

ATP analogues at a glance

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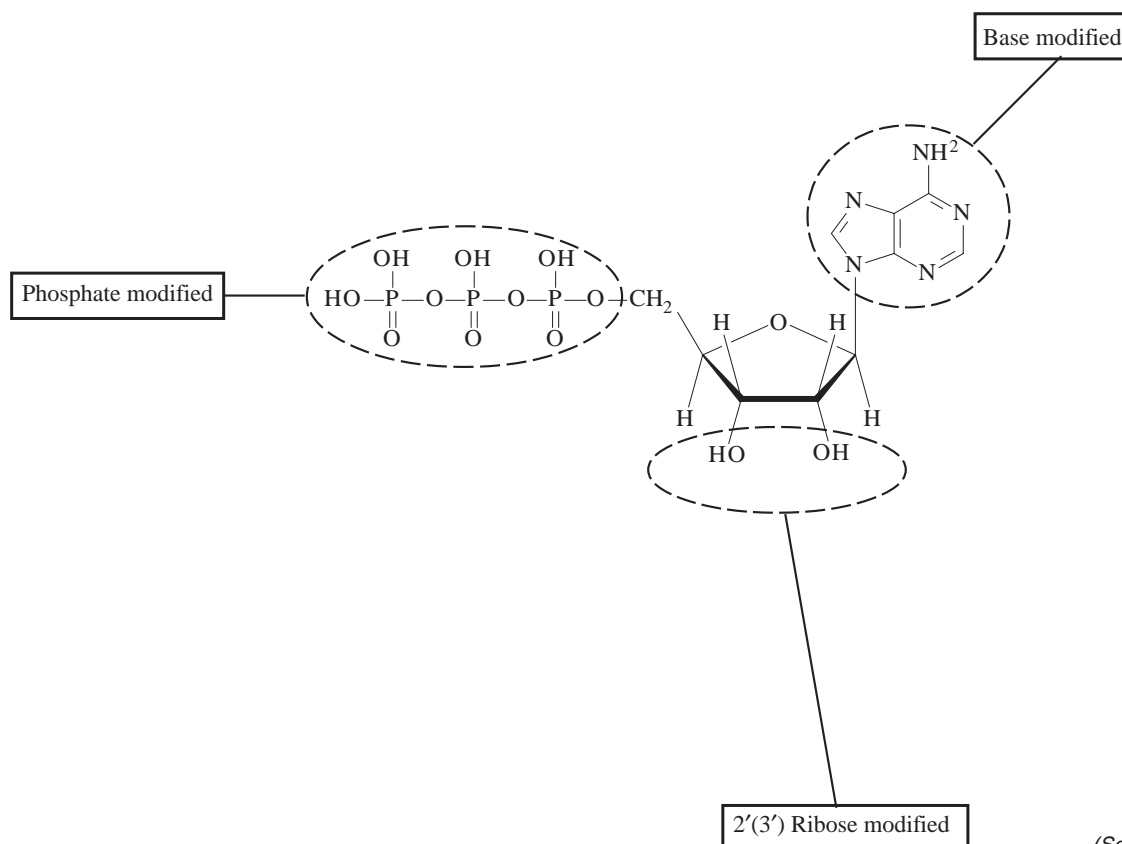
ATP has long been known to play a central role in the energetics of cells both in transduction mechanisms and in metabolic pathways, and is involved in regulation of enzyme, channel and receptor activities. Numerous ATP analogues have been synthesised to probe the role of ATP in biosystems (Yount, 1975; Jameson and Eccleston, 1997; Bagshaw, 1998). In general, two contrasting strategies are employed. Modifications may be introduced deliberately to change the properties of ATP (e.g. making it non-hydrolysable) so as to perturb the chemical steps involved in its action. Typically these involve modification of the phosphate chain. Alternatively, derivatives (e.g. fluorescent probes) are designed to report on the action of ATP but have a minimal effect on its properties. ATP-

utilising systems vary enormously in their specificity; so what acts as a good analogue in one case may be very poor in another. The accompanying poster shows a representative selection of derivatives that have been synthesised and summarises their key properties.

In energy-transducing reactions, ATP is normally hydrolysed between the β and γ phosphate groups, and modification of this region produces slowly hydrolysable or non-hydrolysable analogues (e.g. AP.PNP). These derivatives can be used to assess the role of binding energy in the transduction process. Non-hydrolysable analogues are also useful in crystallographic studies, as are the stable complexes formed between protein-bound ADP and phosphate analogues, such as vanadate. Another route to making a stable ATP state is the use of Co(III) or Cr(III) metal substitutes that display very slow ligand-exchange rates. ATP γ S is hydrolysed in many systems but usually shows a much reduced rate compared with ATP. This has been exploited in kinase/phosphatase studies, because once an amino acid side chain

has been thiophosphorylated it may be resistant to rapid dephosphorylation. Sulphur analogues in the α and β positions give rise to stereoisomers that can be used to probe the specificity of binding sites. Introduction of bulky organic probes on the phosphate chain generally gives poorly binding analogues, but this factor is exploited in caged-ATP derivatives that contain a photolabile derivative (McCray and Trentham, 1989). Flashes of 350-nm light release ATP within milliseconds and can be used to initiate reactions *in vitro* or within cells. Different caging groups have different absorption characteristics and photolysis rates.

