Cocatalysis: Pyrophosphate Synthesis from Acetylphosphate catalysed by a Macrocyclic Polyamine

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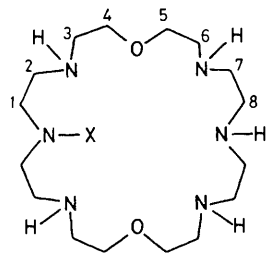
The protonated macrocyclic polyamine [24]-N_6O_2 (**1**) catalyses acetylphosphate hydrolysis and pyrophosphate formation *via* generation of a phosphorylated intermediate (**2**) which transfers a phosphoryl group to a phosphate substrate, thus acting as a bond-forming molecular cocatalyst.

Molecular reagents and enzyme models have been developed which bind substrate species and perform a reaction.[1,2] In order to realize bond-making rather than bond-breaking processes, the presence of several binding and reactive groups is essential. Such is the case for coreceptor molecules, in which subunits may co-operate to bring species together in order to react within a supramolecular complex, thus performing cocatalysis.[3,4]

We have shown earlier that macrocyclic polyamines, in particular the [24]-N_6O_2 macrocycle (**1**), catalyse ATP hydrolysis.[5] We now report that when (**1**) is used as catalyst for the hydrolysis of acetylphosphate (AcP, $MeCOOPO_3^{2-}$), it mediates the synthesis of pyrophosphate, *via* reaction of a phosphorylated intermediate with orthophosphate.

Several earlier studies on the mechanism and catalysis of the hydrolysis of AcP and related compounds have been des-



(1) X = H
(2) X = PO_3^{2-}

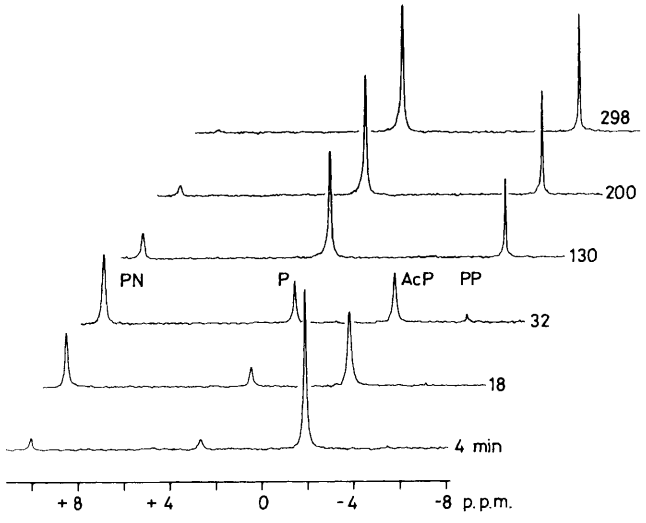


Figure 1. Observation of reaction of AcP in the presence of macrocycle (**1**) by ^{31}P n.m.r. spectroscopy at 81 MHz as a function of time (pH 7.0; 40 °C).† The chemical shifts are given with respect to external 85% H_3PO_4.

cribed.[6—9] In the present work the reaction of AcP in the presence or absence of (**1**) in aqueous solution was followed by ^{31}P and ^{1}H n.m.r. spectroscopy.†

In addition to acetate and phosphate (P), two other species were observed in the hydrolysis of AcP in the presence of (**1**) at pH 7: pyrophosphate (PP; *ca.* 30% conversion of AcP; identified by addition of authentic sodium pyrophosphate) and a transient intermediate (PN) giving a ^{31}P n.m.r. signal at *ca.* +10 p.p.m.; PN first accumulated and then disappeared after all the AcP had reacted, while both P and PP were formed (Figure 1). PP itself slowly gave P.[5] These PP and PN signals were never detected in the absence of (**1**), in the conditions used here.

AcP consumption was accelerated by addition of 1 equiv. of (**1**) (by a factor of *ca.* five, at pH 7.0) and was first-order ($k_{obs.}$ *ca.* 0.035 min^{-1}, pH 7.0). The rate data agree with the reaction taking place predominantly *via* [(**1**)-nH^+, AcP] complexes as the reactive species. The rate showed a maximum at pH ~7 in the presence of (**1**), whereas it is at a minimum in the range pH 5—9 for AcP alone.[6]

