Studies on ligand interactions of human complement factor H

Thesis submitted for the degree of Doctor of

Philosophy at the University of Leicester

By

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Statement of originality

This accompanying thesis submitted for the degree of PhD entitled “Studies on ligand interactions of human complement factor H” is based on work conducted by the author at the University of Leicester mainly during the period between October 2012 and September 2016.

All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references.

None of the work has been submitted for another degree in this or any other university.

Signed: Date:

Abstract

Studies on ligand interactions of human complement factor H

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The work reported in this thesis is mainly biochemical research on the properties of the human immune system plasma protein, complement factor H (FH). A major function of FH is to bind to "foreign" or "altered-host" surfaces, mainly by recognising charge cluster motifs. When bound to a surface, it down regulates activation of the complement system on that surface. Its binding properties towards a range of other proteins and macromolecules, have been examined, mainly by ELISA-style assays. Research then focused on a smaller number of these FH ligands, which appear to bind FH very strongly, and not, as is the usual situation, by charge interactions. These ligands are Adrenomedullin, Trinitrophenyl-derivatised ligands, and dinitrophenol-derivatised ligands (TNP and DNP). The binding and dissociation characteristics of these ligands have been examined, the binding optimised, and it has been shown that TNP and DNP derivatised ligands can be used for affinity purification of FH from human plasma. A factor H homologue in plasma, C4bp, also binds these ligands, but a number of other FH homologues in plasma do not (eg beta2 glycoprotein1). Expression systems have been obtained from other labs to make recombinant segments of FH and recombinant protein expressed in order to narrow down the binding sites on FH for these ligands. Binding sites in 3 regions of FH have been located, and the effects of ligand binding on the complement-regulatory functions of FH have been assessed.

Acknowledgement

After an intensive period of four years, today is the day: writing this note of thanks is the finishing touch on my thesis. It has been a period of intense learning for me, not only in the scientific arena, but also on a personal level. Writing this thesis has had a big impact on me. I would like to reflect on the people who have supported and helped me so much throughout this period.

I would like to express my deep sense of my gratitude to Professor Sim for his supervision, guidance and the encouragement that he showed me during my studies, and I would like to thank Professor Schwaeble for his continuous support and easing the difficulties in carrying out my PhD.

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General introduction

1 Introduction

1.1 The complement system

Complement was discovered in the 1890s to be a cytotoxic effector of antibodies. The name "complement" was used as it "complemented" the effect of antibodies, by lysing bacteria and other cells to which the antibodies were bound. Later it was discovered that it has several antigen-antibody-independent pathways of activation, including the alternative pathway and the lectin pathway, which were discovered in the 1950s, and 1980s, respectively (Sim *et al.,* 2016). Researches in complement revealed that this system consists of approximately 40 proteins, which can be soluble or cell bound. This system forms, with other plasma systems, for instance, blood clotting, fibrinolysis and kinin formation, an amplified response to stimulate the host immune system, which is based on catalytic reactions. Complement system function is involved in many aspects of biology and physiology and it is associated with many inflammatory and immunologic diseases (Markiewski and Lambris, 2007). Its activity controls aspects of acquired and innate immunity. The functions of this system include cell and bacterial lysis, virus neutralization, opsonisation (the promotion of phagocytosis) of particles, such as bacteria, and also host apoptotic and necrotic cell debris, and cleaning the immune system by removing immune complexes from the blood then depositing them in the liver and spleen (Bergseth *et al.*, 2013). Recently, there has been interest in the role of complement in development, especially in the central nervous system, because its activity in cell killing and removal of apoptotic cells is appropriate to the growth and re-modelling of tissue (Li *et al.*, 2016).

Complement activation can be initiated, by a very wide range of stimuli, via the three routes of recognition: classical pathway, lectin pathway and alternative pathway. When complement becomes activated through these pathways multicomponent serine proteases are formed and complement proteins are deposited on the complement-activating particle, resulting in opsonisation, phagocytic clearance or lysis of the particle or cell (Ritchie *et al.*, 2002).

1.1.1 The classical Pathway

This pathway was the first to be discovered, via its association with antibodies. It is commonly activated by large soluble antibody-antigen complexes, or with antibodies bound to appropriate surfaces, for example bacteria. IgM antibodies, and IgG subclasses (human IgG1, IgG2 and IgG3) can activate the classical pathway. The formation of the antibody-antigen complex promotes conformational changes in the Fc part of the IgM molecule, which allows binding of the C1 component of complement. However, the classical pathway is also activated without the presence of antibodies, by direct recognition, by C1, of surface features of bacteria, apoptotic cells, blood clots, etc (Carroll and Sim, 2011). C1 is a macromolecule found in the plasma and it consists of three different proteins (C1q, C1r and C1s), bound to each other via Ca++ ions (see Fig 1.1). C1q has globular heads, which bind to targets. The globular heads are linked together by collagenous helices. It can recognize a very wide range of targets, mostly by weak charge-dependent interactions. It has 6 globular heads, each with 3 lobes, which mediate the target-binding. Because the individual binding affinities are weak (ie between one head-lobe and one single site on a target), C1q has to be bound to two sites at least to generate a binding reaction of sufficient avidity; this binding generates conformational modifications in C1r, which is a proenzyme of a serine protease. The conformation change converts C1r to an active serine protease enzyme, and this enzyme can cleave C1s, which is also a serine protease proenzyme, to the activated C1s protease. Activated C1s works on two substrates C4 and C2 (see fig 1.3). The complement component C4 is a 190 kDa glycoprotein consisting of three polypeptides α, β and γ. The enzyme C1s cleaves C4 (in the α chain) to a small fragment C4a (9kDa) and large part C4b. The larger part C4b attaches to the surface of the complement-activating particle (such as a bacterium or antibody-antigen complex) near the C1 molecule. The binding of C4b is covalent, forming an ester or amide bond with the surface (Carroll and Sim, 2011). C1s also cleaves the serine protease proenzyme C2 to C2a and C2b; the small part C2b diffuses away, while C4b binds to C2a to form C4b2a, which is called the C3 convertase, a protease which activates C3 to form C3a and C3b. C3 is a homologue of C4, but has only two polypeptides, the α and β chains. It is activated by cleavage, like C4, and can bind covalently to the complement-activating particle, near to the C4b2a. When the C3a (9kDa)

is cleaved from the α chain of C3, C3b is generated. One C3 convertase molecule can activate over 200 C3 molecules; it is in fact an amplification step. Some of formed C3b can bind to the complement activator, and one C3b binds covalently to C4b2a to form a new trimolecular complex, C4b2a3b or C5 convertase. In this complex, C3b and C4b form a binding site for C5, and C5 is cleaved by C2a (Carroll and Sim, 2011). Cleavage of C5 is the common step in the three complement activation pathways; as a result of this cleavage C5a and C5b are released. C5b binds to C6 and both of them have the ability to bind to C7. After this binding C7 undergoes conformational changes leading to formation of the C5b67 complex. This complex has affinity to bind to a phospholipid bilayer of a cell or microbial membrane. Further conformational changes occur to C5b67 when C8 binds to this complex, and the α chain of C8 inserts into the target membrane. The last binding happens between C5b678 to C9. This new complex incorporates several copies of C9 to make a complex called the "Membrane Attack Complex" or MAC, which damages the cell membrane and causes cell lysis (Celik *et al.*, 2009).

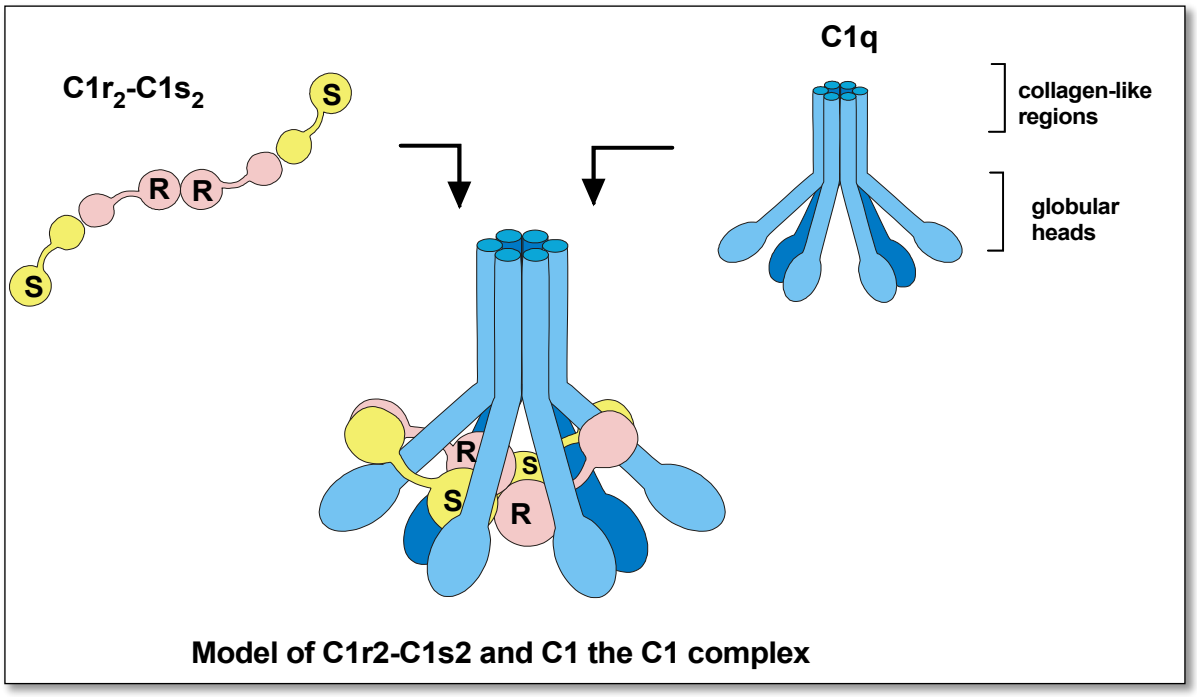


Figure 1-1 The first protein in the classical pathway of complement activation is C1, which is a complex of C1q, C1r, and C1s. C1q is composed of six identical subunits with globular heads and long collagen-like tails.
The tails combine to bind to two molecules each of C1r and C1s, forming the C1 complex C1q:C1r2:C1s2. The heads can bind to the constant regions of immunoglobulin molecules or directly to the pathogen surface, causing a conformational change in C1r, which then cleaves and activates the C1s zymogen. Figure adapted from(Arlaud *et al.*, 1987), with permission.

1.1.2 The Lectin Pathway (Figures 1.2 and 1.3)

This pathway is similar to the classical pathway: the major differences are that C1q is replaced by different recognition proteins, and C1r and C1s are replaced by the "mannose-binding lectin associated proteases" (MASPs), which are homologues of C1r and C1s. The proteins which play a crucial role in target recognition by the lectin pathway are mannose - binding lectin (MBL) and the ficolins (fcn), and the recently-described collectin-11 (CL-11) (Wallis, 2007). There are three ficolins in humans, named H, M and L-ficolin. MBL and CL-11 are collectins (collagenous lectin) and are similar in function and structure to C1q in the classical complement cascade, with 3-lobed globular heads linked together with collagenous helices. The ficolins also have similar quaternary structure (Fig 1.2). The globular heads of C1q bind mostly to charge clusters, but the globular heads of the collectins bind to neutral sugars, such as N-acetyl glucosamine, mannose and fucose, which are present on the surface of many bacteria and other microorganisms, and also on host glycoproteins. The ficolins have globular head domains, which are not homologous to those of C1q or the collectins: instead they have "fibrinogen-like domains" (FBG). The binding specificity of these is not very precisely known, but they bind to acetyl groups, so they recognize, for example, acetylated sugars, such as N-acetyl glucosamine, N-acetyl galactosamine, some Sialic acids. MBL, ficolins and CL-11 have 3-6 globular heads. Instead of C1r and C1s, they bind to any of three proteases zymogens MASP1, MASP2 or MASP3, which are homologues C1r and C1s, and the three MASP proenzymes can be activated when the recognition protein binds to a target surface (Carroll and Sim, 2011). MASP2 cleaves C4 and C2 in a similar way to activation of the classical pathway, and this activation leads to formation of the C3 convertase via binding of C4b to C2a (Fig 1.3). The roles of the proteases MASP1 and MASP3 are still under investigation. Then the rest of activation occurs similarly to the classical pathway (Meri, 2013). Individuals deficient in MBL suffer an increase in infection during their childhood period, before their antibody repertoire is fully developed. This gives evidence about the importance of MBL-lectin pathway for host immunity (Wallis *et al.*, 2010).

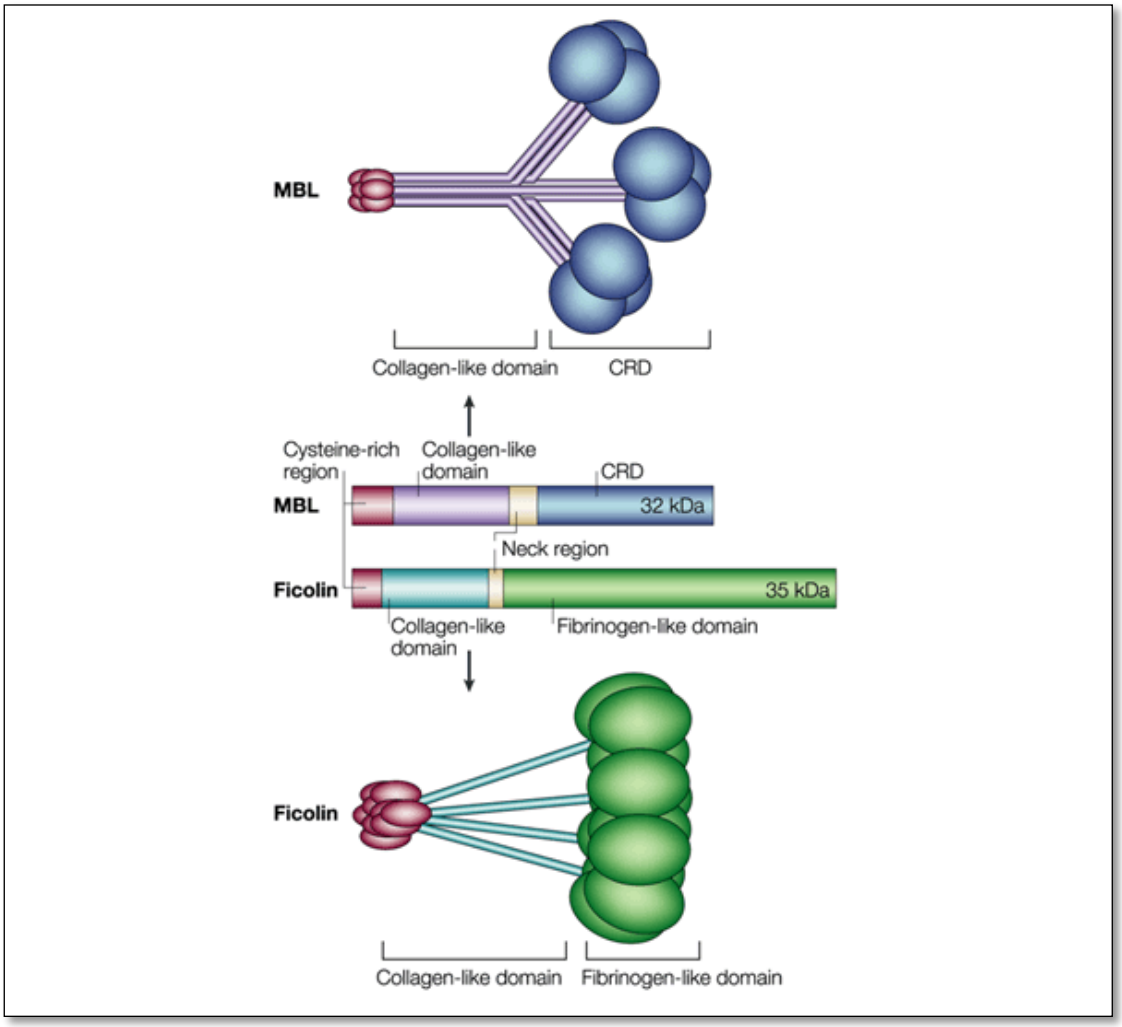


Figure 1-2. Structure of Mannose-binding lectin (MBL) and ficolins.
(MBL) and ficolins are oligomers of structural subunits, each of which is composed of three identical 32-kDa and 35-kDa polypeptides, respectively. CRD= carbohydrate recognition domain. This figure is from (Fujita, 2004).

1.1.3 The Alternative Pathway

The alternative pathway was discovered in the 1950s as an antibody-independent pathway, and so was clearly distinct from the classical pathway, which at that time was thought to be entirely antibody-dependent (Pillemer *et al.*, 1954). It is activated through contact with different substances such as lipoteichoic acid from gram-positive bacterial cell walls, gram negative lipopolyscaccharide (LPS), yeast cell walls (eg zymosan), unidentified targets on many parasites, and is now known to be activated by immune complexes containing IgG and some IgA antibodies. Rabbit red blood cells are used as a convenient activator in assays for human alternative pathway activation (Carroll and Sim, 2011). Activation of this pathway is based on hydrolysis of C3, which circulates in unactivated form in plasma. The thiolester in C3 undergoes very slow hydrolysis to form C3(H2O), which takes up the same conformation as the fragment C3b which arises from proteolytic cleavage of C3, and so can bind to Factor B. Binding of factor B to C3(H2O) produces C3(H2O)B that is a substrate for factor D to form the molecule C3(H2O)Bb, which is itself a C3 convertase. This convertase is homologous to C4b2a of the classical and lectin pathways: C4 is a homologue of C3, and Factor B is a homologue of C2. C3(H2O)Bb will cleave C3 to form C3a and C3b. C3b will bind randomly to any nearby surface, where it can bind to either Factor B or Factor H. If it binds factor H, it will be inactivated by Factor I (see below), and complement activation will go no further. If it binds Factor B, the C3bB complex is activated by factor D to form C3bBb, the major C3 convertase of the alternative pathway. This surface-bound C3 convertase can activate hundreds of molecules of C3, and many of the resulting C3b molecules will bind covalently to the surface, in a cluster around the activating protease. This clustered C3b is important for opsonisation, a process that leads to destruction of microbes due to facilitating their recognition and engulfment via phagocytic cells. Opsonisation requires that the C3b (or its breakdown products iC3b or C3d), binds specifically to C3 receptors on the phagocytic cell membrane. C3b which does not attach to a surface will quickly hydrolyze and catabolize in the absence of a binding surface. As soon as C3b (formed by any of the three pathways) becomes attached to a surface, it can form the alternative pathway convertase C3bBb, and so amplify the turnover and fixation of C3. As happens for the convertase C4b2a, C3bBb can be modified by covalent attachment

of another C3b, forming C3bBbC3b, the alternative pathway C5 convertase. This cleaves C5, which then interacts with C6-C9, to form the MAC, as described above (see Fig 1.3) (Thurman and Holers, 2006).

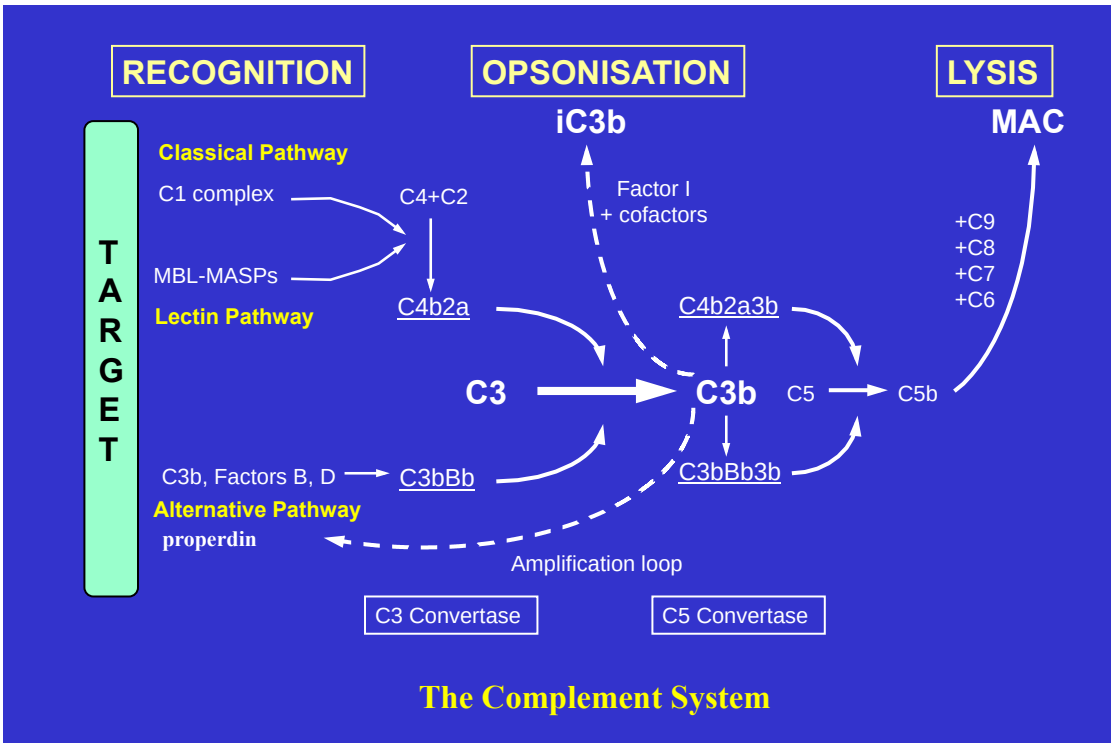


Figure 1-3 The complement system activation pathways; classical, lectin and alternative. The classical pathway is initiated by activation of the C1 components, when C1q binds to the surface of targets such as microbes or apoptotic cells and immune complexes. Lectin pathway activation starts with the binding of MBL-MASP or Ficolin-MASP to carbohydrate and other ligands on the target surfaces. The alternative pathway activation depends on the hydrolysis of C3 in the plasma, which the binds to factor B which is cleaved by factor D. C3 convertase is formed in all pathways, C4b2a in both classical and the lectin pathways and C3bBb in the alternative pathway. This complex cleaves C3 to C3a and C3b and the latter binds to the convertase complex to form C5 convertases, which cleaves C5 to C5a and C5b. C5b will bind to C6, C7, C8 and C9 to form the membrane attack complex (MAC). Figure supplied by Robert B. Sim, Oxford University (copyright holder).

1.2 Regulation of the complement system

Complement is activated when the body comes under attack by microbes (bacteria, fungi and parasites), or when there is (mechanical) damage to tissue. During complement activation microbes and tissue damage products become opsonised by complement components. Complement can be also activated in some other situations (eg, tissue ischemia and reperfusion) and this can lead to complement attack on the host's own tissues.

Therefore, the complement system must be tightly regulated to minimize any host damage. Complement is controlled via both soluble (plasma) and membrane-bound inhibitory proteins (Noris and Remuzzi, 2013) (Figure 1-5). The plasma protein; C1 inhibitor (C1 INH), a member of the SERPIN family (SERine Protease INhibitor) inhibits the autoactivation of C1qrs by binding to proenzymic C1s and C1r (Hughes *et al.,* 2004). More importantly, C1 INH also reacts directly with activated C1r and C1s, and also activated MASP1 and MASP2, to form a tight complex, in which the protease is inactive. (Wouters and Zeerleder, 2015). C1-INH also inhibits the plasma proteases of the kinin and coagulation systems, Kallikrein, Factor XIa and Factor XIIa, so its effects are not confined to regulating the complement system (Cugno *et al.,* 1997).

The C3 and C5 convertases of the complement system are controlled by several structurally-related proteins, which are encoded on a gene cluster on human chromosome 1q32, called the RCA (regulation of complement activation) gene cluster. These proteins are Factor H and C4bp (both soluble proteins) and the membrane-bound CR1 (complement receptor 1=CD35), DAF (decay accelerating factor =CD55) and MCP (membrane cofactor protein =CD47), The C3 and C5 convertases are unstable enzymes, and their subunits dissociate, making them inactive, with a half-life of a few minutes. The RCA proteins can bind to them accelerating their decay: this is called “Decay-acceleration Activity”. Once bound to C3b or C4b, the RCA proteins make them a target for inactivation by the protease Factor I, breaking C4b down to form C4c and C4d, and C3b to form iC3b. This is called “Factor I-cofactor Activity” (Harris *et al.,* 2000). (Figure 1-4)

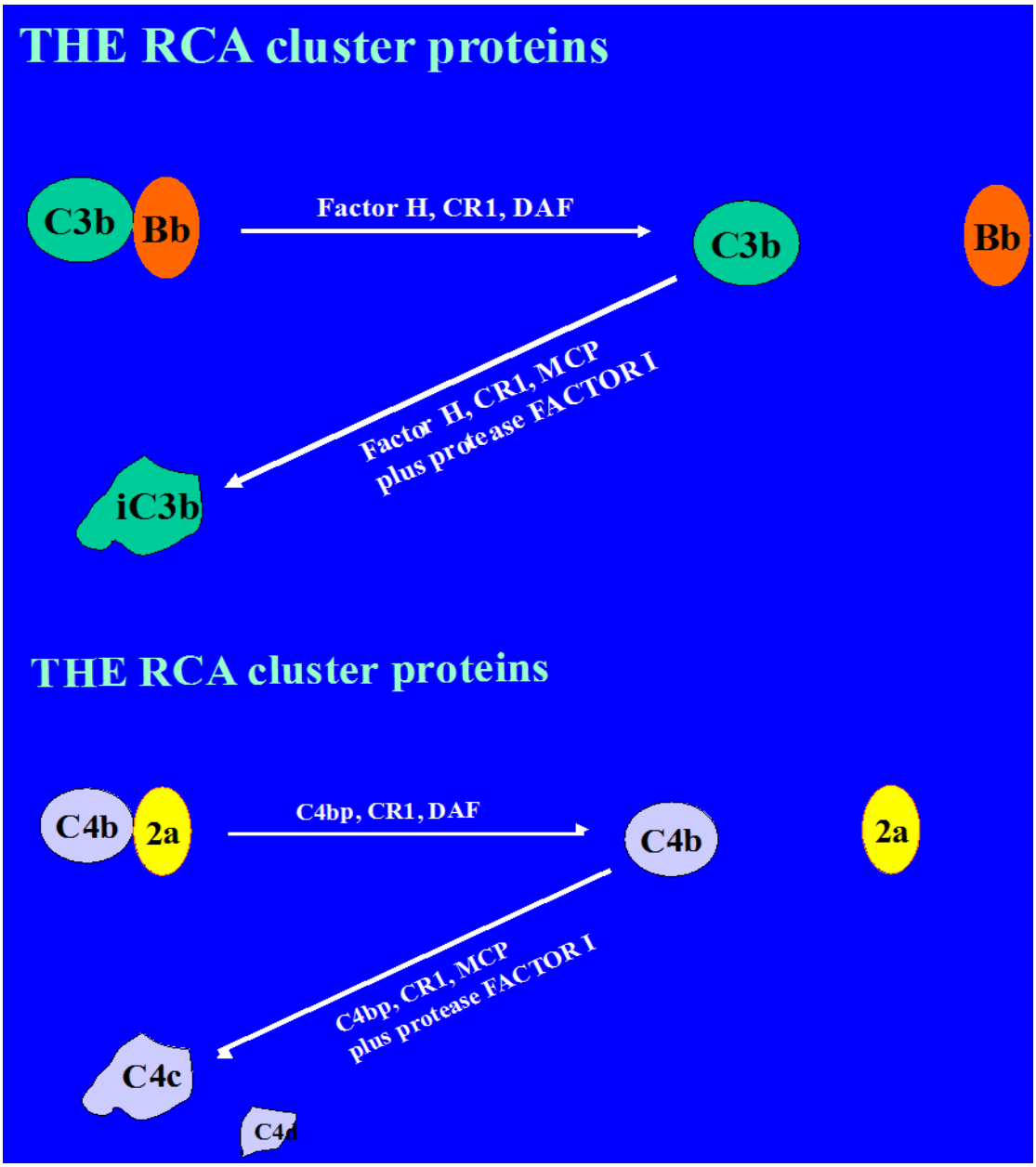


Figure 1-4. complement regulation by RCA cluster proteins.
DAF (or FH or CR1) destabilizes C3 convertases and accelerate the dissociation of C3bBb and C4b2a. For C4b2a, the regulatory protein C4bp acts instead of FH. This is "decay-acceleration activity". These proteins also have Factor I-Cofactor activity: MCP (or FH or CR1) binds to C3b and serves as a cofactor for FI-mediated cleavage and inactivation of C3b. The same happens for C4b, except that again C4bp acts instead of FH. Figure provided by Robert B. Sim, Oxford University (copyright holder).

C4b-binding protein (C4bp) for example, regulates the classical and the lectin pathways. It binds to C4b and accelerates the decay of C4b2a. C4bp works as a cofactor for the cleavage of C4b by factor I (Wenderfer *et al.*, 2007). Factor H is the most abundant fluid phase regulator glycoprotein for the alternative pathway. It has decay acceleration activity. For C3bBb and C3bBbC3b, and acts as Factor I-cofactor for the breakdown of C3b to iC3b. Membrane bound inhibitory regulators complement receptor 1 (CR1) and membrane cofactor protein (MCP) both have the combined activities of C4bp and Factor H, so they act on C3b and C4b, and all the convertases. Decay accelerating factor (DAF) inhibits C3 convertases in both classical and alternative pathways (decay acceleration activity), but has no factor I-cofactor activity. These proteins encoded in the RCA gene cluster are all structurally similar, and are made up of different numbers of CCP domains (Carroll and Sim, 2011) (see further details in section 1.3.1). Also encoded in this gene cluster are 5 factor-H related proteins (FHRs), which also appear to have complement-regulatory activity, the details of which are currently being explored.

The terminal pathway of the complement system (C5-C9, lytic complex) is also regulated. Host cells are protected by CD59 (protectin) on their surface, which binds to C5b-8 complexes and prevents their interaction with C9 (Nesargikar *et al.*, 2012).

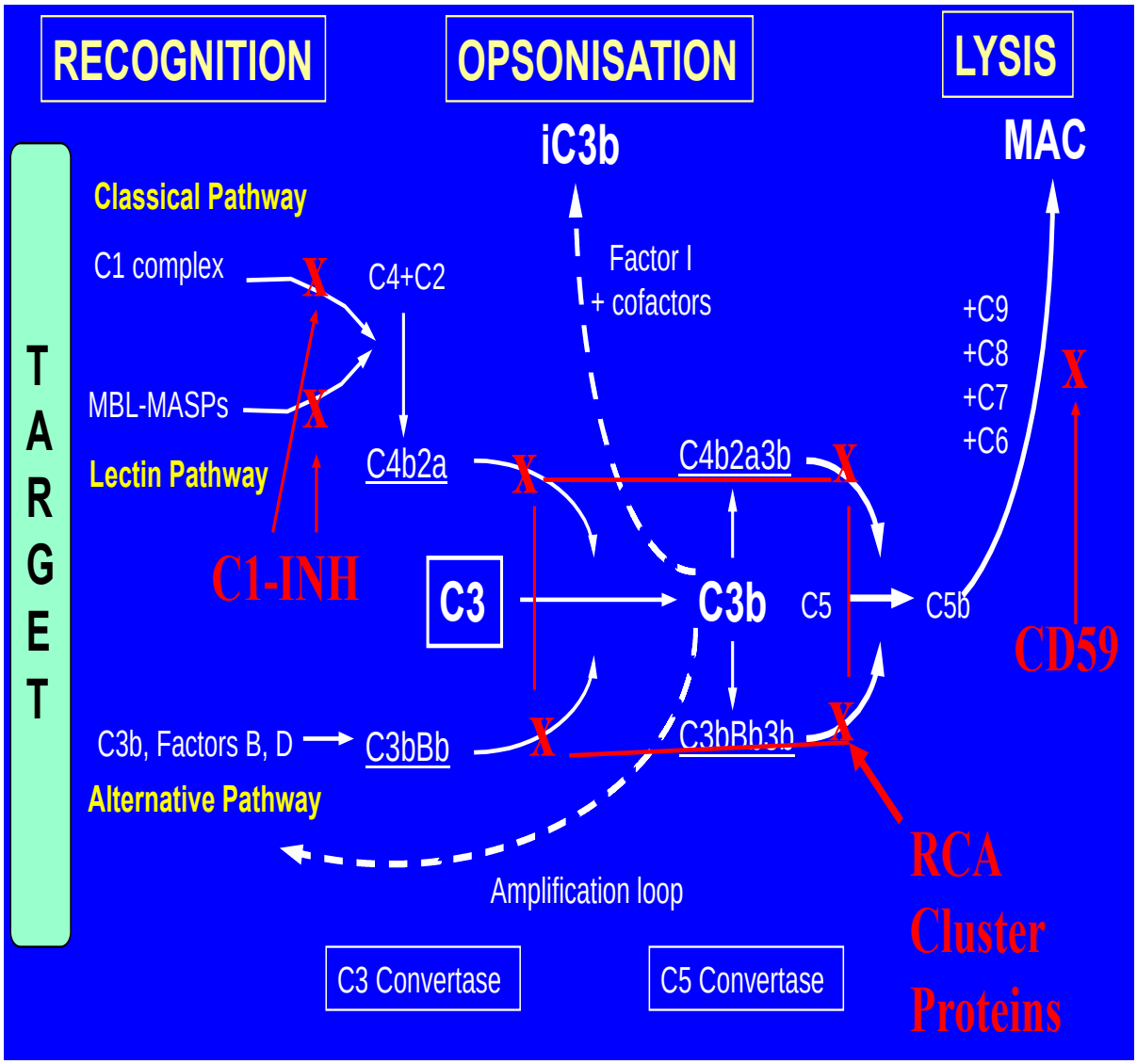


Figure 1-5 The complement system is controlled by several fluid phase and membrane-bound regulators that act at various steps of the activation cascade.
Factor H is the major fluid-phase regulator of the alternative pathway, as it prevents the formation of the C3 and C5 convertases, facilitates the disassembly of already formed convertases and acts as a cofactor for the inactivation (enzymatic cleavage) of C3b by Factor I. Figure provided by Robert B. Sim, Oxford University (copyright holder).

1.3 Factor H (FH)

The complement system is a highly active defense line against microbial infection. Human complement effectively helps to remove dead cells, microbes, debris and any other foreign particles from the body, by recognizing suitable targets and promoting their phagocytosis. Regarding to its defense activity against microbial infections; this system must be carefully regulated to avoid over-consumption of its components and to limit possible damage for the host tissues. Regulation of the complement system occurs by fluid-phase regulators and membrane proteins, of which FH is the most abundant in plasma.

1.3.1 Structure of factor H:

One of the most abundant well-characterised proteins in vertebrate serum is factor H (FH). Factor H was first named by Nilsson and Mueller-Eberhard as β1H globulin. Factor H is a single chain glycoprotein with molecular weight 155 kDa. It circulates in human plasma at a concentration which varies widely between individuals (260-651 μg/ml) (Rodríguez *et al.*, 2004).

Factor H belongs to the factor H family of proteins, which consists of six or more highly related proteins. The main form of factor H is composed of 1213 amino acid residues (see figure 1-7) distributed in 20 repetitive units (domains) called short consensus repeats (SCR) or complement control protein domains (CCP); each domain has ~ 60 amino acids, with two internal disulfide bonds (Cy^{I} –Cys^{III} and Cys^{II} –Cys^{IV}) (Makou *et al.,* 2013). These disulfide bridges are important in maintaining the characteristic structure of the CCP module. The modules are joined together by short linker regions of 3-8 amino acids, and the protein can be visualised as a string of beads (DiScipio, 1992). The Factor H gene has an alternative splicing product called FH-like protein 1 (FHL-1). It is present in plasma at very low concentration in comparison to FH. Its molecular weight is 42 kDa and it consists of 7 CCPs, which are identical to the first 7 CCPs of FH, followed by a short sequence SFTL. (Ripoche et al, 1988) (Figure 1-6). FHL-1 has similar activities in regulating the complement system as FH. (Józsi and Zipfel, 2008). In plasma there are also five FH-

related proteins, FHR1, FHR2, FHR3, FHR4 and FHR5, each of which is encoded by a separate gene on human chromosome 1q32 within the *RCA* (Regulation of Complement Activation) gene cluster. The FHR4 gene has two alternatively spliced products, FHR4A and B. The five *CFHR* genes are located downstream of the *factor H* gene and each *CFHR* gene codes for a plasma protein that is exclusively composed of CCP domains (Figure 1-6). The entire chromosomal segment with the *CFHR* genes is characterized by several large genomic repeat regions, which have a high degree of sequence identity (Skerka *et al.*, 2013).

FHR1 is the most abundant FHR in the plasma. Each of the FHR proteins is composed of 5-9 CCPs and as shown in Figure 1-6, these CCPs are similar, or sometimes near-identical (in amino acid sequence), to CCPs of FH, in the regions FH CCP6-8 and FH CCP19-20. It has been shown that FHRs do not control complement in the same way as FH, but instead they seem generally to "oppose" the action of FH. (Medjeral-Thomas and Pickering, 2016), They have CCP domains very similar to the CCP6-8 and 19-20 domains of FH, through which FH binds to many targets, but they do not have CCPs similar to FH domains 1-4, which is the main region for Factor I-cofactor activity of FH (see section 1.3.3). So it appears that they can compete with FH for binding to some targets, but they lack cofactor activity, so they effectively "inhibit" some effects of FH. However there are some reports of complement-inhibitory activity: FHR1 inhibits cleavage of C5 by binding to C3b (Heinen et al., 2009). FHR2 is made up of four CCP domains, similar to CCPs 6, 7, 19, 20 of FH. FHR2 is reported to inhibit C3 convertase of the alternative pathway but it does not have cofactor activity (Goicoechea de Jorge et al., 2013). FHR3 protein is composed of five CCP domains, and it binds to C3, C3d and heparin. FHR4A protein consists of nine CCP domains, and its functions in complement remain poorly defined but it binds to C3, C-reactive protein (CRP) and a complement modulatory activity in the form of a factor H cofactor enhancing activity was reported (Skerka et al., 2013; Mihlan et al., 2009). FHR 5 also consists of nine CCP domains. FHR5 binds to C3b and C3d so it competes with factor H, furthermore it binds to CRP (McRae et al., 2005). The FHRs form homo-and heterodimers, which makes the study of their functions in vivo very complex.

