The Pyrophosphatase Activity of Pig Kidney Alkaline Phosphatase and its Inhibition by Magnesium Ions and Excess of Pyrophosphate

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1. Pig kidney enzyme resembles other non-specific alkaline phosphatases in its ability to hydrolyse inorganic pyrophosphate (PP_i). 2. Studies of enzyme velocity as a function of PP_i concentration show that Michaelis-Menten kinetics are obeyed when a constant $\text{PP}_i/\text{Mg}^{2+}$ concentration ratio is maintained, but velocity-substrate concentration curves are sigmoid when the concentration of PP_i is increased but that of Mg^{2+} is kept constant. The enzyme is inhibited when the total PP_i concentration is greater than the total concentration of Mg^{2+} . Pyrophosphatase activity is activated by Mg^{2+} , but if the concentration of the metal ion is increased to a value in excess of the total PP_i concentration Mg^{2+} is then strongly inhibitory. 4. It appears that the enzyme is most active towards the complex ion MgPP_i^{2-} . The enzyme probably hydrolyses PP_i^{4-} also, but this is a poorer substrate and its competition with MgPP_i^{2-} leads to inhibition. At high Mg^{2+} concentrations Mg_2PP_i is formed. This complex appears to be a potent inhibitor. 5. Sigmoid plots of v against s and of v against i result from interactions occurring between Mg^{2+} and PP_i^{4-} leading to MgPP_i^{2-} and Mg_2PP_i , and are not indicative of allosteric behaviour.

It now seems to be clearly established that non-specific alkaline phosphatases (EC 3.1.3.1) prepared from a variety of sources will hydrolyse PP_i (Cox & Griffin, 1965; Fernley & Walker, 1967; Moss, Eaton, Smith & Whitby, 1966; Butterworth, 1968*a*; Sussman & Laga, 1968) and nucleoside pyrophosphates (Fernley & Walker, 1967; Eaton & Moss, 1967*b*). Concentrations of Mg^{2+} that give optimum activation of the orthophosphatase activity of alkaline phosphatase preparations strongly inhibit the associated pyrophosphatase activities (Fernley & Walker, 1967; Eaton & Moss, 1967*a*). The mechanism by which Mg^{2+} inhibits pyrophosphatase activity does not seem to have been elucidated, but it has been suggested that the ion may combine with the substrate to form a complex (Eaton & Moss, 1967*a*).

Inorganic pyrophosphatases appear to be inhibited by high substrate concentrations (Robbins, Stulberg & Boyer, 1955; Bloch-Frankenthal, 1954, and references cited therein), and it has been suggested that the true substrate for pyrophosphatase enzymes is the complex ion MgPP_i^{2-} , which is competed for by the inhibitory PP_i^{4-} ion (Robbins *et al.* 1955). Eaton & Moss (1967*a*) found very little activation of pyrophosphatase activity in the presence of low, non-inhibitory, concentrations of Mg^{2+} and so were unable to confirm that the MgPP_i^{2-}

complex is the true substrate for the inorganic pyrophosphatase associated with non-specific alkaline phosphatase.

The present paper is concerned with studies made of the effects of Mg^{2+} and PP_i on the activity of an alkaline phosphatase-pyrophosphatase enzyme preparation in an attempt to find the true substrate and resolve the nature of the inhibition that can occur in the presence of these agents.

MATERIALS AND METHODS

Materials. Fresh solutions of $\text{Na}_4\text{P}_2\text{O}_7$, MgSO_4 and $\text{Co}(\text{NO}_3)_2$ were made up daily, with A.R.-grade reagents. For those experiments in which Ca^{2+} was included, A.R.-grade CaCO_3 dissolved in the minimum of HCl , followed by neutralization, was employed. On mixing with PP_i no precipitation occurred with solutions containing Mg^{2+} or Co^{2+} (Bloch-Frankenthal, 1954), but a precipitate formed in the presence of Ca^{2+} at concentrations exceeding 2.5 mm.

Enzyme preparation. Alkaline phosphatase was purified from pig kidney as described by Butterworth (1968*b*). The isolation procedure included fractional precipitation with acetone and $(\text{NH}_4)_2\text{SO}_4$, gel filtration on Sephadex G-200 and chromatography on DEAE-cellulose, and at each step of the purification the coincidence of orthophosphatase and pyrophosphatase activities suggested that both activities were associated with the same enzyme.

