Kinetics of Alkaline Phosphatase from Pig Kidney

INFLUENCE OF COMPLEXING AGENTS ON STABILITY AND ACTIVITY

By BERND PETER ACKERMANN and JAN AHLERS*

Institut für Biochemie der Universität Mainz, 6500 Mainz, J.-J.-Becher-Weg 28, W. Germany

(Received 17 July 1975)

Metal ion-complexing agents, like KCN, EDTA etc., inactivate alkaline phosphatase of pig kidney. This inactivation is reversible at low concentrations of the complexing agents and irreversible at high concentrations. The reversible inhibition is probably due to removal of Zn^{2+} ions from the active site, where they are necessary for catalytic action, whereas the irreversible inhibition results from the removal of Zn^{2+} ions necessary for preservation of the structure. The inactivation is pseudo-first-order. It depends on the concentration, size and charge of the complexing agents. β -Glycerophosphate and Mg^{2+} ions protect the enzyme from inactivation by complexing agents. Quantitative examination of the effect of substrate leads to a model that is similar to the 'sequential model' proposed by D. E. Koshland, G. Nemethy & D. Filmer (1966) (*Biochemistry* 5, 365–385) to explain allosteric behaviour of enzymes. It describes the sequential addition of two substrate molecules at two active centres of the dimer enzyme. The binding of the substrate molecules is accompanied by changes in the conformation, which lead to stabilization of the enzyme against attack by complexing agents.

Alkaline phosphatase (EC 3.1.3.1) is a metallo-enzyme (Fernley, 1971). The function of metal ions in the structure and activity of the enzyme has mostly been examined for *Escherichia coli* alkaline phosphatase, which contains four Zn^{2+} ions per dimer (Simpson & Vallee, 1968; Lazdunski & Lazdunski, 1969; Lazdunski *et al.*, 1969). Each subunit of the *E. coli* alkaline phosphatase contains one active site with two Zn^{2+} ions, one responsible for activity and the other responsible for stabilization of the structure (Lazdunski *et al.*, 1969; Petitclerc *et al.*, 1970; Trotman & Greenwood, 1971). There is no concrete information available at present on the number and function of Zn^{2+} ions associated with mammalian alkaline phosphatases. Cloetens (1941*a,b*) found that Zn^{2+} ions are essential for activity and Mathies (1958) found that the activity is directly proportional to the Zn^{2+} content. Hiwada & Wachsmuth (1974) reported the presence of two Zn atoms per molecule of pig kidney enzyme, and Fosset *et al.* (1974) showed that calf intestinal alkaline phosphatase contains four atoms of zinc per enzyme molecule.

In contrast with the *E. coli* enzyme, mammalian alkaline phosphatases are activated by Mg^{2+} ions (Fernley, 1971). These ions are able to react with the free enzyme as well as with the enzyme-substrate complex; they are not involved in substrate binding, but increase V (Ahlers, 1974). Other bivalent cations, especially Mn^{2+} , also activate mammalian alkaline phosphatase, but to a lesser extent (Brunel & Cathala,

1973), but, as yet, there are no unambiguous results on the influence of Ni^{2+} , Co^{2+} and Ca^{2+} (Fernley, 1971). This is because it usually has not been examined whether these cations can replace Zn^{2+} or Mg^{2+} ions, and whether they act directly with the enzyme, merely release the true activating ion from a complexing agent present in the medium, or replace an inactivating metal ion which is inhibiting the enzyme. Other metal ions, e.g. Be^{2+} , or the constituent metal ions at higher concentrations, e.g. Zn^{2+} , inhibit alkaline phosphatase (Cloetens, 1941*c*; Klemperer *et al.*, 1949; Hiwada & Wachsmuth, 1974). There is as yet only little information about the kinetics of inhibition (Fernley, 1971). As the alkaline phosphatase from mammalian organs is a metalloprotein associated with two different metal ions, investigation of the effect of complexing agents on the enzyme is difficult. In particular, there is little information on discrimination between the reactions of the complexing agent with the two metal ions, the reversibility of the reaction and the influence of substrate and metal ions on the course of inhibition.