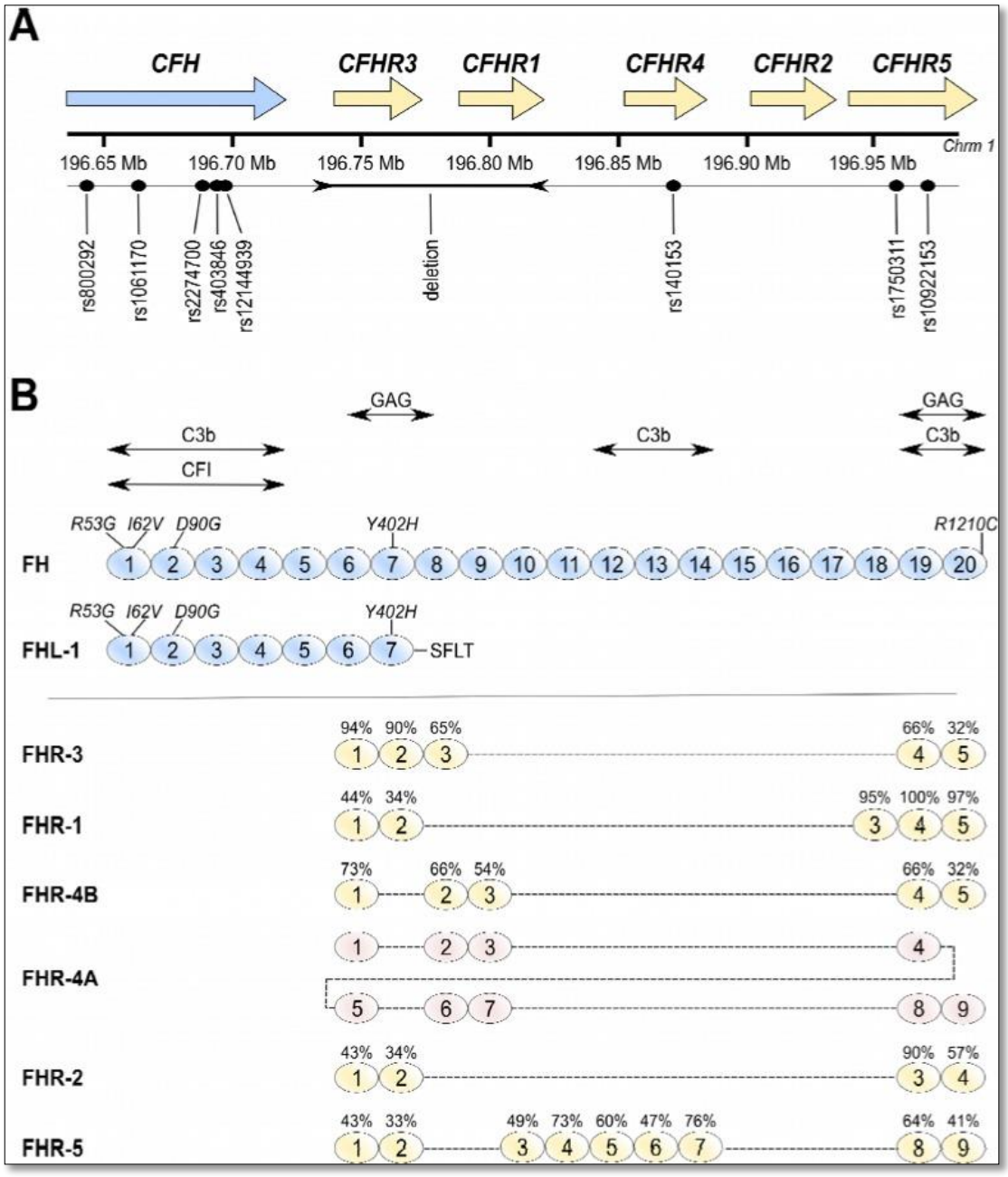


Figure 1-6 Complement factor H (CFH) and complement factor H related protein (CFHR) genes on chromosome 1 and the structures of FH, FHL-1 and FHR proteins.
(A) The CFH and CFHR genes all occupy one stretch of chromosome 1 (1q32) known as the regulators of complement activation gene cluster. In (B), the protein structure (CCP domains) is shown. The FHRs are made up of different numbers of CCPs. As shown in the figure, FHR1 has 5 CCPs, FHR5 has 9. The CCPs in FHRs have a high degree of sequence similarity to some CCPs of FH. The alignment in the figure shows that CCPs 1-3 of FHR3 are most similar to CCPs 6-9 of FH, while CCPs 4 and 5 of FHR3 are most similar to FH domains 19 and 20. Percent amino acid identity with the corresponding FH CCPs is shown. From (Clark and Bishop, 2014)

1.3.2 Factor H functions

Factor H is the main soluble regulator of the alternative pathway of complement. Factor H controls complement activity through several mechanisms: inhibiting the C3 and C5 convertases in the alternative pathway, by competing with factor B for binding to the central component C3b; promoting the disassembly of C3 and C5 convertases by displacing bound Factor Bb; and it acts as a cofactor for factor I in the cleavage of C3b to form iC3b (Kopp *et al.,* 2012). Proteolysis of C3b occurs in the presence of factor H and factor I leading to cleavage in α′-chain of C3b and forming three fragments 3, 43 and 68 kDa (Rodríguez De Córdoba *et al.,* 2004). Factor H works both in the fluid phase and on cell or particle surfaces (Schmidt *et al.,* 2011). On surfaces, factor H can bind to surface-bound C3b, and also to charge clusters on the surface, made up of, for example, sialic acids as well as glycosaminoglycan or sulphated polysaccharides like heparin. (Koistinen, 1993; Rodríguez de Córdoba et al., 2004). This binding to a secondary site on surfaces reinforces its apparent avidity for C3b, and so promotes the action of FH in inhibiting C3 turnover. Thus FH is able to distinguish between activator- and non-activators-surfaces to which C3b is bound: on surfaces with charge clusters recognized by FH, FH binding is enhanced, so C3 turnover is downregulated, while on surfaces lacking such FH recognition sites, alternative pathway activation of C3 will proceed at a greater rate.

Jarva *et al.,* 1999 confirmed that factor H interacts with C-reactive protein, and CRP binds to FH on two binding sites. CRP binds to bacterial surfaces, so can bridge the binding of FH to bacteria. Many pathogens (see section 1.3.4.2) and cancer cells avoid complement activation by mechanisms which allow them to bind FH.

It has been showed that factor H can also regulate the classical pathway through direct competition with C1q, the recognition protein of the classical pathway for binding to several types of targets, including anionic phospholipids such as cardiolipin, lipid A and the bacterium *Escherichia coli* (Tan *et al.,* 2010; Kishore & Sim 2012, Alrashidi, 2015).

1.3.3 Binding sites of factor H:

As mentioned in section 1.3.1 factor H is a single chain composed of 20 tandemly arranged homologous complement control protein modules (CCP units). Many different experimental methods such as, using monoclonal antibodies, proteolytic digestion, expression of recombinant segments of FH, and point mutagenesis have been used to identify and characterise functional domains of factor H (Gordon *et al.,* 1995). The functional sites of factor H have been studied for years, and they are mainly three sites, two of which engage with C3b or C3d. One of these sites, plus another, interacts mainly through charge clusters, with self-surfaces. The sites (shown on Figure 1-6, are CCP 1-4 (interaction with C3b, Factor I), CCP 6-8 (interaction with charge clusters, bacterial proteins and many other targets) and CCP 19-20 (interaction with C3b or C3d, GAGs, sialic acids). Another site marked as a C3b binding site on Figure 1-6, CCPs 12-14, has little evidence to support it. (Schmidt *et al.,* 2008). The amino acid sequences of these regions of FH are shown in Figure 1-7.

1.3.3.1 CCP 1-4:

FH CCP 1-4 is the major site for interaction with C3b and Factor I, so is the location of Factor I-cofactor activity (Jozsi, 2005).

1.3.3.2 CCP 19-20:

FH CCP 19-20 also binds to C3b, and also to iC3b and its cleavage product C3d (Ferreira, Pangburn and Cortés, 2010). In addition CCP19-20 binding to heparin and sialic acid have been described (Blackmore *et al.,* 1996). It has been found through structural analysis of CCP19-20 that there is a group of positively charged amino acids that participate in the interaction with C3b and heparin and also CRP (Perkins and Goodship, 2002). Mutations and polymorphism in CCP19-20 is associated with kidney disease such as hemolytic uremic syndrome HUS (Jozsi, 2005).

1.3.3.3 CCP 6-8:

Factor H has two main GAG-binding sites2, CCP6-8 and CCP19-20 (Clark *et al.*, 2013). Clark et al (2013 also found that recombinant factor H CCP6-8 binds more tightly to highly sulphated heparin than CCP19-20. CCP7 and CCP 20 on factor H carry electropositive surfaces patches that participate in the avid binding of factor H to polyanions such as those on sulphated GAGs (Makou *et al.*, 2013) Additionally, most characterized bacterial FH-binding proteins bind within CCP6–8 or CCP19–20 (Ferreira, *et al.*, 2010).

Figure 1-7 The complete amino acid sequence of FH (Ripoche *et al.*, 1988).
Human complement factor H is a single chain made up of 20 domain called complement control proteins (CCP). Its molecular weight is 155 kDa, and it has 7-8 N-linked glycans. Amino acids 1-18 (top line) represent the signal sequence. In later chapters of this thesis, recombinant proteins representing different regions of FH are used in experiments. These recombinant proteins represent the following CCP modules: FH CCP 1-4 amino acid numbers are 19-264, MW 27800, pI is 5.04, E280 1mg/ml is 1.72; FH CCP 6-8 amino acid numbers are 324-507, MW is 20844, pI is 8.09, E280 1mg/ml is 8.09; FH CCP 19-20 amino acid numbers are 1107-1230, MW is 20056, pI is 875, E280 1mg/ml is 1.945. Mol Wt, pI and E280 values were calculated using the Protparam website available via UNIPROT.

1.3.4 Factor H interactions:

Factor H regulates the alternative pathway of complement system by binding to C3b which results of inhibition the interaction of C3b with factor B and C5 (Soames and Sim, 1997). It also may regulate the complement classical pathway by competing with C1q (C1) for binding to some charge-cluster ligands (Alrashidi, 2015; Tan *et al.*, 2010).

1.3.4.1 Factor H attaches to host cells and ligands:

Factor H binds to cells and surfaces, as factor H has binding sites for GAGs such as heparin and for "polyanions" eg clustered sialic acids. This reveals its important role of discriminating between activators and non-activators (i.e. polyanion rich) surfaces (Józsi *et al.,* 2006). In conditions of strong complement activation, cells and tissues require effective protection against unwanted complement-mediated damage, thus binding of soluble regulator factor H to endothelial cells via cell surface glycosaminoglycans and C3b prevents endothelial damage and acute renal failure in the kidney (Kajander *et al.,* 2011). Additionally, factor H takes part in recognition of host cells by binding to cell markers that occur on the apoptotic cells for instance DNA, annexin II and anionic phospholipids. Factor H can bind DNA and histones when cells are damaged and these ligands become exposed on apoptotic cells (Leffler *et al.,* 2010). Additional studies have reported that factor H binds to the soluble acute phase protein C-reactive protein and the related pentraxin 3: these have shown good ability to bind to apoptotic cells and so bring factor H to the cell surface (Hebecker *et al.,* 2010; Deban *et al.,* 2008). Other host cells for example neutrophils, B lymphocytes, monocytes and platelets contain markers on their surfaces which recruit factor H (Ferreira *et al.,* 2010; Mnjoyan *et al.,* 2008). Furthermore, a study by Martínez et al., (2001) showed that there is an interaction between Adrenomedullin (ADM) and factor H. Adrenomedullin is a ubiquitous peptide hormone, composed of 52 amino acids which can regulate blood pressure, and influence growth, neurotransmission, and inflammation (Martínez *et al.,* 2003). ADM is described in more detail in section 1.4. A specific Adrenomedullin binding protein (AMBP-1), was identified, and later shown to be identical to FH (Martinez et al, 2001, 2003). AMBP-1 facilitates binding of

Adrenomedullin to its receptors and modulates its biological activity (Idrovo *et al.*, 2015). Combination of Adrenomedullin and FH (AMBP-1) has been shown to have protective effects to decrease destruction of ADM by random proteolysis in circulation (Carrizo *et al.*, 2007). It has been found that FH binds also to fibulin (DiScipio *et al.*, 2016).

Table 1-1 Some Factor H ligands, binding sites and potential relevance of the interactions.

Ligand	Biding sites	Relevance
C3 Fragments C3b	CCP 1-4, 6-8	Complement regulation
C3d	CCP 19-20	
Polyanionic molecules: Heparin		Attachment to host cells
Other Glycosaminoglycans	CCP 7,19-20	
Sialic acid		
Pentraxins: C-reactive protein	CCP 7, 8 -11, 19-20	Targeting the activity of FH to pentraxin-binding particles
Pentraxin3	CCP 7, 19-20	
Apoptotic/ necrotic cells: Annexin-II	CCP 6-8	Promoting safe clearance, protection from autoimmunity
DNA	CCP 6-8, 19-20	
Histones	CCP 1-4, 6-8, 8-15	
Extracellular matrix: Fibromodulin	CCP 6-8	Regulation of inflammation
Osteoadherin	?	(e.g., in rheumatoid arthritis)
Chondroadherin	?	
Malondialdehyde	?	Recognition of damaged tissues
Adrenomedullin	CCP 8-11, 12-20	Modulation of Adrenomedullin function

Data from Kopp *et al.*, (2012).

1.3.4.2 Factor H interacts with microbes and other organisms:

The complement system helps in clearance from the body of pathogens and debris that results from damaged cells (Carroll, 1998). Factor H is the major soluble inhibitor for the alternative pathway of complement system. Bacteria and viruses use several host protection mechanisms to escape or limit attack by complement, and one of these mechanisms is binding factor H to their surface to provide resistance to the alternative pathway (Ferreira *et al.,* 2010).

Table 1-2 List of some microbes that bind factor H.

Microbe	**Type**	**Reference**
Neisseria meningitides	Gram –ve bacteria	(McNeil *et al.,* 2013)
Haemophilus influenza	Gram –ve bacteria	(Meri *et al.,* 2013)
Bordetella pertussis,	Gram –ve bacteria	(Amdahl *et al.,* 2011)
Pseudomonas aeruginosa	Gram –ve bacteria	(Kunert *et al.,* 2007)
Streptococcus pneumonia & S. pyogenes	Gram +ve bacteria	(Dave *et al.,* 2004)
Candida albicans	Yeast	(Luo *et al.,* 2009)
Borrelia burgdorferi	Gram –ve bacteria	(Hellwage *et al.,* 2001)
B. hermsii,	Gram –ve bacteria	(Meri *et al.,* 2013)
Salmonella enterica	Gram –ve bacteria	(Ho, Jarva and Meri, 2010)
Escherichia coli	Gram –ve bacteria	(Ho, Jarva and Meri, 2010)
Aspergillus fumigatus	Fungus	(Behnsen *et al.,* 2008)
Plasmodium falciparum	Parasite	(Simon *et al.,* 2013)
Anopheles mosquito gut	Insect	(Khattab *et al.,* 2015)
HIV	Virus	(Ferreira, Pangburn and Cortés, 2010)

1.4 Adrenomedullin (ADM)

ADM was first described in 1993, as a result of its action as a hypotensive peptide (Kitamura *et al.,* 1993). ADM is a 52 amino acid regulatory peptide which has limited homology to CGRP (calcitonin gene-related peptide: 37 amino acids) and amylin (37 amino acids) (Hinson *et al.,* 2000). It has a single disulphide bridge between residues 16 and 21. It is processed from a 185-amino acid precursor, preproadrenomedullin, and an amidated C-terminus is formed during processing. The plasma concentration of ADM is in the 10–20 pMolar range, although measurement is made difficult by the presence of the binding protein, AMBP1 (=FH) which has a plasma concentration of 220–650 g/ml (1.4–4.3 uM), and is therefore about 10exp8-fold more abundant than ADM in plasma. Adrenomedullin is a multifunctional regulatory and vasoactive peptide. ADM like FH is present in most body fluids, eg milk, sweat, cerebrospinal fluid, urine and it has been discovered in human breast, lung, ovarian, pancreatic, prostate and renal cancers, cardiomyocytes, fibroblasts, monocytes, and leukocytes (Martínez *et al.,* 2003; Zhou *et al.,* 2015), although their relative concentrations in these fluids are different from that in plasma (Sim *et al.,* 2008).

ADM is a circulating hormone, although it functions also as a local paracrine and autocrine mediator with multiple biological activities such as vasodilatation, cell growth, regulation of hormone secretion, natriuresis, and antimicrobial effects (Hinson *et al.,* 2000). At specific physiological concentration, ADM was shown to have a crucial role in the systemic and pulmonary circulation. In many cell types, including endothelial and smooth muscle cells of the vascular wall, production and secretion of ADM are increased in response to cellular strain induced by hypoxia and ischemia (Minamino *et al.,* 1995).

Sequence of ADM: Tyr-Arg-Gln-Ser-Met-Asn-Asn-Phe-Gln-Gly-Leu-Arg-Ser-Phe-Gly-Cys-Arg-Phe-Gly-Thr-Cys- Thr-Val-Gln-Lys-Leu-Ala-His-Gln-Ile-Tyr-Gln-Phe-Thr-Asp-Lys-Asp-Lys-Asp-Asn-Val-Ala-Pro-Arg-Ser-Lys- Ile-Ser-Pro-Gln-Gly-Tyr-NH2

(52 aa; 1 disulphide bridge). Adrenomedullin Sequence, reference from Uniprot. (http://www.uniprot.org/uniprot/P35318)

1.5 Protein interactions with small molecules and macromolecules.

In this thesis, the interaction of Factor H with a number of other proteins, other macromolecules (eg heparin) and "small" molecules, typically, <1000 mol wt) is studied and reported.

Proteins interact with other molecules via several different types of non-covalent atomic or molecular interaction. These are summarised at https://en.wikipedia.org/wiki/Non-covalent_interactions, and include:

1: Ionic interactions: these may be between full negative and positive charges (1 full charge = 1 electron), or between partially charged features (polarised groups or dipoles, where charge is unevenly distributed across a covalently-bonded structure). These interactions are strongly influenced by pH (alteration of ionised groups) or by the ionic strength of the buffer/medium. They can be divided into several types, eg:

Electrostatic interactions: these typically involve fully charged amino acid side chains on the protein, interacting with charged (ionisable) groups on the ligand. Examples could include binding of negatively charged side chains of Asp, Glu, or positively charged side chains (His, Lys, Arg) with oppositely charged amino acid side chains on other proteins, or groups such as sulphate, phosphate, on ligands.

Polarised structures without a full net negative or positive charge can also be involved (eg aromatic rings with electron-donating or electron-withdrawing substituents, oxygen or nitrogen atoms with lone pairs of electrons

Hydrogen bonding: this occurs between hydrogen atoms of one molecule of the receptor-ligand pair, and atoms on the other of the pair with a lone pair of electrons, eg, O, N. Amino acid side chains with OH groups, eg Ser, Thr, Tyr may be involved and also NH groups of the peptide bond.

Van der Waals Forces are a subset of electrostatic interactions involving permanent or induced dipoles

Pi-pi interactions: these are formed between stacked aromatic rings, for example of Tyr, Trp, Phe side chains, and could involve aromatic rings of non-protein entities.

2: hydrophobic effect is the tendency of nonpolar substances to aggregate in aqueous solution and exclude water molecules. It describes the spontaneous segregation of water and nonpolar substances, which maximizes hydrogen bonding between water molecules and minimizes contact between water and nonpolar molecules.

In investigating protein-ligand interactions, it is common practice to use change of pH, change of ionic strength, chaotropes and denaturants to investigate features of the binding interactions involved. For example, increasing ionic strength weakens electrostatic interactions, and changing the pH over certain ranges may indicate the types of ionisable groups involved. Increasing salt strength however strengthens interactions which are mainly hydrophobic. Chaotropes interfere with intramolecular interactions mediated mainly by hydrogen bonds, but also van der Waals forces, and hydrophobic effects. Examples of chaotropes include KSCN, KBr, KCl (for detailed explanation see http://www1.lsbu.ac.uk/water/kosmotropes_chaotropes.html).

Protein denaturants can also be used to investigate interactions: these help to distinguish between covalent and non-covalent binding between proteins and potential ligands. An example of this is the use of urea to determine whether macromolecules binding to fibrin are bound non-covalently or have been covalently cross-linked by the action of the enzyme FXIII—see thesis section 2.2.1.3)

Denaturation is the “process of partial or total alteration of the native secondary, and/or tertiary, and/or quaternary structures of proteins or nucleic acids resulting in a loss of bioactivity” (IUPAC definition). Denaturants used include detergents such as SDS, high concentrations of guanidine salts or urea. Loss of the protein native structure should destroy any specific binding interaction.

Aims of thesis:

Factor H has been reported to bind to a wide range of ligands. Currently interest is mainly in bacterial (proteins) ligands and also in charge polymers such as sulphated glycosaminoglycan (GAGs) (Langford-Smith *et al.,* 2015). The aims of this thesis were to examine a range of compounds as potential ligands for factor H, and to select ligands with unusual binding mechanism (ie not mainly ionic) or very high affinity, for further analysis.

In initial work, TNP-BSA and DNP-HSA were selected as ligands in this category, and further experiments showed a potential similarity with Adrenomedullin factor H binding. Further work was therefore focused on characterising TNP, DNP and Adrenomedullin binding, and exploring the use of TNP as an affinity ligand for purifying FH.

A laboratory fire in April 2016 destroyed all reagents and equipment, and thus further refinements of the work, such as affinity measurement were ruled out.

Chapter Two:

Material and methods

2 Materials and methods

2.1 Materials

Chemical name	Supplier
Tris base	Fisher scientific UK limited
Sodium chloride (NaCl)	Fisher scientific
Phosphate buffered saline (Dulbecco's)	Sigma
Polyoxyethylenesorbitanmonolaurate (Tween20)	Fisher scientific
Bovine Serum Albumin (BSA)	Sigma
Ethylenediaminetetraacetic acid (EDTA)	Sigma
2-[4-(2-hydroxyethl-1-piperazine)] ethanesulfonic acid (HEPES)	Sigma
Diethanolamine	Sigma
Sodium phosphate dibasic anhydrous	Fisher scientific UK limited
Sodium hydroxide (NaOH)	Fisher scientific UK limited
N-tetramethylethylenediamine (TEMED)	Sigma
Acrylamide-bisacrylamide (29:1), 30% solution	Sigma
Coomassie Brilliant Blue	Thermo scientific
Urea	Sigma
Goat Anti rabbit IgG alkaline phosphatase conjugate	Sigma A3687
p-Nitrophenyl phosphate (buffered tablets)	Sigma N2770
Fibrinogen (human)	CALBIOCHEM
Thrombin (human)	Sigma

Human Factor XIII	Haematologic Technologies, Inc
Rabbit anti-human FH, purified IgG	MRC Immunochemistry Unit, University of Oxford
HiTrap Protein G Sepharose, 1ml columns	GE Healthcare
CAPS	Fisher scientific
MES	Sigma
Picrylsulphonic acid	Sigma
Dinitrophenyl-human serum albumin (DNP-BSA)	Sigma
N,N,N,N,-tetramethylethylenediamine (TEMED)	Sigma
Guanidine hydrochloride	Sigma
Factor I	Complement Tech
Sodium dodecyl sulfate (SDS)	Sigma
Cholesterol	Sigma
Poly-L-arginine (P4663, 5-15kDa)	Sigma
Cardiolipin	Sigma
Lipoteichoic acid (LTA) *Staphylococcus aureus*	Sigma
CNBr-Activated Sepharose	GE Healthcare
Gelatin	Serva
DNA salmon sperm	Life technologies
Heparin	Sigma
Trehalose	Sigma
L-α-Phosphotidylethanolamine	Sigma
Polyinosinic-polycytidylic acid (P0913)	Sigma
Histone from calf thymus (H9250)	Sigma
Tris-HCl	Sigma
C3b	Comp Tech USA
Adrenomedullin	GeneCust Luxembourg

Human plasma and serum

Human citrated plasma was from TCS Biosciences or from Seralab both in UK. Other samples of EDTA-plasma or serum were from laboratory volunteers.

Human factor H

FH was initially purified from human plasma by the method of Sim *et al.*, (1993) using the monoclonal anti-FH antibody MRCOX23 attached to Sepharose to purify FH. Subsequently an alternative affinity method was optimised, based on TNP-BSA – Sepharose, and this described with optimisation details in section 4.3. Purified FH was also obtained commercially from Comp Tech (Texas, USA).

Bacterial strains and bacterial recombinant proteins

The bacterial strains *Streptococcus pneumonia* (TIGR4 strain), *Streptococcus pneumonia* (D39 strain), recombinant Neuraminidase from *Streptococcus pneumonia* and recombinant Tributyrin esterase from *Streptococcus pneumonia* were kindly supplied by Dr Hasan Yesilkaya, University of Leicester

Human FH segments 1-4 and 19-20 (CCP1-4 and CCP19-20)

Recombinant human FH segments CCP1-4, and CCP19-20 were kindly provided by Dr Stacey Bell and Dr Janet Lovett, University of St Andrews, UK. They were provided as small quantities of purified proteins (<200ug each). Details of their preparation are described fully in Dr Stacey Bell's PhD thesis (Bell 2015). These were expressed in a yeast system, using Pichia pastoris. CCP1-4 was modified by a C-terminal hexahistidine tag, but CCP19-20 was not Histidine-tagged.

The preparation of another recombinant segment of FH (CCP6-8) is described in section 2.15.

2.2 Methods

2.2.1 Binding assays for factor H to different ligands

2.2.1.1 Preparation of fibrinogen/ fibrin coated wells

Human fibrinogen stock solution was made 1mg/ml in phosphate buffered saline (PBS), and to block FXIIIa, which is a contaminant of most fibrinogen preparations, 2mM of iodoacetamide was added to the solution. Fibrinogen was diluted in 0.1M sodium carbonate pH 9.6 (coating buffer) to 50ug/ml. Microtiter plates (Maxisorp™, Nunc, Kamstrup, Roskilde, Denmark) were coated with 100 ul/well of 50ug/ml fibrinogen. Plates were left for one hour at 4°C and after that plates were washed four times with PBS-0.5 EDTA, 0.1% tween 20. To avoid uncoated surfaces on the plates, plates were blocked with PBS-0.5 EDTA, 0.1% tween 20 for two hours at room temperature.

To make fibrin coated plates, fibrin clots were made in the wells of microtiter plates. Normal fibrinogen was prepared without Iodoacetamide and diluted to 100 ug/ml in 20mM HEPES, 120mM NaCl, 5mM CaCl, 0.05mM DTT pH 7.4. Thrombin was diluted to 0.5ug/ml in the same buffer. The reason for adding DTT is to activate FXIII. 50ul of 100ug/ml of fibrinogen was dispensed into wells and left at room temperature for 15 minute. After that 50ul of 0.5ug/ml thrombin was added to the wells and left for 40 minutes at 37°C. Plates then were transferred to 4°C and left for 20min. To remove unbound proteins, plates then were washed four times with PBS-0.5mM EDTA, 0.1% tween 20. Then plates were blocked with 250ul/ well of PBS-0.5 EDTA, 0.1% tween 20 for two hours at room temperature.

2.2.1.2 Binding of factor H to fibrinogen or fibrin-coated wells

Human factor H was serially two-fold diluted in 20mM HEPES, 120mM NaCl, 0.5mM EDTA pH 7.4, dilutions started from 20ug/ml to 0.313ug/ml. Separately, human serum was used in some experiments as the source of FH, using serum serially diluted from a maximum concentration of 20%. Then 100ul/well of each dilution of factor H was loaded into fibrinogen-or fibrin coated wells. Plates were incubated for 1 hour at room temperature, and then washed four times with PBS-0.5mM EDTA, 0.1% tween 20. After that anti FH (Rabbit anti FH polyclonal antibody) was prepared in washing buffer at 1/2500 dilution (corresponding to approx.10ug/ml IgG) and 100ul was added to the wells. Plates were incubated for one hour at room temperature. After washing four times with PBS-0.5mM EDTA, 0.1% tween 20, secondary antibody (Goat anti-Rabbit IgG- alkaline phosphatase conjugate from Sigma A3687) prepared in washing buffer at 1/5000 dilution (100ul per well) was added and the plates were incubated for one hour at room temperature. After the final washing (4x) with PBS-0.5mM EDTA, 0.1% tween 20, substrate p-Nitrophenylphosphate, buffered tablets from Sigma (N2770) was added to the wells (100ul per well), and the plates incubated at room temperature until colour development was judged to be sufficient. Finally, absorbance was read at 405 nm using a Biorad Multiscan Ascent Microtiter ELISA reader.

2.2.1.3 Does FH bind covalently or non-covalently to fibrin clots?

The fibrinogen used to form fibrin in the plates contains FXIII, plasma transglutaminase,

which can cross-link proteins by forming glutamine-lysine isopeptide bonds. This had previously been observed to occur when FH interacts with fibrin (Yuhoi Kang, Janez Ferluga, University of Oxford, personal cxommunication). To discover whether factor H binds covalently or not to fibrin clots, fibrin was formed in microtiter plate wells as described above. Then 100ul/well of 20ug/ml of factor H was added to the coated wells and incubated for 30 min. at 37° C. After washing with washing buffer (PBS-0.5 mM EDTA, 0.1% tween 20), wells were incubated with 200ul/well of denaturants, including 8M urea solution or 0.2M Tris, 8M urea, 2%SDS, 0,05mM EDTA pH 8.0 buffer or washing buffer separately. The denaturants should remove any FH which is not covalently bound. After treating with these solutions, wells were carefully washed 8 times with washing buffer to eliminate denaturant residues. Then primary anti-FH antibody (Rabbit anti FH polyclonal antibody) was added to the wells and later the secondary antibody was added, as described in section 2.2.1.2. After adding the substrate, plate was read at 405 nm.

2.3 Preparation of TNP-BSA

Trinitrophenyl-bovine serum albumin (TNP-BSA) was made as described by Arnold *et al.*, (2005). 10ml of 1% (w/v) BSA dissolved in PBS 0.5 mM EDTA (pH 7.2-7.4), was added to 2ml of 5% (w/v) picrylsulphonic acid solution. To prevent protein precipitation pH was monitored to keep it neutral, and the whole mixture was left for 4 hour at room temperature. TNP-BSA was dialysed extensively against PBS 0.5mM EDTA overnight at 4hC.

2.4 Binding of factor H to DNP-HSA or TNP-BSA coated wells

Microtiter plates were coated with 100ul/well of 100ug/ml of DNP-HSA (Sigma) or TNP-BSA (made as described above) in 0.1M sodium carbonate pH 9.6 and left for one hour at room temperature. Then plates were washed four times with 0.1 M sodium carbonate. After that plates were blocked with 250ul/well PBS-0.5mM EDTA, 0.1% tween 20 for 1 hour at room temperature. After washing three times with the same buffer, purified factor H serially diluted in 20mM HEPES, 120mM NaCl 0.5mM EDTA pH 7.2 (dilution starting from 10ug/ml to 0.313ug/ml) was added to the wells (100ul/well). Human serum as a source of FH was also serially diluted in the same buffer from 20% and the serial dilutions (100ul) added to wells. Wells were incubated with FH or serum dilutions for 1h at room temperature. After that anti –FH (Rabbit anti FH polyclonal antibody) prepared in washing buffer was added to the wells and plates were incubated for one hour at room temperature. After washing four times with PBS-0.5mM EDTA, 0.1% tween 20, secondary antibody Goat anti-Rabbit IgG-alkaline phosphatase conjugate prepared in washing buffer was added and plates incubated for one hour at room temperature. After the final washing with PBS-0.5 EDTA, 0.1% tween 20, substrate p-Nitrophenylphosphate, was added to the wells. Finally, absorbance was read by Microtiter ELISA reader at 405 nm. Antibody dilutions were as in section 2.2.1.2.

2.5 Interactions with other ligands

ELISA microtiter plates were coated with 3ug/well of different ligands: including cardiolipin (CL), cholesterol, Lipoteichoic acid, poly-L-arginine. CL and cholesterol were dissolved in 1:3 v\v chloroform: methanol at 5mg/ml, then diluted in methanol to 30ug/ml for coating the wells. These samples were air-dried in the wells. Poly-L-arginine was dissolved in water, then diluted in the coating buffer to apply 3ug /well. Other ligands were dissolved directly in coating buffer and applied at 3ug/well. Ligands were incubated overnight at 4°C. After that plates were blocked with 300 ul/well PBS with 0.1% porcine gelatin, 0.1% tween 20 and 0.5mM EDTA) to avoid high background. Serial twofold dilutions (100/well) of purified human FH were tested starting from 10ug/ ml. Dilution was done in washing buffer (20mM Tris-HCl, 130 mM NaCl, 0.5mM EDTA, 0,05 % v/v tween 20; pH 7.4) and plates were incubated for 1 hour. Plates were then washed 3 times with washing buffer. After that anti –FH (Rabbit anti FH polyclonal antibody) prepared in washing buffer was added to the wells and plates were incubated for one hour at room temperature. Secondary antibody anti-Rabbit IgG-alkaline phosphatase conjugate was added to the wells after washing, incubated for 1 hr at room temperature, the wells washed (x4) then substrate was added, and plates were read at OD 405 nm using ELISA reader. Antibody dilutions were as in section 2.2.1.2.

2.6 FH binding to bacteria

2.6.1 Preparation of bacteria

Streptococcus pneumoniae strains D39 and TIGR4 (kindly supplied by Dr Hasan Yesilkaya, University of Leicester) were cultured on blood agar plates at 37°C under anaerobic conditions overnight. The bacteria were identified as pneumococci by Gram staining. The colonies were then transferred to brain–heart infusion (BHI) liquid media (Restrepo *et al.*, 2005) for 9–12 hours at 37°C. After centrifugation, the pellet was washed 3 times using PBS–0.1% tween20, and then the bacteria were fixed by 0.5% formalin (formaldehyde) for 1.5–3 hours. The cells were spun down at 4200 rpm for 10 min and the pellet washed 3 times with PBS–0.1% tween20 before it was stored at -80°C until used.

2.6.2 Factor H binding to bacteria

ELISA microtiter plates (MAXISORP) were coated with 100ul of the bacterial suspension (OD550=0.6) in coating buffer and left for overnight at 4°C.To avoid nonspecific binding and to reduce high background, the plates were blocked by 300ul/well 0.1% gelatin, PBS and 0.1% tween 20 for 1 hour at room temperature. Then plates were washed 3 times with washing buffer (20mM tris –HCl, 130mM NaCl, 0.5mM EDTA and 0.05 % v/v tween 20; pH 7.4). The plate was washed 3 times using the washing buffer. Serial two-fold dilutions of purified human Factor H protein or serum as another source for the complement protein were added to the coated wells for 1 hour at room temperature, starting with 15ug/ml of the purified protein or 15% serum. The plate was washed 3 times using the washing buffer. For FH detection, polyclonal rabbit anti-human Factor H, a secondary antibody conjugate and the substrate were used, as in section 2.2.1.2.

2.7 Binding characteristics of FH to TNP-BSA AND DNP-HSA

2.7.1 The effect of salt strength (NaCl concentration) on the interaction between factor H and DNP-HSA or TNP-BSA

To explore the interaction between FH and DNP-HSA or TNP-BSA at different salt concentrations, buffers were made up in 10mM HEPES pH 7.4 with a range of NaCl concentrations [0, 20, 40, 60, 80, 120, 160, 320, 640, 1000mM]. 100ul from each buffer was dispensed into TNP-BSA or DNP-HSA-coated wells (prepared as above) and 100ul (5ug) / well of FH in 10mM HEPES, 120mM NaCl, 0.5mM EDTA pH 7.4 or 100 ul of 20% human serum in the same buffer was added. Plates were left for one hour at room temperature. Plates were washed four times with buffer at same salt strength as the incubation. Subsequently, primary anti-FH antibody (Rabbit anti FH polyclonal antibody) was prepared in washing buffer PBS- 0.5mM EDTA, 0.1%Tween20 at 1/2500 dilution and 100ul added to the wells and incubated for 1 hour at room temperature. Plates were washed three times with washing buffer, then secondary Goat antibody anti-Rabbit IgG- alkaline phosphatase conjugate prepared in washing buffer at 1/5000 dilution was added (100ul per well) and left for one hour at room temperature. Plates were washed three times with washing buffer. After that p-Nitrophenylphosphate, buffered solution was added to the wells. Finally, absorbance was read by Microtiter ELISA reader at 405 nm.

2.7.2 The effect of pH on the interaction between FH and DNP-HSA or TNP-BSA

Plates were coated with DNP-HSA or TNP-BSA and blocked as above. To explore binding between FH and DNP-BSA or TNP-BSA in different pH values, a composite buffer containing 20 mM Na phosphate, 20 mM MES (2-(N-morpholino)ethanesulfonic acid), 20

mM HEPES, 20 mM CAPS (N-cyclohexyl-3-aminopropanesulfonic acid), 120mM NaCl was made up, and the pH values were adjusted in a range (3.5, 4.5, 5.5, 6.5, 6.8, 7.5, 7.8, 8.5, 9.5, 10.5) by using NaOH and HCl. 100ul of each buffer was added to coated wells then 100ul 5ug /well of FH in 10mM HEPES, 130mM NaCl, 0.5mM EDTA pH 7.4 or 100ul of 20% human serum in the same buffer was added separately. Plates were incubated for one hour at room temperature. Plates were washed four times with same pH buffer as first incubation Primary antibody (Rabbit anti FH polyclonal antibody) was added then the secondary anybody, as above. Later after washing and adding the substrate plates were read at 405 nm. To assess final pH during incubation of FH with DNP-BSA or TNP-BSA, 2ml of each of the pH range buffer was mixed together with 2ml of 10mM HEPES, 130mM NaCl, 0.5mM EDTA pH 7.4 and the final pH was measured with a pH meter.

