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EVIDENCE FOR A PROTOPHOSPHATASE CATALYSED CLEAVAGE OF ADENOSINE TRIPHOSPHATE BY A DISSOCIATIVE-TYPE MECHANISM WITHIN A RECEPTOR-SUBSTRATE COMPLEX

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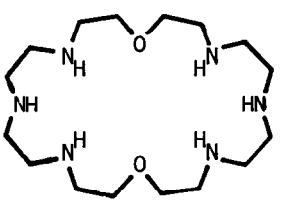
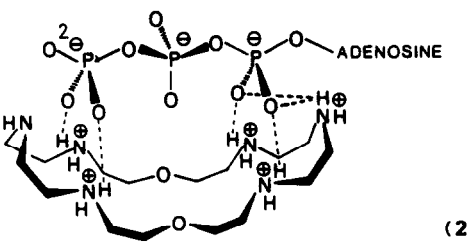
SUMMARY: Analogues of ATP and diadenosine 5',5"'-P_1,P_4-tetraphosphate, Ap_4A, have been used to explore the specificity and mechanism of the proto-ATPase activity of the macrocyclic polyamine [24]-N_6O_2 (1). The results show that (1) has exonuclease-like activity and support a mechanism that is dissociative in character within a pre-associative scheme resulting from receptor-substrate binding.

An exciting development in the field of supramolecular catalysis has been the discovery of a macrocyclic polyamine (1) capable of proto-ATPase, protokinase, and protophosphatase activity.[1-3] The protonated macrocycle acts as an anion receptor and binds nucleotides in the order ATP > ADP > AMP. It is a true catalyst, converting ATP first, rapidly, into ADP and then, more slowly, into AMP and P_i in an effectively pH-independent process, 3<pH<9. In addition, (1) has been shown to catalyse the hydrolysis of acetyl phosphate,[2] in which case there is concommitant formation of pyrophosphate.



(1)



(2)

The mechanisms by which these proto-enzymic processes operate are not clear. It has been suggested that catalysis might involve acidic, electrostatic, and/or nucleophilic components.[1-3] However, the observation of an N-phosphorylated intermediate in the macrocyle-catalysed hydrolysis both of ATP and of acetyl phosphate would seem to demand a nucleophilic pathway.[1-3] The mode of physical association of nucleotide and macrocycle is equally difficult to determine. Three possible binding modes have been considered: involving interactions with P_β,P_γ; P_α,P_β,P_γ; or P_α,P_γ; of which the latter is illustrated (2).

The elucidation of the catalytic mechanism of action of this system is of especial importance because it is capable of such diverse, prototypal enzyme activity and may thus broaden our understanding of enzyme-mediated phosphoryl transfer processes themselves. We have made and characterised a range of nucleotide analogues[4] which have many applications as

mechanistic probes. Through replacement of a labile oxygen bridge by a stable carbon linkage, one can study the reactivity of phosphate anhydrides which otherwise might have been masked in the parent nucleotide. Accordingly, we have carried out a preliminary study on the macrocycle-catalysed hydrolyses of some nucleotide and dinucleotide analogues (Table).

TABLE: Hydrolyses of ATP and other nucleotide analogues by the Macrocycle-[24]-N_6O_2. [a]

Substrate [b]	pH [c]	[Macrocycle]/mM	[Substrate]/mM	$10^5 \times k_{obs}$ /min^{-1} [d]
Appp (ATP)	6.0	0.5	0.5	910 ± 30
AppNHp	6.7	0.5	0.5	370 ± 10
$ApCH_2pp$	6.5	0.5	0.5	99 ± 4
$AppCF_2p$	5.2	0.5	1.0	5 ± 0.5
$AppCCl_2ppA$	6.1	0.5	0.5	<1 [e]

[a] All reactions performed at 60°C in 10mM aquous KCl and carried out in sealed vials (1 - 2.3ml); 10µl aliquots taken for analysis, quenched by cooling to 0°C and immediate dilution by injection into the hplc column.

[b] ATP (Na+ salt) and α,ß-methylene ATP (Li+ salt) from Sigma; adenylyl-imidodiphosphate (AppNHp) from Boehringer Mannheim. Syntheses of ß,γ-difluoromethylene ATP (Na+ salt) and of ß,ß'-dichloromethylene Ap_4A (Na+ salt) have been described previously.[4]

[c] pH was found to vary by less than 3% over the course of the reactions.

[d] Observed first order rate constants were calculated by over-relaxation analysis of data.[5]

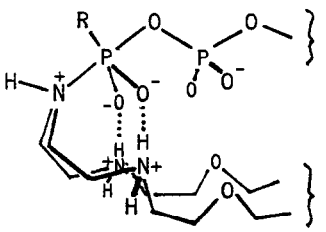
[e] Reaction too slow to measure accurately.