Lazdunski & Ouellet (1962) found non-competitive inhibition of alkaline phosphatase by cysteine after a short preincubation time, whereas Wallach & Ko (1964) observed competitive inhibition and Agus *et al.* (1966) mixed inhibition, both from measurements made without preincubation. Cyanide (Mathies, 1958; Cloetens, 1941*b,c*) and EDTA (Mathies, 1958; Nüske & Venner, 1966; Agus *et al.*, 1966; Conyers *et al.*, 1967; Butterworth, 1968; Schüssler, 1968) have also been used as inhibitors of different mammalian alkaline phosphatases. Csopak

* Present address: Zentralinstitut für Biochemie und Biophysik, 1 Berlin 33, Ehrenbergstraße 26–28, Germany.

& Szajn (1973) found that EDTA bound to the metal-free apo-(alkaline phosphatase) from *E. coli*. Bramley (1975) reported that mouse ovarian alkaline phosphatase could only be inactivated by EDTA in the presence of glycine or other amino acids. Whereas the inhibition by cyanide can be reversed by addition of Zn^{2+} ions (Cloetens, 1941*b,c*; Bartels & Christopher, 1970), the inhibition by EDTA could only be partially reversed by adding Zn^{2+} or $\text{Zn}^{2+} + \text{Mg}^{2+}$ [Agus *et al.* (1966); P. J. Butterworth, unpublished work, cited in Butterworth (1968)]. In the present paper the influence of substrate, Mg^{2+} and Zn^{2+} on the inactivation of alkaline phosphatase from pig kidney by complexing agents is examined.

Experimental

Materials

DEAE-cellulose was obtained from Serva, Heidelberg, Germany; Sephadex was from Pharmacia, Uppsala, Sweden; all other chemicals were from Merck, Darmstadt, Germany.

Pig kidneys were used as the source of alkaline phosphatase.

Methods

The preparation of pig kidney alkaline phosphatase and the determination of enzyme activity was as described by Ahlers (1974, 1975). Under experimental conditions the enzyme was stable for several days.

The test medium for kinetic measurements contained 50 mM- Na_2CO_3 buffer, pH 9.6, as well as different amounts of enzyme, MgCl_2 , ZnCl_2 , various complexing agents and β -glycerophosphate as reported in the Results and Discussion section. The enzyme was preincubated under the conditions given in the Results and Discussion section until equilibrium was achieved. The reaction was then started by the addition of β -glycerophosphate. By continuously recording the amount of phosphate released, it could be established with certainty that the progress curves were linear over the period required for determination of initial rate. Because of continuous recording these results were reproducible with an error of less than 10%. Under optimum conditions the error was less than 3%.

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. The concentration of free metal ion was calculated according to eqn. (1).

$$[\text{Me}^{2+}] = -\frac{[\text{L}]_t - [\text{Me}]_t + K_{\text{MeL}}}{2} + \sqrt{\frac{([\text{L}]_t - [\text{Me}]_t + K_{\text{MeL}})^2}{4} + K_{\text{MeL}} \cdot [\text{Me}]_t} \quad (1)$$

The concentrations of the MeL complex and of the free complexing agent are given in eqns. (2) and (3).

$$[\text{MeL}] = [\text{Me}]_t - [\text{Me}] \quad (2)$$

$$[\text{L}] = [\text{L}]_t - [\text{MeL}] \quad (3)$$

K_{MeL} values were obtained from Sillén & Martell (1964) and corrected for pH 9.6 [$K'_{\text{MeL}} = K_{\text{MeL}}(1 + [\text{H}^+]/K_{\text{HL}})$] as described by Wolf (1973) and Ahlers (1974); $\text{p}K'_{\text{MgNTA}} = 5.7$; $\text{p}K'_{\text{ZnNTA}} = 10.3$; $\text{p}K'_{\text{MgEDTA}} = 8.0$; $\text{p}K'_{\text{ZnEDTA}} = 15.8$; $\text{p}K'_{\text{MgHEDTA}} = 6.6$; $\text{p}K'_{\text{ZnHEDTA}} = 14.1$; $\text{p}K'_{\text{Mgt-CDTA}} = 8.3$; $\text{p}K'_{\text{Znt-CDTA}} = 16.4$.