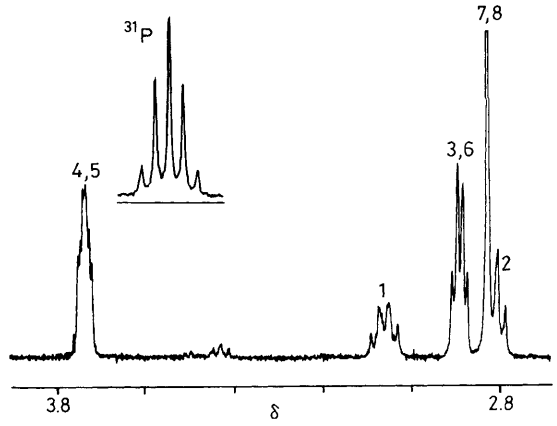


Figure 2. 400 MHz ^{1}H and 162 MHz ^{31}P N.m.r. spectra of the phosphoramidate intermediate (**2**) formed during AcP hydrolysis in the presence of macrocycle (**1**).‡§ The chemical shifts are given with respect to internal trimethylsilyl propylenesulphonate, TMPS (the signal at 3.4 p.p.m. is due to an impurity coming from the extraction procedure; pH *ca.* 13, 20 °C).

With a five-fold excess of AcP, macrocycle (**1**) acted until all substrate had been consumed, showing that the process was catalytic.

Macrocyclic polyamines bind phosphate compounds[5,10,11] and AcP should be complexed by protonated (**1**) in a manner similar to AMP with stability constants of *ca.* 10^3—10^5 (at 25 °C) for the [(**1**)-$4H^+$, AcP] and [(**1**)-$5H^+$, AcP] complexes, which should predominate at pH ~7.[5,12]

The nature of the PN intermediate is of key importance for understanding the present processes.‡ Its ^{1}H and ^{31}P n.m.r. spectra were determined and extensive ^{1}H-{^{1}H} and ^{31}P-{^{1}H} decoupling experiments were performed (Figure 2).§ The downfield position of the ^{31}P signal (~ + 10 p.p.m.) and its quintet structure, resulting from coupling to two CH_2 groups, indicated that PN must be a phosphorylated macrocycle. The proton spectrum and the decoupling experiments allowed unambiguous assignment of the intermediate to a symmetrical monophosphoramidate structure (**2**). Phosphorylation would be expected to occur at a central nitrogen site of the diethylenetriamine subunits of (**1**), since these sites have by far the lowest pK_a values.[13]

The rate of disappearance of PN was first-order ($k_{obs.}$ 0.013 min^{-1}, pH 7.0, 40 °C) and decreased from pH 6 to pH 8, which resembles the behaviour of simple phosphoramidates.[14]

† The acetylphosphate reactions were performed on aqueous solutions (D_2O–H_2O 1 : 9; 2 ml) containing AcP (lithium, potassium salt) and macrocycle (**1**)–6 M HCl (30 mM each) at various pH values adjusted by addition of NaOH or HCl and at 40±3 °C. The reaction was followed by ^{31}P n.m.r. spectroscopy at 81 MHz, with selective proton decoupling of the PN signal (Figure 1). The amounts of the various compounds present in the sample at different times were determined by integration of the ^{31}P n.m.r. signals (±10%). The values of $k_{obs.}$ calculated from the data were reproducible to ±10%.

‡ An aqueous solution of (**1**) and AcP (30 mM each; pH 7) was maintained at 50 °C for 15 min; it was then cooled to 25 °C and the pH raised to 10.5. Addition of *ca.* 1 equiv. $BaCl_2$ precipitates all phosphate containing compound except PN (^{31}P n.m.r. spectrum). After raising the pH to *ca.* 13—14 and evaporating to dryness, the unreacted macrocycle is extracted from the residue with CH_2Cl_2. The remaining residue, which contained the PN compound and acetate, was dissolved in D_2O for the n.m.r. measurements.

§ *Spectroscopic data:* ^{1}H N.m.r. (200 MHz): CH_2O, 2 × t, 4-, 5-H, $J_{H,H}$ 6 Hz; NCH_2CH_2O, 2 × t, 3-, 6-H, $J_{H,H}$ 6 Hz; CH_2NP, q, 1-H, $J_{H,H}$ 9.7, $J_{P,H}$ *ca.* 9.5 Hz; NCH_2CH_2NP, t, 2-H, $J_{H,H}$ 7.3 Hz; NCH_2CH_2N, s, 7-, 8-H. ^{31}P N.m.r. (162 MHz) quintet, $J_{P,H}$ 9.5 Hz; decoupling experiments: {4-, 5-H}, 2 × s, 3-, 6-H; {1-H}, s, 2-H; {3-, 6-H} 2 × s, 4-, 5-H; {2-H}, d, 1-H, $J_{P,H}$ 9.5 Hz; only irradiation of 1-H perturbed the ^{31}P n.m.r. spectrum giving a singlet for the PN signal.

