Nucleoside Pyrophosphatase Activity Associated with Pig Kidney Alkaline Phosphatase

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1. A study was made of the hydrolysis, at pH 9.0, of ATP and ADP catalysed by pig kidney alkaline phosphatase. Both of these nucleoside pyrophosphates are substrates for the enzyme; K_m values are $4 \times 10^{-5} \text{ M}$ for ATP and $6.3 \times 10^{-5} \text{ M}$ for ADP. V_{max} for ADP is approximately double that of ATP. 2. Above 0.1 mM approximately, both ATP and ADP are inhibitory, but the inhibition is reversible by the addition of Mg^{2+} ions to form MgATP^{2-} or MgADP^- complexes. The complexes, besides being non-inhibitory, are also substrates for the enzyme with K_m values identical with those of the respective free nucleotides. 3. Mg^{2+} ions are inhibitory when present in excess of ATP or ADP. The degree of inhibition is greater with ATP as substrate, but with both ATP and ADP a mixed competitive–non-competitive type of inhibition is observed. 4. It is suggested that under normal conditions the enzyme is inhibited by cellular concentrations of ATP plus ADP but that an increase in the concentration of Mg^{2+} ions stimulates activity by relieving nucleoside pyrophosphate inhibition. The properties may be of importance in the regulation of the transport of bivalent cations.

Eaton & Moss (1967b) and Fernley & Walker (1966) showed that alkaline phosphatase (EC 3.1.3.1) preparations from human liver, small intestine and calf intestine hydrolysed nucleoside pyrophosphates at rates comparable with the hydrolysis of orthophosphate esters such as *p*-nitrophenyl phosphate and AMP. From the results of Eaton & Moss (1967b), however, it seemed unlikely that formation of a nucleoside pyrophosphate– Mg^{2+} complex is an obligatory step in the reaction, which differs therefore from most enzymic reactions involving ATP (Cleland, 1967). Although low concentrations of Mg^{2+} ions stimulated activity to a small extent, the bivalent cation was inhibitory at concentrations above 1 mM approximately. A similar pattern of activation and then inhibition by Mg^{2+} ions is seen when alkaline phosphatase acts on PP_i (Eaton & Moss, 1967a; Butterworth, 1968b; Nayudu & Miles, 1969). The activation of inorganic pyrophosphatase by Mg^{2+} has been attributed to the formation of MgPP_i^{2-} as true substrate (Butterworth, 1968b; Nayudu & Miles, 1969) or to the removal of excess of inhibitory PP_i coupled with non-specific activating effects of Mg^{2+} ions (Eaton & Moss, 1967a). The inhibition of inorganic pyrophosphatase activity by higher concentrations of Mg^{2+} ions coupled with the well-known stimulation of orthophosphatase activity by the same metal

ion could be important for controlling the specificity of alkaline phosphatase for orthophosphate and pyrophosphate substrates.

This paper describes an investigation of the kinetics of pig kidney alkaline phosphatase with ATP and ADP as substrates and the effects of Mg^{2+} ions on the nucleoside pyrophosphatase activity. From the results of such studies an attempt was made to assess the possible physiological significance of nucleoside pyrophosphatase properties of alkaline phosphatase in kidney function.

EXPERIMENTAL

Materials. The monosodium salt of ATP and disodium salt of ADP were obtained from the Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. Fresh solutions were made up daily in water as required. A.R.-grade MgSO_4 and tris were obtained from Fisons Scientific Apparatus Ltd., Loughborough, Leics., U.K.

Enzyme. A purified preparation of alkaline phosphatase was obtained from pig kidney by the method described by Butterworth (1968a). The specific activity of the preparation was $25 \mu\text{mol}$ of *p*-nitrophenol released/min per mg of protein when the activity was determined with 3 mM-*p*-nitrophenyl phosphate at 30°C in 0.1 M- NaHCO_3 – Na_2CO_3 buffer, pH 10, and in the presence of 5 mM- MgSO_4 .

Assay of nucleoside pyrophosphatase activity. Reaction

mixtures were of 2 ml volume and contained 1 ml of 0.1 M-tris-HCl buffer, pH 9.0, plus enzyme together with suitable amounts of MgSO_4 , and ATP or ADP contained in 1 ml to give the concentrations of these reactants described in the Results section. The mixtures were incubated at 30°C for 10 min and released P_i was determined by the method of Baginski, Foa & Zak (1967). Activity was expressed as μmol of P_i released/min per ml of enzyme solution. There was some slight interference with the P_i assay at concentrations of ATP and ADP above 1.0 mM approx. This was overcome by constructing a series of standard curves for P_i containing appropriate amounts of ATP or ADP.