Hydrolyses were followed by injecting reaction aliquots into a Zorbax-NH2 analytical hplc column (isocratic elution, pH 6.5, 0.5mM ammonium phosphate mobile phase) and monitoring UV absorbance at 258nm as a function of retention time. Peaks were assigned by comparison with retention times of authentic samples. The change in concentrations of reactants and products with time was followed by gravimetric analysis of UV peak traces from hplc analysis. Substrates with labile ß,γ-phosphate bridges (Table, entries 1-3) were hydrolysed to give ApXpY and P_i (X = O, CH_2; Y = O, NH_2). Both of the other substrates (Table, entries 4 & 5) were slowly cleaved at the α,ß-bridge to give AMP.

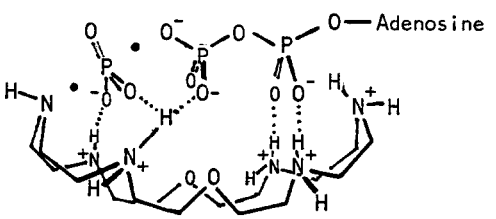
The imido-diphosphate bridge of adenylyl-imidodiphosphate, AppNHp, is cleaved by (1) at a rate slower than, but of the same order of magnitude as, that of the pyrophosphate bridge of ATP. This result is not suprising as this compound is well-known to be a labile analogue of ATP, susceptible to facile, acid-catalysed hydrolysis.[6] The second ATP analogue with a labile ß,γ-linkage, $ApCH_2pp$, is hydrolysed almost an order of magnitude more slowly than is ATP. We have observed a similar retardation in the cleavage of a pyrophosphate adjacent to a methylenebisphosphonate in the utilisation of $AppCH_2p$ by an RNA polymerase,[4a] which can be attributed to the poorer leaving-group ability of the phosphonate oxyanion. However, the most striking result is the relative stability to hydrolysis of the ß,γ-difluoromethylene ATP and of the ß,ß'-dichloromethylene Ap_4A analogues.[7] The resistance of these compounds to cleavage strongly suggests that the macrocycle (1) preferentially catalyses the cleavage of

terminal phosphate groups. This result both identifies the protophosphatase activity of (**1**) as being exolytic and it lends itself to speculation concerning the cleavage of ATP by the macrocycle (**1**): does an associative or a dissociative mechanism operate?

Firstly, let us assume an **associative** S_N2P mechanism and a symmetrical binding mode of nucleotide to the macrocycle, as in (**2**). In this situation, both α- and γ-phosphoryl centres appear to be equally subject to nucleophilic attack by the central amino group (Fig **3**). Consideration of the established preferred dynamics of phosphorus trigonal bipyramidal, TBP, transition states and intermediates[8] suggests two possible origins for the observed selectivity of nucleophilic attack only at the terminal γ-position.[9] On the one hand, there could be a requirement for proton transfer from the attacking amine to a phosphate oxygen (Fig **3**, R = $-O^-$) either prior to attack or in the TBP transition state: the mechanistic advantage for such a process is difficult to gauge.[10] On the other hand, there might be some steric hindrance to the formation of a TBP with an adenosyl group in the equatorial position (Fig **3**, R = Ado-O-). That could give rise to an equatorial/apical interaction, well-known to be unfavourable in phosphorus TBP's when the apical ligand bears a bulky α-substituent.[11] That likelyhood appears remote in this case (Fig **3**).



(Fig **3**)



(Fig **4**)

Alternatively, the selectivity shown here for hydrolysis of a terminal phosphate mono-ester linkage may be interpreted in terms of a **dissociative** mechanism (Fig **4**). Phosphate monoester monoanions typically hydrolyse by an S_N1P mechanism,[12] as has also been argued for the hydrolysis of ATP in solution.[13] In the present case, one might envisage the macrocycle (**1**) catalysing such an S_N1P process by facilitated proton transfer from a P_γ-OH group either to the bridging or to a P_β-oxygen. Such a proton transfer and formation of a "metaphosphate" anion is not possible for non-terminal phosphates.

Although other explanations cannot presently be excluded, it appears that the most reasonable interpretation of the observed pattern of reactivity favours a catalytic mechanism involving a metaphosphate-type, dissociative process.[1,14] The evidence for metaphosphate as an intermediate in the hydrolysis of phosphate monoesters and related compounds has been reviewed critically by Jencks;[15] who concludes that there is no convincing evidence for the existence of a metaphosphate intermediate in dilute aqueous solution but says that a metaphosphate with a lifetime too short to allow diffusion cannot definitely be excluded. In the present situation, there is little distinction between a true dissociative process and a pre-associative mechanism that requires some involvement for the nitrogen nucleophile in the transition state. Even if such nucleophilic participation is not required, it is clear that

the amine is held in close proximity to the incipient metaphosphate species because of the complexation of substrate and catalyst.[15,16] It would appear that chiral phosphate analysis[17] or PIX experiments[18] may prove unable to establish conclusively the difference for the same reasons that such experiments have proved to be ambiguous in enzyme chemistry.

The best mechanistic analysis of these data would suggest that exophosphorolytic activity by the macrocycle (**1**) with ATP and its analogues results from the advantages of a classical "metaphosphate" process being built into a pre-association mechanism as a consequence of the induced intramolecularity brought about by the polycationic nature of (**1**).

Such a conlusion would certainly justify the description of (**1**) as a protoenzyme!

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