2.8 Dissociation of the binding between FH and DNP-HSA or TNP-BSA using different buffers

This experiment is designed to show whether extremes of pH or denaturants will dissociate bound FH from these ligands. Microtiter plates were coated with 100ul of 100ug/ml DNP-BSA or TNP-BSA in 0.1M sodium carbonate pH 9.6 as coating buffer. Plates were incubated for one hour at room temperature. After washing three times with the coating buffer plates were blocked with PBS-0.5mM EDTA, 0.1% Tween 20 for two hours at room temperature. Then 100ul of FH 5ug/well were added to the wells then plates were left for 1 hour at room temperature. Then wells were washed 3 times in washing buffer. Then 100 ul of different solvents were added to the coated wells (buffers at pH 3, 4, 9, 10, (prepared as in section 2.7.2) 8M Guanidine, 3M urea, 6M urea, 0.5% SDS, 1% SDS) and left for 1

hour. After washing (4 x) with PBS-0.5 mM EDTA 0.1% Tween20, the primary antibody (Rabbit anti FH polyclonal antibody) was added, and bound FH was detected as in section 2.2.1.2.

2.9 Inhibition of the binding between FH and DNP-HSA or TNP-BSA using low molecular weight compounds

ELISA plates were coated with 100ul of 100ug/ml DNP-HSA or TNP-BSA and blocked as described above. Compounds (see table 2.1 and further description below) were used at 10mM to check their effect on the binding between FH and DNP-HSA or TNP-BSA (O-phospho-L-tyrosine, N-acetyl-3,5- Dinitro-tyrosine-ethyl ester, ATP, N-formyl-met-leu-phe, bilirubin, *myo*-inositol hexakisphosphate, spermine, para--Nitrophenylacetate, BOC-L-tyrosine, quinidine sulfate, ellagic acid, 3-nitro-L-tyrosine, 5,5-dithio-bis(2-nitrobenzoic acid). Heparin and tRNA and poly Glu-Ala-Tyr were used at 10ug/ml. To test each compound, 100ul of 10mM (or 10ug/ml: see above) of each was dissolved in 20mM HEPES, 120mM NaCl, 0.5mM EDTA pH 7.4 and added to the coated wells. Then human factor H 5ug/well (100ul in 20mM HEPES, 120mM NaCl, 0.5mM EDTA pH 7.4) was added per well; therefore the final concentration of compounds is actually 5mM (or 5ug/ml). Control was included (no inhibitor) but instead contained 100ul of 10mM HEPES, 130mM NaCl, 0.5mM EDTA. Plates were incubated for one hour at room temperature. Plates were washed four times with washing buffer PBS-0.5mM EDTA 0.1% tween20 and

binding was detected with primary and secondary antibodies, as above. After that plates were read at 405nm.

For further information, some properties of the potential inhibitors used are listed below and their structures are shown in table 2.1. : -

The chemical structure and details listed below are from Pubchem and Wikipedia.

Ellagic acid

Ellagic acid is a fused four-ring phenolic compound occurring in many plants. It has OH groups suitable for H-bonding and aromatic rings which can participate in pi-pi interaction. It binds several proteins, including human serum albumin and Factor XII of the blood clotting system.

O- Phospho-L-tyrosine

O-Phospho-L-tyrosine is an amino acid that occurs in endogenous proteins. Tyrosine phosphorylation and dephosphorylation plays a role in cellular signal transduction and possibly in cell growth control and carcinogenesis.

N-formyl-met-leu-phe

This chemotactic peptide is a formylated tripeptide originally isolated from bacterial filtrates that is positively chemotactic to polymorphonuclear leucocytes, and causes them to release lysosomal enzymes and become metabolically activated.

Poly Glu Ala Tyr

Poly(glu(60)ala(30)tyr(10)); this is a synthetic polymer made up of Glutamic acid-alanine-tyrosine, randomly connected in the molar ratio 6:3:1. It would be expected to form ionic

bonds (via Glutamic acid carboxyl group) and pi-pi interactions (via Tyrosine) with proteins.

BOC-L-tyrosine

Boc-Tyr-OH; this is a synthetic derivative of tyrosine, substituted with a tertiary butoxy-carbonyl group attached via the amino group of -L-tyrosine

Quinidine sulfate

Is an optical isomer of quinine, extracted from the bark of the Chinchona tree and similar plant species. This alkaloid has a partly aromatic structure. It dampens the excitability of cardiac and skeletal muscles by blocking sodium and potassium currents across cellular membranes. Quinidine also blocks muscarinic and alpha-adrenergic neurotransmission.

3-nitro-L-tyrosine

3-Nitrotyrosine (NTyr) is formed in vivo in tissue or blood proteins after exposure to nitrosating and/or nitrating agents such as tetranitromethane. Reactive nitrogen species such as peroxynitrite can nitrate specific amino acids, whether free or protein bound, and 3-nitrotyrosine is believed to be one marker of this reaction.

5,5-dithio-bis(2-nitrobenzoic acid)

This compound resembles two nitrophenol molecules held together by a disulphide bridge. It is a standard reagent for the colorimetric determination of reactive sulfhydryl groups and disulfide groups in proteins. The color produced is due to the formation of a thio anion, 3-carboxyl-4-nitrothiophenolate.

ATP

Adenosine Triphosphate is an adenine nucleotide composed of three phosphate groups

esterified to the ribose sugar moiety, found in all living cells. It can interact with macromolecules via charge and aromatic/hydrophobic interactions. Adenosine triphosphate is involved in energy production for metabolic processes and RNA synthesis. In addition, this substance acts as a neurotransmitter.

tRNA

Transfer ribonucleic acid (**tRNA**) is a type of RNA molecule that helps decode a messenger RNA (mRNA) sequence into a protein. **tRNAs** function at specific sites in the ribosome during translation, which is a process that synthesizes a protein from an mRNA molecule. tRNA consists of 76-90 nucleotides (A,G, C, U). The structure has numerous charged (phosphate), hydroxyl and aromatic groups.

Bilirubin

Is a bile pigment that is a degradation product of heme. Bilirubin consists of an open chain of four (aromatic) pyrroles (tetrapyrrole); by contrast, the heme molecule is a closed ring of four pyrroles, called porphyrin. It has OH group substituents. Bilirubin is a yellow breakdown product of normal heme catabolism. Its levels are elevated in certain diseases and it is responsible for the yellow color of bruises

Heparin

Heparin is a heterogeneous glycosaminoglycan. Low molecular weight heparin (LMWH) was used in these studies. It is prepared by nitrous acid degradation of unfractionated heparin of porcine intestinal mucosa origin. It is is an anticoagulant, which binds strongly to many proteins, including the coagulation inhibitor antithrombin III. It increases the rate of inhibition of thrombin by antithrombin III. It is composed of repeating copies of a variably-sulphated disaccharide unit. The most common disaccharide unit is composed of a 2-O-sulfated iduronic acid and 6-O-sulfated, N-sulfated glucosamine, IdoA(2S)-GlcNS(6S). The material used had an average molecular weight of 5000 and about 90% of the material was within the molecular weight range of 2000-9000.

***myo*-inositol hexakisphosphate (Phytic acid)**

Is an intermediate in inositol phosphate metabolism It can be generated from D-myo-Inositol 1, 3, 4, 5, 6-pentakisphosphate via the enzyme inositol-pentakisphosphate 2-kinase (EC:2. 7. 1. 158). Myo-Inositol hexakisphosphate is also known as phytic acid which is abundant in plants. It is highly charged, with 6 phosphate groups. It can be used clinically as a complexing agent for removal of traces of heavy metal ions. It acts also as a hypocalcemic agent. Phytic acid is a strong chelator of important minerals such as calcium, magnesium, iron and zinc, and can therefore contribute to mineral deficiencies in developing countries

Spermine

Is a polyamine (aliphatic chain containing 4 amino groups) formed from spermidine. It is found in a wide variety of organisms and tissues and is an essential growth factor in some bacteria. It is found as a polycation at all pH values. Spermine is associated with nucleic acids, particularly in viruses, and is thought to stabilize the helical structure.

Para—Nitrophenylacetate

4-nitrophenylacetate is the acetic acid ester of p-nitrophenol, commonly used as a substrate for esterase enzymes.

N-acetyl-3,5-Dinitro-tyrosine-ethyl ester

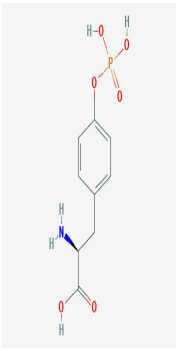
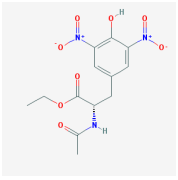
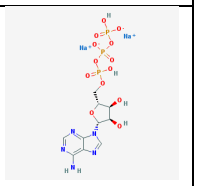
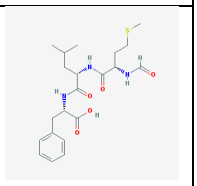
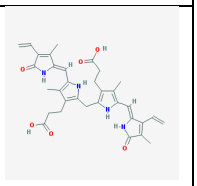
This is a synthetic derivative of tyrosine, and is an analog of 2, 6 dinitrophenol.

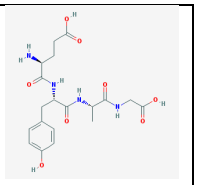
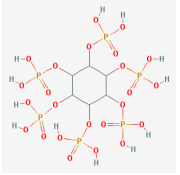
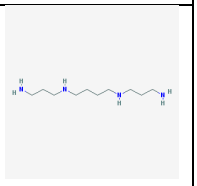
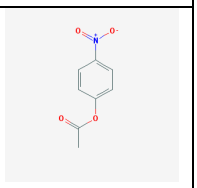
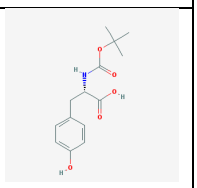
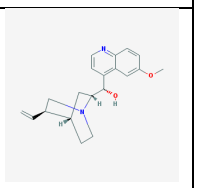
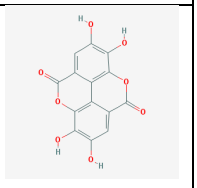
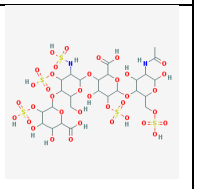
2.9.1 Using the inhibitors at lower concentrations.

Compounds that inhibited the binding successfully were diluted in subsequent experiments to find out whether there was a dose-dependence of inhibition. So they were used in 5

molarities (5, 2.5, 1.25, 0.625, 0.313mM). The rest of the experiment was done as shown above.

Table 2.1

Name of the compound	Molecular formula	Supplier	Solvent	Structure
O-Phospho-L-tyrosine	$C_9H_{12}NO_6P$	Sigma	Water	
N-acetyl-3,5-Dinitro-tyrosine-ethyl ester	$C_{13}H_{15}N_3O_8$	Sigma	Ethanol	
ATP	$C_{10}H_{16}N_5O_{13}P_3$	Boehringer	Water	
N-Formyl-Met-Leu-Phe,	$C_{21}H_{31}N_3$	Sigma	Water	
Bilirubin	$C_{33}H_{36}N_4O_6$	Sigma	Water	

Poly(glu(60)ala(30)tyr(10) (one tripeptide showing 1glu, 1 ala, 1 tyr is shown)		Sigma	25mM Tris pH 7.5	
Myo-inositol hexakisphosphate	$C_6H_{18}O_{24}P_6$	Sigma	Water + acetic acid	
Spermine	$C_{10}H_{26}N_4$	Sigma	Water	
tRNA	------	Fluka	Water	AGCU polymer
para-Nitrophenyl acetate		Aldrich	Methanol	
Boc-L-tyrosine	$C_{14}H_{19}NO_5$	Fluka	Ethanol	
Quinidine	$C_{20}H_{24}N_2O_2$	Sigma	Ethanol	
Ellagic acid	$C_{14}H_6O_8$	Sigma	1M NaOH	
polyglycine				Polymer of glycine
Heparin (2 disaccharide units are shown: heparin is a polymer of the disaccharide structure)	$C_{26}H_{42}N_2O_{37}S_5$	Sigma	Water	

Stock solutions of the compounds were made in the solvents shown in the table, then they were diluted for use in 20 mM HEPES, 120mM NaCl, 0.5mM EDTA pH 7.4.

2.10 Factor H binding to other TNP-derivatised proteins and TNP-amine

TNP-labeled proteins, including TNP-chicken ovalbumin, TNP-Fibrinogen, TNP-gelatin and TNP-bovine gamma globulin (TNP-BGG) were made as described in section 2.2. For TNP incorporation, measurement of the extent of incorporation was done but the results are lost due to the laboratory fire in April 2016. TNP-amine, the reaction product of ammonia with picrylsulphonic acid, was made by preparing 40mM NH_4Cl in 10mM HEPES, 140mM NaCl pH 7.3, adding to it an equimolar quantity of picrylsulphonic acid diluted in the same buffer. The product was assumed to be 20mM (5.86ug/ml) TNP-amine. Microtiter plates were coated for 1 hour at room temperature with 100ul of 100ug/ml TNP-chicken ovalbumin, TNP-fibrinogen, TNP-gelatin, TNP-bovine gamma globulin (TNP-BBG) or 10ug/ml TNP-amine in 0.1M sodium carbonate pH 9.6. Plates were blocked with 250ul/ well of PBS-0.5mM EDTA 0.1% tween 20 for one hour at room temperature. After washing with PBS-0.5mM EDTA, 0.1% tween 20, 100ul/well of 5ug/ml of FH in 10mM HEPES, 120mM NaCl, 0.5mM EDTA pH 7.4 was added to the wells and incubated for 1 hour at room temperature. Then primary and secondary antibodies were added to detect bound FH as described in section 2.2.1.2. After that substrate was added. Finally plates were read with an ELISA plate reader at 405 nm.

2.11 Dose-dependence of binding between factor H and TNP-proteins or TNP-Amine

Microtiter plates were coated with serial dilutions of TNP-proteins or TNP-amine (dilution from 100ug/ml to 0.078ug/ml) in 0.1M sodium carbonate pH 9.4 for one hour. After that plates were washed three times with of PBS-0.5mM EDTA 0.1% tween 20. Then plates were blocked with 250ul/well of PBS-0.5mM EDTA 0.1% tween 20 for one hour, and then washed with the same buffer. Next 100ul/well of 5ug/ml FH in 10mM HEPES, 120mM NaCl, 0.5mM EDTA pH 7.4 was added to the wells and incubated for 1 hour at room temperature. After washing, primary and secondary antibodies were added. Then substrate was added to detect bound FH, as in section 2.2.1.2. Finally plates were read with ELISA plate reader at 405 nm.

2.12 Factor H purification

2.12.1 TNP-BSA affinity chromatography

To isolate factor H from human plasma the procedure of Moreno-Indias *et al.,* (2012) was followed. Human plasma was diluted 1:1 with cold water (4°C) to decrease ionic strength. For the isolation of factor H, 200 ml of diluted plasma was mixed with 20 ml of TNP-BSA-Sepharose, prepared as indicated by Arnold *et al* (2005). The mixture was left at 4 deg C on a slow rotary stirrer for 1 hour. The resin was thoroughly washed with HEPES buffer (10mM HEPES, 60mM NaCl, 0.5mM EDTA pH 7.4). This affinity column was thought to bind mainly factor H, IgG, and IgM (but as shown later, it binds C4bp, which had been mistaken for IgM, due to similar SDS-PAGE mobilities). The resin was placed in a column

and the bound proteins were eluted with high salt buffer (10mM HEPES, 2MNaCl, 0.5mM EDTA pH 7.4) and fractions of 2ml were collected. These fractions were monitored by reading the OD 280 and analyzed by SDS-PAGE.

2.12.2 HiTrap Protein G column

In order to remove IgG from the purified FH, a Hi-Trap Protein G column (1 ml from GE Healthcare) was used. The column was first washed with 5ml of glycine-HCl pH 2.2 then with 5ml of physiological buffer pH 7.4. The factor H factions from TNP-BSA- Sepharose were mixed together and passed into the washed column. The OD of eluted fractions was measured at 280nm. The bound IgG was removed from the column by passing 10 ml of the pH 2.2 glycine-HCl buffer. The column was re-equilibrated in physiological buffer, and the eluted IgG adjusted to ~pH 7 and kept at -20°C. Note that IgG binds very well to the protein G, even although the protein sample is run onto the column in high salt: that is, there is no need to dialyse the sample to reduce salt concentration.

2.12.3 Anti-IgM column

A `rabbit anti-human IgM-Sepharose (4mg IgG / ml of Sepharose) column (10ml) (MRC Immunochemistry Unit, Oxford University) was washed and equilibrated with water, then cleaned with 2 volumes of 3M MgCl then with 2 volumes of water then 2 volumes of HEPES buffer (10mM HEPES, 140mM NaCl, 0.5mM EDTA pH 7.4). Then the factor H sample from HiTrap protein G was passed through the column and fractions of 5ml were collected. OD 280 was measured. This process was done at 4°C.

2.12.4 Concentrating the sample

To concentrate factor H, the sample was centrifuged in a centrifugal filter concentrator (Millipore, 10.000 MWCO).

2.13 Separation of FH from C4bp

The TNP–BSA column binds to FH, IgG IgM, and we found (see results section) that TNP-BSA binds also to C4bp; to attempt to separate FH from C4bp several procedures were followed, including Zinc precipitation and the use of DyeMatrex resins. It would have been logical to separate FH (MW 155kDa) from C4bp (MW >490kDa) by gel filtration on Superose 6 (GE Healthcare) but suitable equipment was not routinely available in the lab.

2.13.1 Separation of FH from C4bp using Zinc Sulphate

Factor H is known to precipitate in the presence of low concentrations of Zn++ (Day and Sim 1986). However higher (several mM) Zn++ precipitates many proteins. It was hoped that differential precipitation of FH and C4bp would be observed.

A TNP-BSA column was loaded with diluted fresh human plasma and washed overnight with 10mM HEPES, 60mM NaCl, 0.5mM EDTA pH 7.4. The column was eluted by using high salt buffer 10mM HEPES, 2M NaCl 0.5mM EDTA pH 7.4. The eluted protein was dialysed against 10mM HEPES, 60mM NaCl, pH 7.4. three times at 4°C. After the dialyses aliquots of the protein were treated with different molarities of final concentration Zinc Sulphate 0.5mM, 1mM, 2mM, 4mM, 5mM, 10mM, 20mM 40mM, 80mM, 160mM. Samples were left at 4°C for three days and checked every few hours for precipitation.

Later they were spun down to separate the precipitate from the supernatant. Then samples (resuspended precipitates and supernatants) were analysed by 10% SDS-PAGE (see section 2.13).

2.13.2 Using Dye Matrix Gels for Factor H purification

DyeMatrex resins (Amicon) contain aromatic dyes attached to agarose. They separate proteins by a mixture of hydrophobic binding and ion-exchange effects. The protein mixture after elution from a TNP-BSA column with 20mM HEPES, 2M NaCl, 0.5mM EDTA pH 7.4 was dialysed against 10mM HEPES, 130 mM NaCl, 0.5 mM EDTA pH 7.4 three times. Protein samples were passed through four different dyematrex gels (blue B, red, orange, green) (Amicon). Columns (each 1 ml) were tested first whether they bind FH. 1 mL protein mixture was incubated in each column for 1 hour at room temperature and after that the column was washed with 1mL of 10mM HEPES, 140mM NaCl, 0.5mM EDTA pH 7.4. Then 1 mL of increasing molarities of KCl in the same buffer, starting from 20mM, then 40mM, 80mM, 120mM 160mM, 300mM, 500mM, 1M, 2M) were passed through the column and fractions were collected. After that, samples were analysed on SDS-PAGE. To remove KCl, fractions were dialysed against PBS three times overnight. Later a larger Dyematrex blue B column of 10mL was made to obtain a reasonable quantity of FH. This has the dye Cibacron Blue F3GA bound to agarose.

2.14 SDS-PAGE

The method of Laemmli (1970) was used for SDS-PAGE analysis. The separating gel was made with 6% or 10 % w/v acrylamide and the stacking gel was 3.0 % w/v acrylamide.

Samples were prepared by diluting them with one volume of loading buffer/dye (1M Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 0.01% bromophenol blue). For reducing the samples 5 % v/v mercaptoethanol was added freshly to the to the loading buffer/dye before using it, and samples were incubated for 5 minutes at 95°C. Non-reduced samples were treated in the same way, but using 20mg/ml iodoacetamide in the loading buffer instead of mercaptoethanol. Molecular weight markers were run under the same conditions. Protein electrophoresis apparatus was set on 150 volts; protein samples were run in Tris-Glycine SDS buffer (Severn Biotech Ltd) until the Bromophenol blue front reached the bottom of the gels. To visualise the protein bands Coomassie Blue R-250 was used as described by (Fairbanks, Steck and Wallach, 1971) (40 % methanol, 10 % acetic acid 50% water, 0.4g/L Coomassie brilliant blue) for 20 minute at room temperature. After that, gels were transferred to de-staining buffer (30% methanol, 10% acetic acid 60% water) three times for 20 minute each. Finally, gels were washed and kept in distilled water.

2.15 Western blotting

An unstained SDS-PAGE gel was soaked in transfer buffer (25mM Tris, 232mM glycine, 20% methanol pH 7.4) for 10 min. A nitrocellulose membrane was soaked in transfer buffer; the gel was placed on the nitrocellulose and fitted in to an electroblot cassette of a Trans-Blot Turbo (BIO-RAD) apparatus. The apparatus was run for 10 min. then the nitrocellulose membrane was blocked with 5 % skim milk in PBS for 1 hour. Later primary anti-FH antibody (Rabbit anti FH polyclonal antibody, 1/2500) (or other primary antibodies) was prepared in skim milk/PBS and incubated with the membrane for 1 hour. After that the membrane was washed with PBS with 0.05 % tween 20 three times for 5 min

each. Then secondary antibody anti rabbit IgG HRP conjugate (Sigma) diluted in blocking buffer was added and incubated for 1 hour with shaking. Then, Luminata Crescendo Western HRP substrate (Merck-Millipore) was added on the nitrocellulose membrane after washing three times. The intensity of the signal was detected after exposing to Fuji X-ray film.

2.16 Preparation of the human recombinant FH segment CCP6-8, with either Histidine or Tyrosine at amino acid position 402.

Small quantities of these materials (about 200ug each) were kindly provided, as purified proteins, by Dr. Simon J. Clark, University of Manchester, and were prepared as described by Clark *et al.*, (2006). Note the position of the amino acid interchange, 402, is in some papers listed as position 384. 384 refers to the position number with signal sequence (18 residues) subtracted.

The expression systems for CCP6-8 were also provided by Dr. Clark, and additional quantities of the recombinant proteins were expressed and purified as described briefly below: [adapted from Clark et al (2006)].

The sequence-verified constructs were supplied in BL21 (DE3) pLysS Escherichia *coli* cells and expressed in *Escherichia coli* and refolded using a method described previously for CCP modules (White *et al.*, 2004). The protein was purified to homogeneity using anion exchange on a 1-ml Mono Q column (Amersham Biosciences, Buckinghamshire, UK) equilibrated in 20 mM CAPS, 130 mM NaCl, 1 mM EDTA, pH 10.0, and eluted with a gradient of 130 mM to 1 M NaCl over 20 min. The collected fractions were analyzed by

SDS-PAGE, and the protein was found to be >98% pure. Protein concentrations were determined by absorption at 280nm.

The record of the SDS-PAGE analysis for the protein purification done in Leicester was destroyed by the lab fire in April 2016. Dr Simon Clark kindly provided the illustrations below to show the stages of purification of the protein.

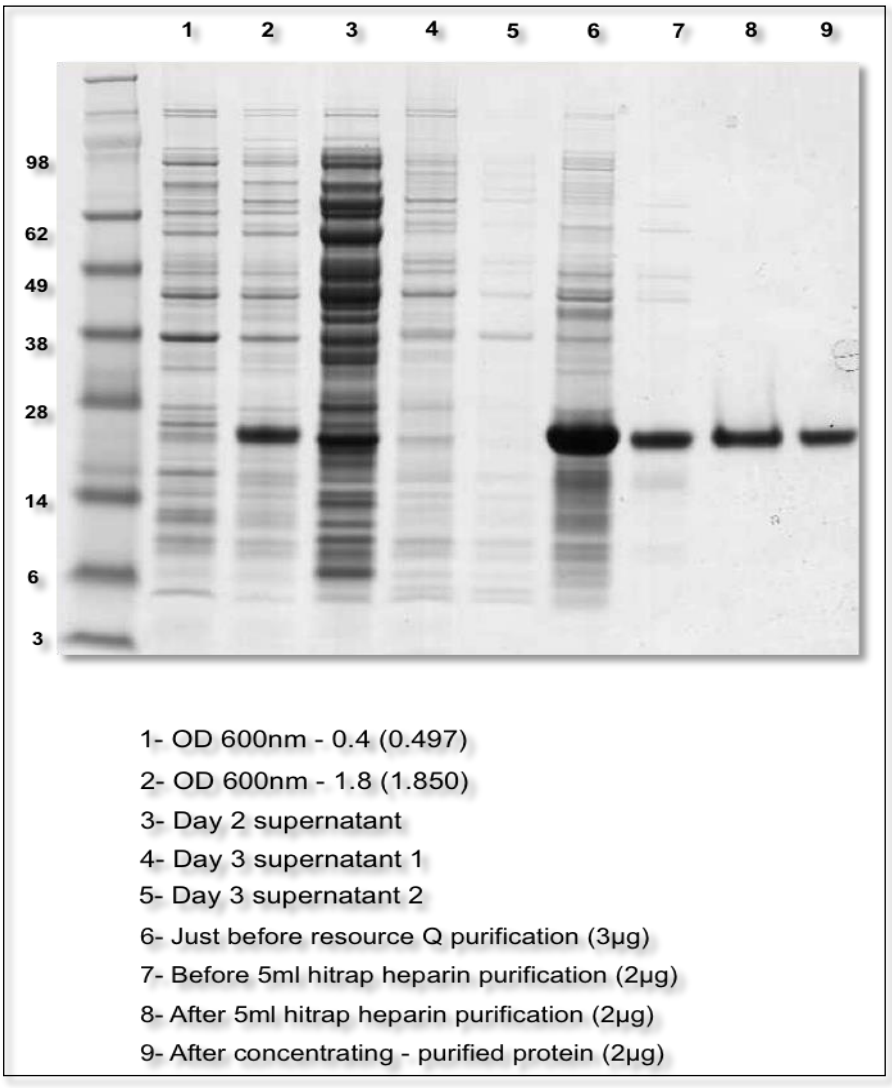


Figure 2-1 Purification of recombinant FH CCP 6-8 Y.
Illustration provided by Dr Simon Clark, University of Manchester. This illustration includes an extra step, heparin chromatography, which was not used by us**.**

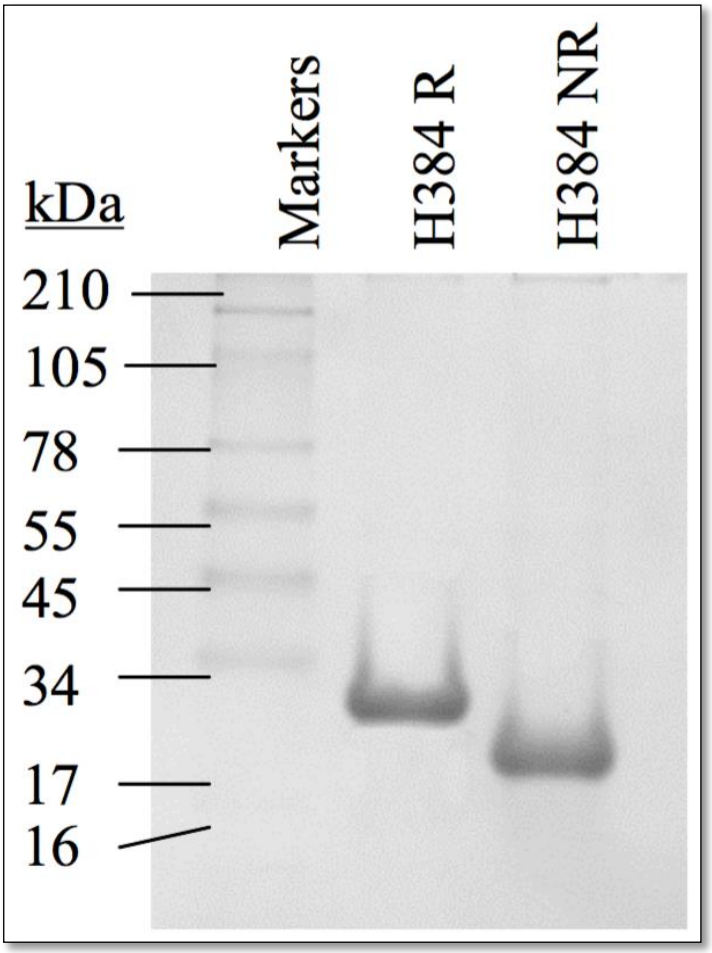


Figure 2-2 Pure CCP 6-8, Histidine at the position 384 (402).
Illustration provided by Dr Simon Clark, University of Manchester.

2.17 Description of Antisera against different CCPs of FH and against C4bp

Antisera were used as whole rabbit serum or in the form of purified (total) IgG. IgG was purified from antisera by triple sodium sulphate precipitation as described below:

1 ml of rabbit antiserum was put in a 1.5 ml Eppendorf tube. Then it was centrifuged for 5 min at full speed (9000g) and at room temperature to remove insoluble debris. The supernatant was collected and then 150 mg of anhydrous sodium sulphate was added to the serum and mixed till the salt dissolved; the serum turned cloudy white, which shows the IgG precipitated. After that the mixture was incubated at 37°C for 40 min, then was centrifuged for 5 min as above. The supernatant was removed and the pellet was dissolved in water to final volume of 2ml. Then 280mg of anhydrous sodium sulphate was added and mixed till it dissolved. After that the mixture was incubated again as above and centrifuged as before. The supernatant was removed and again the pellet was dissolved in 2 ml water, and the whole last step repeated (280 mg of anhydrous sodium sulphate added, mixed till it dissolved, incubated 40 min at 37°C, then centrifuged for 5 min). The pellet was finally re-dissolved in 2 ml PBS with 0.5 mM EDTA, and the OD280 was measured to determine how much protein was present. Normally this procedure yields about 3-6mg 95% pure IgG per 1 ml of serum.

Rabbit polyclonal anti human FH was made by immunising rabbits with FH isolated from human plasma by the method described by Sim et al., (1993) and the purified IgG was provided by the MRC Immunochemistry Unit, Oxford.

Rabbit polyclonal anti human FH CCP6-8, was raised by immunising rabbits with purified recombinant FH6-8 (as described above), and was kindly provided by Dr SJ Clark, University of Manchester.

Rabbit polyclonal anti human FH 3-4 was raised by immunising rabbits with purified recombinant FH3-4. Recombinant FH3-4 was made in a yeast system (*Saccharomyces cerevisiae*), by methods described by Barlow *et al.*, (1991). The protein was made by Dr. Adam Benham MRC Immunochemistry Unit Oxford, and the antiserum provided by the MRC Immunochemistry Unit.

Rabbit polyclonal anti human C4bp was made by immunising rabbits with C4bp isolated from human plasma by a method described by Sim and Sim (1983) and the antiserum was provided by the MRC Immunochemistry Unit, Oxford.

Rabbit polyclonal anti human FHR1 was made by immunising rabbits with FHR1 purified from human plasma by Dr. Marc Fontaine, INSERM U-78, Rouen, France (Fontaine *et al.*, 1989). Since the two C-terminal CCPs of FHR1 are highly homologous to FH CCPs19-20, this antiserum can be used to detect FH CCPs19-20.

2.18 Binding of FHCCP6-8 to TNP- BSA and DNP-HSA coated wells

Microtiter plates were coated with 100ul/well of 100ug/ml DNP-HSA or TNP- BSA in 0.1 M sodium carbonate pH 9.6 for one hour. Then plates were washed three times with PBS-0.5mM EDTA 0.1% tween 20, and then blocked with PBS-0.5mM EDTA 0.1% tween 20 for one hour. Then FHCCP6-8 was serially two-fold diluted in 10mM HEPES, 120mM NaCl, 0.5mM EDTA pH 7.4 and 100ul added to the wells: dilution starting from 5ug/ ml –

0.079ug/ ml. Plates were left at room temperature for 1 hour. After washing, rabbit anti CCP6-8 antibodies in washing buffer were added (1/10000) and incubated for 1 hour. After washing, secondary antibody anti-Rabbit IgG- alkaline phosphatase conjugate in washing buffer was added (1/5000) and incubated for 1 hour. After washing, substrate p-Nitrophenylphosphate, from buffered tablets was added to the wells and OD was read at 405 nm.

2.19 Competition between FH and FHCCP6-8 for binding to TNP-BSA and DNP-HSA

Microtiter plates were coated with 100ul/well of 100ug/ml DNP-HSA or TNP- BSA in 0.1M sodium carbonate pH 9.6 for one hour. Then plates were washed three times with PBS-0.5mM EDTA 0.1% tween20, and after that plates were blocked with PBS-0.5 mM EDTA 0.1% tween20 for one hour. Next a mixture prepared in 10mM HEPES, 120mM NaCl, 0.5mM EDTA pH 7.4 containing 5ug/ml of FH and serial dilutions of FHCCP6-8 dilutions starting from 25 to 0.488ug/ml were loaded at 100ul/well to the wells and plates were incubated at room temperature for one hour. Plates were then washed with PBS-0.5mM EDTA 0.1 % tween20 three times and binding of FH was detected with anti-whole FH antibody (Rabbit anti FH polyclonal antibody) and secondary antibody (anti-Rabbit IgG-alkaline phosphatase conjugate) and substrate p-Nitrophenylphosphate, as described in section 2.2.1.2.

Note that separate tests showed that this primary antiserum recognizes FH CCP6-8 only very weakly.

2.20 Binding assays of Factor H and Adrenomedullin

2.20.1 Optimisation of Adrenomedullin binding to factor H

Antibodies to Adrenomedullin were not available to us; so binding assays were possible only with immobilized Adrenomedullin and fluid-phase FH. In a first experiment Adrenomedullin was serially diluted in order to find out the best concentration for coating the plate to bind to factor H. Microtiter plates (Maxisorp) were coated with 100ul/well of serial dilutions of Adrenomedullin (dilutions starting from 5ug/ml to 0.05ug/ml) in 0.1M sodium carbonate pH 9.6 for one hour at room temperature. Plates were washed and then blocked with 300ul/ well of PBS-0.5mM EDTA 0.1% tween20. Later 5ug/ ml of factor H, 100ul, was added to the coated wells, plates were incubated for one hour at room temperature, then washed 3x with PBS-0.5mM EDTA 0.1% tween20. Then as described in section 2.2.1.2, primary antibody (Rabbit anti FH polyclonal antibody) was added to the wells and plates incubated at room temperature for one hour. Then after washing with washing buffer, secondary antibody anti-Rabbit IgG-alkaline phosphatase conjugate was added and then plates were incubated for one hour at room temperature. Then substrate was added and finally plates were read at 405 nm.

2.20.2 Binding of whole factor H to Adrenomedullin coated wells

Adrenomedullin stock solution (0.1mg/ml) was prepared in water; stock was kept at -20 °C. Adrenomedullin was diluted in coating buffer or other appropriate buffer before use. Microtiter plates were coated with 100ul of 0.5ug/ml of Adrenomedullin in 0.1M sodium carbonate pH 9.6 for one hour at room temperature. Then plates were blocked with PBS 0.5 mM EDTA 0.1% tween20 at room temperature for one hour, then plates were washed with

the same buffer three times. Factor H was serially diluted in 10mM HEPES 120mM NaCl, 0.5mM EDTA pH 7.4, dilutions from 5 to 0.02ug/ml and 100/well were loaded into the wells then plates were left for one hour at room temperature. Plates were washed three times with PBS-0.5mM EDTA 0.1 % tween 20 and then bound Factor H was detected as described in section 2.2.1.2.

2.20.3 Dissociating the binding between FH and Adrenomedullin using different buffers and denaturants

This experiment was designed to explore the best solvent to dissociate the binding between FH and Adrenomedullin, and to compare this with results for TNP-BSA and DNP-HSA. This experiment was done as in section 2.8, above, except that plates were coated with Adrenomedullin, as in 2.20.1 above, and not with TNP-BSA or DNP-HSA.

2.20.4 Inhibition of the binding between FH and Adrenomedullin by using soluble compounds

This experiment was done in the same way as the corresponding study with TNP-BSA or DNP-BSA-coated plates (section 2.9, above), except that plates were coated with Adrenomedullin, as in 2.20.1 above, and not with TNP-BSA or DNP-HSA. The aim of this experiment was to find soluble compounds that can inhibit the binding between FH and Adrenomedullin. Compounds that inhibited the binding at a single dose in the initial test were subsequently re-tested at a range of concentrations to test for dose-dependence of inhibition, using the method described in 2.9.1.

2.20.5 Testing the antisera (anti-CCP 6-8, anti-CCP 3-4 and anti FHR1) to ensure that they will detect CCP6-8, CCP1-4 and CCP 19-20 in ELISAs

Antisera (anti-CCP 6-8, anti CCP 3-4 and anti-FR1 as described in section 2.17) were tested to confirm they could detect the constructs (CCP 6-8, CCP1-4 and CCP19-20). Microtiter plates were coated with 5ug/ml, 100ul/well of these constructs in sodium carbonate pH 9.6 for one hour at room temperature, then plates were washed with PBS-0.5mM EDTA 0.1% tween 20, then blocked with the same buffer at room temperature for one hour. Plates were then washed with same buffer three times and then antisera were serially diluted in PBS-0.5mM EDTA 0.1% tween20, with dilutions from 1/5000 to 1/32000) and 100ul /well was loaded to the wells individually. Then plates were left for one hour at room temperature. Later plates were washed three times with PBS-0.5mM EDTA 0.1% tween20 and then secondary antibody anti-Rabbit IgG-alkaline phosphatase conjugate (as in section 2.2.1.2) was added and then plates were incubated for one hour at room temperature. Then substrate was added and finally plates were read at 405 nm.

