The Influence of Metal Ions on the Orthophosphatase and Inorganic Pyrophosphatase Activities of Human Alkaline Phosphatase

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1. The differential effects of adding Zn^{2+} and Mg^{2+} on the orthophosphatase and inorganic pyrophosphatase activities of human intestinal alkaline phosphatase were studied. 2. In the presence of excess of Zn^{2+} , inorganic pyrophosphatase activity is inhibited. At higher concentrations of pyrophosphate, hydrolysis of this substrate takes place, but is inhibited competitively by the Zn^{2+} -pyrophosphate complex. This complex also acts as a competitive inhibitor of orthophosphate hydrolysis. 3. Excess of Mg^{2+} also inhibits pyrophosphatase action by removal of substrate; at low concentrations, this ion activates pyrophosphatase, as is the case with orthophosphatase. 4. It is concluded that, when interactions between metal ions and pyrophosphate are taken into account, the effects of these ions are consistent with the view that alkaline phosphatases possess both orthophosphatase and inorganic pyrophosphatase activities.

It is now recognized that mammalian alkaline phosphatases (EC 3.1.3.1) can hydrolyse both pyrophosphate and orthophosphate substrates (Cox & Griffin, 1965; Moss, Eaton, Smith & Whitby, 1967; Fernley & Walker, 1967), and it seems probable that a single type of active centre is effective in the hydrolysis of both classes of substrate (Eaton & Moss, 1967*a*; Fernley & Bisaz, 1968). However, Mg^{2+} has a differential action on the two activities of alkaline phosphatase, activating the hydrolysis of orthophosphates but inhibiting that of pyrophosphates (Fernley & Walker, 1967; Eaton & Moss, 1967*a,b*). Similarly, Zn^{2+} inhibits orthophosphatase activity powerfully and non-competitively when present in all except very low concentrations, whereas it exerts a less powerful but competitive inhibition on the hydrolysis of PP_i . It is apparent that the explanation of these differential effects is to be found in interaction of metal ions with pyrophosphate substrates, rather than in the existence of distinct orthophosphatase and pyrophosphatase enzymes or separate active centres. The influence of Zn^{2+} and Mg^{2+} on the two activities of purified human alkaline phosphatase has now been studied in more detail, and the results are compatible with the suggestion that both catalytic activities are properties of a single type of active centre. A preliminary account of some of these results has been presented (Moss, 1969).

MATERIALS AND METHODS

Alkaline phosphatase from human small intestine was purified as described by Moss *et al.* (1967). The hydrolysis of both an orthophosphate substrate, *p*-nitrophenyl phosphate, and PP_i was carried out by incubation with the enzyme preparation in 0.2M-tris-HCl buffer, pH 9.0, at 37° for periods of up to 5 min., the concentration of the enzyme solution being adjusted to give a measurable amount of hydrolysis in this time. Release of *p*-nitrophenol was determined by measurements of E_{400} and the amount of P_i liberated was measured by the method of Delsal & Manhour (1958). Substrate concentrations and additions of MgSO_4 and ZnSO_4 were as noted in the Results section. Enzyme activities are expressed as μmoles of substrate hydrolysed/min./ml. of enzyme solution.

The pH value of 9.0 was chosen because activity towards both substrates can be measured at this pH over a wide range of substrate concentration, and the concentration of buffer is sufficiently high to prevent significant changes of pH on addition of PP_i .

RESULTS

Effect of Zn^{2+} . Addition of Zn^{2+} alters the relationship between reaction velocity, *v*, and substrate concentration, *s*, for hydrolysis of PP_i by intestinal phosphatase from a hyperbolic form to a sigmoid shape (Fig. 1). The steeply rising portions of the curves begin when the molar concentrations of Zn^{2+} and PP_i are approximately equal; below this point there is little release of P_i . The shapes

Fig. 1. Effect of adding Zn^{2+} on the relationship between rate of hydrolysis of PP_1 , v , and substrate concentration, s , by intestinal alkaline phosphatase at pH 9.0. Concentrations of Zn^{2+} : $\circ$, zero; $\bullet$, 0.5 mM; $\triangle$, 1.0 mM; $\blacktriangle$, 2.0 mM; $\square$, 5.0 mM.

of the curves suggest, when Zn^{2+} is in excess and essentially all the PP_1 is present as a Zn^{2+} - PP_1 complex, either that this complex is not a substrate of the enzyme or that its hydrolysis is inhibited by Zn^{2+} . When the molar ratio of PP_1 to Zn^{2+} is greater than unity the appearance of free PP_1 results in rapid enzyme activity.