Assay of pyrophosphatase activity. The reaction mixtures

were of 2ml. volume and contained 1ml. of enzyme in 0.1M-tris-HCl buffer, pH 8.6, plus suitable amounts of $\text{Na}_4\text{P}_2\text{O}_7$ and MgSO_4 contained in 1ml. to give the concentrations of these components described in the Results section. After incubation for 10 min. at 30° the reaction was stopped by the addition of 3ml. of 0.34M-sodium acetate buffer, pH 4.0, and the liberated P_i was measured by the method of Delsal & Manhour (1958). Similar reaction mixtures from which enzyme had been omitted were incubated to serve as controls. All reaction mixtures were set up in duplicate. Enzymic activity was expressed in μmoles of P_i released/min./ml. of enzyme solution. With enzyme diluted in 0.1M-tris-HCl buffer, pH 8.6, to give an activity of approx. 0.5 unit when measured under optimum conditions, the rate of release of P_i was found to be constant for incubation times up to 30 min. under all conditions described in the Results section.

Concentrations of reactive species. The stability constants given by Lambert & Watters (1957) for the formation of MgPP_i^{2-} and Mg_2PP_i are as follows:

$$K_{\text{MgPP}_i^{2-}} = \frac{[\text{MgPP}_i^{2-}]}{[\text{Mg}^{2+}][\text{PP}_i^{4-}]} = 10^{5.4}$$

$$K_{\text{Mg}_2\text{PP}_i} = \frac{[\text{Mg}_2\text{PP}_i]}{[\text{Mg}^{2+}][\text{MgPP}_i^{2-}]} = 10^{2.4}$$

Thus it appears that, when the concentration of PP_i is greater than the total Mg^{2+} concentration, practically all of the Mg^{2+} is in the form of the complex ion MgPP_i^{2-} and consequently the concentration of free Mg^{2+} is almost zero. Under these conditions the concentration of 'free' PP_i will be given by the total PP_i concentration minus the total Mg^{2+} concentration. With excess of Mg^{2+} the complex Mg_2PP_i is formed, and in the presence of a large excess of Mg^{2+} practically all the PP_i in the system will be present in the form of Mg_2PP_i .

RESULTS

The effect of increasing PP_i concentration on the reaction velocity measured at four fixed Mg^{2+} concentrations is shown in Fig. 1. With a Mg^{2+} concentration of 1.0mM a plot of v against s was sigmoid, but at 0.1mM- Mg^{2+} Michaelis-Menten kinetics appeared to be obeyed. At intermediate Mg^{2+} concentrations there seemed to be a linear relationship between v and s . For each of the curves further increase in PP_i concentration beyond that shown on the plots led to inhibition of enzymic activity. Replotting the results obtained at 1.0mM- and 0.5mM- Mg^{2+} in the form of $\log[(V-v)/v]$ against $-\log s$ (Hill, 1910) gave plots that were linear at low values of s with a slope of 1.

Increasing the concentrations of PP_i and Mg^{2+} together to maintain constant $\text{PP}_i/\text{Mg}^{2+}$ concentration ratios gave velocity-substrate concentration curves that conformed to the Michaelis-Menten pattern. By plotting s/v against s , straight-line plots were obtained that enabled an apparent K_m to be calculated for each of the ratios (Fig. 2). At the $\text{PP}_i/\text{Mg}^{2+}$ concentration ratio 1:1, K_m was $2 \times 10^{-4}\text{M}$,

Fig. 1. Effect of increasing PP_i concentration on the pyrophosphatase activity of pig kidney alkaline phosphatase-pyrophosphatase determined at four different Mg^{2+} concentrations: $\circ$, 1.0mM- Mg^{2+} ; $\bullet$, 0.5mM- Mg^{2+} ; $\blacktriangle$, 0.25mM- Mg^{2+} ; $\triangle$, 0.1mM- Mg^{2+} .

but K_m decreased as the ratio was raised and for the $\text{PP}_i/\text{Mg}^{2+}$ concentration ratio 20:1 a value of $2 \times 10^{-5}\text{M}$ was obtained. The maximum velocity also fell as the ratio was increased.

The concentration at which PP_i inhibited enzymic activity depended on the concentration of Mg^{2+} in the assay mixture. An excess of PP_i was needed before inhibition occurred. Fig. 3 shows plots obtained at four different Mg^{2+} concentrations. The inhibition curves at 1.0mM- and 0.5mM- Mg^{2+} appeared to be biphasic, and when the results obtained at all four Mg^{2+} concentrations were plotted in the form of $1/v$ against i (Dixon, 1953) straight lines were not obtained. The $\text{PP}_i/\text{Mg}^{2+}$ concentration ratio required for maximum pyrophosphatase activity depended on the total Mg^{2+} concentration; the ratio increased from 1.25:1 at 1.0mM- Mg^{2+} to 2.0:1 at 0.1mM- Mg^{2+} (Fig. 3).