Further, the influence of Mg^{2+} concentration on Zn^{2+} binding and of Zn^{2+} concentration on Mg^{2+} binding was taken into account as described in the Appendix of Ahlers (1974) and in Wolf (1973).

All values are averages of two or more experiments performed in duplicate.

Symbols and abbreviations

$t_{1/2}$, time necessary for 50% inhibition; *t*-CDTA, *trans*-cyclohexane-1,2-diaminetetra-acetate; Me, metal ion; HEDTA, *N'*-(2-hydroxyethyl)ethylenediaminetriacetate; NTA, nitrilotriacetate; L, ligand. All other symbols and abbreviations have been defined by Ahlers (1974, 1975).

Results and Discussion

Possible reactions of cysteine, cyanide and EDTA with the alkaline phosphatase

Inhibition of alkaline phosphatase by cysteine, cyanide or EDTA may be the consequence of one of the following reversible reactions. (1) The complexing agent lowers the concentration of free Zn^{2+} ions so that the native E-Zn complex dissociates in a monomolecular reaction. (2) The complexing agent removes the Zn^{2+} ion from the enzyme in a bimolecular reaction. (3) The complexing agent aggregates with the enzyme-bound Zn^{2+} and prevents substrate binding or conversion or induces a particular conformation. (4) The complexing agent does not react with the Zn^{2+} in the active site but with a metal ion at another site of the enzyme and induces a conformational change. In this connexion it is noteworthy that Csopak & Szajn (1973) observed binding of EDTA to the metal-free apoenzyme.

Irreversible inhibition may be due to one of the following reactions. (5) After reactions (1) and (2) the enzyme is denatured in a monomolecular reaction. (6) If the enzyme contains two classes of sites for Zn^{2+} , then, after elimination of the Zn^{2+} ion that is essential for catalytic activity, the Zn^{2+} ion that is essential for preserving the structure is removed, causing irreversible denaturation of the enzyme. (7) After reaction (3) the Zn^{2+} ion is removed, causing irreversible changes in the enzyme.

Effect of the concentration of the complexing agent on the time-course of inactivation

To decide between the possible reversible reactions (1)–(4) we examined the velocity of inactivation (v) as a function of EDTA concentration. To maintain a constant Zn^{2+} concentration we used a constant ratio of $[\text{ZnEDTA}]/[\text{EDTA}] = 1:1$, resulting in a concentration of $0.11 \mu\text{M}-\text{Zn}^{2+}$. The enzyme was preincubated at pH 9.6 and 30°C with different concentrations of $\text{ZnEDTA}/\text{EDTA}$. At various times a sample was taken and the activity was determined by adding β -glycerophosphate and different amounts of MgCl_2 to achieve a final concentration of free Mg^{2+} of 4 mM . In Fig. 1 $\log v$ is plotted against the incubation time for various concentrations of EDTA. The straight lines obtained with different slopes show that the inactivation is pseudo-first-order and thus really second-order. As the EDTA concentration is much larger than the enzyme concentration the concentration of the complexing agent does not change

Fig. 1. Plots of $\log v$ versus time of preincubation with various concentrations of EDTA

The enzyme was preincubated at pH 9.6, 30°C and a concentration of free Zn^{2+} ions of $0.11 \mu\text{M}$ with EDTA at the following concentrations: $\bullet$, 0.1 mM ; $\circ$, 0.4 mM ; $\square$, 1.0 mM ; $\triangle$, 4.0 mM ; $\blacktriangle$, 10.0 mM . Enzyme activity was determined after different preincubation times. The assay was done at pH 9.6 and 30°C in an assay system containing $10 \text{ mM}-\beta$ -glycerophosphate and $4 \text{ mM}-\text{MgCl}_2$.

significantly in the course of the reaction, and thus no deviation from the straight lines can be seen.