Concentrations of reactants. Dissociation constants for MgATP^{2-} and MgADP^- complexes were taken to be $1.37 \times 10^{-5} \text{ M}$ and $2.5 \times 10^{-4} \text{ M}$ respectively (O'Sullivan & Perrin, 1964). Thus for mixtures of Mg^{2+} with ATP^{4-} and ADP^{3-} it was assumed that negligible amounts of the free pyrophosphates existed in solution when Mg^{2+} was equimolar with, or in excess of, the total nucleotide concentration. Similarly, with excess of ATP^{4-} or ADP^{3-} all Mg^{2+} in the system was assumed to be complexed. 'Free' Mg^{2+} concentration, when the metal ion was in excess, was calculated by subtracting the total nucleotide concentration from the total Mg^{2+} concentration.

Determination of kinetic parameters. Michaelis and inhibitor constants and maximum velocities were calculated by statistical treatment of data plotted in the form of s/v against s (Wilkinson, 1961). Calculations were carried out on an Olivetti Programma 101 desk computer. The equivalent data are shown in the Results section, however, as double-reciprocal plots (Lineweaver & Burk, 1934), since inhibition patterns are often easier to interpret visually from such plots.

RESULTS

The rate of release of P_i from ATP and ADP was constant for up to 20 min under the conditions of the assay. Although alkaline phosphatase hydrolyses the phosphate groups of ATP and ADP sequentially (Moss & Walli, 1969), it can be assumed that the initial reaction rates represent the hydrolysis only of the terminal phosphate groups of the nucleotides, since under the conditions used in this study the amount of product formed would have been far too small to have a significant effect on the reaction rate.

As found for the hydrolysis of PP_i by alkaline phosphatase (Butterworth, 1968b; Nayudu & Miles, 1969), the effect of substrate on the initial reaction velocity is modified by the presence of Mg^{2+} ions. Fig. 1 shows the dependence of velocity on increasing ATP concentration at fixed total Mg^{2+} ion concentrations. The activity increased steeply as ATP concentrations were raised at all the Mg^{2+} concentrations tested until substrate inhibition by ATP became apparent. Increasing the concentration of Mg^{2+} displaced the 'optimum' ATP concentration to higher values, but did not appear to affect the velocities at the optima, i.e. the maximum

velocities at 0.1 mM-, 0.5 mM- and 1.0 mM- Mg^{2+} were the same but the substrate concentrations at which the maximum was reached differed. The rate of hydrolysis of ADP responded rather similarly to alterations in total Mg^{2+} concentration, but with this substrate the peak activity at 1 mM- Mg^{2+} was greater than at lower concentrations of the cation (Fig. 2).

Fig. 1. Effect of increasing ATP concentration on the rate of its hydrolysis by alkaline phosphatase determined at four different Mg^{2+} concentrations: $\circ$, zero Mg^{2+} ; Δ , 0.1 mM- Mg^{2+} ; $\bullet$, 0.5 mM- Mg^{2+} ; $\square$, 1.0 mM- Mg^{2+} .

Fig. 2. Effect of increasing ADP concentration on the rate of its hydrolysis determined at three different Mg^{2+} concentrations: $\circ$, zero Mg^{2+} ; $\bullet$, 0.5 mM- Mg^{2+} ; $\square$, 1.0 mM- Mg^{2+} .

The enzyme hydrolysed ATP and ADP in the absence of Mg^{2+} ions. Plots of v against s of results obtained with each substrate at concentrations low enough to avoid substrate inhibition were hyperbolic, and plots of $1/v$ against $1/s$ were linear, indicating that Michaelis-Menten kinetics were obeyed. Fig. 3 shows double-reciprocal plots obtained with ATP alone, with Mg^{2+} and ATP maintained at equimolar proportions and with constant amounts of free Mg^{2+} ions. The apparent K_m values for ATP^{4-} and MgATP^{2-} were identical at $4 \times 10^{-5} \text{ M}$, but the apparent $V_{\max}$ was lower for MgATP^{2-} than for ATP^{4-} . Free Mg^{2+} was inhibitory and all plots of results obtained in the presence of Mg^{2+} intersected at a point to the left of the $1/v$ axis, indicating a mixed competitive-non-competitive type of inhibition. Fig. 4 shows the results of a similar kind of experiment performed with ADP as substrate, and free Mg^{2+} again acted as a 'mixed' inhibitor. The values for apparent K_m of ADP^{3-} and MgADP^- were the same, as also were the respective values for $V_{\max}$. A comparison of the $V_{\max}$ values for ATP^{4-} and ADP^{3-} showed that ADP^{3-} is hydrolysed at approximately twice the rate of ATP^{4-} .