2.21 Binding of FHCCP6-8 H/384 or FHCCP6-8 Y/384 to Adrenomedullin coated wells

Plates were coated with Adrenomedullin and blocked as above (section 2.20.2). Then two constructs of recombinant FHCCP6-8, ((Clark *et al.*, 2006) kindly given by Dr Simon Clark), namely FHCCP6-8 H/384 or FHCCP6-8 Y/384 were serially diluted in 10mM HEPES, 120mM NaCl, 0.5 mM EDTA pH 7.4 and loaded in the wells, 100ul/well (dilutions from 20ug/ml to 0.04ug/ml) and plates were incubated for 1 hour. After washing with PBS-0.5mM EDTA 0.1% tween 20, rabbit anti FHCCP6-8 (1/10000) was added and

plates were incubated for one hour at room temperature. Then after washing secondary antibody anti-Rabbit IgG-alkaline phosphatase conjugate (as in section 2.2.1.2) was added then plates were left for one hour at room temperature. Later plates were washed three times with washing buffer and finally substrate was loaded and OD was read at 405 nm.

2.22 Competition between FH and FHCCP6-8Y for binding to Adrenomedullin coated wells

Adrenomedullin coated wells were prepared and blocked as in 2.20.2 above. For the competition assay, wells were loaded with a mixture of 5ug/ml factor H and serial dilutions of recombinant protein FHCCP6-8Y (dilutions from 25 to 0.0488ug/ml) prepared in 20mM Hepes, 120mM NaCl, 0.5mM EDTA pH 7.4. Final concentration of FH is 2.5ug/ml and for FHCCP6-8Y is 12.5ug/ml to 0.024418 ug/ml) Plates were incubated for one hour at room temperature. After washing with PBS-0.5mM EDTA 0.1% tween20 three times, primary antibody (Rabbit anti FH polyclonal antibody) was added and bound FH was measured as in section 2.1.2.

2.23 Binding of constructs CCP1-4 and CCP19-20 to Adrenomedullin, DNP-HSA and TNP-BSA

Microtiter plates were coated with Adrenomedullin or DNP-HSA or TNP-BSA individually and blocked as above. After that 100ul of 5ug/ ml CCP1-4 or CCP19-20 were added separately and plates were left for one hour at room temperature. After washing three times with PBS-0.5mM EDTA 0.1% tween20, anti CCP 3-4 91/10000) was added to detect

CCP1-4, and anti FHR1 (1/10000) was added to detect CCP19-20. Plates were left for one hour at room temperature. Then plates were washed three times with washing buffer then secondary antibody (anti-Rabbit IgG- alkaline phosphatase conjugate 1/5000) was added and plates left for one hour at room temperature. Plates were washed and substrate was added, and finally plates were read at 405 nm.

2.24 Cleavage of C3b in the presence of factor I and factor H

A functional test for FH is to monitor the cleavage of C3b to form iC3b. Cleavage is the action of FI, and the reaction works only if FH is present (Sim and Sim, 1983; Sim *et al.*, 1993). The cleavage is observed by SDS-PAGE, and the rate of cleavage is proportional to the activity of FH, if FH is not present in excess.

Reactions of 20ul were prepared in HEPES buffer containing 5ug of C3b with 1ug of factor I and 10ng of factor H, all in 2mM HEPES, 120mM NaCl, 0.5mM EDTA pH 7.4. These samples were incubated for different time periods (2, 3, 5, 24, 48 hour) to select the best cleavage of C3b α chain. Reaction was stopped by adding 20ul of stopping buffer (0.2M tris-HCl, 2% SDS, 8M urea pH 8.2) to each sample and the sample run, reduced, on 10% acrylamide SDS-PAGE.

2.25 Do Adrenomedullin or DNP-HSA or TNP-BSA inhibit factor H function?

To determine whether the reaction described in 2.24 above is influenced by these FH ligands, the following was done. Reactions of 20ul were prepared in 20mM HEPES, 120mM NaCl, 0.5mM EDTA pH 7.4 as above, containing 1ug of factor I and 10ng of factor H and a gradient of Adrenomedullin concentration [ranging from 1000ng -0.06ng/

reaction], or a gradient of DNP-HSA or TNP-BSA [0.25ng - 0.0125ng/ reaction] s. Samples were incubated at room temperature for 30 minute and then 5ug C3b was added to each reaction and samples were incubated at 37 °C for three hours. After that, reaction was stopped by adding an equal volume of 0.2M tris-HCl, 8M urea, 2% SDS, pH 8.2 buffer), and then samples were run, reduced, on SDS-PAGE.

Chapter three

Results

Binding of Factor H to a Range of Ligands

3 Binding of Factor H to a Range of Ligands

Factor H is known to interact with many ligands, as summarized in the introduction. The binding of factor H to a range of chemically diverse ligands was tested, to identify ligands that might justify further investigation. As shown below, Cardiolipin, fibrinogen, TNP-BSA, DNP-HSA and several others were confirmed or identified as strong binders. Testing the binding of factor H to these ligands was done by using an ELISA technique.

3.1 Binding assay of FH to Fibrinogen and fibrin coated wells

3.1.1 Binding of FH to human fibrinogen

To investigate whether Factor H binds to human fibrinogen an assay was designed, by coating microtiter plates with human fibrinogen. This experiment was done as in section 2.2.1.2. The results, shown in Fig 3.1 shows good binding between factor H and human fibrinogen.

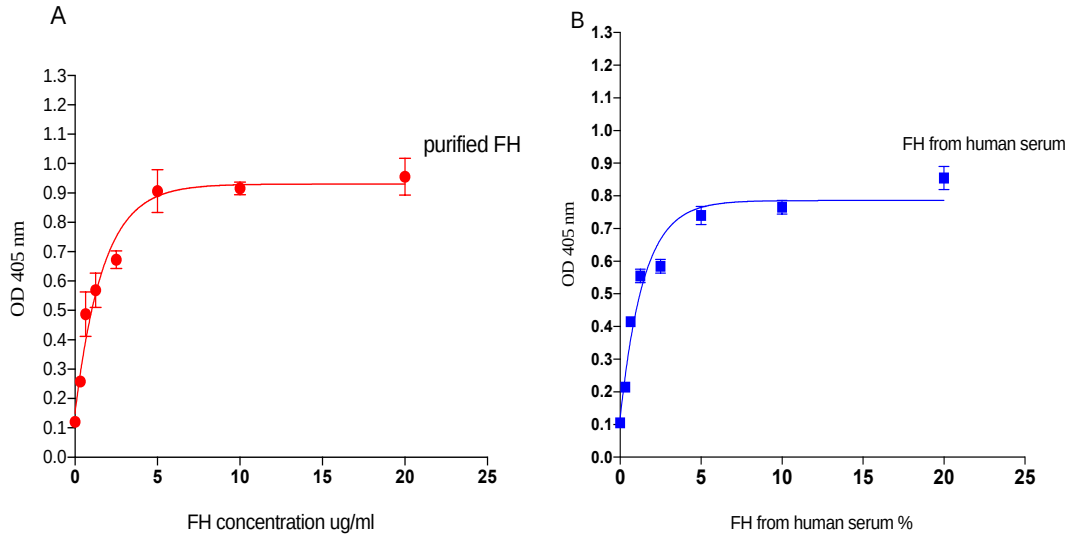


Figure 3-1 Binding of FH to fibrinogen.
A shows binding of purified FH to fibrinogen-coated wells, B shows binding of FH from human serum to fibrinogen coated-wells Plates were coated with 50 ug/ml Fibrinogen and then serial dilutions of FH were added. Purified FH binds strongly with apparent saturation above 5 ug /ml and FH from human serum shows similar binding but with a slightly lower plateau than the purified FH: possibly this indicates another serum protein is binding to the fibrinogen and decreasing the number of binding sites for FH. The experimental data shown are the average and range of 3-4 experiments.

3.1.2 Binding of FH to fibrin

To investigate whether purified FH and FH from human serum interact with fibrin clots, fibrin coated wells were prepared as described in methods section 2.2.1.1. From figure 3.2 it can be seen that, as with fibrinogen (Fig 3.1) purified factor H binds with a slightly higher plateau to fibrin-coated wells in comparison with factor H from human serum. Again this may indicate the presence of competing proteins in the serum.

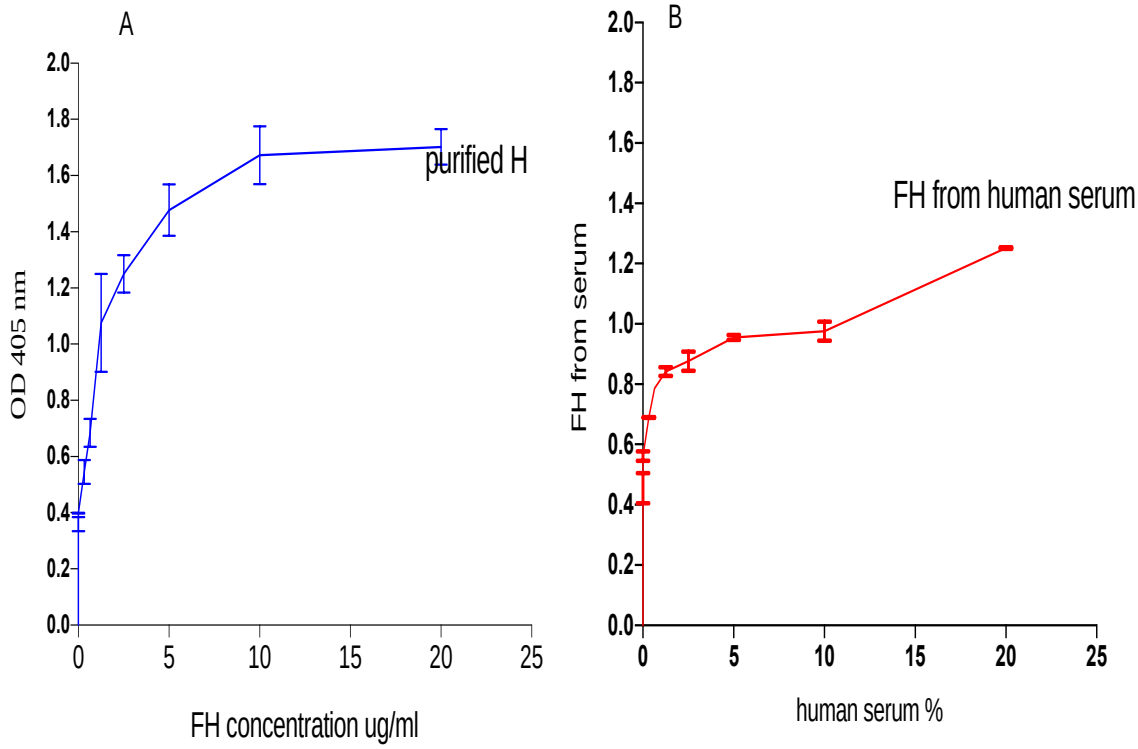


Figure 3-2 Binding of FH to prepared Fibrin clots.
A shows purified FH and B FH from human serum. Fibrin coated wells were prepared and after that serial dilutions of purified FH and FH from human serum were added to the wells. The experimental data shown are the average of 3-4 experiments.

3.1.3 Does FH binds covalently or non-covalently to fibrin clots?

Dr Yu-Hoi Kang has shown that FH can become covalently cross-linked to fibrin clots, by the action of coagulation factor XIIIa (Ferluga, *et al.,* 2016). An experiment was designed to see if the binding of Factor H to the fibrin clot is (partially) covalent; i.e. is FXIII involved in crosslinking. This experiment relies on the observation that nearly all-commercial batches of fibrinogen have a small content of FXIII, which could be activated when thrombin was used to convert fibrinogen on the plate to fibrin. Factor H binding to fibrin clots was carried out in plates as described above, and then the wells were washed with denaturant solutions to see if FH was removed. Results are shown in Fig 3.3

Surprisingly, FH was not removed by 8M urea, but it was removed by SDS-+ Urea. The result is therefore inconclusive: 8M urea would be expected to remove noncovalently bound FH, and this suggests the FH is partially covalently bound. However, SDS+urea removes nearly all signal for FH, which may indicate that it was not covalently bound, or that SDS binding obscured the epitopes for antibody recognition. Destruction of all of these protein reagents in a laboratory fire (April 2016) prevented further investigation.

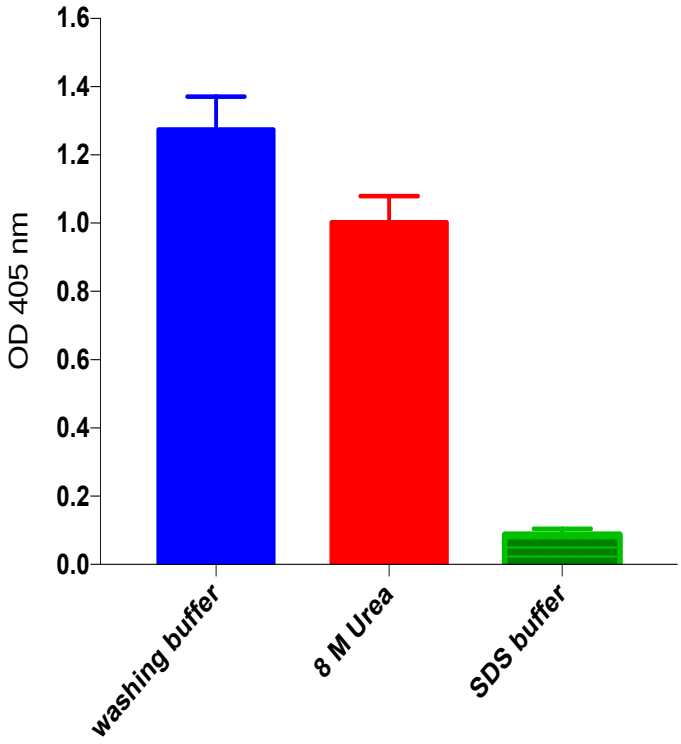


Figure 3-3 Factor H was tested to determine whether it binds covalently or not to fibrin coated wells.
Fibrin clots were formed then factor H was bound to these wells. After washing the wells first with normal washing buffer (PBS-0.5 mM EDTA 0.1 % Tween 20) some of the wells were incubated with 8 M urea, or the gel sample buffer, 2% SDS, 8M urea, 0.2M Tris, pH 8.0, or washing buffer for 40 minutes. Wells were then washed again with washing buffer to remove denaturant, and bound FH was detected with antibodies, as usual. Factor H was still associated with fibrin clots after treating with 8 m urea, but treating the wells with SDS solution has entirely removed FH. The experimental data shown are the average of 3-4 experiments.

3.2 Binding assays of FH to TNP-BSA and DNP-HSA coated wells

Arnold et al (2005) reported that FH bound to a TNP-BSA-Sepharose affinity column and Moreno Indias et al (2012) reported using TNP-BSA-Sepharose as an affinity medium for purifying FH from goat serum. To see whether factor H binds to TNP-BSA coated on a well, and also to DNP-HSA, which is more conveniently commercially available (Sigma), microtiter plates were coated with TNP-BSA or DNP-HSA as described in methods section 2. 4, then serial dilutions of purified human FH, or human serum as a source of FH, were added to the coated wells.

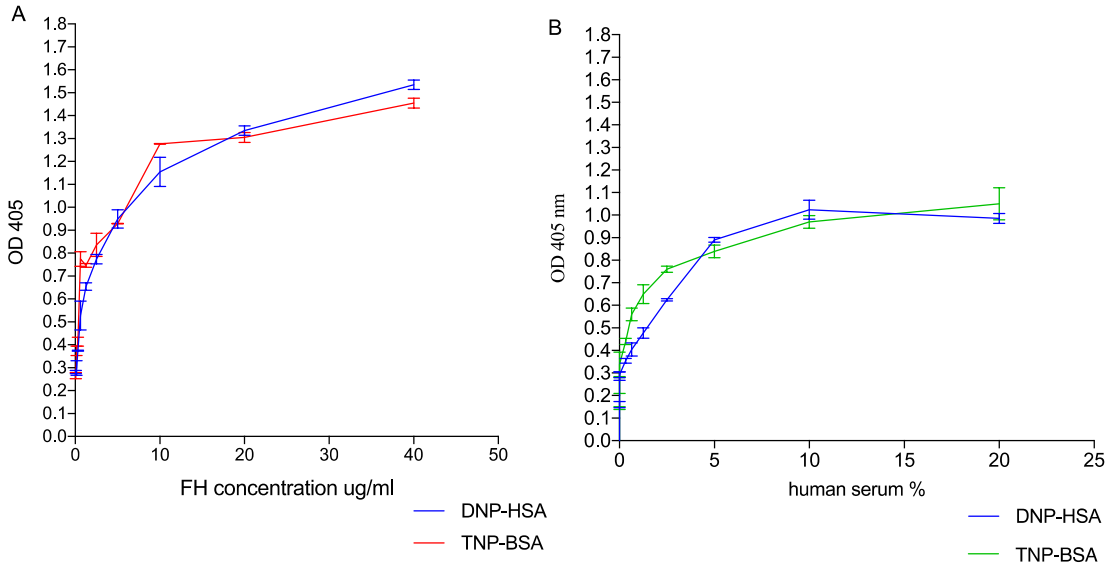


Figure 3-4 Binding of factor H to TNP-BSA and DNP-HSA.
A shows purified FH and B shows FH from human serum. Plates were coated with TNP-BSA or DNP-HSA then serial dilutions of purified FH were added to the plates. The graph shows good binding of FH to both TNP-BSA and DNP-HSA. This binding reaches a plateau at about 10 ug/ml The experimental data shown are the average of 3-4 experiments.

This result Fig 3.4 indicates that FH binds very similarly to both ligands. The TNP used here is a 2,4,6 Trinitrophenyl group, whereas the DNP is a 2,4 dinitrophenyl, indicating that the nitro group at the 6 position may contribute little to the binding to TNP-BSA. Binding from serum has a lower plateau than seen with purified FH. This suggests competition from

other serum proteins. As described below (section 4.3), C4bp was later identified as the major competing protein.

3.3 Interactions with other ligands

Some of the following experiments were done in collaboration with Hanan Alrashidi, who was studying C1q binding to a similar range of ligands (Alrashidi, 2015). Ligands as shown in table 3.1, and including cardiolipin, poly-L-arginine, *Streptococcus pneumonia* whole bacteria and Lipoteichic acid [LTA] *staphylococcus*) were chosen to investigate a wide range of ligand types. Results for some are shown graphically below (figures 3.5 to 3.8, and results for all summarized in table 3.1. The binding of both purified FH and FH from serum was investigated. The experiments were done as described in section 2.5.

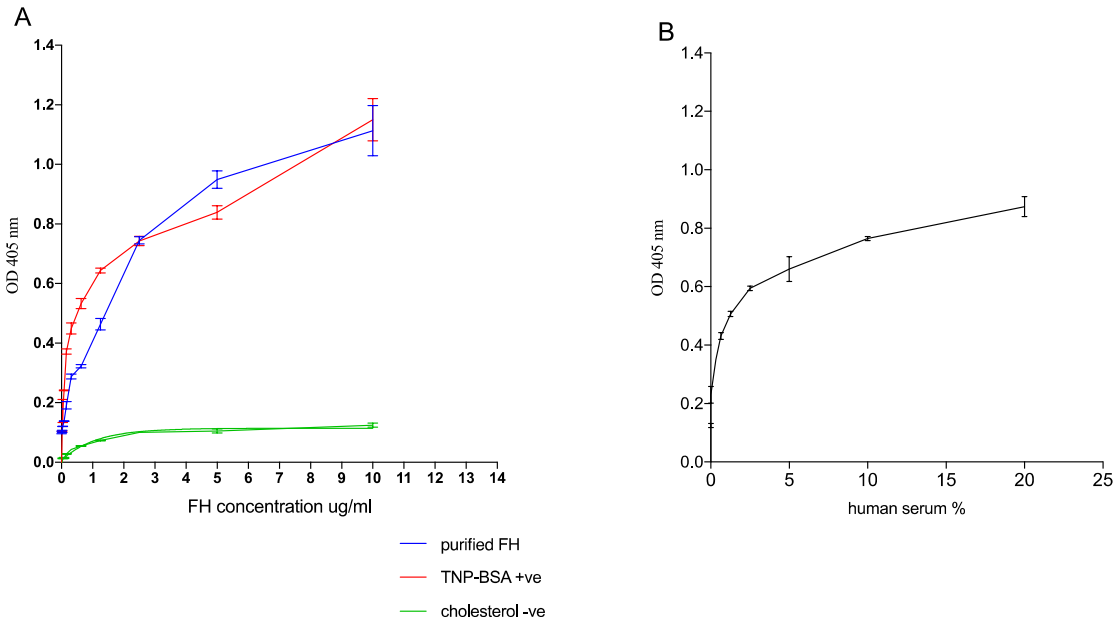


Figure 3-5 Factor H binds to cardiolipin.
A) purified FH protein is shown binding to cardiolipin (blue) beside the positive control (TNP-BSA-coated wells (red)) and the negative control (cholesterol-coated wells (green)) while B illustrates the binding of FH in human serum to cardiolipin. Microtiter plates were coated with 3ug/well of Cardiolipin, cholesterol or TNP-BSA separately overnight. The coated wells were blocked with blocking buffer for an hour at RT. The wells were incubated with serial dilutions starting at 10 ug/ml purified FH in the washing buffer, for an hour at RT. Plate was washed, and then bound FH was detected with Rabbit anti-FH and secondary alkaline phosphatase antibody. The purified FH shows a similar magnitude of binding to cardiolipin, compared to the positive control (ie similar number of binding sites on well), but lower affinity (judged by the slope). The experimental data shown are the average of 3-4 experiments.

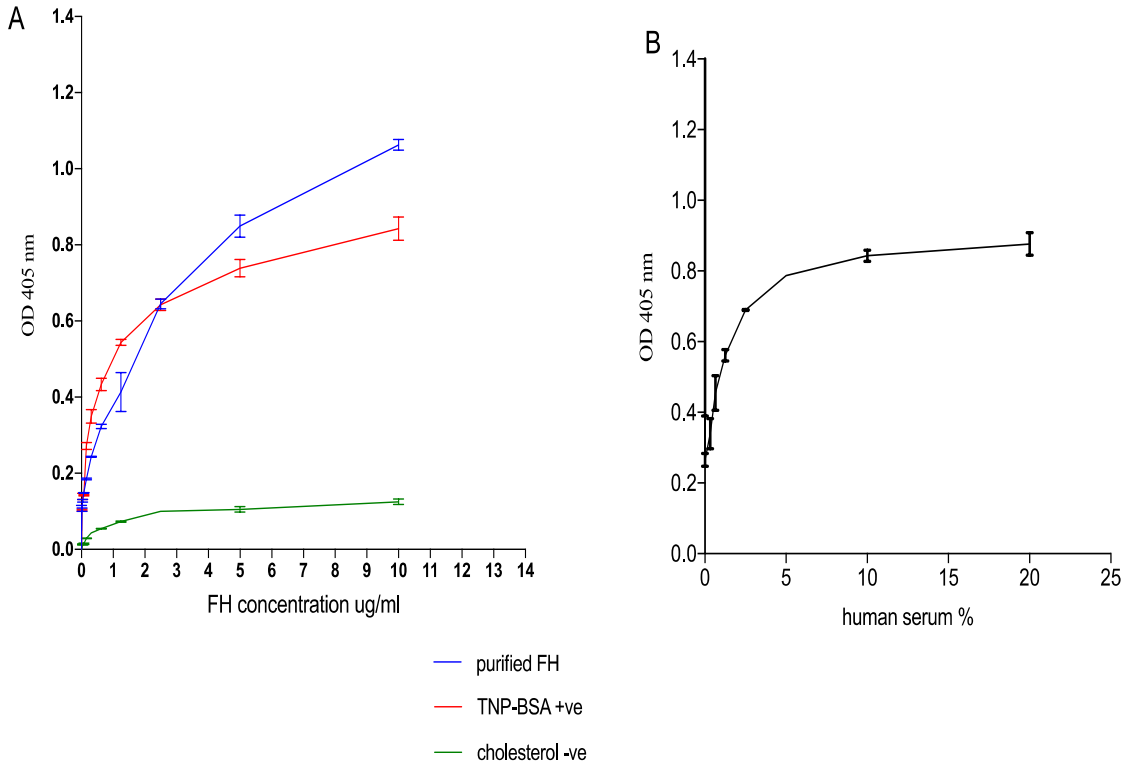


Figure 3-6 Factor H binds to poly-L- arginine.
A shows the binding of the purified FH to poly-L-arginine (blue) beside the positive and the negative control while B illustrates the binding of FH in human serum to poly-L-arginine, which are the same as in fig 3.5. Microtiter plates were coated by 3ug/well of Poly-L-arginine overnight, and the assay was done as in fig 3.5. The purified FH gives a higher level of binding to the poly-L-arginine than to the positive control (TNP-BSA) but with lower affinity. The experimental data shown are the average of 3 experiments.

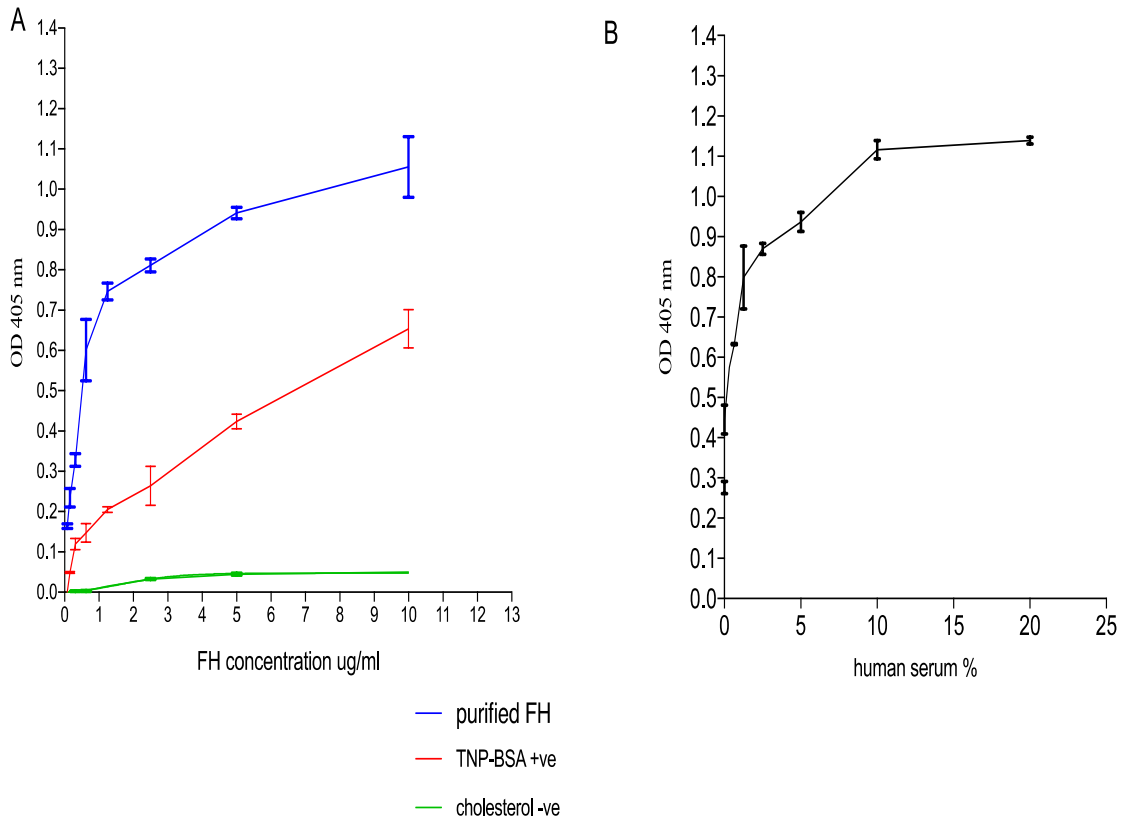


Figure 3-7 Factor H binding to *Streptococcus pneumonia* (D39 strain).
The graph shows (A) the binding of FH as purified protein (blue) beside the positive and the negative controls while B indicates the binding of FH to the bacteria from human serum. Formalin-fixed S. pneumonia (strain D39) was coated on microtiter plates. Binding of purified FH and Fh from serum to the whole bacterium is very high, with very high affinity. The experimental data shown are the average of 3 experiments

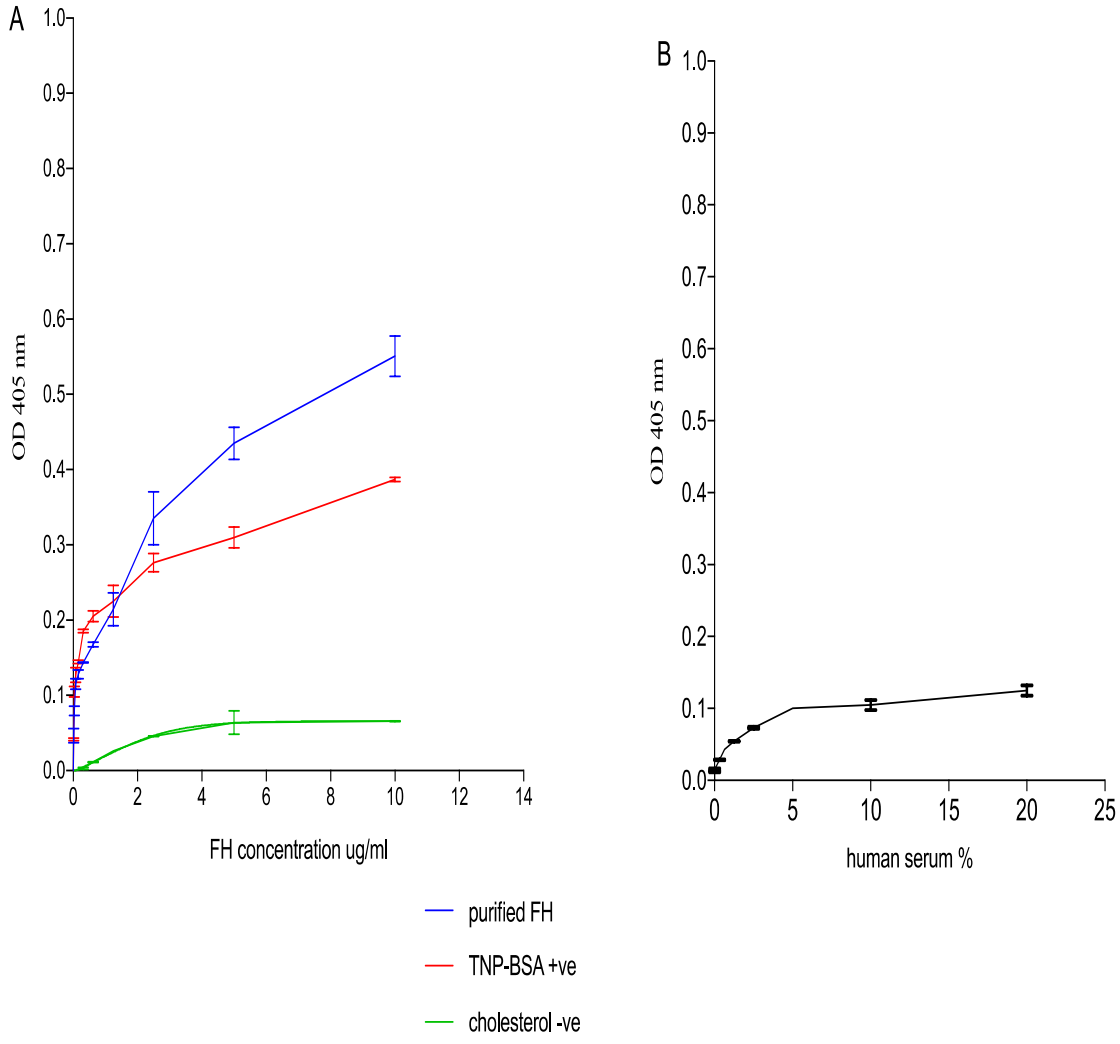


Figure 3-8 Factor H binding to LTA.
A) shows binding of the purified FH (blue)with positive and negative controls. B illustrates the binding of FH in human serum to LTA. Microtiter plates were coated with 30ug/ml of Lipoteichoic acid (LTA) The purified FH gives higher binding than to the positive control, and with similar affinity. Binding from serum is much lower, suggesting that other serum components may block FH binding sites. The experimental data shown are the average of 3experiments

Table 3-1 FH binding to different ligands

Ligand	Binding	
	FH purified	FH from serum
Cardiolipin	****	****
Cholesterol	0	0
TNP-BSA	****	****
DNP-HSA	****	****
Fibrin	****	***
Poly-L-arginine	****	****
Streptococcus pneumonia (whole bacteria) (D39 strain)	****	****
Streptococcus pneumonia (TIGR4 strain)	****	****
Trehalose	0	0
Phosphatidylethanolamine (PE)	0	0
Lipoteichoic acid	***	*
Immune complexes (HSA and sheep IgG anti-HSA)	0	0
Poly-L-lysine	**	**
DNA	**	**
Polyinosinic-polycytidylic acid	0	0
Heparin	**	0
Histone	0	0
Tributyrin Esterase of *S. Pneumonia* (recombinant protein)	**	**
Neuraminidase of *S. Pneumonia* (recombinant protein)	0	0

0 = No binding; * or ** = Low binding; *** = Moderate binding; ****= High binding

3.4 Binding characteristics of FH to TNP-BSA and DNP-HSA

It was decided to explore further the interaction with TNP-BSA and DNP-HSA, as these ligands showed very high binding affinity to factor H and there is little published data on their binding properties. As noted above, (Arnold *et al.,* 2005) reported that FH bound to a TNP-BSA-Sepharose affinity column and Moreno Indias et al (2012) reported using TNP-BSA-Sepharose as an affinity medium for purifying FH from goat serum. Yu *et al.,* (2014), also described the use of this affinity material to purify FH, but none of these papers investigated binding characteristics more extensively.

3.4.1 Effect of salt strength (NaCl concentration) on the interaction between factor H and DNP-HSA or TNP-BSA

Binding of factor H to TNP-BSA and DNP-HSA coated wells was examined above at moderate salt strength (approximately 120mM NaCl). Factor H in buffer with ten different ionic strengths (NaCl concentrations of 0, 20, 40, 60, 80, 120, 160, 320, 640, 1000mM) was incubated with TNP-BSA and DNP-HSA coated wells as described in section 2.7.1. The binding of FH from serum was also studied. Very high binding of factor H for both TNP-BSA and DNP-HSA was observed at low salt concentration, 20 mM NaCl. Binding at 320 mM NaCl concentration was less 50 % of that at 20mM. From the graphs in figures 3.9 and 3.10 it can be seen that by increasing the ionic strength the binding is dramatically decreased. Binding is however, still above zero at 1M NaCl. These results indicate that the binding does have an electrostatic/ionic component, although the persistence of binding at high salt suggests additional nonionic interactions

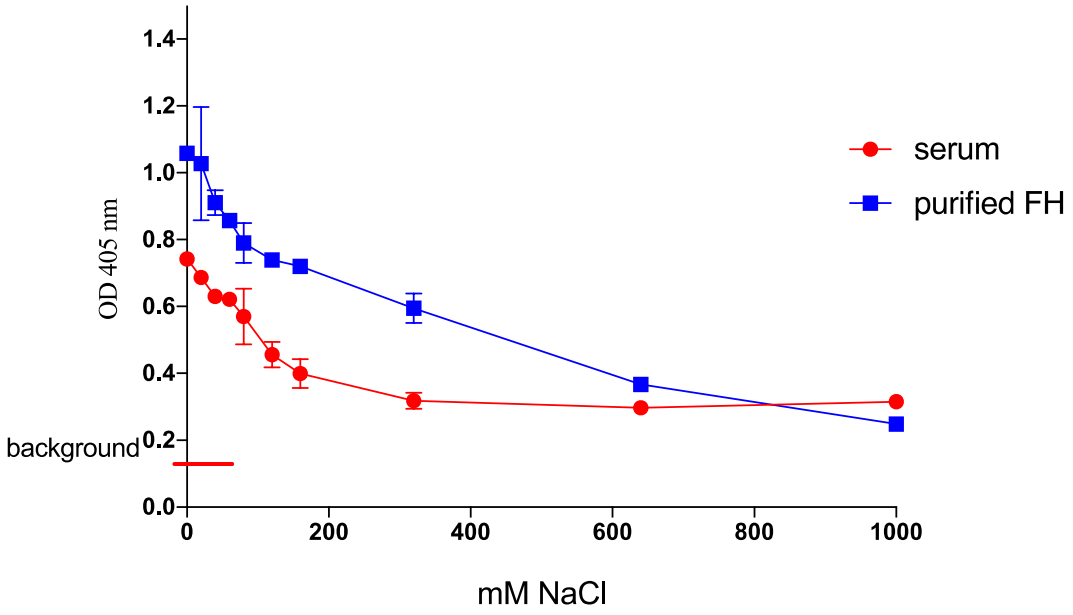


Figure 3-9 Effect of salt concentration on binding of FH to TNP-BSA.
Plates were coated with TNP-BSA 10 ug/well of purified FH or 20% human serum were added to the coated wells in presence of different NaCl concentrations. From the graph it can be seen that good binding occurs at low salt strength, and the binding becomes weaker by increasing the ionic strength. The background was measured from samples where no FH but only antibodies were added. The experimental data shown are the average of 3 experiments.