The following rate equation can be deduced.

$$\log \frac{[\text{ZnE}]}{[\text{ZnE}]_0} = -k_2 \cdot [\text{L}]_0 \cdot t / 2.303 = \log v - \log v_0 \quad (4)$$

where $[\text{ZnE}]_0$ is the concentration of the catalytically active $\text{Zn}^{2+}-\text{E}$ complex at $t=0$ and $[\text{L}]_0$ is the concentration of the complexing agent at $t=0$.

If the assumption that the inactivation of alkaline phosphatase is a consequence of a bimolecular reaction between enzyme and EDTA is correct, then the slope of the straight lines from Fig. 1 should be a function of EDTA concentration. To prove this, the slopes of the straight lines from Fig. 1 were plotted against EDTA concentration (Fig. 2). It can be seen that this assumption is indeed correct.

Effect of the size and charge of the complexing agent on the rate of inactivation

In preliminary experiments (not shown) we found that the small complexing agents cysteine and cyanide react rapidly with the enzyme, whereas the larger EDTA molecule reacts much more slowly. Therefore one can assume that the target of these inhibitors, and thus the active centre of pig kidney alkaline phosphatase, is not exposed, but is rather in the interior of the protein molecule. To obtain more information on the dependence of the rate of inactivation on the size and charge of the complexing agent, we examined the effect of various complexing agents on alkaline phosphatase. For a better comparison we only used derivatives of aminoacetic acid, namely t-CDTA, EDTA, HEDTA and NTA.

Alkaline phosphatase was preincubated at 25°C and pH 9.6 with either $1 \text{ mM}-\text{ZnL} + 1 \text{ mM}-\text{L}$ or with $2 \text{ mM}-\text{L} + 0.1 \mu\text{M}-\text{ZnL}$. The total concentration of complexing agents was 2 mM in all cases. The concentration of free Zn^{2+} ions varied according to the pK_{ZnL} values of the different complexing agents and to the ratio $[\text{ZnL}]/[\text{L}]$. After different times of

Fig. 2. Plot of the slopes of the straight lines from Fig. 1 against EDTA concentration

Fig. 3. Plots of $\log v$ versus time of preincubation

The enzyme was preincubated at pH 9.6 and 25°C with 1 mM-NTA + 1 mM-ZnNTA (■), 1 mM-t-CDTA + 1 mM-Zn-t-CDTA (□), 2 mM-t-CDTA (○) or 2 mM-NTA (●). Enzyme activity was determined after different preincubation times. The assay was done at pH 9.6 and 25°C in an assay system containing 10 mM- β -glycerophosphate and 4 mM-MgCl₂.

Fig. 4. Plots of $\log v$ versus time of preincubation

The enzyme was preincubated at pH 9.6 and 25°C with 1 mM-EDTA + 1 mM-ZnEDTA (■), 2 mM-EDTA (○), 1 mM-HEDTA + 1 mM-ZnHEDTA (●) or 2 mM-HEDTA (□). Enzyme activity was determined after different preincubation times. The assay was done at pH 9.6 and 25°C in an assay system containing 10 mM- β -glycerophosphate and 4 mM-MgCl₂.

preincubation, samples were taken and the activity was determined by adding β -glycerophosphate and MgCl₂. In Figs. 3 and 4, $\log v$ is plotted against the time of incubation for different complexing agents and different Zn²⁺ concentrations.