Values for the substrate inhibition constants of ATP^{4-} and ADP^{3-} were calculated from plots of $1/v$ against s (Dixon & Webb, 1964, p. 77). Such plots were linear at substrate concentrations which were high compared with the Michaelis constant (Fig. 5), and extrapolated values for the inhibition constants were $7 \times 10^{-4} \text{ M}$ for ATP^{4-} and $5 \times 10^{-4} \text{ M}$

for ADP^{3-} . Substrate inhibition did not occur with MgATP^{2-} or MgADP^- .

The effect of increasing Mg^{2+} concentration was studied at fixed concentrations of substrate (Fig. 6). With 0.1 mM-ATP , Mg^{2+} was inhibitory at all concentrations tested, but at higher ATP concentrations some activation was demonstrable until Mg^{2+} was present in excess and became inhibitory. The results obtained with 1.0 mM- and 0.5 mM-ATP showed that some inhibition of activity occurred at Mg^{2+} concentrations below 0.1 mM ; further addition of cation appeared to stimulate activity, however. With ADP the decline

Fig. 4. Plots of $1/v$ against $1/s$ for the hydrolysis of ADP^{3-} (○), of MgADP^- (△) and of MgADP^- in the presence of a constant excess of 0.5 mM-Mg^{2+} (●) or 1.0 mM-Mg^{2+} (□).

Fig. 3. Plots of $1/v$ against $1/s$ for the hydrolysis, by alkaline phosphatase, of ATP^{4-} (○), of MgATP^{2-} (△) and of MgATP^{2-} in the presence of a constant excess of 0.5 mM-Mg^{2+} (●) or 1.0 mM-Mg^{2+} (□).

Fig. 5. Plots of $1/v$ against s to show substrate inhibition by ADP^{3-} (▲) and by ATP^{4-} at various concentrations of MgATP^{2-} : ○, zero MgATP^{2-} ; △, $0.1 \text{ mM-MgATP}^{2-}$; □, $0.25 \text{ mM-MgATP}^{2-}$; ●, $0.5 \text{ mM-MgATP}^{2-}$.

in activity at high Mg^{2+} concentrations was much less than with ATP (Fig. 7).

For ATP and ADP hydrolysis under conditions where Mg^{2+} is equimolar or is in excess (e.g. the results shown in Fig. 3), the effective substrate species is MgATP^{2-} or MgADP^- and the mechanism describing the situation may be represented as shown in Scheme 1.

Assuming rapid equilibration of all enzyme-

Fig. 6. Effect of increasing Mg^{2+} ion concentration on ATP hydrolysis determined at four different concentrations of ATP: $\circ$, 0.1 mM-ATP; $\triangle$, 0.25 mM-ATP; $\square$, 0.5 mM-ATP; $\bullet$, 1.0 mM-ATP.

Fig. 7. Effect of increasing Mg^{2+} ion concentration on ADP hydrolysis at four different concentrations of ADP: $\circ$, 0.1 mM-ADP; $\triangle$, 0.25 mM-ADP; $\square$, 0.5 mM-ADP; $\bullet$, 1.0 mM-ADP.

ligand complexes the rate equation of the mechanism (Frieden, 1964) is given by:

$$v/V = \frac{s(1 + k'[\text{Mg}]/kK_3)/(1 + [\text{Mg}]/K_3)}{s + K_1(1 + [\text{Mg}]/K_2)/(1 + [\text{Mg}]/K_3)}$$

The k/k' ratio was determined from a comparison of maximum velocities at zero and 100 mM excess of Mg^{2+} ions. For ATP hydrolysis k' , i.e. the rate constant for the breakdown of Mg^{2+} -E- MgATP^{2-} complex, appeared to be zero, thus allowing the rate equation to be simplified. Such a simplification is not valid with ADP, however, because the k/k' ratio was found to be approx. 12. The various constants in the rate equation pertaining to MgATP^{2-} hydrolysis were calculated as suggested by Frieden (1964) and the values are shown in Table 1, together with kinetic parameters obtained for ATP^{4-} and ADP^{3-} hydrolysis and substrate inhibition.