The present PN intermediate, (**2**) is the same as that detected in the hydrolysis of ATP catalysed by macrocycle (**1**), thus confirming previous observations and the mechanistic discussion.[5]

The catalysis of AcP hydrolysis by macrocycle (**1**) is expected to occur with initial complexation of the substrate followed by cleavage of bound AcP, with both electrostatic catalysis by the ammonium sites and nucleophilic catalysis by an unprotonated nitrogen site, as indicated by the efficient PN formation and the characteristic pH dependence of the AcP hydrolysis rate (maximum at pH ~7; see above). No acylation of (**1**) was detected.

PP formation is by far the most significant feature of the processes studied here. On the basis of the results summarized above and of supporting experiments (see below), the following sequence of steps may be proposed for the catalytic formation of PP. (a) Substrate (AcP) binding by the protonated molecular catalyst (**1**), equation (1). (b) Phosphoryl transfer within the supramolecular complex giving the phosphorylated intermediate (**2**), equation (2). (c) Binding of HPO_4^{2-}, equation (3). (d) Phosphoryl transfer to P with PP formation, equation (4). (e) Product dissociation and release of the free receptor for a new catalytic cycle, equation (5).¶

$$\text{AcP} + (\mathbf{1}) \rightleftharpoons [(\mathbf{1}), \text{AcP}] \quad (1)$$

$$[(\mathbf{1}), \text{AcP}] \rightarrow (\mathbf{2}) + \text{OAc}^- \quad (2)$$

$$(\mathbf{2}) + \text{P} \rightleftharpoons [(\mathbf{2}), \text{P}] \quad (3)$$

$$[(\mathbf{2}), \text{P}] \rightarrow [(\mathbf{1}), \text{PP}] \quad (4)$$

$$[(\mathbf{1}), \text{PP}] \rightleftharpoons (\mathbf{1}) + \text{PP} \quad (5)$$

Steps (c) and (d) are supported by the following experiments: when an excess (3—4 fold) of ^{18}O-labelled phosphate[15] (~80% total enrichment) was added to a PN‡ solution and the mixture incubated in conditions similar to an AcP hydrolysis experiment (pH 7, 50 °C, 15 min), the PP formed was ^{18}O-labelled (isotopically-shifted PP ^{31}P n.m.r. signals[16]); when increasing amounts of Na_2HPO_4 were added to a PN solution obtained from AcP hydrolysis, the amount of PP produced increased and showed saturation behaviour (maximum conversion of PN into PP of ~ 60% for $>5 \times 10^{-2}$ M P added); competitively bound anionic species like oxalate^{2-}, AMP^{2-}, or ADP^{3-} acted as inhibitors, decreasing the rate of AcP consumption and the amount of PP produced. Further mechanistic studies are in progress.

The formation of PP is in competition with the hydrolysis of (**2**) to P and (**1**) (AcP hydrolysis pathway) and can only occur after some P has been produced, so that a 20—30% conversion is remarkable; as expected it becomes more efficient when extra P is added.‖ Similar studies with other macrocyclic polyamines showed that both PN and PP formation are under marked structural control.[18] Diethylenetriamine itself gave neither PN nor PP.

The fact that (**1**) is a ditopic coreceptor[3] containing two diethylenetriamine subunits may be of special significance for both PN and PP formation. These subunits may co-operate in binding AcP and activating it for phosphoryl transfer *via* the ammonium sites, in providing an unprotonated nitrogen site for PN formation as well as in mediating phosphoryl transfer from the phosphoramidate group to the bound $H_2PO_4^{2-}$ substrate. Thus, (**1**) would combine, in the same molecule, electrostatic with nucleophilic catalysis in a defined structural arrangement, providing conditions suitable for PP synthesis.

Numerous enzyme-catalysed phosphoryl transfer reactions occur *via* a phosphorylated enzyme intermediate.[19] A pyrophosphate-dependent acetate kinase catalyses the same AcP + P ⇌ PP + acetate process as macrocycle (**1**),[20] and pyrophosphate replaces ATP in a number of enzymatic energy-conserving and transferring reactions.[21]

Macrocycle (**1**) effects a two-step phosphoryl transfer process forming from AcP, a phospho-catalyst intermediate, which reacts with either water or P. When water is the substrate, (**1**) shows prototypical phosphatase behaviour, *i.e.* it behaves as a proto-phosphatase. When P is the substrate, (**1**) displays proto-kinase activity. This bond-making process is of special significance since it extends supramolecular reactivity to cocatalysis, mediating specific *synthetic* reactions within the supramolecular entities formed by coreceptor molecules.

Received, 9th April 1985; Com. 469

¶ (**1**) and (**2**) designate also the protonated states of these macrocyclic polyamines.

‖ Solvolysis of 0.4 M AcP gave traces of PP in water and *ca.* 15% PP in 7.3 M $NaClO_4$.[7] Phosphoramidate esters are used as reagents for the synthesis of pyrophosphates in organic media.[17]

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