Fig. 3 shows the results obtained with the complexing agents of largest (t-CDTA) and smallest (NTA) molecular size. The velocity of inactivation is indeed much lower in the presence of t-CDTA than in the presence of NTA. The size of the complexing agent is therefore important in the inactivation of alkaline phosphatase. If only the concentration of free Zn²⁺ were important, then inactivation with t-CDTA would be faster than that with nitrilotriacetate. As expected, an exception results from the experiments with ZnNTA-NTA buffer, as a free Zn²⁺ concentration of 50 μ M is achieved by this metal buffer, which is high enough to prevent dissociation of the Zn-E complex.

Fig. 4 shows the results obtained by using two complexing agents of similar size. In this case, one would expect that the concentration of free Zn²⁺ would be decisive for the rate of inactivation and

therefore that the alkaline phosphatase would be inactivated more rapidly by EDTA than by HEDTA. As can be seen from Fig. 4, the opposite takes place. This can be explained by the difference in charge of the complexing agents used. HEDTA has two negative groups at pH 9.6, whereas EDTA has three. The enzyme protein possesses more negative than positive charges at alkaline pH, so that a (complexing-agent) anion can less readily reach the active site, the more negative charges it bears.

When comparing the rates of inactivation by the same complexing agent but at different concentrations of Zn²⁺ we get the same results in all cases; at low concentrations of free Zn²⁺, which also means a higher concentration of free complexing agent, alkaline phosphatase is inactivated more rapidly. Our results show that alkaline phosphatase is slowly inactivated when the concentration of free Zn²⁺ ions is significantly lower than 50 μ M. Complexing agents accelerate the rate of inactivation as a result of a bimolecular reaction with the enzyme, and this rate depends on the concentration and size of and the number of charges on the complexing agent.

Fig. 5. Dependence of k , the rate of inactivation by EDTA, on the substrate concentration

The enzyme was preincubated at pH 9.6 and 30°C with 1 mM-EDTA and various substrate concentrations. The activity was determined at different times at pH 9.6 and 30°C in an assay system containing 2 mM-MgCl₂ and 10 mM- β -glycerophosphate. k was obtained from the straight lines of $\log v$ -versus-time plots for various substrate concentrations. $\circ$, Measured values; —, values calculated according to eqn. (7) with $k_E = 0.196 \text{ M}^{-1} \cdot \text{s}^{-1}$; $k_{ES} = 0.07 \text{ M}^{-1} \cdot \text{s}^{-1}$; $k_{ES_2} = 0$; $K_S = 16 \mu\text{M}$; $K_{S_2} = 1.0 \text{ mM}$.

Protection from inactivation by complexing agents in the presence of substrate

Conyers *et al.* (1967) observed stabilization of alkaline phosphatase by substrate. Further, the two functional Zn²⁺ ions of alkaline phosphatase from *E. coli* dissociate less readily in the presence of increasing amounts of P_i (Simpson & Vallee 1968; Lazdunski *et al.*, 1969). However, these effects have not yet been quantitatively examined.

To examine quantitatively the effect of substrate on the rate of inactivation we incubated the alkaline phosphatase at 30°C and pH 9.6 with 1 mM-EDTA and various amounts of β -glycerophosphate. Under these conditions, the substrate is not decomposed by the enzyme as no free Mg²⁺ ions are present in the assay. At different times, samples were taken and the activity was determined by adding Mg²⁺ and substrate to final concentrations of 1 mM-MgCl₂ and 10 mM- β -glycerophosphate respectively. Plotting $\log v$ against the incubation time (not shown) we obtained straight lines from 0.5–7 h, indicating that not only in the absence, but also in the presence, of substrate the rate of inactivation is pseudo-first-order. The slopes of the straight lines are a function of the substrate concentration. It is noteworthy that β -glycerophosphate concentrations of $K_m/200$ already lead to significant deceleration of the rate of inactivation.

From the slopes of the straight lines the reaction rate constants k were calculated and plotted against the substrate concentration (Fig. 5). The observed effect of substrate can be explained either by the assumption that Zn²⁺ ions are within the substrate-binding site or that the substrate molecule produces a conformational change in the alkaline phosphatase molecule. In the following we will examine whether the results shown in Fig. 5 are in accordance with models commonly used to explain conformational changes. Of course, this can at best be only an analogy because we have examined the influence of substrate on the stability of the enzyme, whereas the models describe the relationship between addition of substrate, conformation and activity of the enzyme.