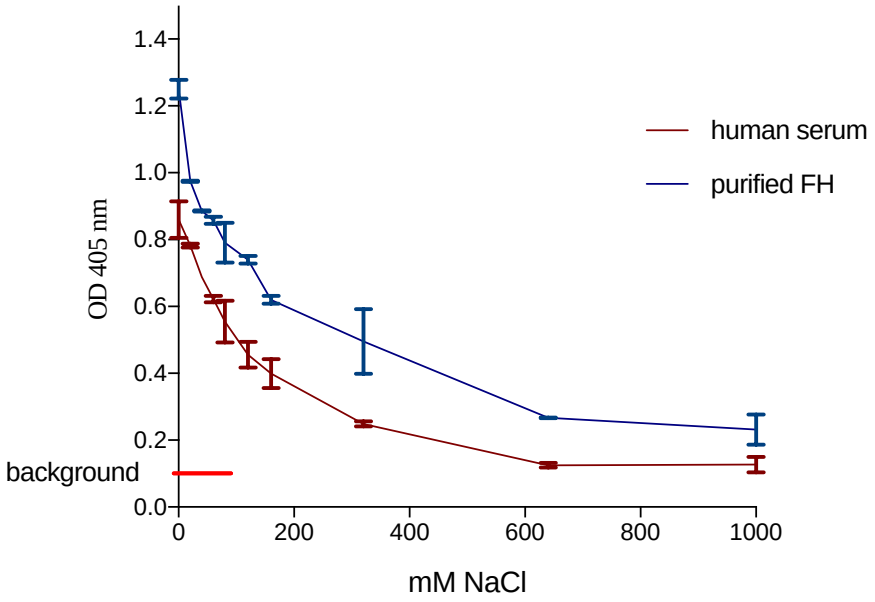


Figure 3-10 Effect of salt concentration on the interaction between FH and DNP-HSA.
Plates were coated with DNP-HSA; purified FH 10 ug/well or 20% serum were added to the coated wells in the presence of different salt concentrations. Binding between DNP and FH decreases with increasing salt strength. The background was measured from samples where no FH but only antibodies were added. The experimental data shown are the average of 3 experiments.

3.4.2 Effect of pH on the interaction between FH and DNP-HSA or TNP-BSA

The binding free energy of protein-ligand interaction can be influenced dramatically by pH of the solution. Binding of factor H to TNP-BSA and DNP-HSA was examined at different pH values, as described in section 2.7.2. Results are very similar for DNP and TNP and are shown in figures 3.11 and 3.12. The best binding for purified FH occurs at pH values from 6-8. Binding falls off sharply below and above these values The shape of the curve for purified FH resembles a classical bell shape in which the rise and fall of binding is thought to be caused by ionization of amino acids side chains, such as Histidine (pH 5-7) and lysine (pH 8-9). This confirms the indication from salt-strength experiments that electrostatic interactions are involved in the binding. The shape of the curve for FH from serum is different, suggesting participation of other proteins or macromolecules (eg lipoproteins), which may compete with FH for binding, such that the competition varies with pH. Alternatively FH may bind wholly or partially via other serum ligands at the higher pH range. The pH of samples containing serum was re-verified after addition of serum. This was done by making large-volume mixtures for use with a pH meter.

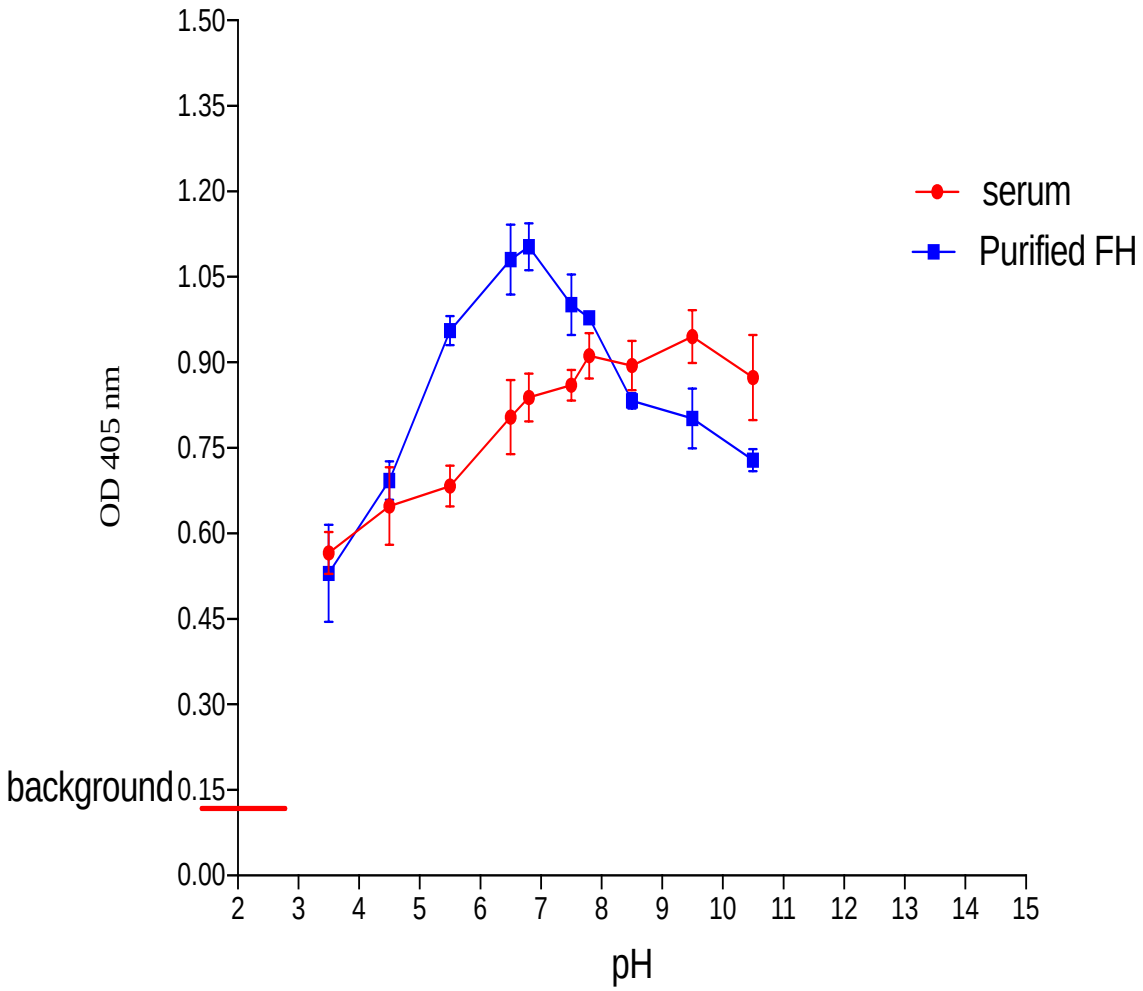


Figure 3-11 Effect of pH on the binding between TNP-BSA and FH.
Plates were coated with TNP-BSA and then purified FH and FH from human serum were added to the wells in presence of different pH buffers. For purified FH the best binding occurs at neutral conditions while it deceases at low and high values. The background was measured from samples where no FH but only antibodies were added. The experimental data shown are the average and range of 3 experiments.

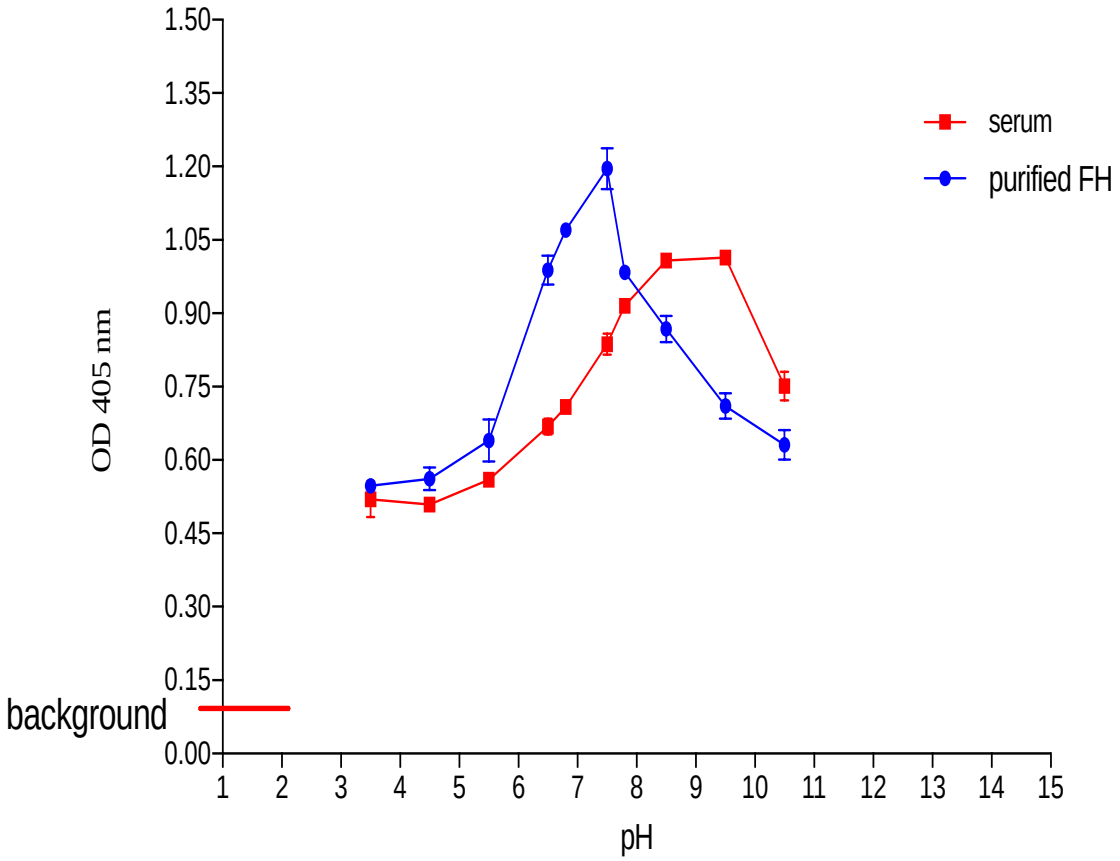


Figure 3-12 Effect of pH on the binding between DNP-HSA and FH.
Plates were coated with DNP-HSA and then purified FH and FH from human serum were added to the wells in the presence of different pH buffers. Again for purified FH the best binding occurs at neutral condition while it deceases at low and high values. The background was measured from samples where no FH but only antibodies were added. The experimental data shown are the average of 3 experiments.

3.5 Dissociating the binding between FH and TNP-BSA or DNP-HSA using different buffers and denaturants

The previous results have showed that factor H binds strongly to TNP-BSA and DNP-HSA. This means the TNP-BSA can be used to bind FH from human serum. The aim of designing this experiment is to explore the best solvent to dissociate the binding between FH and TNP-BSA, so that FH could be efficiently eluted from an affinity column made with these ligands. ELISA plates were coated with TNP-BSA; then FH was added to the coated wells. For dissociating the binding between FH and TNP-BSA, several solvents were added to individual wells, as described in section 2.8. Although strong denaturants have been used to release the bound factor H from TNP-BSA coated wells, none were really efficient to dissociate the binding. About 35% of bound FH was removed by using 0.5% SDS, in addition, 1% SDS and 8M Guanidine removed bound FH slightly. There are some differences in results between TNP and DNP, but this could be due to several factors, e.g. the degree of substitution of the BSA or HSA, and this was not investigated further. Repeated washing of the wells with solvent may be a better way to imitate the conditions of elution from an affinity column. SDS is not desirable for use as an eluant, as it is difficult to remove from the eluted proteins. Urea would be a better choice. It is notable that the high and low pH buffers do not have much effect on the bound FH, although as seen above, the initial binding is sensitive to pH.

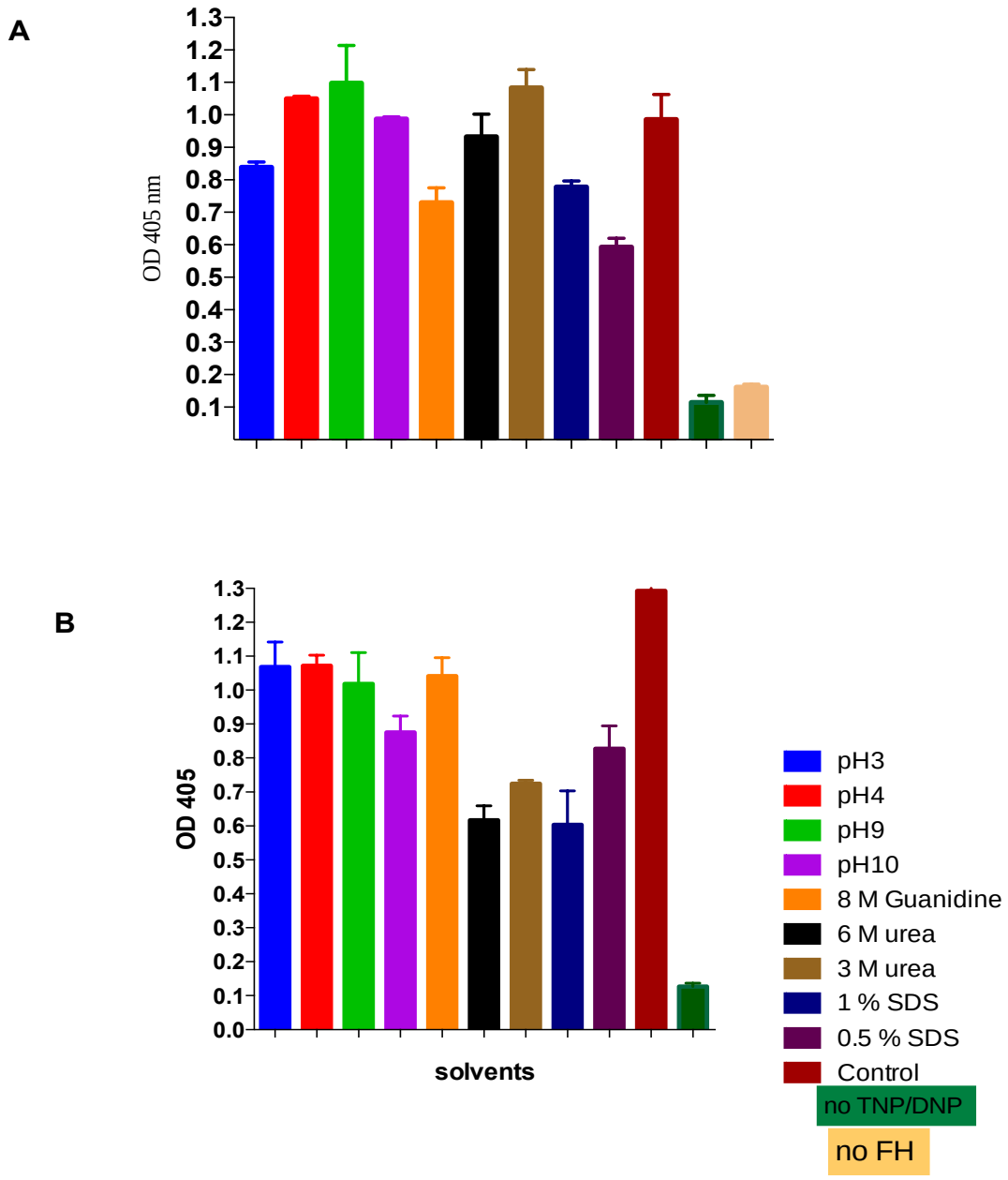


Figure 3-13 Dissociation of the binding between (A) factor H and TNP-BSA and (B) FH and DNP-HSA by using solvents.

Factor H was bound to TNP-BSA or DNP-HSA coated wells, after that wells were treated for one hour with solvents and denaturants to elute the bound FH. Remaining FH was detected by antibodies. Positive control presents normal binding of FH to the ligands (untreated with solvents) From the graph it can be seen that the binding between FH and TNP-BSA is very strong although it is partially removed using urea or SDS. The error bars represent mean ± SD of three independent experiments.

3.6 Comments on other factor H related proteins that may bind to TNP-BSA-Sepharose beads

In an attempt to examine whether other factor H related proteins or FH homologues might bind to TNP-BSA (for example, factor H like protein FHL-1 and Factor H related proteins FHRs1-5), diluted human serum was passed through a TNP-BSA column as described in section 2.12.1. The figure 4.5 (next chapter) shows four main proteins eluted from TNP-BSA-Sepharose with high salt buffer (1M NaCl): IgG, HSA, Factor H and C4b- binding protein (C4bp). The immunoglobulin IgG and possible traces of IgM were removed by using specific columns to trap them (section 4.3). C4bp was separated from FH by passing the mixture through a dyematrex gel (blue B column) and using a gradient of KCl as mentioned in section 4.3. In Fig 3.14 a western blot of the eluate from TNP-BSA-Sepharose, developed with anti-C4bp, shows a big protein in non-reducing conditions (.>245 kDa) and a protein at 72 kDa in reducing conditions, which corresponds to C4bp α-chain. This protein was detected by using anti-C4bp antibody. Therefore, C4bp, as well as FH, binds to TNP-BSA. On fig 4.5 no bands corresponding to FHRs 1-5 or FHL-1 are seen, but these are low abundance proteins so they would be unlikely to be visible easily by Coomassie Blue staining. No western blot (with anti-FH) was done on the TNP eluate, so the presence or absence of FHRs1-5 and FHL-1 is unknown. Two other proteins are of interest: beta2 glycoprotein 1 and FXIII B chain, which are both homologues of FH. The latter is a low abundance protein, so would not likely be seen by Coomassie Blue stain. However beta2 glycoprotein 1 is an abundant serum protein and it is not visible in the eluates (as judged by SDS-PAGE as in fig 4.5 and further SDS-PAGE analysis (not shown) of purification stages. Although beta2 glycoprotein 1 shares some properties with FH, such as binding to anionic phospholipids (Kertesz *et al.*, 1995; Ferluga *et al.*, 2014), it does not bind to TNP-BSA or DNP-HSA.

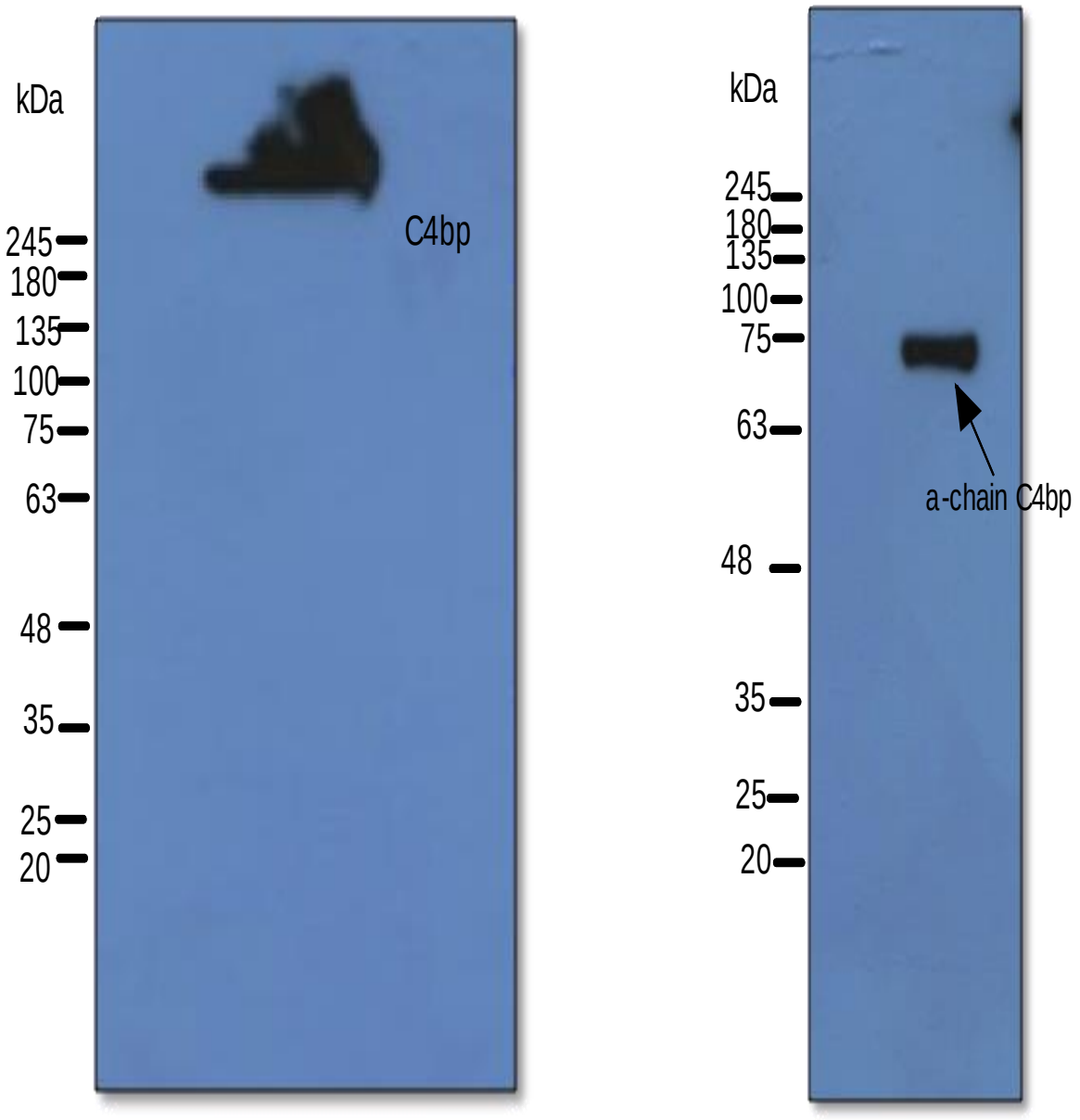


Figure 3-14 western blot film showing C4bp.
This shows SDS-PAGE separation of material eluted from a TNP-BSA-Sepharose column. The proteins were transferred to nitrocellulose, which was incubated with anti-C4bp antibodies. The results identify C4bp as a TNP-BSA-binding protein. The most common isoform of C4bp is composed of seven identical covalently-linked α-chains and some molecules also have a β-chain, C4bp migrates as a 570 kDa band under non-reducing conditions (left) while in reducing conditions (right) a band of about 72 kDa appears which represents α-chain

Chapter four

Results

Further studies on binding to TNP and DNP

4 Further studies on binding to TNP and DNP

4.1 Binding of FH to other TNP-labelled proteins

It has been shown that factor H binds strongly to TNP-BSA and DNP-HSA; this is high affinity binding. To ensure that the binding was to the DNP or TNP substituents, and was not dependent on the albumins, an experiment was set up to explore the behavior of factor H towards some other TNP-labeled proteins, such as TNP-chicken ovalbumin, TNP-Fibrinogen, TNP-gelatin and TNP-bovine gamma globulin (TNP-BGG) and also to the simple amine derivative of TNP, TNP-amine. The TNP-group was added to proteins as described in methods section 2.10. Binding of factor H to TNP-proteins was tested by using microtiter plates coated with TNP-albumin, TNP-Fibrinogen, TNP-gelatin, TNP-Bovine gamma globulin and TNP-amine. Non-derivatised proteins were used as controls. It can be seen from the graph in figure 4.1 that factor H binds consistently well to all the TNP-derivatised proteins and also to the TNP-amine. The binding to non-derivatised proteins was low except to fibrinogen. Concentration-dependence of binding of FH to the same derivatised proteins is shown in Fig 4.2. The maximum FH binding does vary between the different ligands, but this is most likely due to different degrees of substitution of the proteins with TNP, and (especially for TNP-amine), different quantities of ligand bound to the well.

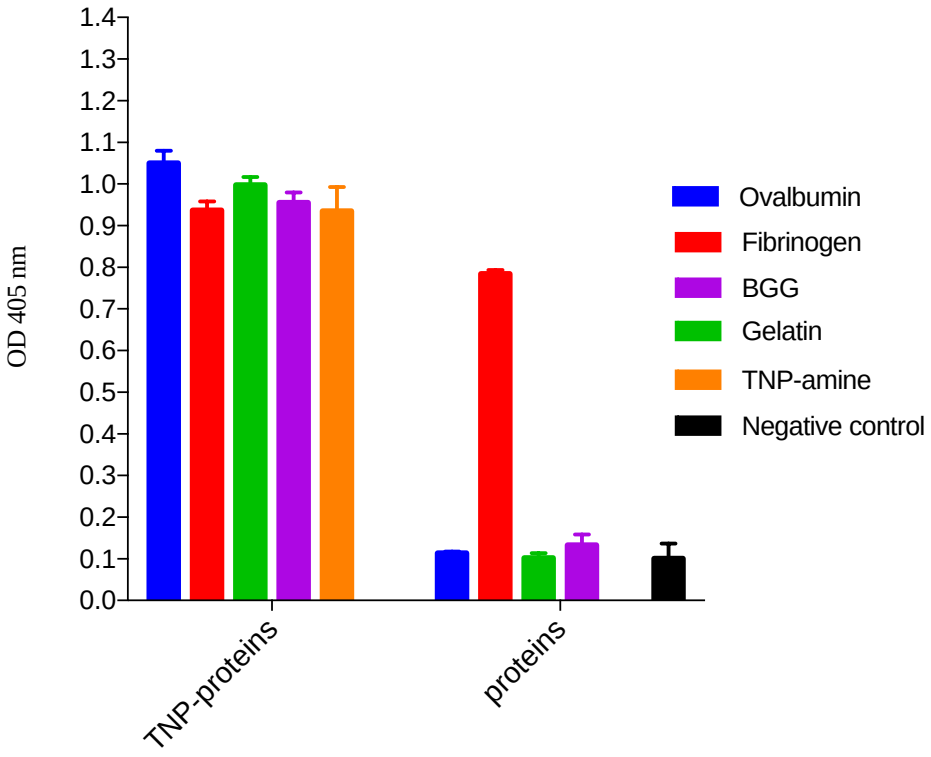


Figure 4-1 FH binds to TNP-proteins and TNP-amine.
The TNP-proteins were used at 100 ug/ml, 100ul/well while 10ug/ml was used for TNP-amine to coat plates; then FH (5 ug/ml) was added to the plates, and binding detected as described above. Non-derivatised proteins at the same concentrations were used to compare the binding to FH with TNP- proteins. The negative control wells were coated with TNP-BSA but received no FH. The experimental data shown are the average and range of 3 experiments.

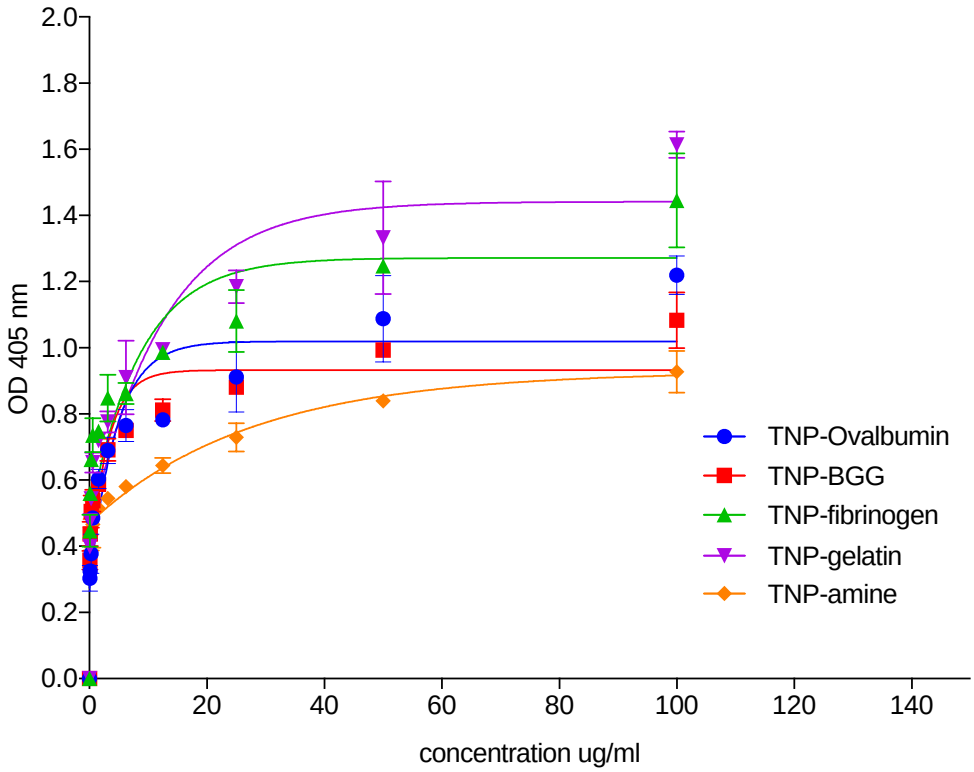


Figure 4-2 Concentration-dependence of FH binding to TNP-proteins and TNP-amine. These materials were used at 100 ug/ml, 100 ul/well to coat plates, except for TNP-amine, 10ug/well; then FH serial dilutions were added to the plates. The graph here shows that binding becomes less when decreasing the protein concentrations, and it can be said the binding is concentration dependent. The experimental data shown are the average and range of 3 experiments.

4.2 Inhibition of the binding between FH and DNP-HSA, using soluble inhibitors

Previous results showed that factor H binds strongly to TNP-BSA and DNP-HSA, and this binding is hard to dissociate by using high /low pH or even by detergents or denaturants (see figure 3.13). In this experiment we wanted to see whether some soluble proteins, amino acids, nucleic acids and other compounds could inhibit the binding of factor H to DNP-HSA. Plates were coated as usual with DNP-HSA, and after blocking, test compounds were added to wells at final concentration 5mM (O- phospho-L-tyrosine, N-acetyl-3, 5- Dinitro-tyrosine-ethyl ester, ATP, N-formyl- met-leu-phe, bilirubin, *myo*-inositol hexakisphosphate , Spermine, para-Nitrophenyl acetate, BOC-L-tyrosine, quinidine

sulfate, ellagic acid, 3-nitro-L-tyrosine, 5,5-dithio- bis(2-nitrobenzoic acid)). In addition, heparin, tRNA and poly-(Glu-Ala-Tyr) were used at 10 ug/ml . Purified FH (5 ug/well) was added. Plates were incubated for 1 hour at room temperature. It can be seen from figure 4.3 that the compounds phosphotyrosine, ATP, bilirubin, spermine, ellagic acid, BOC tyrosine, all cause a high degree of inhibition of binding, up to about 80%. Other compounds, including poly-Glu-ala-tyr, N-acetyl dinitrotyrosine ethyl ester, F-met-Leu-Phe (NMFL), *myo*-inositol hexakisphosohate, tRNA and quinidine sulfate have intermediate effects, and nitrotyrosine, p-nitrophenol acetate, 2-nitrobenzoic acid, 2,4-dinitrophenol acetate and heparin have little effect. In a further experiment, compounds that showed good inhibition were used at lower concentrations to explore how they behave in a lower dose see figure 4.4.

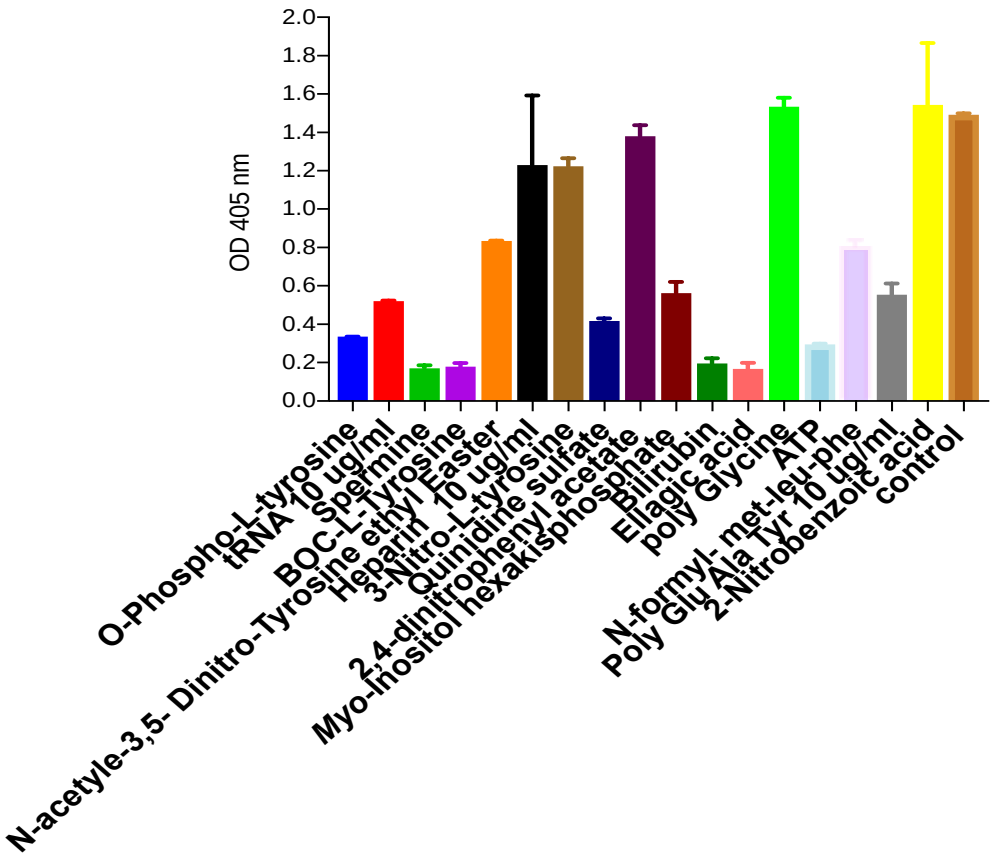


Figure 4-3 Inhibition of the binding between DNP-HSA and FH.
Several compounds were incubated with Factor H and DNP-HSA, then the binding of FH to DNP-HSA was assessed, as described in section 2.9. The experimental data shown are the average and range of 3 experiments.

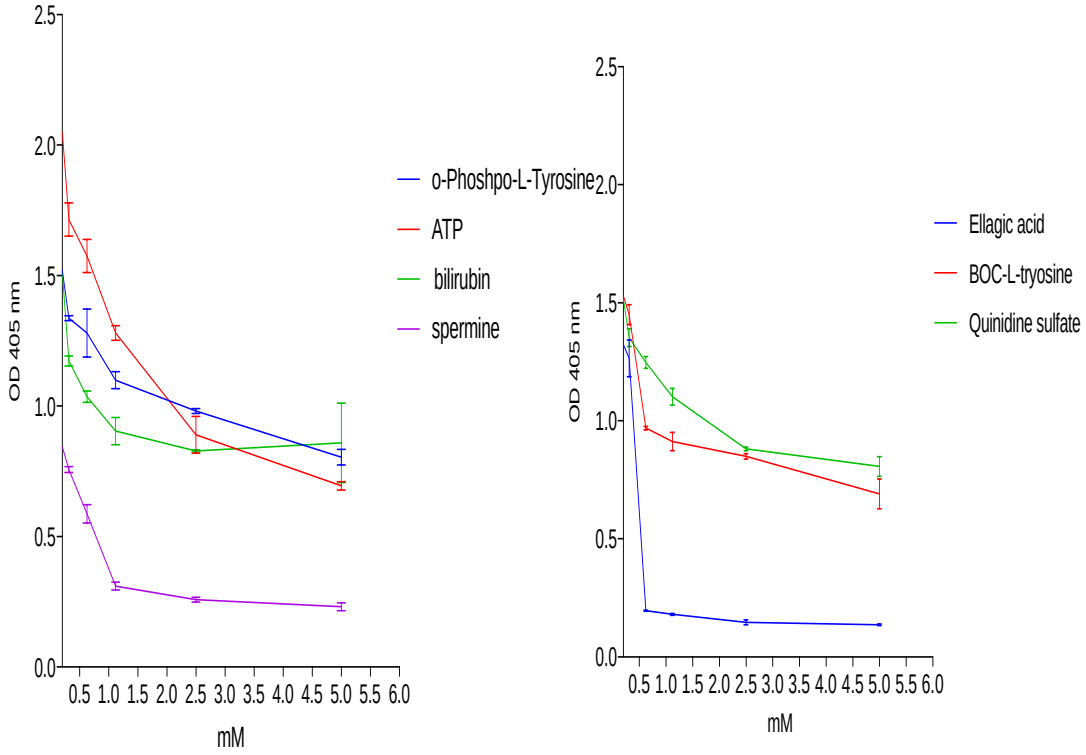


Figure 4-4 Dose-dependence of inhibition of factor H binding to DNP-HSA.
The best inhibitors selected from the data in figure 4-3 were tested at various concentrations, as in section 2.9.1. From the graph it can be seen that there is dose-dependence of inhibition. The experimental data shown are the average and range of 3 experiments

Table 4-1 Compounds and their similarity in structure to TNP/DNP and their inhibition of factor H binding to DNP-HSA

Compound	Similarity in structure to TNP/DNP	Inhibition of binding to DNP
O-Phospho-L-tyrosine	**	***
N-acetyl-3,5-Dinitro-tyrosine-ethyl ester	***	*
ATP	–	***
N-Formyl-Met-Leu-Phe	*	*
Bilirubin	–	****
Poly (glu(60)ala(30)tyr(10)	*	**
Myo-inositol hexakisphosphate	–	**
Spermine	–	****
tRNA	–	**
para-Nitrophenyl acetate	**	–
Boc-L-tyrosine	*	****
Quinidine	–	****
Ellagic acid	–	****
polyglycine	-	-
Heparin	–	–
2-Nitrobenzoic acid	*	***
2-dinitrophenyl acetate	****	–

Note that compounds with stronger structural similarity are poor inhibitors. The stronger inhibitors are likely to have different binding mechanisms from DNP or TNP but possibly bind to overlapping sites. ─ = No inhibition, *= weak inhibition, **= moderate inhibition, ***= good inhibition, **** = strong inhibition. The structural similarity is based simply on the presence or absence of a phenol structure (aromatic ring with OH substituent). The presence of nitro substituent was judged to confer (extra) similarity.