DISCUSSION

These observations seem to establish that uncomplexed ATP^{4-} and ADP^{3-} are substrates for pig kidney alkaline phosphatase at pH 9.0 although it could be argued perhaps, that sufficient Mg^{2+} ions

Scheme 1.

Table 1. Summary of the kinetic data obtained with pig kidney alkaline phosphatase

All the kinetic constants were determined at pH 9.0 and 30°C. The mean values $\pm$ S.D. of four determinations are given.

Constant	$10^5 \times$ Value of constant (M)
K_m of ATP^{4-} and MgATP^{2-} (K_1)	4.0 ± 0.4
K_m of ADP^{3-} and MgADP^-	6.3 ± 0.8
ATP^{4-} inhibition	70 ± 12
ADP^{3-} inhibition	50 (single determination)
K_2	14.3 ± 3.5
K_3	122 ± 5.1
K_4	33.4 ± 11

are present as contaminants in the reaction mixtures to form effective complexes with ATP^{4-} and ADP^{3-} . The enzyme is subjected to exhaustive dialysis during its preparation so that any ions remaining would need to be very tightly bound. No information appears to be available for the amounts of bound Mg^{2+} to highly purified alkaline phosphatase preparations, but it has been estimated that human placental enzyme contains 2 to 3 atoms of zinc per enzyme molecule (Harkness, 1968) and it is possible that this is the upper limit of the amount of bivalent cations that can be tightly bound. The purity of the substrates is estimated by the manufacturers to be 99% and even if all the remaining 1% was in the form of Mg^{2+} ions, this amount, together with that already enzyme-bound, would appear to be too small to account for any enzymic activity attributable to MgATP^{2-} or MgADP^{-} at concentrations of the substrates up to 0.05 mM.

The enzyme also appears to bind MgATP^{2-} and MgADP^{-} as tightly as the free nucleotide pyrophosphates, but the metal ion complex of ATP is hydrolysed less rapidly than is free ATP itself. The major difference, as far as alkaline phosphatase is concerned, between complexed and uncomplexed pyrophosphates seems to reside in substrate inhibition behaviour, excess of MgATP^{2-} and MgADP^{-} being non-inhibitory. Thus the position of the apparent optimum substrate concentration is dependent on the total concentration of Mg^{2+} ions, because ATP^{4-} can be converted into the non-inhibitory metal ion complex whenever Mg^{2+} ions are in excess. The metal ion complexes do not seem to compete with the free pyrophosphates for the inhibitory site, since the inhibition constant for ATP^{4-} is unaffected by the presence of various amounts of MgATP^{2-} . The binding of ATP^{4-} and ADP^{3-} to the enzyme may involve the phosphate groups involved in the formation of metal ion complexes (Bruce & Benkovic, 1966). Pig kidney alkaline phosphatase probably contains zinc atoms essential for activity (Mathies, 1958), and the inhibitory pyrophosphate complexes may be formed through chelation of important zinc atoms by free pyrophosphate groups of ATP^{4-} and ADP^{3-} .

An increase in activity on adding Mg^{2+} ions is almost certainly the result of a relief of ATP^{4-} or ADP^{3-} inhibition rather than a direct activation by Mg^{2+} ions. The slight initial inhibitory effect of Mg^{2+} ions seen in some of the plots in Fig. 6 may arise from a combination of direct inhibition by Mg^{2+} ions coupled with conversion of some of the available ATP^{4-} into the less favourable substrate MgATP^{2-} before any significant removal of bound inhibitory ATP^{4-} occurs. Similar results have been described by Beck (1967) for the activity of ribonucleotide reductase as a function of Mg^{2+} ion concentration.

Nayudu & Miles (1969) reported mixed competitive-non-competitive inhibition of the inorganic pyrophosphatase activity of mouse duodenal alkaline phosphatase by Mg^{2+} ions, and argued that the competitive portion of the inhibition may arise because metal ion binds to the active site to form a bridge for pyrophosphate (Dixon & Webb, 1964, p. 427). Since free ATP^{4-} and ADP^{3-} can bind to the enzyme and be broken down, it is unnecessary to assume a bridging role for the metal ion, and it seems more likely that Mg^{2+} ions bind to a separate locus on the enzyme, thereby influencing both the formation and breakdown of the ES complex. Brunel & Cathala (1971) found a non-competitive activation of brain and intestinal alkaline phosphatase by Mg^{2+} ions indicative of a separate binding site for the metal ion. The kinetics of the inhibition seem to indicate that the presence of substrate diminishes the affinity of Mg^{2+} ions approximately eightfold.

