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Hydrolysis of Synthetic Pyrophosphoric Esters by an Isoenzyme of Apyrase from *Solanum tuberosum*

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A highly purified isoenzyme of apyrase obtained from potatoes (*Solanum tuberosum* var. Pimpernel) exhibits a low specificity for the organic moiety of synthetic pyro- and tri-phosphates. Methyl di- and tri-phosphates were hydrolysed at higher rates than ADP and ATP, but their K_m values were also higher. Steric hindrance at the carbon atom linked to the pyrophosphate chain decreases both binding and maximum rate, whereas length or polarity of the organic chain do not have systematic effects. t-Butyl diphosphate, inorganic pyrophosphate, adenosine 5′-[α,β-methylene]triphosphate and adenosine 5′-[β,γ-methylene]triphosphate are competitive inhibitors of the hydrolysis of ATP and ADP.

Plant tissues contain an ATP diphosphohydrolase (EC 3.6.1.5) which has been called apyrase (Meyerhof, 1945). The enzyme from potato tuber has been studied extensively (Molnar & Lorand, 1961; Valenzuela *et al.*, 1973). It exists in more than one molecular form, and at least two isoenzymes have been identified (Traverso-Cori *et al.*, 1970). Apyrases hydrolyse the terminal phosphoryl group of ADP or the two terminal groups of ATP, the final products being AMP plus respectively 1 or 2mol of P_i/mol of AMP. The two isoenzymes identified so far (Traverso-Cori *et al.*, 1970) differ in the relative rates of hydrolysis of ATP and ADP.

Specificity studies (Cori *et al.*, 1965) have shown that the structural requirements for the organic moiety of the substrate are not very critical. Apyrase splits the di- and tri-phosphates of natural ribo- and deoxyribo-nucleosides as well as phenylpropyl di- and tri-phosphate (Miller & Westheimer, 1966) at comparable rates. Thiamin pyrophosphate has also been reported to be a substrate (Kiessling, 1956). Changes in the ionic moiety of the substrate molecule affect enzyme activity more drastically: adenosine tetraphosphate is split at about 5% of the rate of ATP (Liébecq *et al.*, 1962), and esterification of the pyrophosphate group by a second substituent (adenosine, morpholidate, nicotinamide, ribose etc.) completely suppresses the ability of the enzyme to attack the pyrophosphate bond.

PP_i has been shown to be a very poor substrate and an inhibitor of hydrolysis of nucleoside pyrophosphate, but no quantitative kinetic data are available.

Potato apyrase has been used as a tool to establish the structure of synthetic or biosynthetic allylic pyrophosphates (Cardemil & Cori, 1973). Because they were establishing structure, these authors did not perform any kinetic measurements, but incubated the substrate to complete hydrolysis, establishing that the pyrophosphates were split and the phosphomonoesters were not attacked.

These facts built up a picture of potato apyrase as a rather unspecific organic pyrophosphatase, which requires a terminal ionic moiety in its substrate. However, the evidence obtained so far has not covered a wide enough spectrum of compounds to warrant this view. All the compounds tested so far have rather large organic moieties, as in ribotides, or double bonds, as in prenyl pyrophosphates (Cardemil & Cori, 1973). Further, all these specificity studies were performed with a partially purified apyrase preparation from potatoes obtained from commercial sources.

It was found (Traverso-Cori *et al.*, 1970) that the enzyme material used for early specificity studies (Cori *et al.*, 1965) was quite heterogeneous, and contained variable proportions of isoenzymes of apyrase. It was possible to isolate one isoenzyme if potatoes of a single variety were used as source of enzyme. Substrate specificity for ATP and ADP varied from one isoenzyme to another.

Thus it seemed worthwhile to explore some further aspects of the substrate specificity of one highly purified isoenzyme of potato apyrase, by using a range of different organic substituents. It also seemed desirable to test substrates in which the charge of the

pyrophosphate moiety could be altered without introducing large steric factors, as in phosphono derivatives. It was expected that these studies would provide a clearer picture of the enzyme–substrate interaction of this pyrophosphohydrolase.

Experimental

Chemicals

All chemicals were reagent grade. ATP, ADP, adenosine 5′-[α,β-methylene]triphosphate, adenosine 5′-[β,γ-methylene]triphosphate, sodium triphosphate, methylene diphosphonic acid and DEAE-Sephadex A-25 were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. The optically active alcohols (+)- and (−)-butan-2-ol were purchased from Norse Laboratories, Santa Barbara, CA, U.S.A.