Introduction of spectroscopic probes (absorption, fluorescent, EPR and NMR probes) is best done through the adenosine or ribose groups, depending on the specificity of the particular binding site. Although ATP absorbs strongly in the UV light (259 nm) range, this signal is usually masked by protein absorbance and cannot be exploited in spectroscopic studies. The adenine ring can be modified to shift the absorption to



(See poster insert)

>300 nm (e.g. 2-SH-ATP), but, in general, fluorescent derivatives provide more-sensitive probes. Among the apparently subtlest of changes is the substitution of an adenosine with a fluorescent formycin ring. However, the slightly longer C-C bond that connects to the ribose results in this analogue preferentially existing in the *syn* conformation, in which the base is positioned over the ribose, rather than the extended *anti* conformation, which is required by most protein-binding sites. In any event, this naturally occurring nucleoside base has not been available from commercial sources for several years. Substitution of groups in the 8 position of adenine also tends to favour the *syn* conformation.

In general, fluorescent derivatives require the addition of at least one aromatic ring, whereas two or more ring systems are required to shift the excitation into the visible region, as might be needed for optical microscopy. Under these circumstances, the probe may be larger than the ATP moiety. Changes in association rate constants of several fold are almost inevitable from diffusion considerations alone, but this is of minor energetic significance and can usually be tolerated. However, in many cases, a bulky fluorophore will interfere with subsequent binding interactions with the protein and this can be more significant.

The N⁶-amino group of adenine is not particularly reactive, and only a few analogues have been made through its derivatization. However, if an amino group is introduced into the ribose, either directly (by replacing one of the hydroxyl groups) or through a linker such as ethylenediamine, then the resultant compounds can be precursors for a host of derivatives. A wide variety of amine reactive probes that can be used specifically to label the precursor analogue are commercially available. Recently, the EDA-ATP intermediate has been employed as a starting point for a number of fluorescent analogues that have been used to investigate motor proteins (Jameson and Eccleston, 1997; Bagshaw, 1998). In particular, derivatives such as Cy3-EDA-ATP can readily be detected at the single

molecule level by laser microscopy (Oiwa et al., 2000). Luminescent probes based on long-lifetime emitting species, such as terbium (Tb), have also been made with the EDA linker. These analogues have particular use in fluorescent resonance energy transfer (FRET) studies, because gated detection can reduce nonspecific signals from short-lifetime fluorophores.

One complication of derivatizing the ribose hydroxyls is that a mixture of 2' and 3' isomers will be generated. These can convert via a cyclic 2', 3' intermediate. Indeed TNP-ATP exists in solution as a deprotonated Meisenheimer compound in which the TNP moiety crosslinks the 2' and 3' hydroxyl oxygens. In the case of ant-ATP and mant-ATP, the 2' and 3' isomers can be separated by chromatography, but, at pH 7, they re-equilibrate within several minutes. The carbomyl esters, as found in EDA-ATP derivatives, re-equilibrate much more slowly (over several hours at pH 7); so the properties of the separate isomers can be investigated (Oiwa et al., 2000). One way to avoid the complication of mixed isomers is to make the EDA derivative from nucleotides based on 2' deoxyribose.

Magnetic resonance probes can be produced in an analogous manner to fluorescent probes by incorporation of paramagnetic nitroxide spin labels (TEMPO derivatives). Alternatively, the fluorine atom provides an NMR probe in which there is little perturbation of the ATP molecule.

ATP analogues have also been prepared as affinity labels to locate nucleotide-binding sites within primary structures of proteins. One problem that often arises is that of distinguishing genuine sites from nonspecific labelling by the reactive functional group. A useful strategy is first to trap a photoaffinity probe (e.g. 8-azido-ADP, Bz₂-ADP) at the nucleotide-binding sites (e.g. as a vanadate complex) and remove the excess ATP analogue before illumination to activate the reaction (Cremo and Yount, 1987). The production of a biotin-ATP analogue opens the way for further derivatisation with avidin and hence a useful probe for

identifying nucleotide-binding sites at low resolution by electron microscopy.

The ease of synthesis of ATP analogues varies widely: some require specialist chemical skills and facilities, whereas others can be made by straightforward labelling reactions under aqueous conditions. Commercial availability has an important influence on the use of analogues, particularly because there is no way to predict the effectiveness of an analogue in a novel biological system accurately, and therefore a wide range of analogues may need to be screened. Useful sources of ATP analogues can be found at www.probes.com, www.sigmaaldrich.com, www.calbiochem.com, www.dojindo.com and www.biochem.boehringer-mannheim.com.

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