4.3 Factor H purification

TNP-BSA-Sepharose has been used to purify FH from plasma/serum as reported by Moreno-Indias *et al.,* (2012) and Yu *et al.,* (2014) However the procedure has not been optimized and potential contaminants are not identified with certainty. We set out to modify and improve the procedure. Factor H was purified from human serum according to Moreno-Indias's *et al.,* (2012) procedure. The plasma was diluted 1:1 with cold water (4°C) to decrease ionic strength. For the isolation of factor H, 200 ml of diluted plasma was mixed with 20 ml of TNP-BSA-Sepharose, prepared as indicated by Arnold *et al*., (2005). The mixture was left at 4°C on slow rotary stirrer for 1 hour. The resin was thoroughly washed with HEPES buffer (10mM HEPES, 60mM NaCl, 0.5mM EDTA pH 7.4). This affinity column binds mainly factor H, IgG, and, it was thought, IgM. The resin was placed in a column and the bound proteins were eluted with the high salt buffer (10 mM HEPES, 2M NaCl, 0.5mM EDTA pH 7.4). To remove the contaminant immunoglobulin (IgG) a Hi-Trap protein G column was used and a rabbit anti-human IgM-Sepharose column used to eliminate IgM. Then samples from each step were run on SDS-PAGE. On figure 4.5 can be seen the major proteins eluted from TNP-BSA-Sepharose. After several repetitions of this procedure, it was realized that the anti-IgM column did not seem effective, as it removed very little protein. It was concluded that either the column had lost binding capacity, or the protein that looked like IgM on SDS-PAGE, was not IgM. A plasma protein which looks like IgM on SDS-PAGE is C4bp. C4bp run in reduced conditions has a band at about 75 kDa, similar in mobility to IgM heavy chain. In non-reducing conditions, C4bp is a disulphide-linked oligomer of about 570kDa, while IgM is about 900 kDa or greater—so both would stay at the top of an SDS-PAGE gel. IgM also has light chain (25 kDa) but this coruns with IgG light chain and C4bp also has a faint band at ~45 kDa (the beta chain), but it is present in low quantity. To investigate further, the TNP-BSA-Sepharose eluate was analysed by Western blot using anti-C4bp, and it was confirmed that the major contaminant was C4bp, not IgM (see section 3.6 and associated figure 3-14).

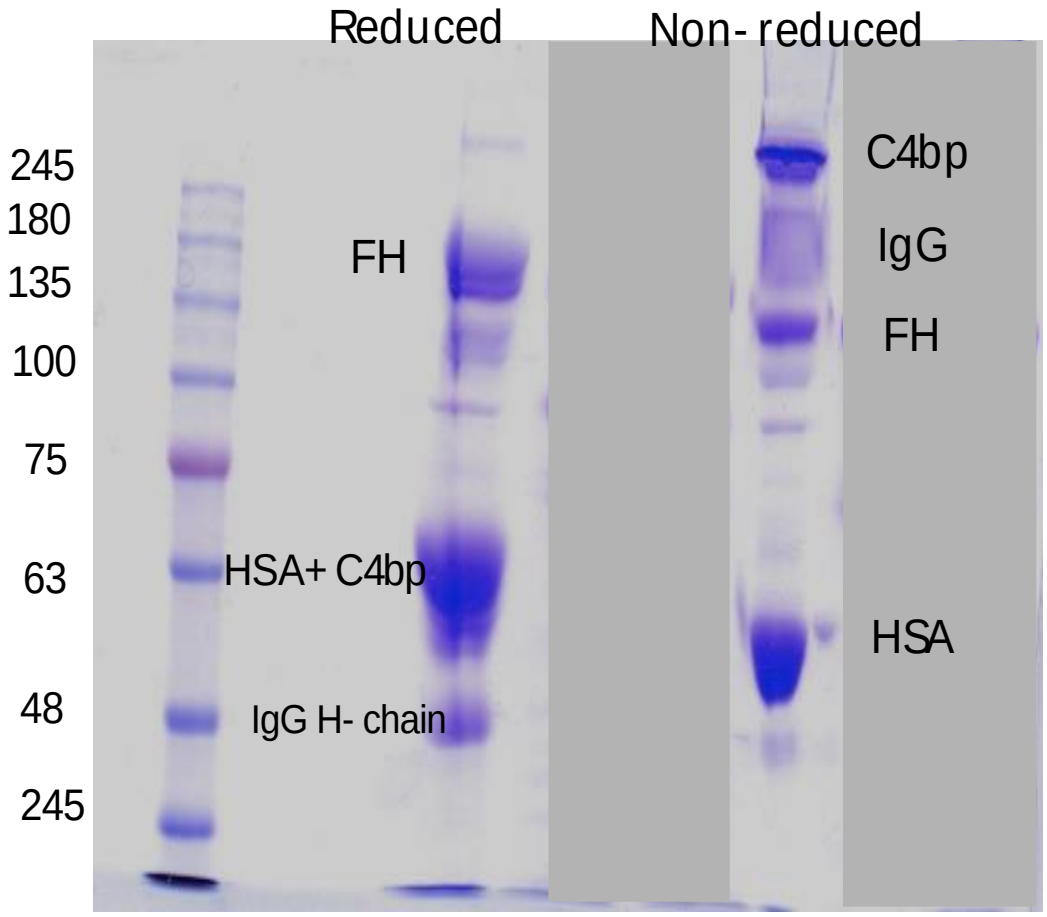


Figure 4-5 SDS-PAGE analysis of a stage in the purification of factor H.
Materials eluted from TNP-BSA-Sepharose analysed by 10 % SDS-PAGE as described in methods and the gel was stained with Coomassie Blue. Note that the presence of albumin (HSA) in the eluate is simply an indication that the resin wash not washed sufficiently, and care was taken to wash more extensively in subsequent preparations.

Several methods were applied to separate C4bp from FH. Normally this could be done by gel filtration, because of the size difference between the two proteins (570 vs 155 kDa) but this requires concentrating the material to low volume then making multiple repeat separation runs. A more convenient method was thought to be an advantage.

4.3.1 Using Zinc Sulphate to selectively precipitate FH

Eluted material from TNP-BSA-Sepharose was dialysed three times against PBS at 4°C. After that, aliquots of the protein were incubated with different concentrations of zinc sulphate (0.5mM, 1mM, 2mM, 4mM, 5mM, 10mM, 20mM 40mM, 80mM, 160mM) as described in Methods section 2.13.1. Aliquots were checked every 8 hours to see the cloudy appearance of protein precipitation. A centrifugation step then later was followed to separate the supernatant from precipitate. Precipitates were resuspended in PBS-5mM EDTA. Samples (supernatants and resuspended precipitates) were run (unreduced) on 10% SDS-PAGE. The basis of this procedure is that FH is known to precipitate in low molarities of Zn++ (<1 mM) and it was hoped that C4bp would remain in solution, or at least precipitate at a different concentration. However in this case factor H and C4bp have both precipitated at 1 mM. The analysis of the supernatants suggest C4bp was less completely precipitated at low Zn++, but the difference was not enough to form the basis of a separation.

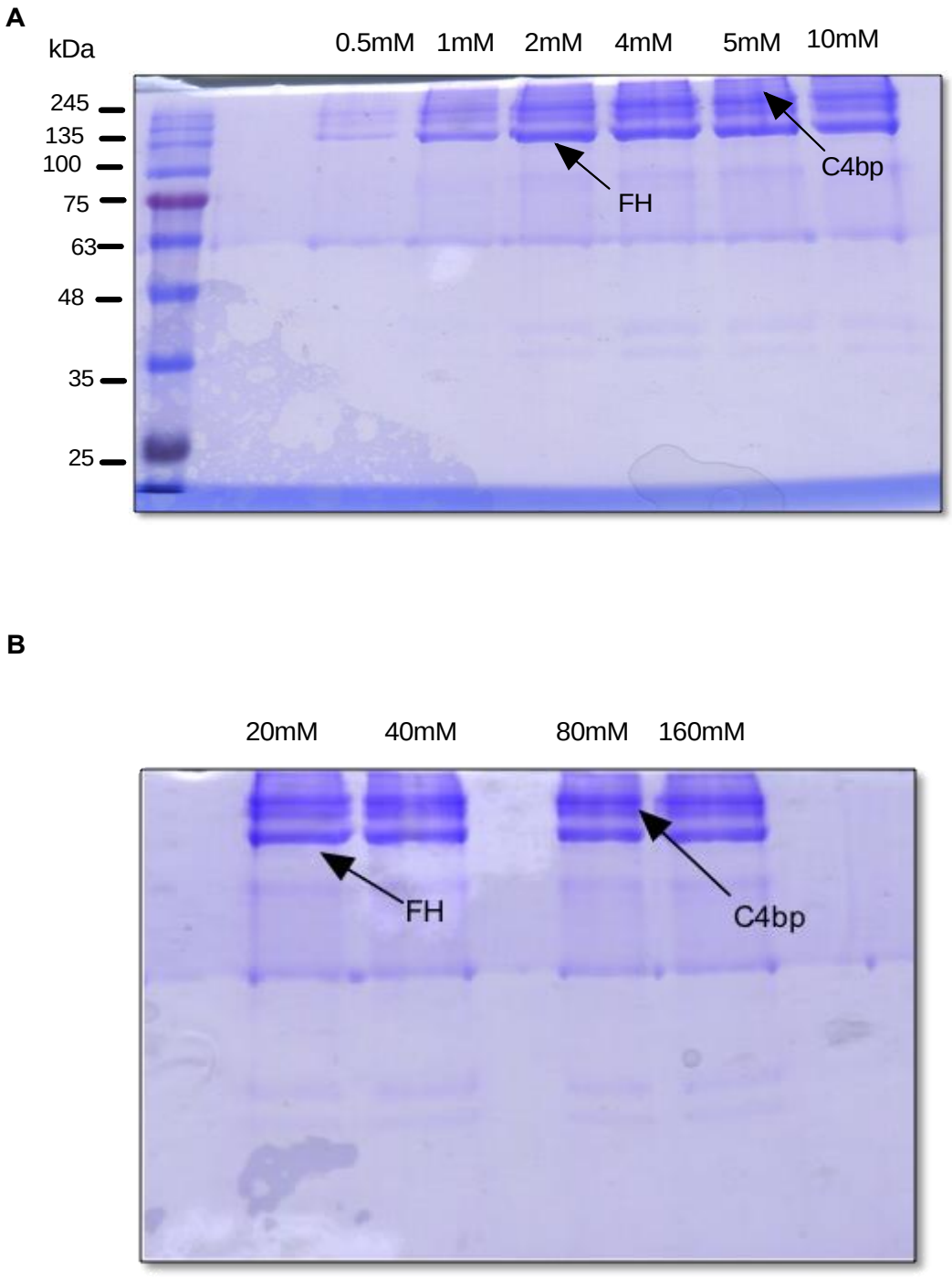


Figure 4-6 A and B. SDS-PAGE analysis of precipitates of FH and C4bp in the presence of Zn++.
Precipitate samples were run (unreduced) after incubation with different molarities of ZnSO4. Lane 1; the molecular weight marker of broad range 20 kDa- 245 kDa. Lanes 2-10 are samples of protein that precipitated with different molarities of zinc sulphate.

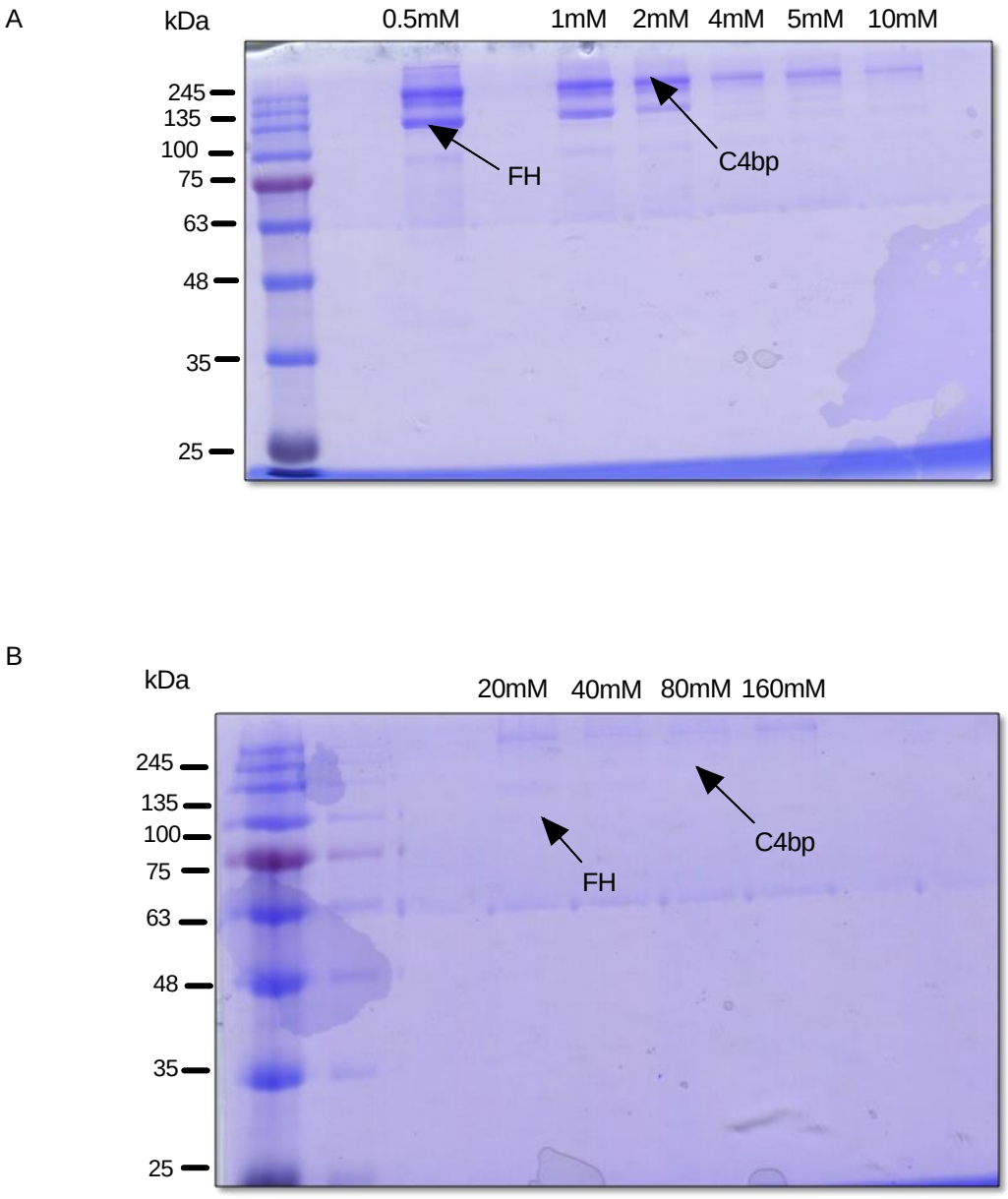


Figure 4-7 A and B. SDS-PAGE analysis of supernatants of FH and C4bp incubated with Zn++.

Supernatant samples were run (unreduced) after incubation with different molarities of ZnSO4. Lane 1; the molecular weight marker of broad range 20 kDa- 245 kDa. Lanes 2-10 are sample of proteins remaining in the supernatant after incubation with different molarities of zinc Sulphate.

4.3.2 Using DyeMatrix Gels (immobilized dyes) for Factor H purification

Protein after elution from TNP-BSA Sepharose with 20mM HEPES, 2M NaCl, 0.5 mM EDTA pH 7.4 was dialysed (3 x) against 10mM HEPES, 130mM NaCl 0.5mM EDTA pH 7.4. Protein samples were passed through the protein G and anti-IgM columns. Then samples of the protein were tested on five different dyematrex gels (1 ml columns) (Blue A, Red A, orange A, Green A and Blue B) provided by Amicon USA. Columns were tested first to find out if they bind FH. One mL of protein solution was incubated in the columns for 1 hour at room temperature and after that the column was washed with 1 mL of 10 mM HEPES, 140 mM NaCl, 0.5mM EDTA pH 7.4. Next 1 mL of increasing molarities of KCl starting from 20mM, then 40mM, 80mM, 120mM 160mM, 300mM, 500mM, 1M, 2M, all in 10mM HEPES, 0.5mM EDTA, pH 7.4, were passed through the columns and 1 ml fractions were collected. After that, samples were run on SDS-PAGE. To remove KCl from protein, fractions were dialysed against PBS three times (before SDS-PAGE analysis). The Blue B column was the only one found to provide separation of FH and C4bp. The other columns all bound both proteins, but there was no separation when they were eluted. Later a larger DyeMatrex blue B column (10 mL) was made to purify enough FH. From the SDS-PAGE analysis, very good separation of factor H from C4bp was obtained (see figures 4.8 and 4.9). Factor H is a single band, which runs at about 155 kDa under reducing and non-reducing conditions. However, C4-binding protein consists of seven SS-linked identical chains (α-75 kDa) and some molecules of C4bp also have one copy of a small β-chain (45 kDa). So unreduced C4bp is > 500 kDa. As seen in the figures below (Figure 4.8 and 4.9), on Matrex blue B, C4bp binds to the column but is eluted cleanly with 20mM KCl. FH elutes at higher KCL concentrations, and “trails” over a wider KCL concentration range. The separation is not complete, as there is some overlap, but it is sufficient to provide samples of both proteins at high purity.

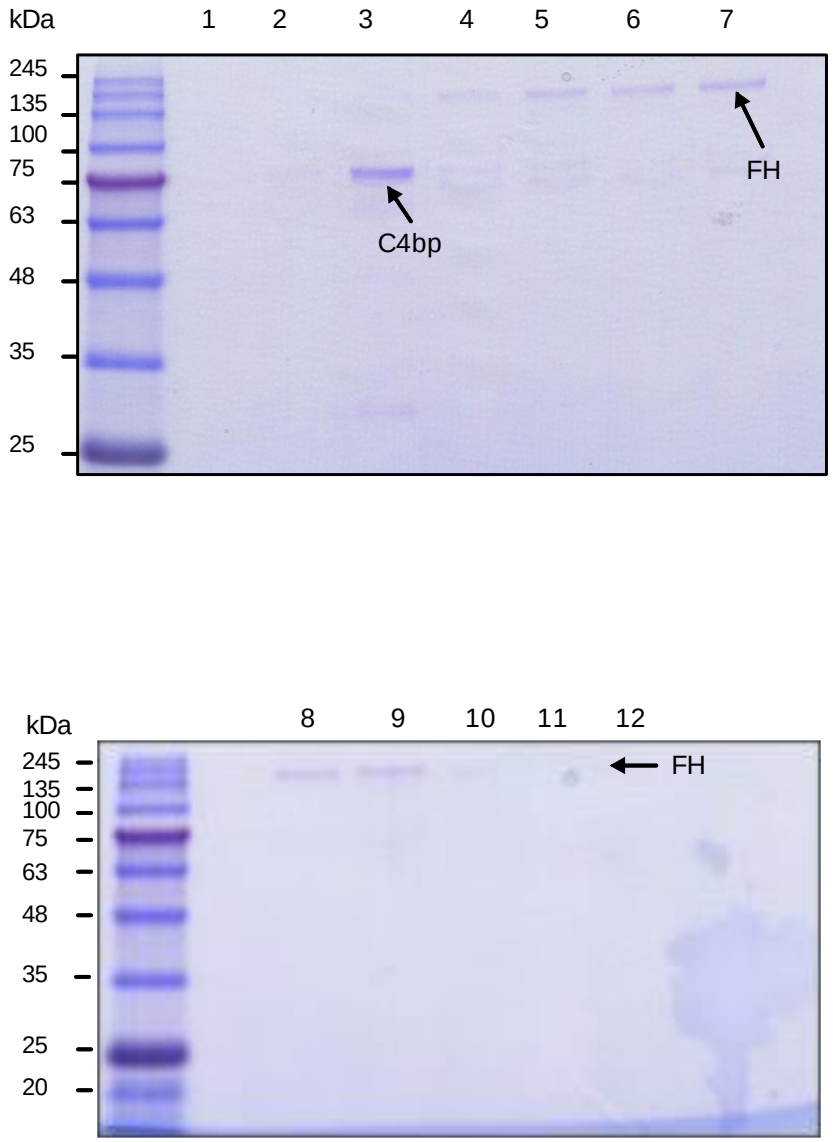


Figure 4-8 Factor H separation on Matrex Blue B, SDS-PAGE analysis under reducing conditions.

A Matrex blue B column was loaded in 10mM HEPES, 130mM NaCl, 0.5mM EDTA, pH 7.4, the protein left in contact for 1h, then the column washed with the loading buffer then with increasing concentration of KCl in the same buffer. Lanes 1 and 2; elution with loading buffer. Lane 3; washing with 20 mM KCl. Lane 4; 40 mM KCl Lane 5; 80 mM KCl Lane 6; 120 mM KCl Lane 7; 160mM KCL. Lane 8; 300 mM KCL Lane 9; 500 mM KCL Lane 10; 1M KCL. Lane 11; 2M KCL. FH mw is 155 kDa while C4bp α-chain is 70 kDa under reducing conditions. The β- chain of C4bp is usually too weak to be seen on a gel**.**

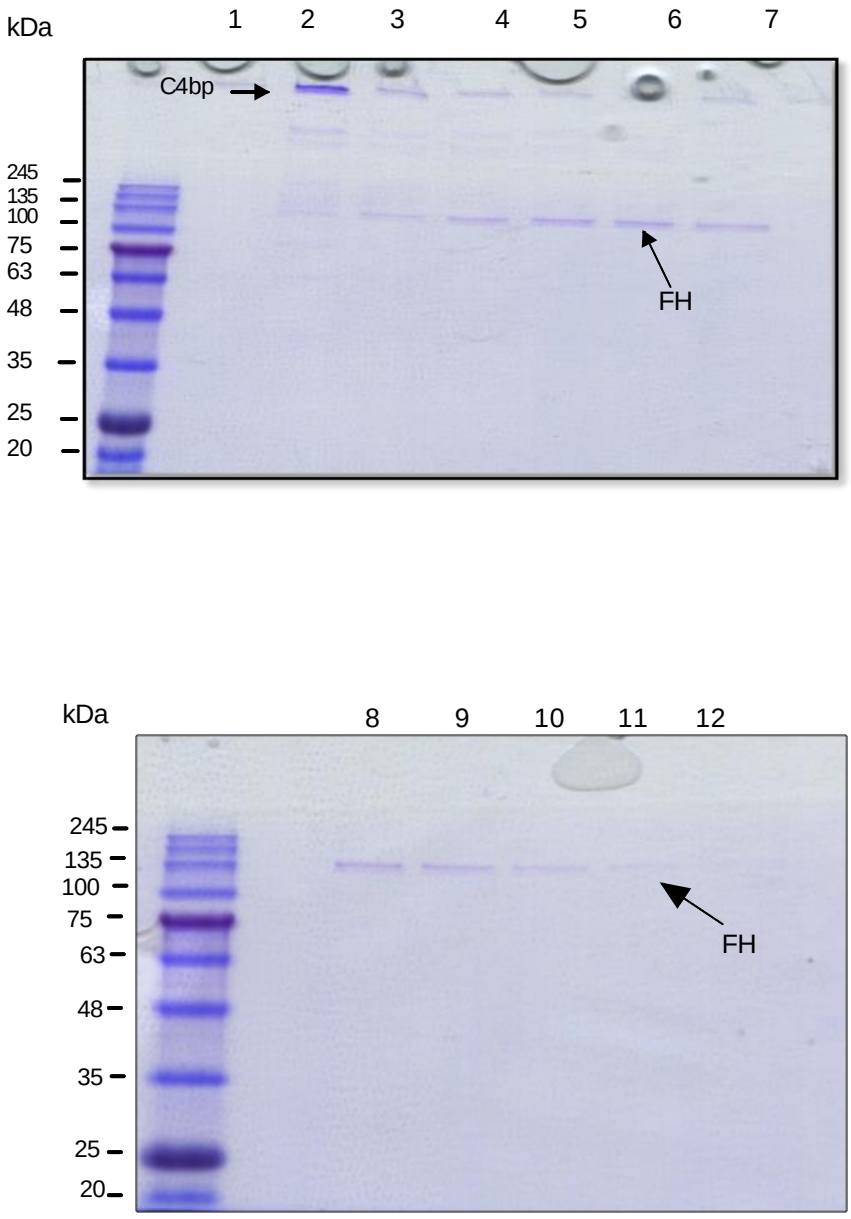


Figure 4-9 Factor H separation on Matrex Blue B, SDS-PAGE analysis under non-reducing conditions.

As in figure 4.8 above, a blue B column was loaded in 10mM HEPES, 130mM NaCl, 0.5mM EDTA, pH 7.4, the protein left in contact for 1h, then the column washed with the loading buffer then with increasing concentrations of KCl in the same buffer. Lane 1; elution with loading buffer. Lane 2; washing with 20 mM KCl. Lane 3; 40 mM KCl. Lane 4; 80 mM KCl. Lane 5; 120 mM KCl m Lane 6; 160mM KCL. Lane 7; 300 mM KCL Lane 8; 500 mM KCL Lane 9; washing with 1M KCL. Lane 10 band 11; 2 M KCl; lane 12, empty**.**

4.3.3 Western blotting of the collected fractions

After collecting FH fractions that looked pure on SDS-PAGE and clear from any contaminant C4bp, samples were run on SDS-PAGE and then tested by western blot, with anti-FH (polyclonal) as the primary antibody. It can be seen from the western blot film in figure 4-10 that only one clear single band with approximate molecular weight 150 kD which represents factor H with molecular weight 155 kD is visible

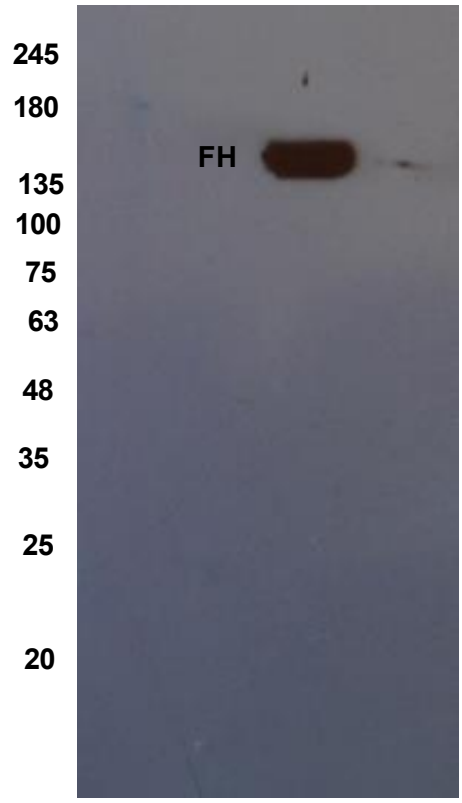


Figure 4-10 Western blot film shows factor H as a single clear band at MW 150kD.

4.4 Exploring the binding sites on FH for TNP or DNP

At this stage we had no indication whether TNP or DNP is binding to FH via a single site on FH or via multiple sites. To explore this, recombinant segments of FH were used. Initially only the recombinant 3-domain segment CCP6-8 was available (small quantities kindly provided by Dr Simon Clark, University of Manchester or larger quantities made as described in methods section 2.15, by the method of Clark *et al.*, 2006). At a late stage in the project, additional recombinant proteins, CCP1-4 and CCP19-20 were kindly provided by Drs Janet Lovett and Stacey Bell, University of St Andrews (Bell, 2015). Additional CCP15 and CCP15-16 were available, but were destroyed in the lab fire of April 2016.

4.4.1 Binding of FHCCP6-8 to TNP- BSA and DNP-HSA coated wells

An expressed factor H FHCCP6-8 was used to find out whether this region of FH has the ability to bind to TNP- BSA and DNP-HSA. ELISA plates were coated with TNP-BSA or DNP-HSA individually. Then FHCCP6-8 was serially diluted and added to the coated wells: dilution started from 5ug/ ml – 0.0079ug/ml. The binding of CCP6-8 was detected by using polyclonal rabbit anti recombinant CCP6-8 and secondary antibody, anti-rabbit IgG-alkaline phosphatase conjugate. From figure 4.11 it can be seen that the recombinant FHCCP6-8 binds to both TNP-BSA and DNP-HSA. However, saturation is not reached at 5ug/ml, suggesting a relatively low affinity. 5ug/ml of CCP-6-8 would be equivalent in molar terms to about 33ug/ml of full-length FH, and as seen in chapter 3, figure 3.4, FH binding to TNP/DNP reaches saturation at about 10ug/ml FH.

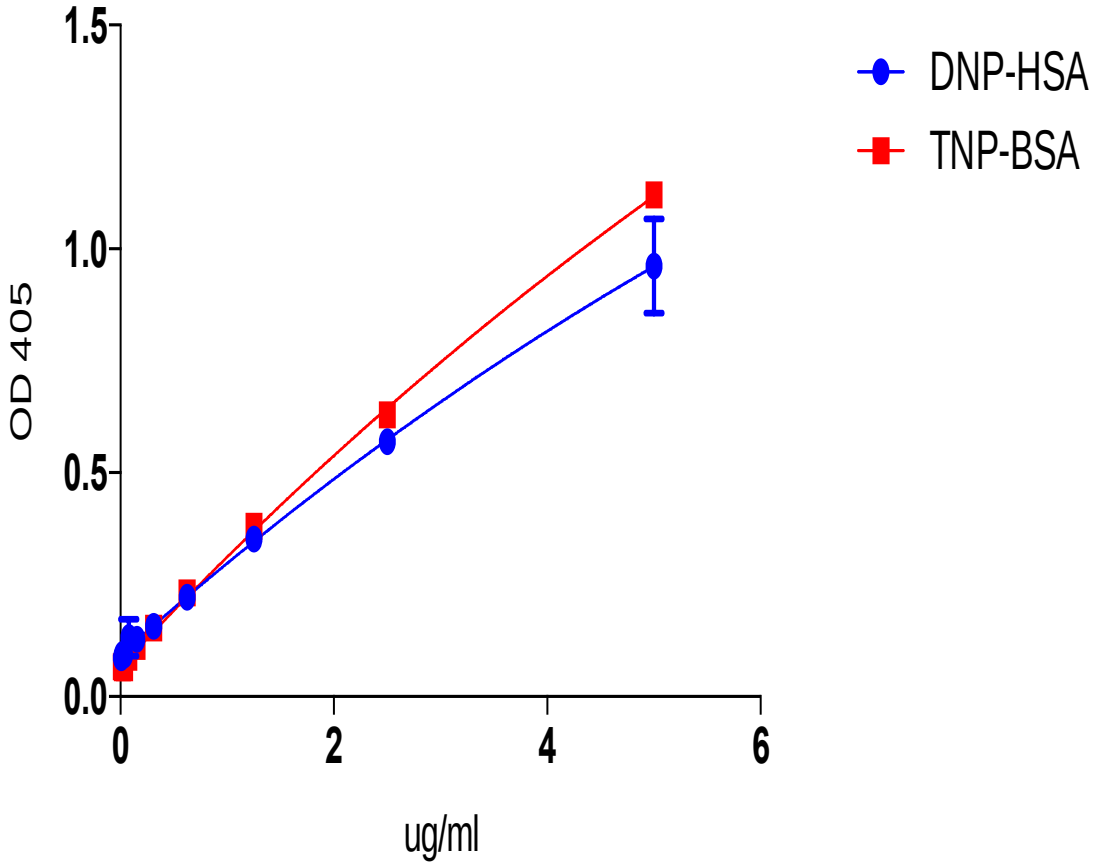


Figure 4-11 Binding of FHCCP6-8 to TNP-BSA and DNP-HSA.
This short segment FHCCP6-8 binds to TNP-BSA and DNP- HSA. The experimental data shown are the average of 3 experiments.

4.4.2 Competition between FH and CCP6-8 for binding to TNP-BSA and DNP-HSA

From the previous results it has been shown that FH binds to TNP-BSA and DNP-HSA and it has been shown that FHCCP6-8 also binds to TNP-BSA and DNP-HSA. In a further experiment we wanted to investigate whether there is more than one binding site on FH for TNP-BSA or DNP-HSA. Therefore, a competition assay was applied to reveal this binding. The usual coating of TNP-BSA or DNP-HSA was done on ELISA plates. After that a mixture of factor H containing 5ug/ml of FH and serial dilutions of FH CCP6-8 starting from 25ug/ml – 0.488ug/ml was added to the coated wells, as described in section 2.19.

Binding was detected by using anti whole FH antibody. From the results it can be said that at high concentration of FHCCP6-8, (25 ug/ml) there is very little inhibitory effect, which means that FHCCP6-8 is not the only binding site on FH for TNP or DNP. This is consistent with CCP6-8 having relatively low affinity for TNP and DNP. Even if, at 25ug/ml, CCP6-8 can saturate all the TNP or DNP on the plate, one or more other binding sites on FH must be able to displace the CCP6-8, by binding with higher affinity.

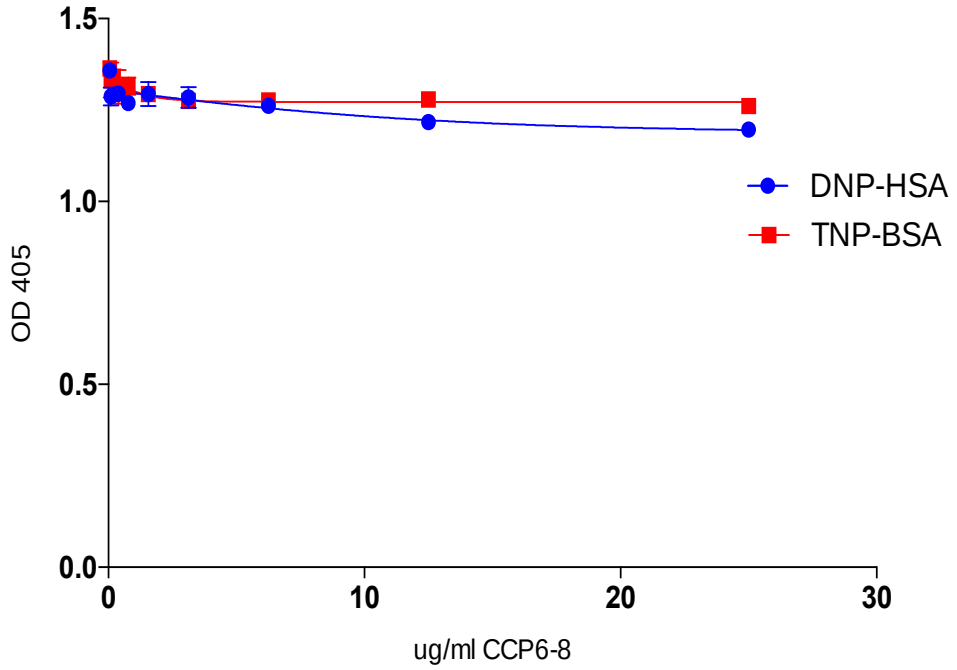


Figure 4-12 Competition between FH and FHCCP6-8 for binding to TNP-BSA and DNP-HSA.
This experiment was done as described in section 2.19. FHCCP6-8 did not compete effectively with factor H for binding indicating there is more than one binding site on factor H for TNP-BSA and DNP-HSA. The experimental data shown are the average of 3 experiments.

4.4.3 Binding of other FH segments to TNP or DNP

As noted above, recombinant CCP1-4 and 19-20 became available at a late stage in the project. Only a small quantity was available, but these were used in tests of binding to TNP-BSA and DNP-HSA. The results are shown in chapter 5, figure 5.10 where comparison with binding to Adrenomedullin is shown in the same figure. BOTH CCP1-4 and CCP19-20 were shown to bind to TNP and DNP, but there was insufficient material to explore affinity or competition binding. However it can be concluded that TNP and DNP bind to FH at least 3 sites, namely CCP1-4, CCP19-20 and CCP6-8, of which the last has quite low affinity.

Chapter 5

Results

Binding of FH to Adrenomedullin (ADM)

5 Binding of FH to Adrenomedullin (ADM).

A survey of the literature on FH-Adrenomedullin interaction (Sim *et al.*, 2015) suggested that the binding interaction with ADM is unusual, as shown by (Pío *et al.*, 2001). They used a western-blot based binding assay, and showed that it was difficult to dissociate ADM from the nitrocellulose-bound FH. Binding was dissociated by denaturants or the chaotrope 3M NaSCN, but not by 4M NaCl, or by acid at pH 2.5. Most ligand binding by FH is ionic in nature, such as binding to glycosaminoglycans, C3b, Factor I, anionic phospholipids and sialic acids (Sim *et al.*, 2015) so would be eliminated by high salt. ADM binding is, therefore, distinctive and presumably has a strong hydrophobic component. Even in 3M NaSCN, dissociation was slow and incomplete. The binding of FH to TNP and DNP has dissociation and hydrophobic characteristics superficially similar to this: and so it was considered worthwhile to investigate the similarities of binding to ADM and to TNP/DNP.

The ADM used in these experiments was synthetic, purchased from a peptide-synthesis company (GeneCust (Europe) based in Luxembourg). The product was correctly amidated at the C-terminal and >70% pure, but other (limited) analysis data supplied by GeneCust was destroyed in the lab fire of April 2016.

5.1 Binding assays of factor H to Adrenomedullin

5.1.1 Optimisation of FH binding to Adrenomedullin

The aim in these experiments is to see whether human factor H and recombinant segments of FH bind to AM. Preliminary experiments were done to determine a suitable plate coating concentration for AM.

First Adrenomedullin was serially diluted to explore the best concentration for binding to factor H. In figure 5.1 it can be seen Adrenomedullin binds better at low concentrations so 0.5ug/ml was selected as a suitable concentration to use in the experiments.