Hill plots of $\log[(V-v_i)/v_i]$ against $\log i$, where V is the velocity at the optimum $\text{PP}_i/\text{Mg}^{2+}$ concentration ratio, v_i is the velocity of the inhibited reaction and i is the free PP_i^{4-} concentration, gave straight lines whose slopes were greater than 1.6, suggesting that co-operative interactions between inhibitor-binding sites may have occurred. Heating of the enzyme at 55° for 10 min. in 0.05M-tris-hydrochloric acid buffer, pH 8.6, before inhibition experiments with PP_i^{4-} had no effect on the shape of the inhibition curves. The activity of the enzyme preparation was decreased by about 25% by the

Fig. 2. Effect of PP_i concentration on pyrophosphatase activity determined at constant $\text{PP}_i/\text{Mg}^{2+}$ concentration ratios: $\circ$, 1:1 (K_m $2 \times 10^{-4}\text{M}$, $V_{\max}$ 0.61 unit); $\bullet$, 2:1 (K_m $1.3 \times 10^{-4}\text{M}$, $V_{\max}$ 0.51 unit); $\triangle$, 5:1 (K_m $0.6 \times 10^{-4}\text{M}$, $V_{\max}$ 0.24 unit); $\blacktriangle$, 20:1 (K_m $0.2 \times 10^{-4}\text{M}$, $V_{\max}$ 0.12 unit). The results are plotted in the form of s/v against s .

Fig. 3. Inhibition of pyrophosphatase activity by PP_i at four fixed Mg^{2+} concentrations: $\circ$, 1.0 mM- Mg^{2+} ; $\bullet$, 0.5 mM- Mg^{2+} ; $\blacktriangle$, 0.25 mM- Mg^{2+} ; $\triangle$, 0.1 mM- Mg^{2+} .

heating procedure. Preincubation of the enzyme with 3M-urea in 0.05M-tris-hydrochloric acid buffer, pH 8.6, for 20 min. at 30° did not affect the inhibition behaviour, nor did the inclusion of urea at

Fig. 4. Inhibition of pyrophosphatase activity by Mg^{2+} at four fixed PP_i concentrations: $\circ$, 1.0 mM- PP_i ; $\bullet$, 0.5 mM- PP_i ; $\blacktriangle$, 0.25 mM- PP_i ; $\triangle$, 0.1 mM- PP_i .

concentrations up to 3M in the reaction mixtures. The inclusion of 25 mM-borate in the reaction mixtures caused a decrease in total activity, but did not change the inhibition pattern obtained with PP_i^{4-} . Borate is a competitive inhibitor of alkaline phosphatase (Zittle & Della Monica, 1950), but there was no evidence to show that borate competed with PP_i^{4-} for a specific inhibitor-binding site on the enzyme distinct from the substrate-binding active site.

The addition of Mg^{2+} considerably activated the pyrophosphatase properties of the enzyme preparation, but once the amount of added Mg^{2+} exceeded the total PP_i concentration the metal ion was strongly inhibitory (Fig. 4). Both Co^{2+} and Ca^{2+} resembled Mg^{2+} in their effects on activity, but the degree of activation at lower concentrations of the metal ions was not so great as that brought about by Mg^{2+} . At higher concentrations their inhibitory effects were nearly identical with those of Mg^{2+} . Plots of velocity against total Mg^{2+} concentration at four fixed PP_i concentrations were sigmoid (Fig. 4), and curves were obtained when the results were replotted in the form of $1/v$ against i .

The inhibition results were plotted according to the Hill equation, the activity at equimolar concentrations of Mg^{2+} and PP_i being substituted for V in the expression $(V - v_i)/v_i$. Straight-line plots were obtained (Fig. 5) of slope approx. 1.4. Treatment of the enzyme with 3M-urea or preheating at 55° for 10 min. did not affect the shape of the Mg^{2+} -inhibition plots. Similar results were also obtained with Mg^{2+} when the experiments were repeated in the presence of 25 mM-borate.

Fig. 5. Hill plots of the inhibition of pyrophosphatase activity by excess of Mg^{2+} . The value chosen for V was that of the activity at zero excess of Mg^{2+} . $\circ$, 1.0 mM- PP_i ; $\bullet$, 0.5 mM- PP_i ; $\blacktriangle$, 0.25 mM- PP_i ; $\triangle$, 0.1 mM- PP_i . The slope of plots is approx. 1.4.

DISCUSSION

Sigmoid velocity-substrate concentration and velocity-inhibitor concentration curves and Hill plots with slopes greater than unity are often taken to be indicative of the co-operative interactions between substrate molecules or inhibitor molecules that are a feature of allosteric enzymes (Monod, Changeux & Jacob, 1963), but, as has been pointed out by Denburg & Deluca (1968), in systems that require metal ions and have substrates that are chelating agents it is important to exclude anomalous effects as a result of chelation before attributing allosteric behaviour to an enzyme. The pyrophosphatase activity associated with pig kidney alkaline phosphatase falls into the category of enzymes discussed by Denburg & Deluca (1968).