Synthetic substrates

(*a*) *Methyl triphosphate.* This was prepared (H. Brintzinger, personal communication) by very slowly adding a 1.5-fold excess of dimethyl sulphate to a 0.2M solution of sodium tripolyphosphate at room temperature (20°C) in an automatic titration instrument (Radiometer type TTT 1c), which maintained the pH value between 8.0 and 8.5 by the addition of 8M-NaOH. After completing the addition of dimethyl sulphate, stirring was continued until no further changes in pH were observed.

The reaction mixture was applied to a column (0.8cm×84cm) of Dowex 1 (X8; 100–200 mesh) previously equilibrated with 0.1M-NH_4HCO_3. The phosphorylated compounds were eluted by a linear gradient (0.1–1.0M) of NH_4HCO_3. Methyl diphosphate emerges at 0.5M-NH_4HCO_3 and methyl triphosphate at 0.6M. The column was monitored by measuring acid-labile phosphate (Umbreit *et al.*, 1959) in the fractions.

The collected samples were concentrated in a rotatory evaporator under vacuum until the pH was 6.5 (Cardemil & Cori, 1973). This procedure removes most of the bicarbonate. The overall yield of methyl triphosphate was 15%.

(*b*) *Organic pyrophosphates.* Alcohols were pyrophosphorylated by a modification (Popják *et al.*, 1962; Cardemil & Cori, 1973) of the procedure of Cramer & Bohm (1959), which uses bis(triethylammonium) phosphate as phosphorylating agent in the presence of trichloroacetonitrile. The crude reaction mixture contains inorganic pyrophosphates and the mono- and pyro-phosphate esters of the alcohol used. Sometimes triphosphates were found in quantities too small to make this procedure useful for their preparation.

Inorganic pyrophosphates interfere with the successful separation of the organic esters. It was found convenient to treat the crude reaction mixture with partially purified inorganic pyrophosphatases from yeast (Heppel, 1960) before the separation on DEAE-Sephadex. The enzymic preparation used has a specific activity of 5.1μmol/min per mg of protein and was devoid of phosphomonoesterase, adenosine diphosphatase and adenosine triphosphatase activities. Complete hydrolysis of inorganic pyrophosphates was achieved in 3h at room temperature at pH8.0 in the presence of 5mM-$MgCl_2$ and 3.3mg of pyrophosphatase/ml. The sample was diluted about 2-fold to prevent inactivation of the pyrophosphatase by side products of the Cramer & Bohm (1959) reaction. Hydrolysis of pyrophosphates was followed by determination of P_i (Fiske & SubbaRow, 1925).

After treatment with pyrophosphatase, the reaction mixture was applied directly to a column (1.6cm×84cm) of DEAE-Sephadex A-25 equilibrated with 0.05M-NH_4HCO_3 and eluted with a linear gradient of the same salt between 0.05 and 0.5M (Oster & West, 1968). The final yield of the synthesis varied between 10 and 15%. The phosphomonoesters were also obtained by this procedure.

(*c*) *Benzyl triphosphate.* This was synthesized from the corresponding pyrophosphates by using the procedure of Khorana & Todd (1953) and separated as described above. The yield was 15%.

Identification of the product of synthesis

All the synthesis products used as substrates met the following criteria.

1. Elementary analysis (University of Concepción, Department of Organic Chemistry).
2. Infrared analysis (Faculty of Chemical Sciences, Department of Organic Chemistry).
3. Paper chromatography (Cramer & Bohm, 1959). All the synthetic substrates used exhibited a single spot on descending paper chromatography in propanol/NH_3 (sp.gr. 0.880)/water (6:3:1, by vol.).
4. Absence of P_i (below 2% of the sample).
5. Absence of PP_i, measured as P_i liberated by treatment with inorganic pyrophosphatase.
6. Ratio of total acid-labile phosphate (Umbreit *et al.*, 1959). This ratio was 2.0 for all the pyrophosphates and 1.5 for the triphosphates. Only for t-butyl pyrophosphate was this ratio 1.0, because the C–O bond in the tertiary phosphoester is easily split by acid, yielding alcohol plus PP_i.
7. Ratio of total phosphate to alcohol after splitting of the phosphomonoester bond with 50μg of *Escherichia coli* alkaline phosphatase (Worthington Biochemical Corp., Freehold, NJ, U.S.A.) as described by Beytía *et al.* (1969). This ratio was 2 or 3, as expected for the pyro- and tri-phosphates.

