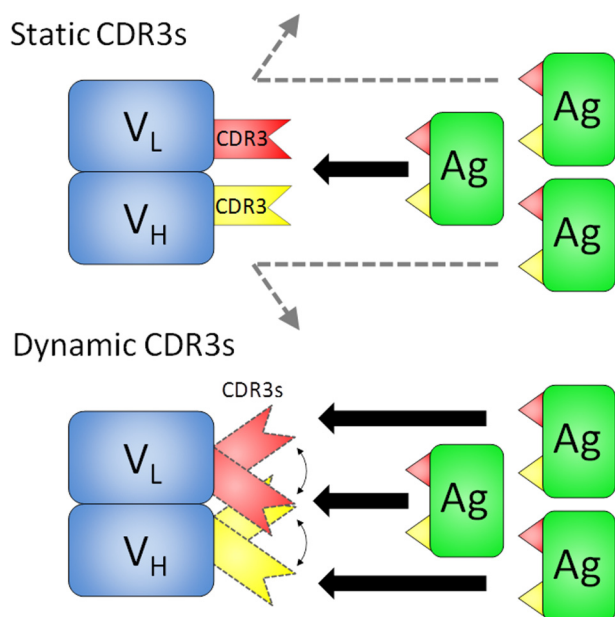


FIGURE 10. Conformational heterogeneity at high affinity protein-protein interfaces facilitates a soft capture, protein docking mechanism. In the absence of a dynamic interface (top), the binding of two proteins occurs across a very restrictive, near perfect set of orientations for the interacting partners. In contrast, conformational heterogeneity and slow dynamics at the core of the binding site enable productive binding over a range of close, but suboptimal relative orientations. This facilitates the formation of high affinity protein complexes on a timescale consistent with biological processes.

affinity protein-protein interaction surfaces across a wide range of biological processes, which is consistent with a potentially important role for the soft capture, protein-protein docking mechanism in high affinity complex formation (7–13).

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