The enzyme is inhibited by both excess of PP_i and excess of Mg^{2+} . The greater the concentration of Mg^{2+} the greater is the departure from Michaelis-Menten kinetics. Co-operative binding of substrate molecules that could lead to sigmoid velocity-substrate concentration curves seems to be excluded by other tests, e.g. no change in properties can be demonstrated after heating or after incubation with urea. Such treatment would probably 'desensitize' an allosteric enzyme with concomitant loss of co-operativity (Monod *et al.* 1963). Borate, which might conceivably compete with PP_i for an allosteric site, did not change the nature of the inhibition. The presence of excess of Mg^{2+} , which obtains at low PP_i concentrations, inhibits enzymic activity, but as the concentration of PP_i is raised the

decreasing excess of Mg^{2+} concentration results in less inhibition of activity. The variation in the amount of inhibition as the concentration of PP_i is changed is reflected in sigmoid plots of v against s . When PP_i and Mg^{2+} concentrations are increased together to maintain a constant ratio then a variation in the amount of inhibition is absent and Michaelis-Menten kinetics apply. Thus alkaline phosphatase differs from the pyrophosphatase of mouse liver cytoplasm studied by Horn, Bornig & Thiele (1967), which seems to fit into the category of true allosteric enzymes.

It has been suggested that the true substrate for erythrocyte pyrophosphatase is the complex ion MgPP_i^{2-} (Bloch-Frankenthal, 1954; Robbins *et al.* 1955; Pynes & Younathan, 1967). Eaton & Moss (1967a) found that the pyrophosphatase activities associated with intestinal and liver alkaline phosphatases were not so dependent on Mg^{2+} , and concluded that PP_i is not bound to these enzymes in the form of a Mg^{2+} complex. Pig kidney alkaline phosphatase-pyrophosphatase is activated to a greater extent by bivalent metal ions than the enzymes studied by Eaton & Moss (1967a), but, as with their enzymes and unlike erythrocyte pyrophosphatase (Bloch-Frankenthal, 1954; Robbins *et al.* 1955), excess of Mg^{2+} is strongly inhibitory. From the present results it appears that pig kidney pyrophosphatase is most active towards MgPP_i^{2-} , but, as there is some demonstrable activity in the absence of Mg^{2+} , and a large excess of PP_i^{4-} is not totally inhibitory, it is probable that PP_i^{4-} is also a substrate for the enzyme but is hydrolysed more slowly. With both PP_i^{4-} and MgPP_i^{2-} in the reaction mixtures there will be competition for the enzyme, resulting in an inhibition of activity as a result of the binding of the poorer substrate PP_i^{4-} . A competitive action by PP_i would not, by itself, give rise to the complex inhibition curves found experimentally. Pig kidney alkaline phosphatase is, however, a Zn^{2+} -containing enzyme (Mathies, 1958), and it is possible that PP_i chelates with Zn^{2+} and that this interaction is responsible for some of the inhibition brought about by PP_i .

An increase in the concentration of Mg^{2+} has at least three effects on the enzyme reaction mixture: (a) the concentration of MgPP_i^{2-} , which is the probable substrate, falls; (b) the concentration of Mg_2PP_i , which may be inhibitory, rises; (c) free Mg^{2+} may accumulate and react directly with the enzyme. The inhibitory effect of Mg^{2+} probably derives from a combination of these effects. That Mg_2PP_i is a potent inhibitor is suggested by the finding that for optimum enzymic activities the total PP_i concentration needs to be slightly greater than the total Mg^{2+} concentration. Under these conditions the concentration of Mg_2PP_i will be minimal. At equal concentrations of Mg^{2+} and PP_i

there will be some Mg_2PP_i present, sufficient perhaps to cause some inhibition of activity. Erythrocyte pyrophosphatase does not seem to be strongly inhibited by Mg_2PP_i (Pynes & Younathan, 1967). Free Mg^{2+} also seems to be inhibitory towards alkaline phosphatase-pyrophosphatase. When the total metal ion concentration is many times greater than that of total PP_i , further addition of Mg^{2+} appears to lead to a further decrease in enzymic activity (Fig. 4), yet such an addition would have negligible effect on the concentration of Mg_2PP_i already present.

The effects on the reaction velocity of pig kidney alkaline phosphatase-pyrophosphatase of interactions between PP_i and bivalent metal ions may be important physiologically. The activity of the enzyme could be cellularly controlled by a feedback mechanism that does not rely on the development during evolution of special regulatory binding sites on the enzyme molecule. Also, variations in the concentration of bivalent metal ions in the vicinity of the enzyme could be effective in regulating the specificity of the enzyme for P_i and PP_i substrates.

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