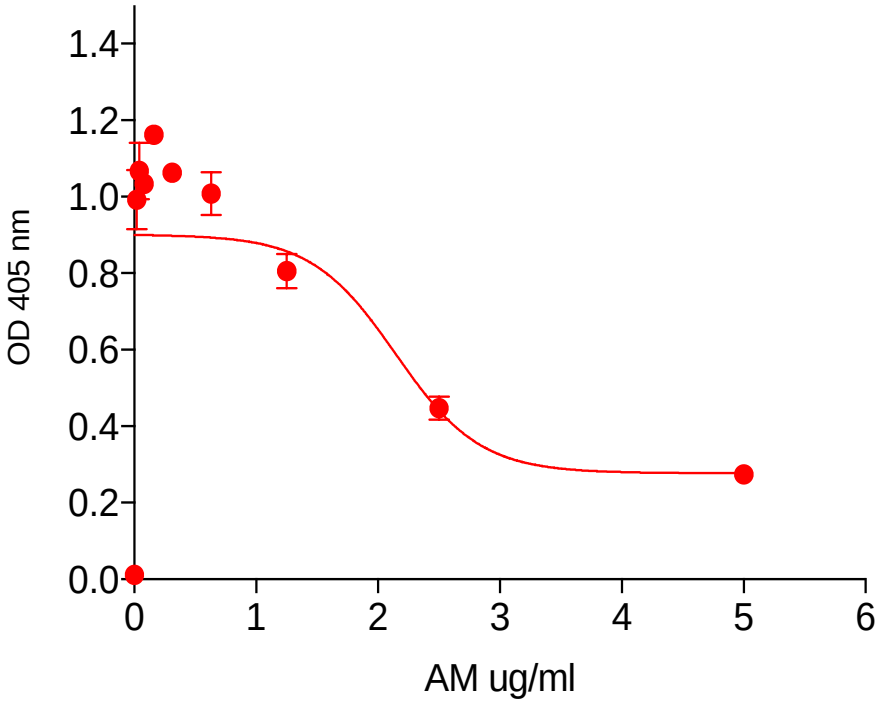


Figure 5-1 Figure 5.1 Optimisation of ADM coating for binding FH.
Adrenomedullin was serially diluted and added to microtiter plates, dilutions starting from 5 ug/ml – 0.05ug /ml. After washing and blocking, 5ug/ml of factor H was added to the coated wells. After 1 hr incubation and washing, primary and secondary antibodies were added. Adrenomedullin binds better in lower concentrations to FH. The experimental data shown are the average of 3 experiments.

5.1.2 Binding of whole factor H to Adrenomedullin coated wells

Human factor H was tested for its binding to Adrenomedullin. ELISA plates were coated with ADM at the optimum concentration determined above (0.5 ug/ml), then human factor H was serially diluted and added to coated wells. Figure 5.2 shows that factor H binds in a dose-dependent manner, and that saturation is reached at about 3 ug/ml FH.

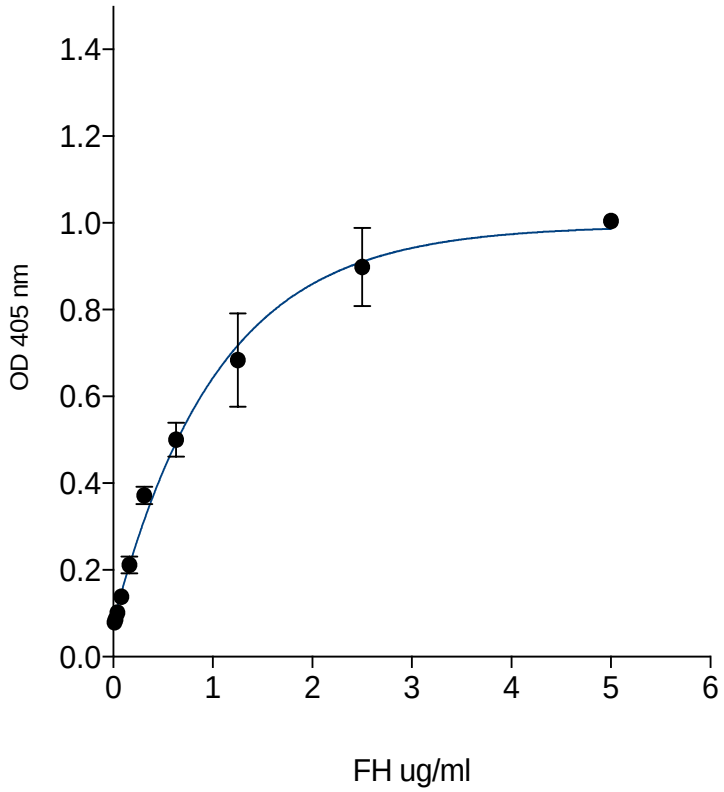


Figure 5-2 Binding of whole factor H to Adrenomedullin.
Factor H was serially diluted (dilutions from 5 ug/ml- 0.02 ug/ml) and then added to 0.05 ug/ml ADM coated wells. From the figure above it can be said that binding of FH to ADM is dose-dependent. The experimental data shown are the average of 3 experiments.

5.1.3 Dissociating the binding between FH and Adrenomedullin using different buffers and denaturants

Previous results in the literature imply that factor H binds strongly to AM. The next experiment was designed to explore the best solvent to dissociate the binding between FH and ADM, and to compare this with results for TNP and DNP, which were shown in chapter 3 (Figure 3.13). ELISA plates were coated with ADM; then FH was added to the coated wells and left to bind. Unbound FH was washed away, and then various solvents were added to wells, as mentioned in methods section (2.20.3).

These were the same solvents as used for TNP and DNP (Figure 3.13). For convenience in comparing the results, the TNP and DNP results are shown again in Figure 5.3 (A shows TNP, B shows DNP, and C shows the results with ADM). Although strong denaturing solvents were used to release the bound factor H from ADM coated wells, none were really effective in dissociating the binding. About 50% of bound FH was eliminated by using 1% SDS or 6M urea. 8M Guanidine removed only about 15 %. The results are quite similar to those for TNP-BSA and DNP-HSA, but low pH seemed slightly more effective for ADM.

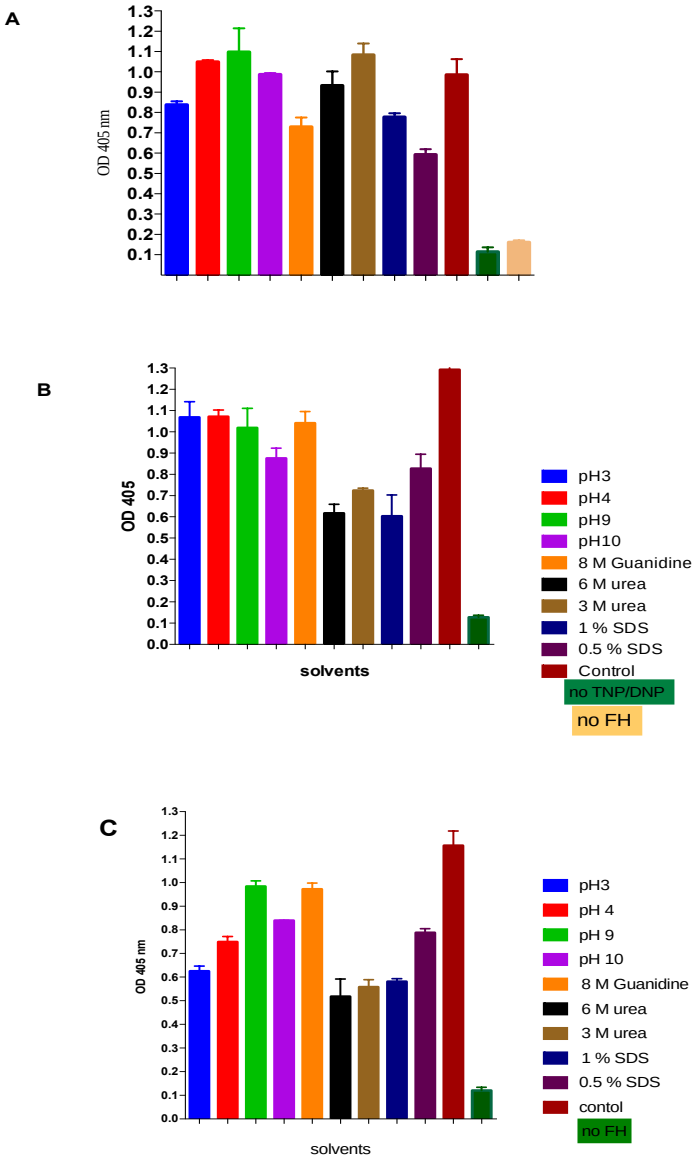


Figure 5-3 Dissociation of the binding between FH and (A) TNP-BSA and (B) DNP-HSA and (C) ADM by using different solvents.

Factor H was added to TNP-BSA, DNP-HSA or ADM- coated wells, after that wells were washed and treated for one hour with solvents and denaturants to elute the bound FH. Remaining FH was detected by antibodies. Positive control represents normal binding of FH to the three ligands (washed only with HEPES buffer). From the graphs it can be seen that dissociation of the binding between FH and Adrenomedullin by different solvents is quite similar to the effects seen with TNP and DNP. Urea and 1% SDS are the best "eluents" in each case. Extremes of pH are not effective, although the FH-ADM binding is more disrupted at low pH than is the binding to TNP or DNP. The experimental data shown are the average of 3 experiments.

5.1.4 Inhibition of the binding between FH and Adrenomedullin by using soluble compounds.

Seventeen soluble compounds were tested to examine their activity on the binding between FH and ADM. Experiments were done as described in sections 2.20.4, 2.9 and 2.9.1. As shown in figure 5.4 some of these compounds gave a high degree of inhibition of the binding. These were spermine, heparin, quinidine sulphate, *myo*-inositol hexakisphosphate, and ellagic acid. Bilirubin and 2,4 DNP had little effect, while others gave intermediate effects. To compare these results with what was found for inhibition of binding of FH to DNP-HSA,figure 4.3 from chapter 4 is repeated below (see figure 5.5). The spectrum here is quite different however: the most effective inhibitors of the DNP-FH interaction are phosphotyrosine, ATP, bilirubin, BOC-tyrosine, and also ellagic acid and spermine. Only the last two named inhibited both binding reactions well. The spectrum of inhibition is therefore quite different, suggesting that AM and DNP do not bind to the same sites on FH, although they may bind to the same general regions, eg to the same CCPs.

Ellagic acid is a large polyphenol, uncharged but with multiple OH groups, which is reported to bind to many proteins and to DNA (Whitley *et al.,* 2003), so its effect is likely to be quite "nonspecific". Spermine is a non-aromatic "polycation" (2 positive charges), while myoinositol hexakisphosphate is a non-aromatic polyanion. These may interfere with initial binding contacts, which may be ionic. Heparin is known to bind to FH CCPs 6-8 and 19-20 and possibly other sites, and it interferes with ADM binding, but surprisinly not with DNP binding since, as shown in chapter 4, DNP/TNP binds to CCPs6-8 and 19-20. Bilirubin, a tetrapyrrole with positive and negative charges, is an inhibitor of DNP-FH binding, but has no effect on ADM-FH. This experiment was done as described in sections 2.8 and 2.20.3. Further experiments were done to examine the concentration-dependence of the compounds that gave substantial inhibition at the single dose shown and the results are in figure 5.6. Dose-dependence is clear for ellagic acid, myoinositol hexakisphosphate, but the inhibition curve is rather "flat" for heparin and spermine.

In table 5-1, a comparison of inhibitors of FH binding to ADM, and to DNP is shown.

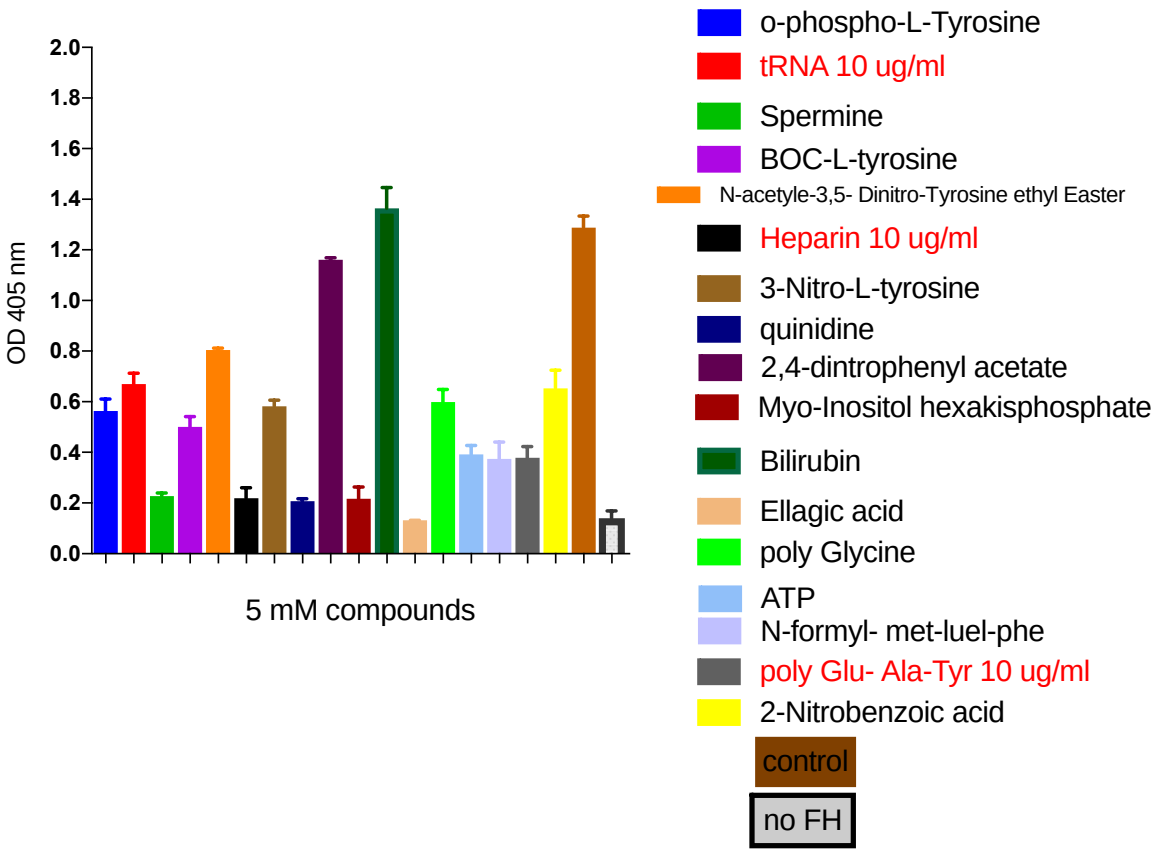


Figure 5-4 Inhibition of the binding between FH and Adrenomedullin.
ELISA plates were coated with ADM and compounds were tested at final concentration 5mM (except those indicated as 10ug/ml) to see if they could inhibit FH-ADM binding. The control (see figure) represents uninhibited binding, and the "no FH" column represents background signal with no FH present. . The experimental data shown are the average of 3 experiments

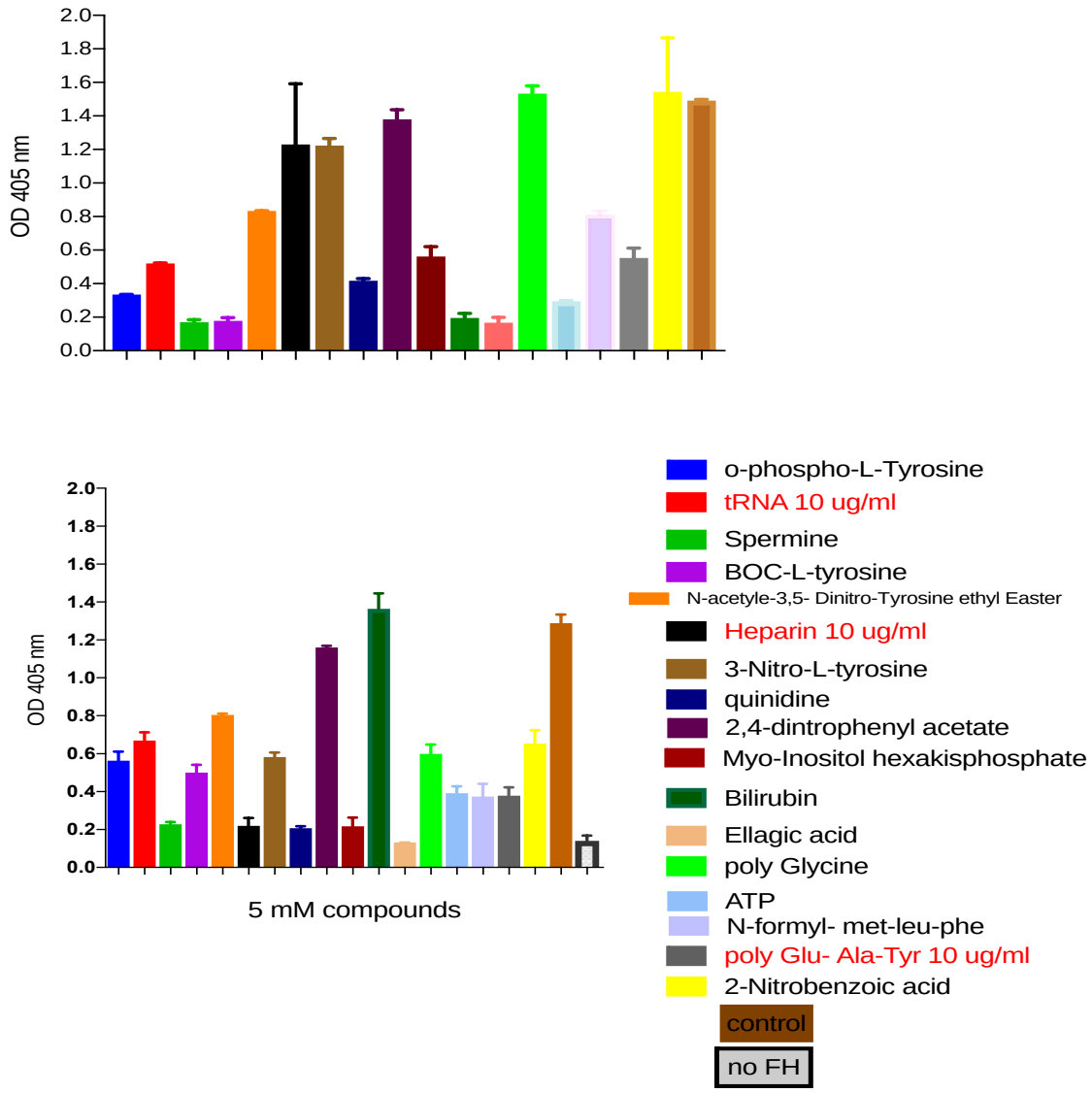


Figure 5-5 Comparison of the inhibition of binding of FH to DNP, with that to ADM.
The lower figure is the inhibition of binding to AM, and is a repeat of figure 5.4. The upper part of the figure shows inhibition of binding to DNP-BSA and is a repeat of figure 4.3.

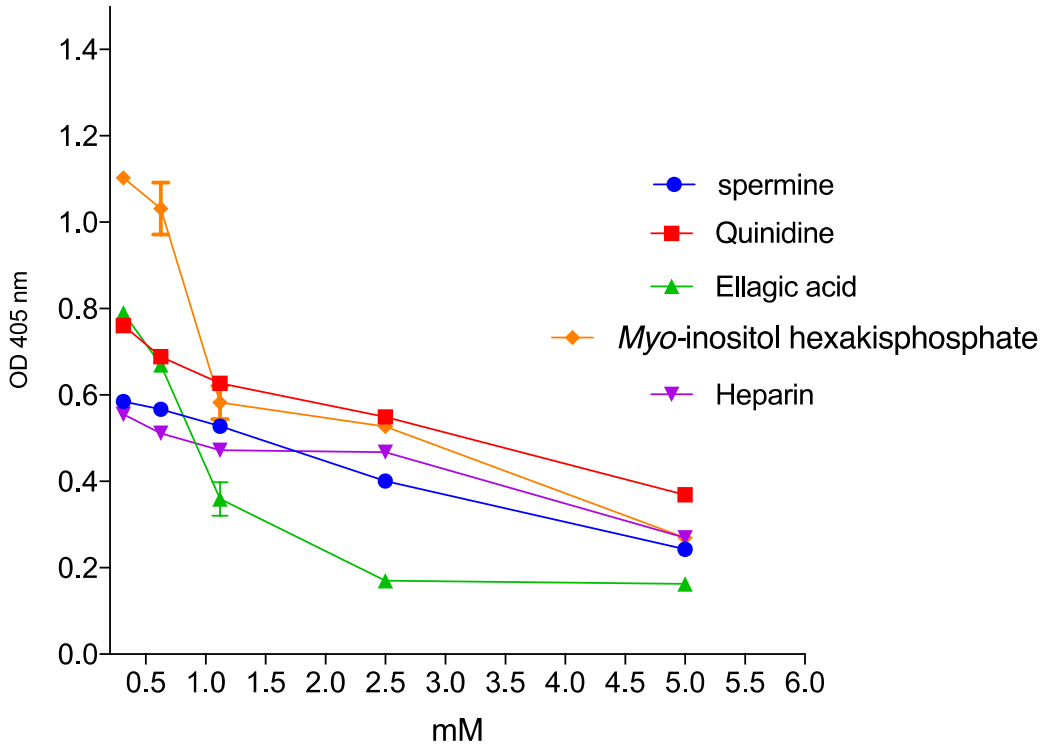


Figure 5-6 Dose-dependence of inhibition of FH binding to ADM.
Selected inhibitors were tested in serial 2-fold dilutions, starting at 5mM (or 10 ug/ml for heparin). From the graph it can be seen binding becomes less at higher inhibitor concentrations, indicating concentration dependence. The experimental data shown are the average of 3 experiments.

Table 5-1 Comparison of inhibition of binding to DNP and binding to ADM

Compound	Inhibition of binding to DNP	Inhibition of binding to ADM
O-Phospho-L-tyrosine	***	***
N-acetyl-3,5-Dinitro-tyrosine-ethyl ester	*	***
ATP	***	***
N-Formyl-Met-Leu-Phe	*	***
Bilirubin	****	–
Poly (glu(60)ala(30)tyr(10)	**	***
Myo-inositol hexakisphosphate	**	****
Spermine	****	****
tRNA	**	**
para-Nitrophenyl acetate	–	ND
Boc-L-tyrosine	****	***
Quinidine	****	****
Ellagic acid	****	****
polyglycine	-	**
Heparin	–	****
2-Nitrobenzoic acid	***	**
2-dinitrophenyl acetate	–	*

– = No inhibition, *=weak inhibition, **= moderate inhibition, ***= good inhibition, ****= strong inhibition, ND= not done.

The table shows approximate effectiveness of inhibition of binding of FH to DNP (same data as in Table 4.1) and to ADM (taken from the results in fig 5.4.). There are large differences in inhibition of binding to the 2 ligands by dinitrotyrosine, f-Met-Leu-Phe, heparin, myoinositol hexakisphosphate and bilirubin. Phosphotyrosine, ATP, spermine, BOC-tyrosine, quinidine sulphate, ellagic acid all inhibit both bindings quite effectively. However the large differences strongly suggest either that DNP and ADM do not have shared binding sites on FH, or that some of the inhibitory effects seen are due to inhibitor binding to DNP-HSA or ADM, and not to FH.

5.2 Testing of the antisera (anti-CCP 6-8, anti-CCP 3-4 and anti-FHR1) to ensure that they will detect CCP6-8, CCP1-4 and CCP 19-20 in ELISAs

The test shown in figure 5.7 was done to determine a suitable concentration of the antibodies to give a good signal in ELISAs and to examine whether these antibodies are able to recognise these fragments. This experiment was done as described in methods section 2.20.5.

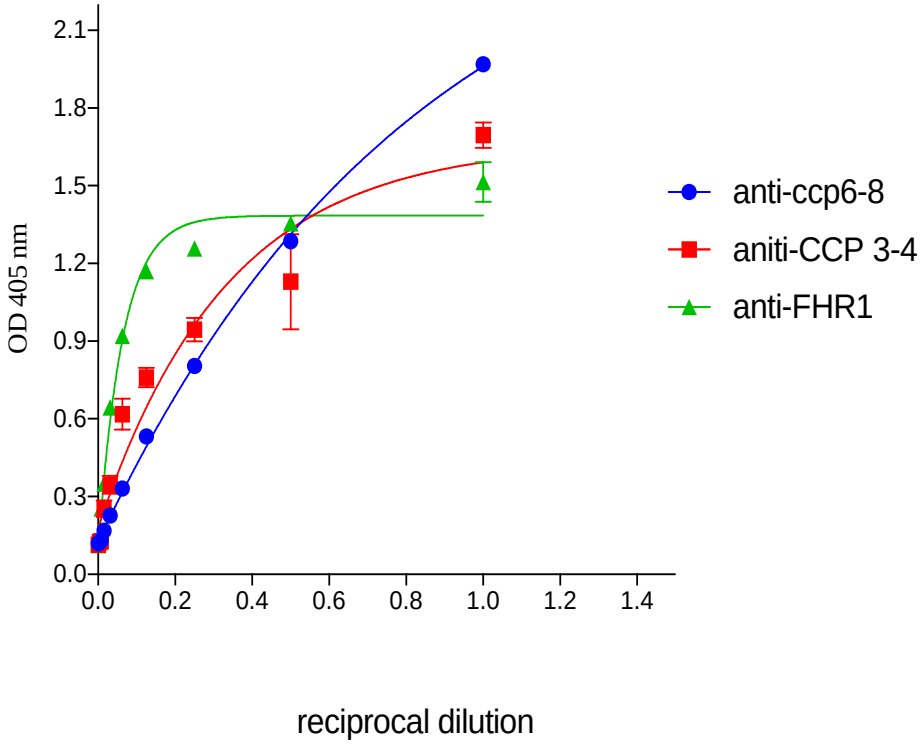


Figure 5-7 Testing of antisera (anti-CCP 6-8, anti CCP 3-4 and anti FHR1) to fragments (CCP6-8, CCP1-4 and CCP 19-20).
ELISA plates were coated with 5ug/well CCP6-8, or CCP1-4 or CCP19-20 then the compatible antibodies were added to the coated wells in serial dilutions, starting at 1/10000. The same secondary antibody, anti-rabbit IgG, AP conjugate was used for all. The antisera show good recognition towards the constructs. Cross-reactivity was not explored. The experimental data shown are the average of 3 experiments. The x-axis shows reciprocal dilution, which corresponds to 10000 divided by dilution factor= reciprocal dilution. Thus 10000-fold dilution =1, 20000 fold dilution =0.5, etc.

5.2.1 Binding of FHCCP6-8/H402 or FHCCP6-8/Y402 to Adrenomedullin coated wells

A limited quantity of both variants of CCP6-8, containing either histidine or tyrosine at amino acid position 402 were available from Dr Simon Clark, Manchester University. Note in some papers, the 402 amino acid position is identified as 384: this is the same position, but numbered with the leader sequence subtracted.

Recombinant proteins FHCCP6-8/H402 and FHCCP6-8/Y402 were serially diluted from 20ug/ml maximum in 10mM HEPES, 120 mM NaCl, 0.5mM EDTA and they were added to ADM coated wells. Adrenomedullin coated wells were prepared with 0.5 ug/ml ADM in coating buffer. Dilutions were loaded onto Adrenomedullin-coated wells and incubated for 1 hour at RT. After washing, rabbit anti FHCCP6-8 was added (1/5000) and after incubation for 1 h and washing, secondary antibody conjugate, and subsequently substrate, was added. FHCCP6-8/H402 and FHCCP6-8/Y402 have exactly similar behavior in binding to ADM. So the Tyr/ His interchange has no influence on binding ADM.

It was also confirmed that the recombinant CCP6-8 protein made in Leicester behaved identically to the materials provided by Dr Simon Clark.

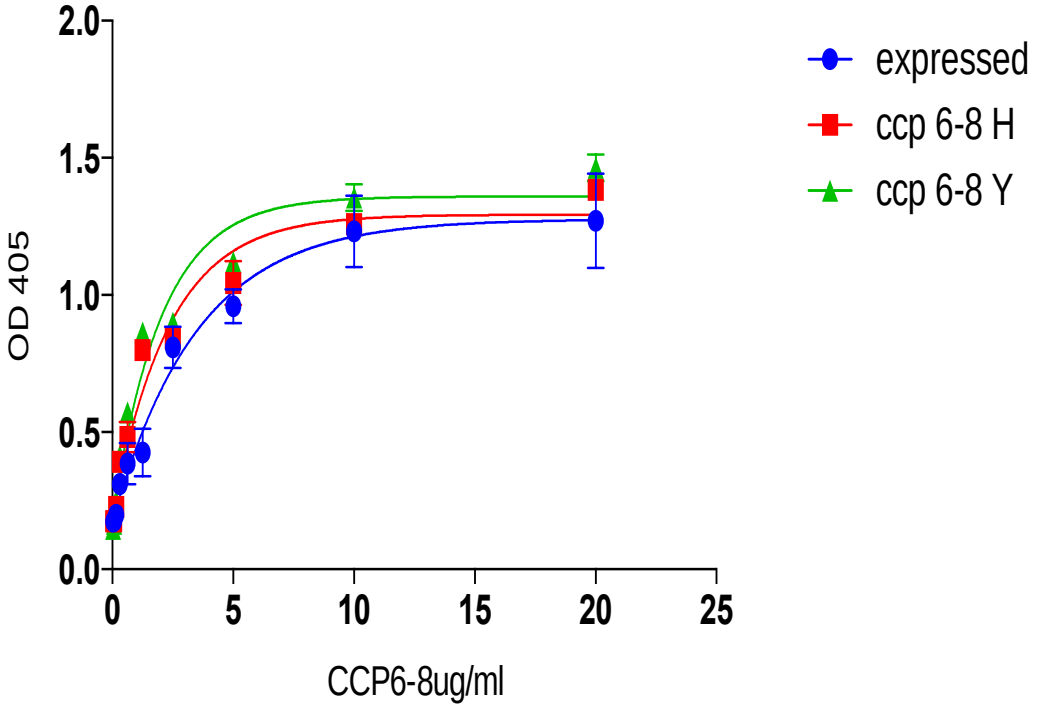


Figure 5-8 Binding of FHCCP6-8 H/402 or FHCCP6-8 Y/402 to Adrenomedullin coated wells.
Both recombinant proteins bind to ADM almost identically. The experimental data shown are the average of 3 experiments. CCP6-8Y and CCP6-8H were proteins donated by Dr Simon J Clark, Manchester University. "Expressed" refers to the protein made in Leicester, which was the CCP6-8Y form.

5.2.2 Competition between FH and FHCCP6-8 for binding to Adrenomedullin coated wells

Adrenomedullin coated wells were prepared as described in section 2.22. For the competition assay a mixture of factor H (5ug/ml) and serial dilutions of recombinant protein FHCCP6-8Y (made in Leicester), dilutions from 25 to 0.0 488ug/ml, were prepared in 10mM HEPES, 120mM NaCl, 0.5mM EDTA. This mixture was loaded into the ADM-coated wells and incubated for 1 hour at RT. After washing, rabbit anti factor H antibody was added followed later by anti-rabbit IgG AP conjugate. From figure 5.9 it can be seen that there is very substantial inhibition of the binding of FH to ADM by FHCCP6-8. Therefore, it appears that CCP6-8 may be the highest affinity-binding site on FH for ADM. It is unlikely to be the only binding site on FH as half-maximal inhibition requires a 3-4-fold molar excess of CCP6-8, and the inhibition seen in the figure does not appear to reach 100%.

This is in contrast to the findings with FH binding to TNP/DNP (figure 4.12), where CCP6-8 does bind TNP/DNP, but with affinity too low to compete with FH for binding in an experiment of this type

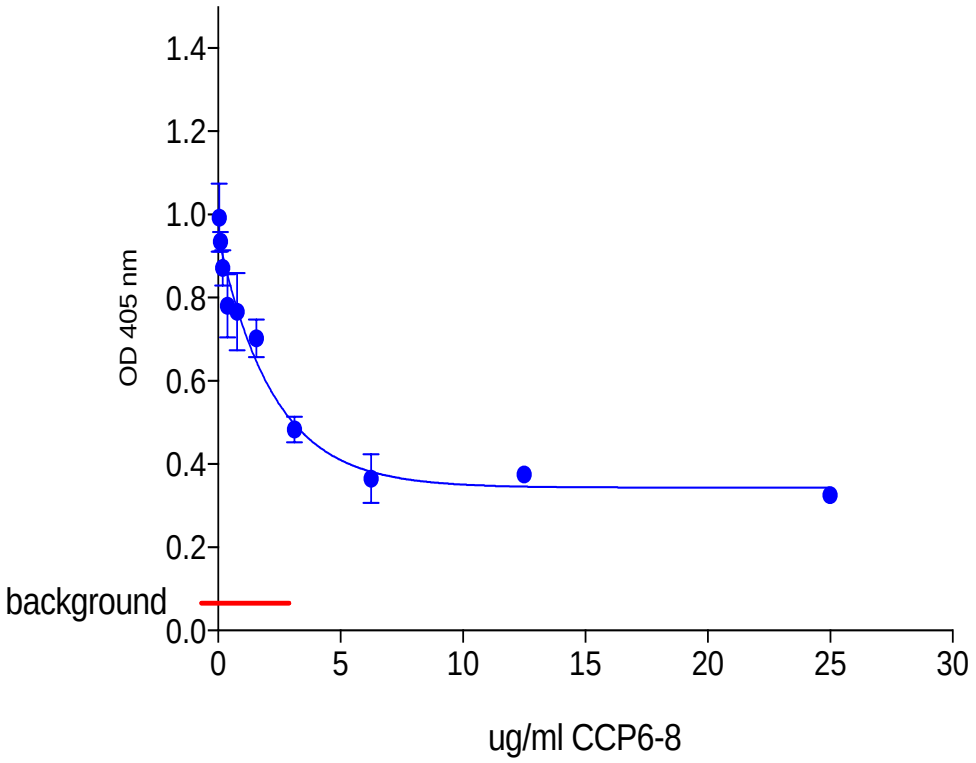


Figure 5-9 Competition between FH and FHCCP6-8 for binding to Adrenomedullin.
This experiment was done as described in section 2.22. and shows that recombinant FHCCP6-8 competes with factor H for the binding sites on Adrenomedullin.
At high concentration of FHCCP6-8 more inhibition occurs. The background was measured from samples where no FH but only antibodies were added. The inhibition appears incomplete, relative to the background, possibly indicating more than 1 binding site on FH for ADM. The experimental data shown are the average of 3 experiments. Half-maximal inhibition occurs at 2-3 ug/ml CCP6-8, which is a molar excess of about 3-4-fold over FH (5ug)

5.2.3 Binding of constructs CCP1-4 and CCP19-20 to Adrenomedullin, DNP-HSA and TNP-BSA

Our previous results showed that the recombinant CCP6-8 binds effectively to Adrenomedullin. It also binds to DNP-HSA and TNP-BSA, but with low affinity. To obtain data about the other available recombinant CCPs of factor H an experiment was designed to find out whether CCP1-4 and CCP19-20 could bind to Adrenomedullin and to DNP or TNP albumins. At a late stage in this research, limited quantities of recombinant CCPs 1-4 and 19-20 were kindly provided by Drs Stacey Bell and Janet Lovett, St Andrews University. Plates were coated with Adrenomedullin or DNP-HSA or TNP-BSA then constructs were added separately to the coated wells at a single concentration of 5ug/well (in triplicate). The quantities of CCP1-4 and 19-20 available were not sufficient for more extensive testing. Results show that CCP 1-4 binds to DNP-HSA and TNP-BSA but not to Adrenomedullin, however, CCP19-20 binds to all of the materials.

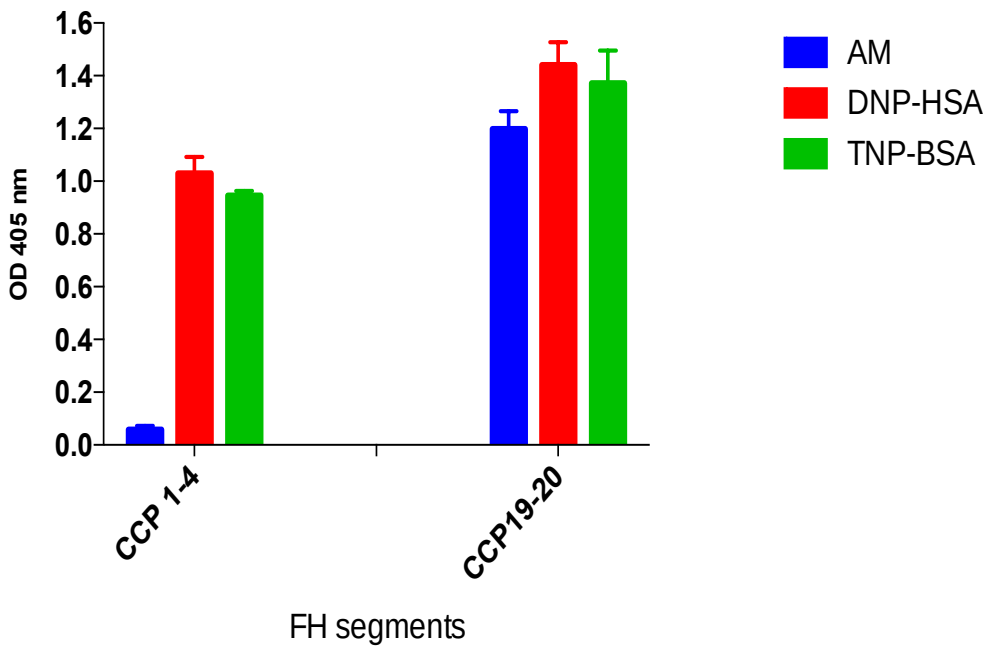


Figure 5-10 Binding of CCP1-4 and CCP19-20 to Adrenomedullin, DNP-HSA and TNP-BSA. ELISA plates were coated with those materials and then constructs of factor H were presented. CCP19-20 shows good binding to these materials, while CCP 1-4 binds only to DNP-HSA and TNP-BSA but not to Adrenomedullin. The experimental data shown are the average of 3 experiments. On the figure, AM=adrenomedullin

5.2.4 Use of antibodies to influence binding reactions of FH

It was considered that potentially the polyclonal antibodies against CCP3-4 or CCP6-8 or the anti-FHR1 might inhibit (singly or in combination) the binding of FH to DNP/TNP or ADM. A preliminary experiment was done (figure 5.7), but unfortunately loss of all the reagents in the lab fire, April 2016, prevented any follow-up. Microtiter plates were coated with Adrenomedullin, or DNP-HSA or TNP-BSA. FH was then added to the plates, and unbound FH washed away. The anti CCP3-4, and anti-FHR1 were added to the wells, to see if A) they could detect the bound FH; or B) they could displace the bound FH. From the graph it can be seen that factor H can be detected by anti CCP3-4 and anti FHR1, and so they probably do not displace the FH.