The curves can be redrawn to originate from zero velocities at equimolar Zn^{2+} and PP_1 concentrations and the 'effective PP_1 ' concentration obtained for each curve by subtracting an amount equal to the Zn^{2+} concentration from the total PP_1 . The resulting curves show a residual inhibition when compared with the activity observed without addition of Zn^{2+} and plots of s/v against s show this to be of a competitive type (Fig. 2). This observation can be accounted for by assuming that the Zn^{2+} - PP_1 complex, which is probably the ion $\text{ZnP}_2\text{O}_7^{2-}$ and which is assumed to be essentially equal in concentration to the total Zn^{2+} concentration when PP_1 is in excess, is a competitive inhibitor of pyrophosphatase activity. If this explanation is correct, and if orthophosphatase and pyrophosphatase activities are properties of a single enzyme, the complex should also inhibit orthophosphate hydrolysis. This was demonstrated by adding equimolar amounts of Zn^{2+} and PP_1 to the p -nitrophenyl phosphatase assay system, with the results shown in Fig. 3. To minimize the possibility of interference by free Zn^{2+} , a slight excess of PP_1 was added to the extent of 5% over the exactly equimolar ratio. Competitive inhibition was observed, but the calculated values for K_i were not identical; these were approx. $0.5 \times 10^{-3} \text{ M}$ for the inhibition of PP_1 hydrolysis and $2 \times 10^{-3} \text{ M}$ for orthophosphatase inhibition. However, K_m values for the two activities differ by an order of magnitude at this pH value (Eaton & Moss, 1968), so that the precision

Fig. 2. Plots of s/v against effective s derived from Fig. 1, showing competitive inhibition by presumed $\text{ZnP}_2\text{O}_7^{2-}$ complex ion. Inhibitor concentrations: $\circ$, zero; $\bullet$, 0.5 mM; $\triangle$, 1.0 mM; $\blacktriangle$, 2.0 mM. K_i is approx. $0.5 \times 10^{-3} \text{ M}$. (The effective substrate concentration is obtained by subtracting an amount equal to the Zn^{2+} concentration from each concentration of PP_1 .)

Fig. 3. Effect of adding equimolar amounts of Zn^{2+} and PP_1 on the relationship between the rate of hydrolysis of p -nitrophenyl phosphate at pH 9.0 by intestinal alkaline phosphatase and substrate concentration, plotted as s/v against s , showing competitive inhibition by presumed $\text{ZnP}_2\text{O}_7^{2-}$ complex ion. Concentrations of PP_1 and Zn^{2+} : $\circ$, zero; $\bullet$, 4 mM. K_i is approx. $2 \times 10^{-3} \text{ M}$.

of the estimates of K_i is probably not of the same order for the two substrates.

The influence of the Zn^{2+} - PP_1 complex on orthophosphate hydrolysis may be contrasted with the effect of Zn^{2+} alone on this activity, which is of a non-competitive or mixed type.

Effect of Mg^{2+} . Addition of Mg^{2+} alters the shape of the curves obtained by plotting v against s for the inorganic pyrophosphatase activity of intestinal

phosphatase in a similar way to the addition of Zn^{2+} . However, the steeply rising portions of the curves are not reached until the concentration of PP_1 exceeds about half that of Mg^{2+} . Above this ratio activation of the rate of hydrolysis is seen. Precipitation of $\text{Mg}_2\text{P}_2\text{O}_7$ was not evident in these experiments.

DISCUSSION

In the interpretation of these results it has been assumed that under the conditions of the experiments the metal ions will be almost entirely in the form of complexes with PP_1 when the latter is in excess, and values for the binding constants for these ions by PP_1 (Sillén, 1964) indicate that this is probably the case.

A mixture of Zn^{2+} and PP_1 inhibits both inorganic pyrophosphatase and orthophosphatase activities in a similar manner. This inhibition is of competitive type and appears to be due to the complex ion $\text{ZnP}_2\text{O}_7^{2-}$. The effect on inorganic pyrophosphatase activity of adding Mg^{2+} is less easy to interpret, since activation of the enzyme also occurs. However, it can be concluded that high concentrations of Mg^{2+} inhibit PP_1 hydrolysis by the removal of substrate, apparently as $\text{Mg}_2\text{P}_2\text{O}_7$ even though no

precipitate of this salt may be observed. As the substrate concentration is increased to overcome this inhibition hydrolysis of PP_1 begins to take place at a rate faster than that observed when no Mg^{2+} is added; thus pyrophosphatase activity is enhanced by Mg^{2+} as is orthophosphate hydrolysis, and there is presumably enough free Mg^{2+} to produce this effect even when PP_1 is in excess. The effects of both Zn^{2+} and Mg^{2+} are therefore compatible with the conclusion that this alkaline phosphatase possesses both orthophosphatase and inorganic pyrophosphatase activities.

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