The presence of inorganic pyrophosphates was excluded by the absence of P_i liberation by yeast

pyrophosphatase (Heppel, 1960). The ratio of total to acid-labile phosphate was measured by comparing P_i liberated after wet ashing and hydrolysis in 1M-HCl (Umbreit *et al.*, 1959) with the amount liberated by 10min acid hydrolysis. P_i thus liberated was measured by the method of Fiske & SubbaRow (1925) or Ernster *et al.* (1950).

Esterified alcohol content was measured by treating the sample with 1M-HCl at 100°C followed by neutralization with 1M-NaOH and incubation with alkaline phosphatase from *E. coli* (Beytía *et al.*, 1969). The procedure could not be used for t-butyl pyrophosphate. The alcohols liberated were extracted with *n*-hexane and measured by g.l.c. on a column (0.635cm×200cm) of Chromosorb W (60–80 mesh) coated with 2% poly(ethylene glycol) adipate. The temperature was set between 120 and 190°C according to the alcohol. The mass peaks were compared with those obtained by injection of authentic alcohols.

Apyrase preparation

An apyrase isoenzyme of high adenosine triphosphatase/adenosine diphosphatase ratio was prepared from the Pimpernel variety of potato obtained by clonal selection (Valenzuela *et al.*, 1973). The enzyme had a specific activity of 1210μmol of P_i released/min per mg of protein for ATP, and of 100 for ADP (ratio 12). All experiments were performed with the same batch of enzyme preparation. This isoenzyme will be called 'Pimpernel apyrase' below. The activity decayed by 13%/month during storage.

Protein was determined by the method of Warburg & Christian (1941).

Incubation procedure

The synthetic substrates were incubated at 30°C in the presence of 50mM-potassium succinate buffer, pH6.0, containing 5mM-$CaCl_2$.

Enzyme and substrate concentration and time of assay were adjusted to obtain initial velocities within the sensitivity of the analytical procedure used. Substrates were used in concentrations ranging from 0.1 to 16mM.

The enzymic reaction was stopped by use of the acid/molybdate reagent (Traverso-Cori *et al.*, 1970), and P_i was measured by the procedure of either Fiske & SubbaRow (1925) or Ernster *et al.* (1950).

K_m and $V_{max.}$ were calculated by the method of least squares. The method of Dixon was used to calculate inhibition constants (Dixon & Webb, 1964).

Results and Discussion

Stoicheiometry

When apyrase was incubated with synthetic organic pyrophosphates for extended periods of time, it liberated an amount of P_i equivalent to 50% of the total phosphorus present in the sample. With triphosphate, the amount of P_i liberated was two-thirds of the total. This shows that apyrase behaves towards synthetic pyro- and tri-phosphates as it

Table 1. *Kinetic parameters of the hydrolysis of various substrates of potato apyrase*

All experiments were run at least in duplicate. Individual rate determinations differed by less than 10%. There was a maximum dispersion of 20% between parameters calculated from different experiments. For experimental details see under 'Incubation procedure' in the Experimental section. The numbers in parentheses after the substrate indicate the range of concentrations used to obtain the kinetic parameters.

Substrates (mM)	K_m (mM)	$V_{max.}$ (μmol/min per mg of protein)	$V_{max.}/K_m$
ADP (0.2–2.0)	0.25	100	400.0
Benzyl diphosphate (0.09–1.8)	0.41	98	329.0
Methyl diphosphate (0.1–4.0)	2.0	272	136.0
Ethyl diphosphate (0.2–4.0)	1.0	249	249.0
2-Aminoethyl diphosphate (0.2–6.0)	5.0	432	86.4
n-Butyl diphosphate (0.8–5.0)	2.4	155	64.0
n-Octyl diphosphate (0.3–3.8)	0.9	105	117.0
Isopropyl diphosphate (0.2–4.0)	2.9	51	17.6
(±) -But-2-yl diphosphate (0.5–8.0)*	7.0	58	8.3
(−) -But-2-yl diphosphate (0.5–8.0)*	8.0	57	7.1
(+) -But-2-yl diphosphate (0.5–8.0)*	8.0	47	5.9
t-Butyl diphosphate (0.8–16.0)	8.1	33	4.1
ATP (0.1–2.0)	0.06	1210	20200
Benzyl triphosphate (0.1–2.0)	0.11	1020	9230
Phenylpropyl triphosphate (0.08–2.0)	0.43	610	1420
Methyl triphosphate (0.25–1.5)	1.05	2940	2800
Dimethylallyl triphosphate (0.05–2.2)	1.68	4	2.4