(a) According to the 'induced fit theory' (Koshland, 1958) the addition of the substrate molecule induces a conformational change:

where $\circ$ is the conformation of the free enzyme and $\boxed{S}$ is the conformation of the ES complex. Our results suggest that the ES complex reacts more slowly with EDTA than does the free enzyme. Thus the rate constant for inactivation k is composed of the different rate constants k_E and k_{ES} of the inactivation of E and ES respectively. Calculating the dependence of k on the substrate concentration (not shown) we get a curve that does not fit the results shown in Fig. 5. Thus we have to take a more complex model into account.

(b) The 'sequential model' (Koshland *et al.*, 1966) postulates the sequential addition of two (or more) substrate molecules to one enzyme molecule:

The reaction of the enzyme with the substrate molecules leads to conformations ES and ES₂. These complexes react less readily with EDTA than does the free enzyme. Thus the rate constant k of the total inactivation is composed of the rate constants k_E , k_{ES} and k_{ES_2} .

$$k = \frac{(k_E \cdot K_S \cdot K_{S_2}) + (k_{ES_2} \cdot K_{S_2} \cdot [S]) + (k_{ES_2} \cdot [S]^2)}{(K_S \cdot K_{S_2}) + (K_{S_2} \cdot [S]) + [S]^2} \quad (7)$$

k_E can be calculated from the results presented in Fig. 5 for $[S] = 0$ and k_{ES_2} for $1/[S] = 0$. The other constants k_{ES} , K_S and K_{S_2} were systematically varied by means of a computer program until the best fit between the calculated and the measured values for k was obtained. With $k_E = 0.196 \text{ M}^{-1} \cdot \text{s}^{-1}$, $k_{ES} = 0.07 \text{ M}^{-1} \cdot \text{s}^{-1}$, $k_{ES_2} = 0$, $K_S = 16.0 \mu\text{M}$ and $K_{S_2} = 1.0 \text{ mM}$, the solid line shown in Fig. 5 was drawn. From this it can be seen that the hyperbolic function

Table 1. *Inhibition of alkaline phosphatase by EDTA, cyanide or cysteine under various conditions*

All incubations were carried out at pH9.6 and 30°C.

Expt. no.	Conditions of incubation	$t_{\frac{1}{2}}$ (h)
1	2 mM-EDTA+2 mM-MgCl ₂ + 1 mM-ZnCl ₂	No inhibition
2	2 mM-EDTA+1 mM- β -glycero- phosphate	5.7
3	2 mM-EDTA+2 mM-MgCl ₂	1.2
4	2 mM-EDTA	1.0
5	2 mM-cyanide+2 mM-MgCl ₂ + 0.25 mM-ZnCl ₂	6.2
6	2 mM-cyanide+5 mM- β -glycero- phosphate	0.8
7	2 mM-cyanide+2 mM-MgCl ₂	9.0
8	2 mM-cyanide	0.1
9	1 mM-cysteine+2 mM-MgCl ₂ + 0.25 mM-ZnCl ₂	50
10	1 mM-cysteine+1 mM- β -glycero- phosphate	0.3
11	1 mM-cysteine+2 mM-MgCl ₂	14.7
12	1 mM-cysteine	0.1

(7) fits the measured results perfectly. Thus the 'sequential model' is best able to explain our results. It implies that the first substrate molecule is bound to one active site with a different dissociation constant from that of the second substrate molecule binding to the other active site or to a regulatory site. Interestingly, the dissociation constant of the second substrate molecule, K_{s_2} , is identical with the K_m value evaluated by Ahlers (1975).