If alkaline phosphatase reacts with ATP and/or ADP *in vivo* then it is likely that the reaction is of a phosphotransferase type, important perhaps in absorption, or that the enzyme resembles other membrane-bound adenosine triphosphatases coupled with ion transport. A metabolically isolated hydrolysis of ATP probably does not occur in cellular metabolism (Banks, 1969). The possible identity of alkaline phosphatase with a Ca^{2+} -stimulated adenosine triphosphatase in intestinal brush-border cells has been discussed (Norman, Mircheff, Adams & Spielvogel, 1970; Haussler, Nagode & Rasmussen, 1970). If the cellular concentration of ATP plus ADP is assumed to be approx. 5 mM (Krebs, 1964) and if a similar concentration is present at the location of the enzyme, then it is clear from the results of the present investigation that the enzyme will be inhibited to a significant extent in the absence of Mg^{2+} ions. The total Mg^{2+} ion concentration of mammalian kidney is generally taken to be approx. 12.5 mM, but the non-protein-bound cellular concentration is almost certainly much lower than this. For brain tissue the free concentration is estimated to be 1.5 mM (Bachelard & Goldfarb, 1969), and if a similar value is assumed for kidney it is therefore probable that alkaline phosphatase is often partially inhibited by intracellular nucleotide concentrations. If a role for alkaline phosphatase should be found in tubular reabsorption of bivalent cations, then the apparent stimulation of activity by Mg^{2+} ions brought about by the release of ATP inhibition could be important. Similarly, an excess of free intracellular Mg^{2+} could limit further transport by a negative-feedback type of mechanism.

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REFERENCES

- Bachelard, H. S. & Goldfarb, P. S. G. (1969). *Biochem. J.* **112**, 579.
- Baginski, E. S., Foa, P. P. & Zak, B. (1967). *Clinica chim. Acta*, **15**, 155.
- Banks, B. E. C. (1969). *Chem. Br.* **5**, 514.
- Beck, W. S. (1967). *J. biol. Chem.* **242**, 1983.
- Bruice, T. C. & Benkovic, S. (1966). *Bio-organic Mechanisms*, vol. 2, p. 172. New York: W. A. Benjamin Inc.
- Brunel, C. & Cathala, G. (1971). *Biochim. biophys. Acta*, **235**, 106.
- Butterworth, P. J. (1968a). *Biochem. J.* **108**, 243.
- Butterworth, P. J. (1968b). *Biochem. J.* **110**, 671.
- Cleland, W. W. (1967). *A. Rev. Biochem.* **36**, 77.
- Dixon, M. & Webb, E. C. (1964). *Enzymes*, 2nd ed. London: Longmans, Green and Co.
- Eaton, R. H. & Moss, D. W. (1967a). *Biochem. J.* **102**, 917.
- Eaton, R. H. & Moss, D. W. (1967b). *Biochem. J.* **105**, 1307.
- Fernley, H. N. & Walker, P. G. (1966). *Biochem. J.* **99**, 39p.
- Frieden, C. (1964). *J. biol. Chem.* **239**, 3522.
- Harkness, D. R. (1968). *Archs Biochem. Biophys.* **126**, 503.
- Haussler, M. R., Nagode, L. A. & Rasmussen, H. (1970). *Nature, Lond.*, **228**, 1199.
- Krebs, H. A. (1964). *Proc. R. Soc. B*, **159**, 545.
- Lineweaver, H. & Burk, D. (1934). *J. Am. chem. Soc.* **56**, 658.
- Mathies, J. C. (1958). *J. biol. Chem.* **233**, 1121.
- Moss, D. W. & Walli, A. K. (1969). *Biochim. biophys. Acta*, **191**, 476.
- Nayudu, P. R. V. & Miles, P. L. (1969). *Biochem. J.* **115**, 29.
- Norman, A. W., Mircheff, A. K., Adams, T. H. & Spielvogel, A. (1970). *Biochim. biophys. Acta*, **215**, 348.
- O'Sullivan, W. J. & Perrin, D. D. (1964). *Biochemistry, Easton*, **3**, 18.
- Wilkinson, G. N. (1961). *Biochem. J.* **80**, 234.