* The (+), (−) or (±) signs indicate the optical activity of the asymmetric alcohol used to synthesize the pyrophosphate.

does towards natural nucleotides, i.e. by hydrolysing the pyrophosphate bonds without attacking the phosphomonoesters formed. This was also tested directly with the monophosphates obtained as by-products in the chemical synthesis, which were not substrates for this enzyme. The hydrolysis of inorganic sodium tripolyphosphate was never complete. Only one-third of the total phosphate was liberated within 7h by this isoenzyme of apyrase.

Structure of the organic moiety of the substrates

Table 1 shows that all organic di- and tri-phosphates tested were split by the Pimpernel isoenzyme of potato apyrase. Adenosine 5′-[α,β-methylene]triphosphate and adenosine 5′-[β,γ-methylene]triphosphate were not attacked.

The maximum rates were comparable with or higher than those of the corresponding nucleoside pyrophosphates, except for substrates with branching at C_1. In the extreme case of t-butyl pyrophosphate, incubation time and enzyme concentration had to be increased 6–10-fold to obtain measurable quantities of P_i. The enzyme exhibited Michaelis kinetics for all substrates tested, and in this discussion it will be assumed that K_m is a parameter that correlates with affinity of the enzyme for the substrate (Atkinson, 1966).

Some of the synthetic substrates were hydrolysed at higher maximum rates than ADP or ATP (Table 1). This could be interpreted in terms of a higher efficiency of the final hydrolytic step of the enzymic reaction, i.e. higher values of $k_{cat.}$. All K_m values were higher for the synthetic substrates than for ADP or ATP, and the changes in K_m were larger than those for $V_{max.}$. The data in Table 1 also show that $k_{cat.}$ cannot be the only rate constant affected by the structure of the substrate. If the higher K_m values were caused by this sole factor, Michaelis kinetics would require a hyperbolic relationship between $V_{max.}$ and $V_{max.}/K_m$ at a fixed enzyme concentration. The large fluctuations of this ratio, unrelated to $V_{max.}$ show that this is not the case and that the other kinetic constants must differ substantially for each substrate. It may be thus concluded that some changes in the structure of the substrate lead to an increased rate of hydrolysis while the binding to the enzyme decreases. It is not possible at this moment to envisage simple relationships between structural features, such as chain length or polarity of the organic moiety of the substrate, and binding or maximum rate.

On the other hand, steric hindrance at C-1, the carbon atom attached to the pyrophosphate moiety, decreased both binding and maximum rate, as seen by comparing ethyl, isopropyl, but-2-yl and t-butyl pyrophosphates. $V_{max.}/K_m$ was decreased by 60-fold in going from ethyl pyrophosphate to t-butyl pyrophosphate. Rates of hydrolysis of s-butyl pyrophosphates were closer to those of the tertiary ester than to those of the primary ester, stressing the importance of steric hindrance both in binding and in the catalytic step. A similar hindrance seems to apply with dimethylallyl triphosphate, where the inspection of Fischer molecular models shows that the planar *gem*-dimethyl groups cannot be accommodated in regions that may accommodate a benzyl or an adenosyl group.

The kinetic parameters of enantiomeric but-2-yl pyrophosphates were practically identical, showing that the active site does not have strict stereochemical requirements for the two relatively small groups attached to the chiral C-2.

The concentration range used for some substrates led to an excess of pyrophosphate over Ca^{2+} in the extreme values. However, no deviation from a linear double-reciprocal relationship was observed, and thus it may be concluded that the lower $V_{max.}$ values observed for but-2-yl and t-butyl diphosphate were not due to an excess of substrate over metal.