Effect of Mg²⁺ and Zn²⁺ on the rate of inactivation by EDTA, cyanide and cysteine

When investigating the effect of Mg²⁺ ions on the rate of inactivation, one has to consider that changing the concentration of Mg²⁺ at constant concentration of a complexing agent such as EDTA, leads to a change in the concentrations of free Zn²⁺ ions. Therefore it is advantageous to use a complexing agent such as cyanide, which does not bind Mg²⁺ ions. To obtain information on the effect of Mg²⁺, for comparison with the effect of substrate, on the rate of inactivation, we incubated alkaline phosphatase at pH9.6 and 30°C with EDTA, cyanide or cysteine under the conditions given in Table 1. The activity was determined after various time-intervals. In the presence of Mg²⁺+Zn²⁺, alkaline phosphatase is not inhibited by EDTA (Expt. 1). This may be due to one of two effects of Mg²⁺, an increase in the concentration of free Zn²⁺ ions or protection of the active site by Mg²⁺ ions, which can also be seen in Expt. 3. Thus Mg²⁺ has an effect similar to that of the substrate (Expt. 2), which has been discussed above. This

Fig. 6. *Reversible and irreversible inhibition of alkaline phosphatase by EDTA*

The enzyme was preincubated for 16h at pH9.6 and 21°C with various concentrations of EDTA. Then the activity in a sample was determined at pH9.6 and 21°C in an assay system containing 10mM- β -glycerophosphate and 2mM-MgCl₂ (●). Another sample of the preincubated enzyme was further incubated for 10h at pH9.6 and 21°C with 15mM-ZnNTA and 5mM-MgNTA; the activity was then determined as described above (○). If the inhibition by EDTA was reversible these activities should approach 100%.

effect can also be seen in Expt. 7, where a complexing agent that does not react with Mg²⁺ ions was used, so the observed effect must be due to direct interaction of the Mg²⁺ ions with the enzyme molecule.

By comparing the results of Expts. 2 and 3, 6 and 7 and 10 and 11, one can see that the action of substrate and MgCl₂ discussed above are principally the same when quite different complexing agents have been used. The results of Expts. 1 and 4, 5 and 8 and 9 and 12 show that Zn²⁺ ions stabilize alkaline phosphatase under all experimental conditions. Further, these results show that inhibition of pig kidney alkaline phosphatase by cyanide and cysteine is due to their reaction with Zn²⁺ ions and not to their reaction with disulphide bridges in the enzyme.

Reversible and irreversible inactivation of alkaline phosphatase by complexing agents

To discriminate between reversible and irreversible reactions of complexing agents with kidney alkaline phosphatase, the enzyme was preincubated for 16h at pH9.6 and 21°C with various concentrations of EDTA, cysteine or cyanide. The activity was then determined at pH9.6 and 21°C in one sample, and another sample was incubated with 15mM-ZnNTA and 5mM-MgNTA for 10h at the same pH and temperature; the activity was then determined as before. In Fig. 6 the activity is plotted against -log (EDTA concentration). A typical dissociation curve is obtained when the enzyme is incubated with EDTA in the absence of Mg²⁺ and Zn²⁺. The primary activity could be restored by adding Zn²⁺ and Mg²⁺ at EDTA concentrations lower than 0.1mM. Thus

inhibition of alkaline phosphatase by small concentrations of EDTA is reversible. At EDTA concentrations higher than 0.1 mM, where the reversible inactivation is nearly complete, we find only partial reactivation, which diminishes with increasing EDTA concentration. An exception was the inhibition by MgEDTA, which was reversible even at high concentrations of the inhibitor (not shown). An explanation for this is the relatively high concentration of free Zn^{2+} ions in the presence of high Mg^{2+} concentrations, which prevents irreversible removal of Zn^{2+} ions from the enzyme.

These results indicate the presence of two different Zn^{2+} ions per active centre, which are bound by the enzyme with different strengths. The reversibly bound Zn^{2+} ions must have catalytic properties, whereas the irreversibly bound Zn^{2+} ions are essential for structural stability. Similar results are reported by Lazdunski *et al.* (1969), Petitclerc *et al.* (1970) and Trotman & Greenwood (1971) for *E. coli* alkaline phosphatase.