Further experiments, pre-treating FH with mixtures of the 3 antibodies (in the form of purified IgG) might be a feasible way of confirming the regions of FH involved in binding.

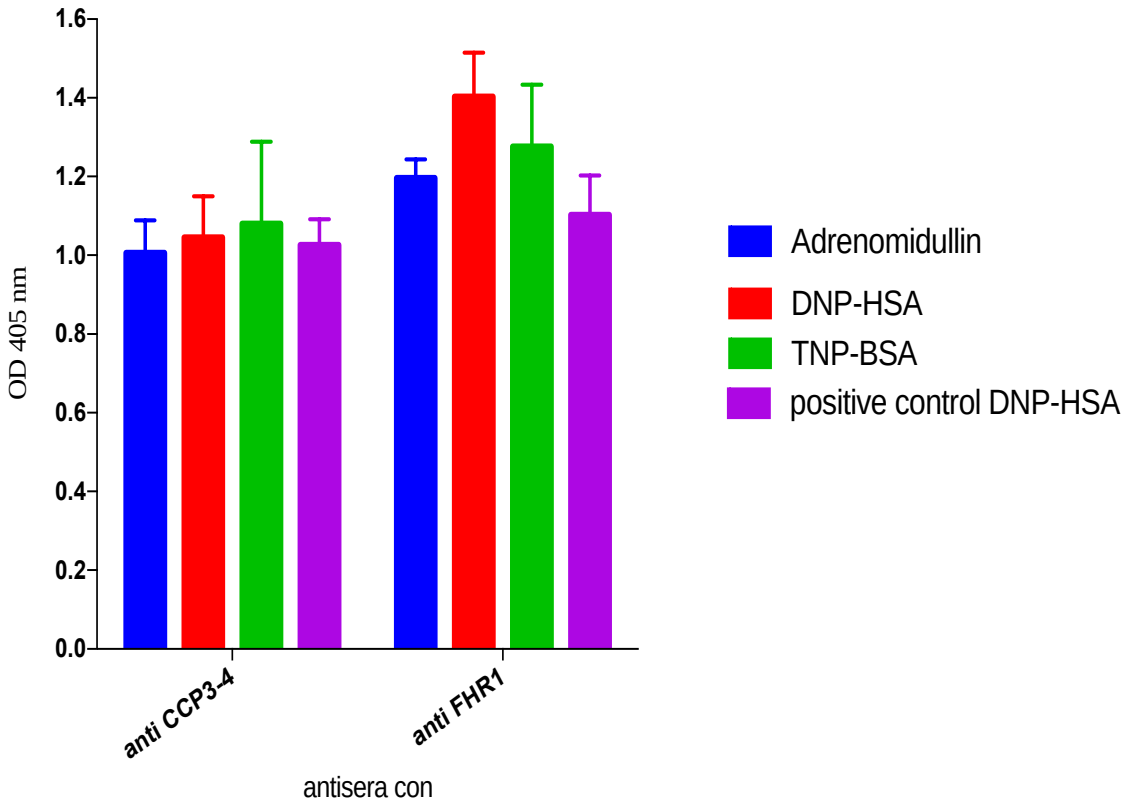


Figure 5-11 Confirmation that anti CCP3-4 and anti-FHR can bind to ligand bound FH.
Plates were coated with ADM, or DNP-HSA or TNP-BSA as in previous experiments. Then 5 ug/ml of FH was added to the wells and incubated for 1 hr. After washing, binding of factor H was detected by two different polyclonal antibody preparations:anti CCP-3-4 and anti FHR1 (both purified IgG) were used at 1/5000, (approximately equal to 10 ug/ml). Binding of FH is detected by both antibodies. It can be said here that these antibodies can bind to ligand- bound FH but they probably do not significantly displace FH. The experimental data shown are the average of 3 experiments.

5.3 Do Adrenomedullin, DNP-HSA and TNP-BSA inhibit factor H function?

5.3.1 Adrenomedullin.

Our findings showed that factor H binds strongly to Adrenomedullin, DNP-HSA and TNP-BSA. To investigate whether these materials have any effect on factor H function an experiment to test the fluid-phase factor I-cofactor function for the breakdown of C3b was set up. This procedure (based on Sim and Sim, 1983) measures the cleavage by factor I of C3b to form iC3b. This reaction requires FH as a nonenzymic cofactor, and within limits, the rate of reaction is dependent on FH concentration. The cleavage is observed by SDS-PAGE analysis of reduced C3b/iC3b as described in methods section 2.24. Firstly, optimizing the cleavage time of C3b was done by incubation, in a volume of 20ul, a mixture of 5ug of C3b with 1 ug of factor I and 10 ng of factor H all in 20mM HEPES, 120mM NaCl, 0.5mM EDTA pH 7.4, for five time points: 2, 3, 5, 24 and 48 hour. From figure 5.12 the best time at which α-chain is substantially but not completely cleaved, was selected (3 hours). After choosing the optimal time of incubation another experiment was set up, this time adding Adrenomedullin, DNP-HSA or TNP-BSA to the reaction. Thus factor H and Factor I were pre-incubated at the beginning with serial dilutions of Adrenomedullin, or DNP-HSA or TNP-BSA for 30 min at room temperature, and after that C3b was added to the mixture and incubated to at 37°C for 3 hr. Reaction was stopped by adding an equal volume of gel sample buffer (0.2M tris, 8M urea, 2% SDS, pH 8.2), then samples were run, reduced on SDS-PAGE (see figures 5. 13 and 5.14)

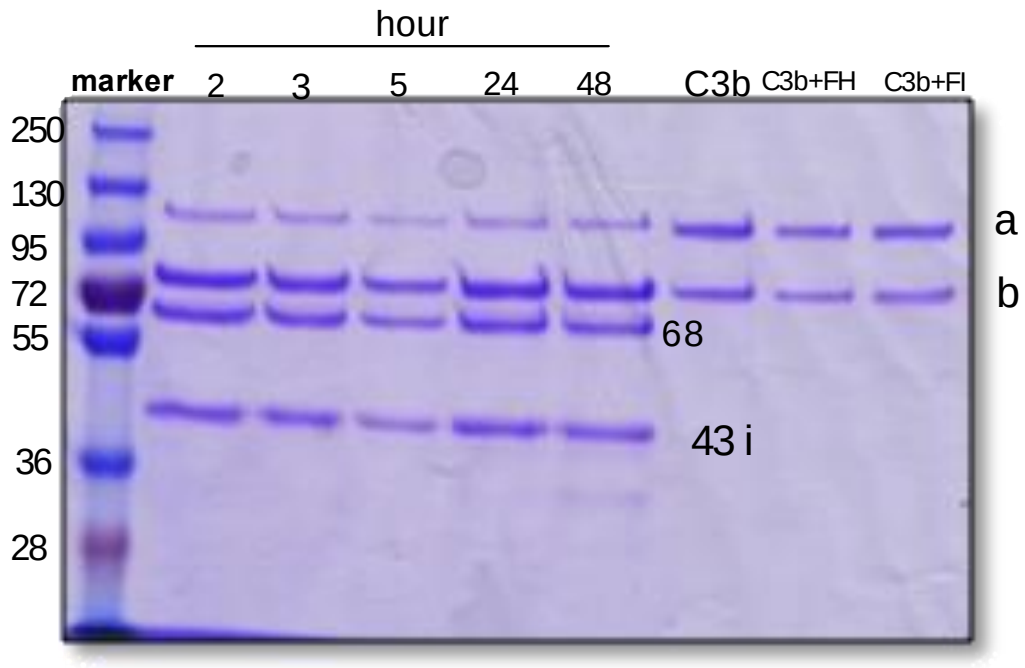


Figure 5-12 Optimizing C3b cleavage by factor I in the presence of factor H.
Inactivation of C3b by FI requires cofactor function of FH. α-Chain of C3b molecule is cleaved producing two shorter chains: α 68 and α 43. Cleaving of α-Chain was incomplete after 48 hour of incubation, probably because a proportion of the C3b was denatured (inactive). A suitable degree of cleavage of α-Chain occurs at 3 hours. The lanes correspond to 2, 3, 5, 24, 48 hr incubation. On the right are 3 negative controls incubated for 48h: C3b represents C3b with no FH or FI, C3b+FH contains no FI, and C3b+FI contains no FH. The quantities of FH and FI in the mixture are not sufficient to be seen with Coomassie Blue staining. The alpha, and beta chains of C3, and the 68 and 43kD fragments of iC3b are shown.

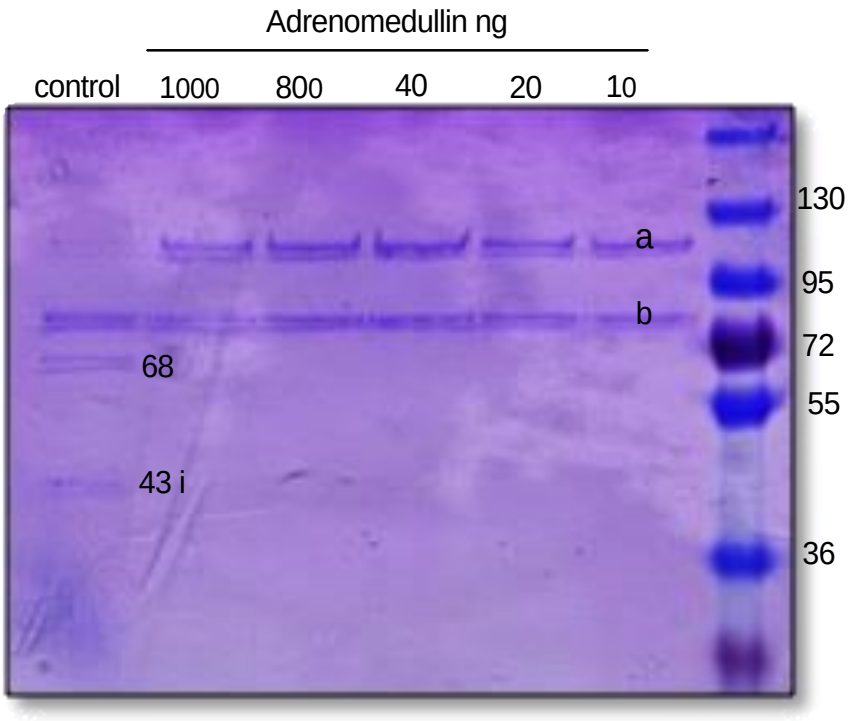


Figure 5-13 Inhibition of factor H function by Adrenomedullin.

Serial dilutions of Adrenomedullin were added to a mixture of FI and FH for 30 minutes. Then C3b was added and the reaction continued for 3hr. Here FH concentration is 10ng per reaction mix, and the minimum concentration of ADM is 10ng/reaction. Since ADM has mol wt about 5.7kDa and FH is 155 kDa, the ADM is at a minimum of 25-30-fold molar excess**.** The control (left) has 0 ADM, and the quantity of ADM (ng) is shown for each lane (top). . The alpha, and beta chains of C3, and the 68 and 43kD fragments of iC3b are shown.

In Figure 5.13, it can be seen that with no ADM (the control) C3b is almost completely converted to iC3b. However at all concentrations of ADM tested, cleavage is completely inhibited. To examine dose dependence more closely, a new experiment was set up using less Adrenomedullin. The gel (figure 5.14) shows that at the 2 higher doses of

Adrenomedullin C3b cleavage is completely stopped, but at lower doses, close to one to one molar ratio, (Factor H: Adrenomedullin), inhibition is incomplete.

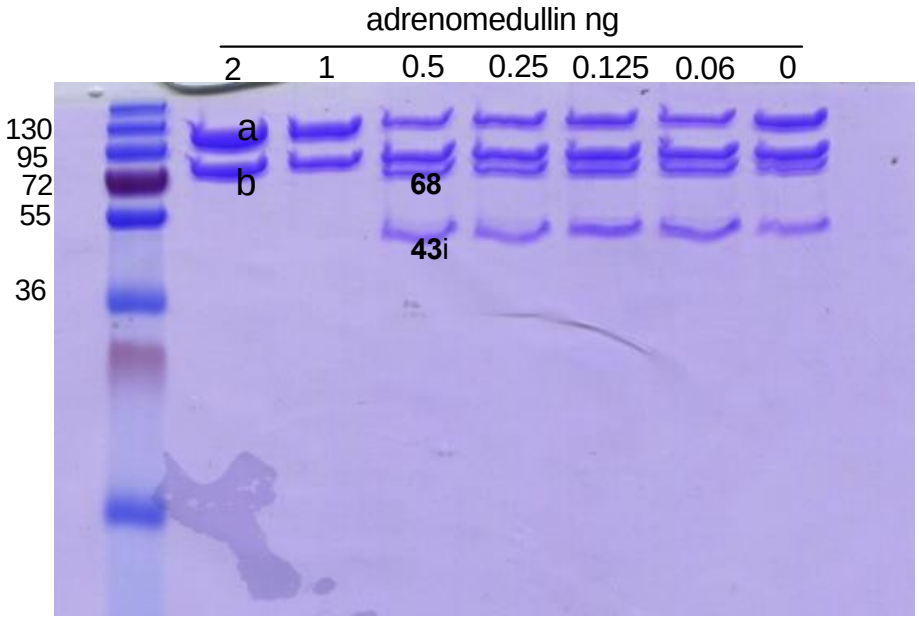


Figure 5-14 Dose dependent inhibition of C3b cleavage by adrenomedullin.
Factor H was incubated with serial dilutions of ADM including the near equivalent of a 1:1 molar ratio. At dose where FH molecules are fewer than ADM complete inhibition occurs, but where molecules numbers of FH and ADM are similar or FH is in excess, cleavage occurs. Here 2 ng ADM represents about a 5-fold molar excess over FH, and at 0.25 ng ADM, there is <1 molecule of ADM per 1 molecule of FH.

5.3.2 TNP and DNP.

Further similar experiments were done to assess inhibition by DNP-HSA or TNP-BSA. As shown in figure 5.15, inhibition by DNP albumin is exceptionally potent. At concentrations where there is only about 1 molecule of TNP or DNP albumin per 400 FH molecules, inhibition is still almost complete. This strongly suggests that the inhibition occurs because the TNP/DNP albumins cause aggregation (clustering) of the FH, and perhaps has nothing to do with blockage of specific binding sites. The commercial product, DNP-HSA (Sigma) is specified as having 30-40 DNP per molecule of albumin. This means that one molecule of the DNP albumin could bind a large number of FH molecules, potentially more than 20,

if the FH bound only by the highest affinity site. These clusters could be cross-linked by other DNP-albumin molecules to form very large (and inactive) aggregates. Aggregation alone would decrease activity by altering kinetics of diffusion in the solution. Similar results were obtained for TNP-BSA, but the gels were lost in the lab fire of April 2016.

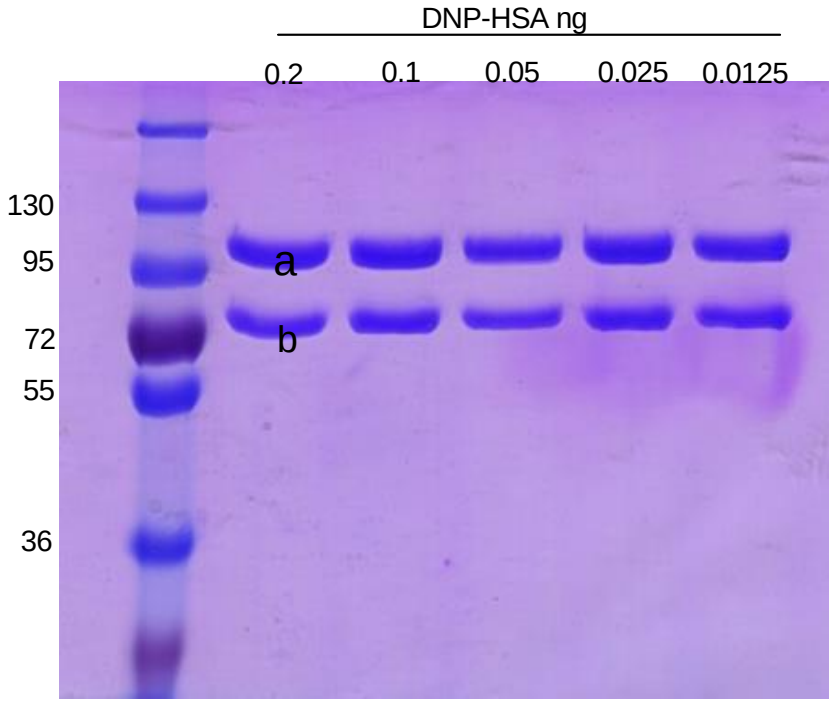


Figure 5-15 Inhibition of factor H function by DNP-HSA.
Serial dilutions of DNP-HSA were added to the mixture of FI and FH for 30 min. After that 5 ug of C3b was added to the mixture and incubated at 37 °C for 3 hours. It can be seen from the gel that DNP-HSA prevents cleavage of C3b by factor I in presence of FH. In this figure the CONTROL (no inhibitor) was the same as that in Fig 5.14 (ie same tube, same experiment). Here there are 10ng FH per reaction. DNP-HSA has mol. wt approx. 75kDa, so 5ng of DNP-HSA would represent equal numbers of molecules of FH and DNP-HSA. 0.25ng DNP-HSA therefore represents only 1 molecule of DNP-HSA per 20 molecules of FH, and 0.0125 ng represents only 1 molecule of DNP-HSA per 400 molecules of FH.

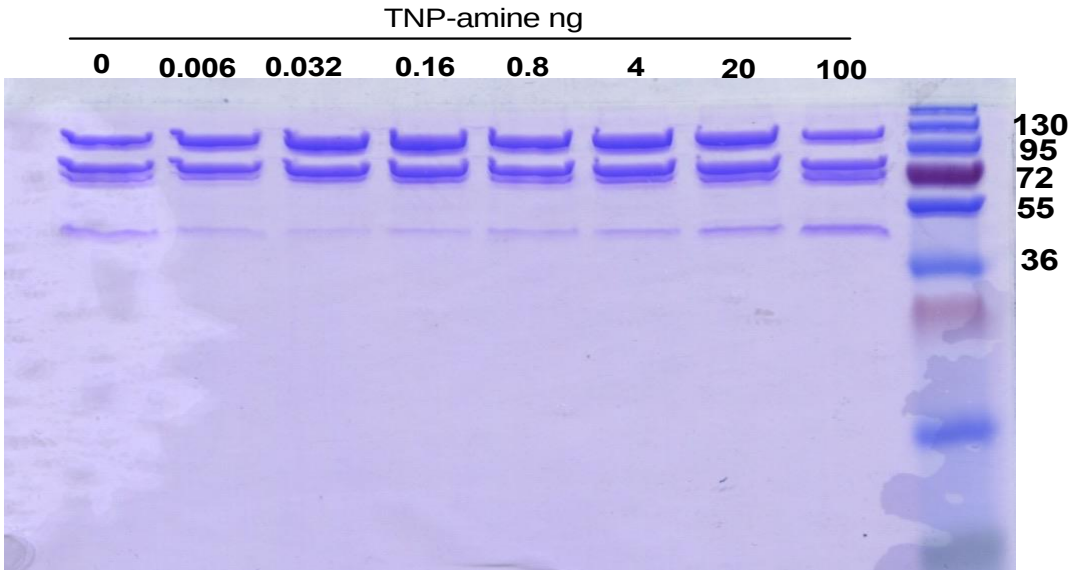


Figure 5-16 Inhibition of factor H function by TNP-amine.
Dilutions of TNP-amine were added to the mixture of FI and FH for 30 min. After that 5 ug of C3b was added to the mixture and incubated at 37 °C for 3 hours. It can be seen from the gel that TNP-amine has negligible effect on the cleavage of C3b. Here there are 10ng FH per reaction, and the maximum quantity of TNP-amine used represents about 600-fold molar excess.

The concept that the inhibition seen in figure 5.15 is due to aggregation and not to blockage of specific sites is supported by results obtained with TNP-amine (Figure 5.16). Here, even at several hundred-fold molar excess, there is little effect on C3b cleavage. As shown earlier, TNP-amine can bind FH, but presumably occupation of the TNP binding sites by a large molar excess of TNP-amine does not inhibit FH cofactor activity.

Chapter 6

Discussion

6 Discussion

6.1 FH binding to multiple ligands

6.1.1 Fibrinogen/Fibrin

In chapter 3 FH binding to multiple diverse ligands was explored. Fibrinogen/fibrin and TNP/DNP were explored in more detail. The binding to fibrinogen/fibrin has been shown to occur during the clotting of plasma and it has been speculated that it may be recognition of sulphation of fibrinogen: a proportion (about 30%) of circulating fibrinogen is sulphated (Sim *et al.,* 2008). The physiological role of binding to fibrin clots is not known, but could be involved with clearance (eg of small clots from circulation) or with limiting complement activation/inflammation by clots, which bind C1q and activate complement (Sim *et al.,* 2008).

Purified FH and FH from human serum were used to test whether other serum proteins have an influence on this interaction. Purified FH and FH from human serum show good binding to human fibrinogen. In an early study Horstmann *et al.,* (1992) indicated that FH binds to fibrinogen with high affinity using both plasma and purified FH. Binding of FH to fibrin-coated wells also has been examined; in chapter 3 purified FH shows greater binding to fibrin than binding of FH from human serum, and that difference in binding may be due to the interaction of other proteins in the serum with fibrin. Similar findings were obtained by Kang, (2006), who showed that factor H becomes covalently associated with fibrin clots by the action of FXIIIa (reported in Sim *et al.,* 2008). This was shown partly by using urea and SDS-urea washes. In the clot-urea wash assay shown in chapter 3, clots were firstly washed with normal physiological non-denaturant buffer followed secondly by denaturant containing 8 M urea and more than 80% of factor H remained bound. This suggests the proportion of factor H that is covalently bound is very high. However, washing with SDS-urea buffer did remove the FH signal, but this may have been due to insufficient wash-out of SDS.

6.1.2 2,4,6-trinitophenyl-bovine serum albumin (TNP-BSA) and 2,4-Dinitrophenyl-Human Serum Albumin (DNP- HSA)

Binding of FH to DNP and TNP-derivatised ligands has not been explored in much detail previously. DNP and TNP have a long history in immunology as HAPTENS. TNP and DNP are haptens used for antibody production because of their high immunogenicity (Mongini *et al.,* 1981). The term, "hapten," was coined by Landsteiner and Jacobs and is derived from the Greek "hapten", meaning, "to fasten." Haptens are low molecular weight (LMW; <1000 Daltons) chemicals that must bind to a carrier molecule to be antigenic. The carriers are usually proteins that bind covalently to the LMW species (Pichler *et al.,* 2006). The main reason for using covalent binding of a hapten to a protein is to study the immune recognition of the hapten in immunological studies but substantial evidence exists for this to be a prominent mechanism through which chemicals and drugs or their metabolites become antigenic (Chipinda *et al.,* 2011). Haptens have been used to boost immune responses to antigens, to study allergies including contact dermatitis (ACD) and inflammatory bowel disease (IBD) and to induce autoimmune responses, viral wart regression, and even antitumor immunity. Haptenated protein (bovine serum albumin (BSA) or ovalbumin (OVA)) were mainly used to induce strong immune responses in animal models to help unravel the basics of T- and B-cell-mediated responses (Paul *et al.,* 1967). Immunized BSA-tolerised rabbits with DNP-modified BSA producing antibodies to the dinitrophenyl (DNP)-BSA conjugate, BSA alone, and DNP alone, suggesting potential cross-reactive responses. Classically, B-cells are known to recognize the DNP-BSA conjugates via membrane bound IgM, process them, make antibody against the DNP, and present the BSA to CD4+ T-cells. These abilities of haptens have made them a tantalizing molecule for use in several settings. Some hapten-mediated responses are correlated to drug-induced autoimmune reactions. When a drug is metabolized, its metabolites can form strong haptens, which bind self-protein and sometimes elicit autoimmune responses (Chang and Gershwin, 2010). Hapten-carrier conjugates have been used in the past as drug-abuse

therapies, inducing an immune response against the drug of interest. Haptens have also been used to create autoimmune models in mice, such as IBD (Erkes and Selvan, 2014). Haptens are <1 kDa in size and elicit an immune response when bound to a carrier protein, including tolerised antigen. Haptens are not immunogenic by themselves, as they are too small to be recognized by the immune system. Most haptens are electrophilic compounds that covalently bind to nucleophilic residues creating new antigenic epitopes (Chipinda *et al.,* 2011).

TNP-BSA and DNP-BSA both bind C1q and activate complement (Alrashidi, 2015), and as shown here, they both bind FH. The activation of complement by these haptens probably has a strong role in uptake of TNP and DNP labelled proteins by the immune system, to produce an immune response against TNP or DNP. The regulation of complement activation by FH binding to the TNP/DNP may also influence cellular uptake, as has been observed by Kang *et al.,* (2012).

In this thesis, for the first time, the binding of C4bp to TNP and DNP albumins has been shown, and three binding sites on FH for TNP or DNP have been found: CCC1-4, CCP6-8 (low affinity) and CCP19-20. Inhibition of FH cofactor activity (in the fluid phase) has been shown, but this seems likely to be due mainly to cross-linking/aggregation of FH.

6.2 Factor H binding to other ligands

Binding to cholesterol: in this study cholesterol was used as a negative control for Factor H as it is known that it has no ability to bind factor H (Tan *et al.,* 2010).

Binding to anionic phospholipids: Anionic phospholipids are known complement classical pathway activators. Cardiolipin (CL), a negatively charged phospholipid found abundantly in mitochondrial membranes binds C1q and activates the classical pathway in an antibody-independent manner (Tan *et al.,* 2010). (Kertesz *et al.,* 1995) has reported that factor H binds strongly to cardiolipin. This finding is completely similar to our results in the current study, where factor H binds also strongly to cardiolipin. Anionic phospholipids are

negatively charged molecules and it has been shown that Factor H has binding sites for negatively charged polyions (Puurunen *et al.*, 1995).

Polycations: factor H also has high affinity of binding to poly-L-arginine.

Bacteria: Whole bacteria showed high binding of factor H. *Streptococcus pneumonia* strains D39 and TIGR4 were used. These bacteria express several proteins which are known to bind FH, such as Pneumococcal surface protein C (PspC) and choline-binding proteins (CBPs) that can interact with several human proteins for example factor H. Pneumococcal binding to FH has been attributed to choline-binding protein A (CbpA) of *S. pneumonia* and its allelic variants, all of which are surface-exposed proteins (Lu *et al.*, 2006). The S. pneumonia capsule can inhibit several aspects of host immunity, including neutrophil extracellular traps and both complement-dependent and complement-independent neutrophil phagocytosis (Hyams *et al.*, 2010). Bacterial pathogens can resist the body defense by means of evolving different mechanisms; one of the ways to avoid the complement defense is by binding to factor H (Jarva *et al.*, 2002). It has been reported that factor H binds to Psp on *S. pneumonia* strain D 39 through CCP 6-10 and CCP 13-15 (Dave *et al.*, 2001). In addition, in a study by Hammerschmidt *et al.*, (2007) further binding regions of factor H to PspC were localised to CCP8-11 and CCP 19-20.

Lipoteichoic acid (LTA): Lipoteichoic acid is an important component of gram positive bacteria cell walls (Seo *et al.*, 2008). In Figure 3.8 factor H shows high binding to LTA, and the binding of factor H to LTA might be one of the strategies to avoid the complement activation.

6.3 Adrenomedullin.

Below is a summary of what was known of FH-ADM interaction before the work reported in this thesis, based on the review by Sim *et al.*, (2015)

Adrenomedullin is a multifunctional regulatory and vasoactive peptide originally isolated from human pheo-chromocytoma by a Japanese group Kitamura et al (Kitamura,

Kanagawa, *et al.,* 1993). Adrenomedullin (ADM) is a 52–amino acid peptide with a single disulphide bridge between residues 16 and 21 and with an amidated tyrosine at the carboxy terminus, which presents structural homology with the calcitonin gene-related peptide and with amylin. (Hinson *et al.,* 2000) ADM overexpression has been discovered in human breast, lung, ovarian, pancreatic, prostate and renal cancers, cardiomyocytes, fibroblasts, monocytes, and leukocytes (Zhou *et al.,* 2015). Adrenomedullin is synthesised as part of large processor, called preproadrenomedullin (Kitamura, Sakata, *et al.,* 1993). ADM is a circulating hormone; although it functions also as a local paracrine and autocrine mediator with multiple biological activities such as vasodilatation, cell growth, and regulation of hormone secretion, natriuresis, and antimicrobial effects (Hinson *et al.,* 2000). At physiological concentration, ADM was shown to have a crucial role in the systemic and pulmonary circulation, in many cell types, including endothelial and smooth muscle cells of the vascular wall, production and secretion of ADM are increased in response to cellular strain induced by hypoxia and ischemia (Minamino *et al.,* 1995),

While complement researchers in the 1970s-1990s were examining the details of FH structure, function and activity within the complement system, another research group were examining FH under a different name: adrenomedullin-binding protein (AMBP) and later as AMBP1. Cuttitta and colleagues, while optimising assays for Adrenomedullin (ADM), observed anomalies in the assay when serum/plasma was present, and showed that the anomalous behaviour was due to the binding of ADM to a plasma protein. They showed, using a Western- blot assay, that a protein of 120 kDa (blotted onto nitrocellulose, after non-reducing SDS-PAGE) from human serum bound radioiodinated or fluoresceinated ADM. Proteins of the same size bound human ADM in the sera of calf, pig, goat, dog, mouse, chicken, guinea-pig, sheep, rabbit and human. The binding protein was named AMBP1 (Pio *et al.,* 2001) and was subsequently shown to be (in humans) identical to FH. .

6.3.1 Binding site for AM on FH

An assay of binding of fluoresceinated synthetic ADM to microtitre-plate-immobilised FH was used to explore the binding site on FH for ADM. Recombinant segments of FH, and

antibodies to FH were used, to determine if they interfered with binding. It was found that CCP8–20 and CCP15–20 of FH inhibited binding in a manner quantitatively similar to intact FH. FHL-1, which corresponds to CCP1–7, did not inhibit significantly. An anomaly in the results was that the monoclonal antibodies MRCOX23 and 24, which both bind to single epitopes in FHL1, did inhibit binding, as did polyclonal anti-FH. Using smaller segments of FH, CCP15–18, 19–20 and 8–11 were all found to inhibit weakly. Thus the binding of ADM to FH appears to be complex, and the authors suggested a high affinity site in CCP15–20, which possibly required interaction with more than one CCP, or required the junction of CCP18–19. A low affinity site was suggested for CCP8–11. Overall affinity was expressed as having dissociation constant in the 100–200nM range, weaker than the binding of ADM to its cellular receptors (dissociation constant in the 1–10 nM range). The related peptides CGRP and amylin did not interfere with binding, and nor did C3b, although in other work (see below) ADM was found to have an effect on rate of the FH-dependent cleavage of C3b by Factor I. The binding interaction is unusual, as shown by Pio *et al.,* (2001): they used a western-blot based binding assay, and showed that it was very difficult to dissociate ADM from the nitrocellulose-bound FH. Binding was dissociated by denaturants 1% SDS, 3 M guanidine-HCl, or the chaotrope 3 M NaSCN, but not by 4 M NaCl, or by acid pH (2.5). Most ligand binding by FH is ionic in nature, such as binding to glycosaminoglycans, C3b, Factor I, Anionic phospholipids and sialic acids (Soames and Sim, 1997; Tan *et al.,* 2010; Kishore and Sim, 2012), so would be eliminated by high salt. ADM binding is, therefore, distinctive and presumably has a strong hydrophobic component. Even in 3 M NaSCN, dissociation was slow and incomplete

The work in this thesis identifies 2 sites on FH for binding of ADM: these are CCP19-20 and CCP6-8. These are consistent with previous work indicating the larger regions 8-20 and 15-20, and the weaker interaction of 8-11. Consistently with previous results, CCP1-4 were found not to bind ADM. However, our findings on effect of ADM on FH cofactor activity are not in agreement with previous work, in which only relatively small effects on activity were seen. In contrast, in figure 5.13, very potent inhibition by ADM is seen.

6.3.2 The binding site for FH on AM:

The binding of ADM to FH could be investigated further by synthesising sequence variants of ADM, but this has not yet been done. Recent reduced cost of peptide synthesis may permit such studies. Pio *et al.*, (2001) showed that only the intact 52-amino acid ADM bound to FH in their western blot assay. Peptide segments 1–12, 13–52 and other smaller segments were unable to compete out the binding of intact AM. It was suggested on the basis of variable activity of radioiodinated ADM that tyrosines (at positions 1, 31 and 52) in ADM formed part of the binding site.

6.3.3 Reciprocal effects of binding on FH and ADM activities

Binding of ADM to FH is reported (as summarized by Sim et al, 2015), to have effects on the activities of both proteins. It was reported that in a fluid-phase assay of C3b conversion to iC3b, in the presence of factors H and I, addition of ADM increased the rate of reaction. However, this was evident only at a large molar excess of ADM over FH (about a 30:1 excess). CGRP, used as a control, had no effect. Since ADM does not bind to the region of FH involved in C3b and Factor I binding (CCPs 1–5), it could be considered possible that ADM alters the folding of FH by cross-linking sites at CCPs 8–11 and 15–20. Barlow and colleagues have suggested that alteration of the folding of FH in a similar way "activates" FH by altering the apposition of C3b and C3d binding sites in CCPs 8–12 and 19–20, respectively. However as noted above, our findings on effect of ADM on FH cofactor activity are not in agreement with previous work, in which only relatively small stimulatory effects on activity were seen. In contrast, in figure 5.13, very potent inhibition by ADM is seen.

Sim et al (2015) also summarized published effects of FH on some activities of ADM. ADM elevates cAMP in fibroblasts, and coincubation of the cells with FH and AM (in molar ratios from 0.5:1 up to 2:1) caused about a 2-fold increase in cAMP, compared with using AM alone. It has been considered whether FH influenced the rate or extent of ADM

binding to the cells (possibly by stabilising a conformation of ADM, or by providing a secondary interaction with the cell surface, via FH), but they showed that FH had little effect on the association or dissociation rates of ADM from the cell surface. FH inhibited the antimicrobial effect of ADM on E. coli in an agar plate radial diffusion assay, but here the effect may be due to the slower diffusion rate of an ADM-FH complex compared to ADM alone.

ADM appears to be relatively unstable in circulation and is degraded by proteases. Matrix metalloprotease 2 was identified as a likely candidate for degradation of ADM in circulation. The presence of FH inhibits cleavage of ADM by this protease, and this finding is currently the best explanation for the ability of FH to increase the activity of ADM in several experimental systems. This may be a major factor, but is unlikely to be the only mechanism by which FH modulates the effects of ADM. In plasma, since FH is in very large excess, it would be expected that circulating ADM would nearly all be in complex with FH. The unusual dissociation properties noted above suggest that the complex is very stable and long-lived, but these properties might be influenced by the medium on which the assay was performed (nitrocellulose). The circulating half-life of ADM has been estimated as only 22 min but this measurement may be made more complex by the effect of the binding protein on the assay used.

On the basis of the above information on ADM-FH interactions, we explored the possibility that AM binding was similar to TNP/DNP binding. It was confirmed that both binding reactions were of high affinity and difficult to dissociate with denaturants, extremes of pH, etc. It was shown that AM binds to at least 2 sites on FH, namely CCP6-8 and CCP19-20, but not to CCP1-4, which is compatible with the published results summarized above. However, TNP/DNP binds to all of these segments, although affinity for CCP6-8 is weak.

It was found that ADM was a very potent inhibitor of the fluid-phase cofactor activity of FH, in contrast to the previous published results, reported above. TNP/DNP-albumins were also very potent inhibitors, but it seemed unlikely that the two inhibitors acted by similar

mechanisms: this would need further work with more highly defined TNP/DNP oligomers (see below) for confirmation.

Low molecular weight inhibitors of binding to ADM and to TNP/DNP were tested, and the spectrum of inhibition was not very similar, again demonstrating that ADM binding and TNP/DNP binding are not comparable, except perhaps in affinity.

Potential further work:

The strong binding of TNP and DNP albumins to FH, and their inhibition of FH function is potentially useful, for example in experimental systems where the effect of eliminating FH function is being studied. It is intriguing that TNP and DNP albumins appear to bind very selectively to FH and C4bp, two proteins with closely-related functions. Potentially the binding of TNP and DNP to these is via a functionally-important site. However, the experiments (chapter 5) on inhibiting FH function with TNP-BSA suggested that the main effect could have been aggregation of FH, with many binding sites on each TNP-BSA, and at least three binding sites per FH molecule. It will be necessary therefore, to find out more sophisticated details of interaction, to use more highly defined TNP ligands, such as TNP "monomers", such as could be made by reacting picrylsulphonic acid with glycine, or "dimers" made using lysine, or glycyl-lysine. Possibly TNP-BSA has effects by cross-linking different sites on one FH molecule, and these could be explored by making TNP "dimers" of different lengths, which could be based on $(gly)_x$ lys peptides. Such work was planned earlier, but was halted by the lab fire of April 2016. Defined TNP oligomers of this sort, bound to FH recombinant segments, would also be amenable to structural analysis (X-ray crystallography) to define the mode of binding.

The strong binding to ADM should also be investigated further. It was striking that commercially synthesized ADM worked easily in these binding studies, so that sufficient ADM could be obtained cheaply for crystallographic studies. The physiological significance of FH-ADM complexes has been reported by others as summarized above, but the reported research has not been taken up by other research groups, and so needs further independent confirmation.

It was hoped that defined TNP oligomers and ADM could be used in studies to determine binding affinities, by, eg surface plasmon resonance, but again plans to do this were stopped after the laboratory fire.

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Addendum:

Parts of my thesis have been already published. (See manuscript attached)