Inhibition by phosphonate analogues of ATP

Both phosphonate analogues of ATP competitively inhibited the hydrolysis of this substrate (Table 2). This fact agrees with observations on the effect of adenosine 5′-[β,γ-methylene]triphosphate on ATP hydrolysis by myofibrillar adenosine triphosphatase (Moos *et al.*, 1960) or by heavy meromyosin (Yount *et al.*, 1971). The resemblance, however, is only qualitative. Adenosine 5′-[β,γ-methylene]triphosphate is a mixed type inhibitor (Dixon & Webb, 1964) of heavy-meromyosin adenosine triphosphatase, with K_i values from 17 to 500 times the K_m for ATP, whereas apyrase was inhibited competitively by the same compound, and its K_i was only 30 times the K_m. Again, the role of phosphonates in binding

Table 2. *Inhibitors of adenosine triphosphatase and adenosine diphosphatase activity of potato apyrase*

The enzyme was incubated as described in Table 1 and in the Experimental section, in the absence or in the presence of inhibitors in a concentration range of 1–2mM. Inhibition was purely competitive in all cases. All the experiments were run at least in duplicate.

Inhibitors	Adenosine triphosphatase activity K_i (mM)	Adenosine diphosphatase activity K_i (mM)
Adenosine 5′-[α,β-methylene]triphosphate	1.0	0.23
Adenosine 5′-[β,γ-methylene]triphosphate	1.35	0.91
t-Butyl diphosphate	1.61	1.47
PP_i	0.33	0.21

metals (Moos *et al.*, 1960) cannot be ruled out at present.

Contrary to our expectations, adenosine 5′-[α,β-methylene]triphosphate was not split by potato apyrase, in spite of the fact that the terminal pyrophosphate bond behaved like that of ATP towards acid hydrolysis, i.e. we found that 1 mol of P_i/mol of the phosphonate was liberated from the latter by treatment with 1 M-HCl at 100°C. Its effectiveness as a competitive inhibitor does not differ greatly from that of adenosine 5′-[β,γ-methylene]triphosphate.

The inability of apyrase to split adenosine 5′-[α,β-methylene]triphosphate could be explained by the findings of D. R. Trentham, H. G. Mannherz & J. F. Eccleston (personal communication), who have shown that a fragment of myosin adenosine triphosphatase splits adenosine 5′-[α,β-methylene]triphosphate at a rate of 0.67×10^{-3} times the rate of cleavage of ATP, although the binding rate to myosin of ATP and of the phosphonates differed only by a factor of 30.

PP_i, which is a competitive inhibitor, may also occupy the active site with only one of its phosphoryl groups, but it may not induce an adequate fit of this isoenzyme (Koshland, 1964) to align the catalytic site with the O–P–O bond to be split. However, the role of pyrophosphate in binding Ca^{2+} ions, which are required for maximum rate, should be explored before accepting this interpretation.

PP_i and t-butyl diphosphate have the same K_i whether ATP or ADP was the substrate, suggesting that each inhibitor forms a single enzyme–inhibitor complex. K_i thus reflects the dissociation constant of this single complex.

On the other hand, the K_i values of the phosphonate analogues of ATP are different depending on the substrate used, especially for adenosine 5′-[α,β-methylene]triphosphate. The inhibitor may bind to different sites of the enzyme and thus form more than one species of enzyme–inhibitor complex, which might have different dissociation constants. If ATP and ADP also bind to distinct amino acids at the active sites, as suggested by the different protective effect of these two substrates from inactivation by tetranitromethane (Valenzuela *et al.*, 1973), it is conceivable that the interaction between substrate and enzyme–inhibitor complex may be also different.

Methylene diphosphonic acid, which lacks both the organic moiety and the adequate polarity and geometry in the bridge between the two phosphoryl groups of the ligand, is neither substrate nor inhibitor at concentration ranges between 0.8 and 2 mM.

The specificity of Pimpernel apyrase is rather low for the organic moiety of the substrate. When this moiety is absent, the ligand may still be bound as an inhibitor (PP_i). The structure of the pyrophosphate moiety seems to be more important for catalysis, but substitution of the P–O–P by a $P–CH_2–P$ group as in phosphate analogues of ATP does not prevent binding. In the absence of an organic portion and with a modified pyrophosphate moiety, the resulting molecule is neither bound nor attacked, as occurs with methylene diphosphonate.

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