We obtained very similar results when either cysteine or cyanide was used as complexing agent. This similarity between inhibition by cysteine, cyanide and EDTA is further evidence that cyanide and cysteine inhibit pig kidney alkaline phosphatase only in their capacity as complexing agents. Cyanide therefore is the most suitable complexing agent as it does not form complexes with Mg^{2+} ions, i.e. the apparent dissociation constant of $\text{Zn}(\text{CN})_4$ is independent of Mg^{2+} concentration.

We thank Dr. H. U. Wolf for helpful suggestions and valuable discussion and Miss H. Köth for technical assistance.

References

- Agus, S. G., Cox, R. P. & Griffin, M. J. (1966) *Biochim. Biophys. Acta* **118**, 363–370
- Ahlers, J. (1974) *Biochem. J.* **141**, 257–263
- Ahlers, J. (1975) *Biochem. J.* **149**, 535–546
- Bartels, W. & Christophersen, J. (1970) *Hoppe-Seyler's Z. Physiol. Chem.* **351**, 213–226
- Bramley, T. A. (1975) *Biochem. J.* **147**, 259–265
- Brunel, C. & Cathala, G. (1973) *Biochim. Biophys. Acta* **309**, 104–115
- Butterworth, P. J. (1968) *Biochem. J.* **108**, 243–246
- Cloetens, R. (1941a) *Biochem. Z.* **307**, 352–365
- Cloetens, R. (1941b) *Biochem. Z.* **308**, 37–39
- Cloetens, R. (1941c) *Biochem. Z.* **310**, 42–53
- Conyers, R. A. J., Birkett, D. J., Neale, F. C., Posen, S. & Brudenell-Woods, J. (1967) *Biochim. Biophys. Acta* **139**, 363–371
- Csopak, H. & Szajn, H. (1973) *Arch. Biochem. Biophys.* **157**, 374–379
- Fernley, H. N. (1971) *Enzymes* **4**, 417–447
- Fosset, M., Chappelet-Tordo, D. & Lazdunski, M. (1974) *Biochemistry* **13**, 1783–1787
- Hiwada, K. & Wachsmuth, E. D. (1974) *Biochem. J.* **141**, 283–391
- Klemperer, F. W., Miller, J. M. & Hill, C. J. (1949) *J. Biol. Chem.* **180**, 281–288
- Koshland, D. E. (1958) *Proc. Natl. Acad. Sci. U.S.A.* **44**, 98–104
- Koshland, D. E., Nemethy, G. & Filmer, D. (1966) *Biochemistry* **5**, 365–385
- Lazdunski, C. & Lazdunski, M. (1969) *Eur. J. Biochem.* **7**, 294–300
- Lazdunski, M. & Ouellet, L. (1962) *Can. J. Biochem.* **40**, 1619–1639
- Lazdunski, C., Petitclerc, C. & Lazdunski, M. (1969) *Eur. J. Biochem.* **8**, 510–517
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Mathies, J. C. (1958) *J. Biol. Chem.* **233**, 464–466
- Nüske, R. & Venner, H. (1966) *Biochem. Z.* **346**, 226–243
- Petitclerc, C., Lazdunski, C., Chappelet, D., Mouling, A. & Lazdunski, M. (1970) *Eur. J. Biochem.* **14**, 301–308
- Schüssler, H. (1968) *Biochim. Biophys. Acta* **151**, 383–393
- Sillén, L. G. & Martell, A. E. (1964) *Chem. Soc. Spec. Publ.* no. 17
- Simpson, R. T. & Vallee, B. L. (1968) *Biochemistry* **7**, 4343–4350
- Trotman, C. N. A. & Greenwood, C. (1971) *Biochem. J.* **124**, 25–30
- Wallach, D. P. & Ko, H. (1964) *Can. J. Biochem.* **42**, 891–896
- Wolf, H. U. (1973) *Experientia* **29**, 241–249