Kinetics of Alkaline Phosphatase from Pig Kidney

MECHANISM OF ACTIVATION BY MAGNESIUM IONS

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(Received 28 December 1973)

The mechanism of activation of alkaline phosphatase (EC 3.1.3.1) from pig kidney by Mg^{2+} ions was investigated with the aid of kinetic measurements. Mg^{2+} ions are essential for enzyme activity. The following model (Scheme 1 of the text) for the reaction of enzyme, substrate and Mg^{2+} ions was derived:

The binding of the substrate to the enzyme is independent of the binding of the activator, and vice versa. Mg^{2+} must therefore play a part in the substrate decomposition. It is not possible to determine whether the Mg^{2+} ions are involved directly in the catalytic process, or whether they act as regulatory effectors. Because of the strong affinity existing between the alkaline phosphatase and Mg^{2+} , it is necessary to adjust the metal-ion concentration with the aid of a metal buffer. In the Appendix the necessary equations are derived for calculating the concentration of free metal ions in a system with several different metal ions. A FORTRAN IV program for solving these equations and for graphic presentation of the results has been deposited as Supplementary Publication SUP 50030 at the British Library (Lending Division) (formerly the National Lending Library for Science and Technology), Boston Spa, Yorks. LS 23 7 BQ, U.K., from whom copies may be obtained on the terms indicated in *Biochem. J.* (1973), 131, 5.

Alkaline phosphatase (EC 3.1.3.1) is a metal-containing enzyme. It requires metal ions both for preservation of its structure and for its enzymic activity (Fernley, 1971). The enzyme extracted from *Escherichia coli* contains 4 zinc atoms/molecule, two of which are very firmly bound and probably have structure-preserving properties, whereas the others are of importance for the catalytic process (Reynolds & Schlesinger, 1969; Simpson & Vallee, 1968; Lazdunski & Lazdunski, 1969; Lazdunski *et al.*, 1969). Zinc may be replaced by cobalt, copper, cadmium, nickel or manganese (Applebury & Coleman, 1969; Lazdunski *et al.*, 1969; Petitclerc *et al.*, 1970; Csopak & Falk, 1970), but only the cobalt enzyme shows catalytic activity (about one-sixth of that of the native enzyme). Similar results

were obtained with alkaline phosphatase from rabbit intestine (Clark & Porteous, 1965) and dog intestine (Nguyen-van Thoai, 1947).

Alkaline phosphatase from *E. coli* is not activated by Mg^{2+} ions. Since an enzyme molecule contains one to two atoms of magnesium, besides two to four atoms of zinc (Plocke *et al.*, 1962; Simpson & Vallee, 1968), and since heat inactivation is decreased when Mg^{2+} ions are in the assay, one can assume in this case that the Mg^{2+} ions have a structure-stabilizing effect. For alkaline phosphatase from mammalian tissue, the main influence of Mg^{2+} ions is on the enzymic activity (Müller *et al.*, 1971; Cloetens, 1941). There are as yet no unequivocal results on the role of Mg^{2+} ions in the catalytic process. First it was presumed that Mg^{2+} ions form a complex with the phosphoric acid ester and this complex is the active substrate (Mathies, 1958). Albers *et al.* (1962) showed by means of kinetic measurements that the

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Mg^{2+} ions react first with the enzyme. The activated enzyme- Mg^{2+} complex can then bind and convert the substrate. Albers *et al.* (1962) found that the non-activated enzyme can also decompose the substrate, though more slowly; hence the system possesses residual activity. Brunel & Cathala (1973) reported similar results.

Since there is no definitive data available in the literature on binding constants of alkaline phosphatase for Mg^{2+} ions, the apparent residual activity could be the result of traces of Mg^{2+} in the enzyme preparation or in the reagents, particularly if the binding constant for magnesium was relatively large.

Since Mg^{2+} ions play an important role in alkaline phosphatase activity and since there is little conclusive information about the mechanism of action of these ions on the enzyme, in the present paper I examine the function(s) of Mg^{2+} ions in alkaline phosphatase activity by kinetic analyses. From the data obtained, a model for the reaction of enzyme, substrate and activator ions is deduced.

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 source for the alkaline phosphatase.

Methods

Alkaline phosphatase was prepared from pig kidney by using the autolysis procedure of Albers & Albers (1935). The enzyme was further purified from the crude autolysis extract by ethanol fractionation, DEAE-cellulose ion-exchange chromatography and gel filtration over Sephadex G-200. The purified enzyme preparation had a specific activity of $30 \mu\text{mol}/\text{min per mg}$ of protein.

By adding Mg^{2+} ions and by maintaining a constant Zn^{2+} concentration (by using nitrilotriacetate-Zn buffer) in all preparatory steps, it was possible to stabilize the enzyme completely. Even the enzyme fractions that had been purified by column chromatography could be stored in solution at 4°C for several months without any loss of activity. Thus kinetic measurements could be made under reproducible conditions.

Inherent in the use of organic solvents in the preparatory work is the possibility of bringing about irreversible changes in the structure of the enzyme. Because of this, a comparative enzyme preparation

was made in which organic solvents were not used (B. P. Ackermann & H. U. Wolf, personal communication). The alkaline phosphatase obtained without organic solvents behaved, in all kinetic measurements taken, in the same way as alkaline phosphatase prepared by the above procedure.

Determination of enzyme activity

The conditions for enzyme activity measurements are reported in the Results section. The test volume was 10ml throughout, the temperature 30°C . The test medium for the kinetic measurements contained 50mm-sodium carbonate buffer, pH9.51, 4mm-nitrilotriacetate, 2mm- ZnCl_2 and between $20 \mu\text{M}$ - and 1.8mm - MgCl_2 . To permit measurements under optimal conditions, the enzyme concentration was varied. Since the activity was directly proportional to the concentration of the enzyme (J. Ahlers, unpublished work), all results could be related to the same concentration of enzyme.

Sodium β -glycerophosphate served as substrate. Enzyme, activator and buffer were incubated at 30°C in the reaction flasks. After the reaction had been started by adding the substrate, an autoanalyser was used to draw off the reaction mixture and to add trichloroacetic acid to stop the reaction.

The reaction was closely observed by continuously measuring the amount of phosphate released by the method of Fiske & SubbaRow (1925), as revised by Lacy (1965). The development of the colour and the measurement of its extinction was as described by Wolf (1972).

Care was taken to ensure that the quantity of β -glycerophosphate hydrolysed amounted to 5% and never exceeded 10% of the total substrate concentration. By the continuous recording of the amount of phosphate released, it could be established with certainty that progress curves were linear over the period required for the determinations of initial rate.

Determination of protein concentration

Protein was generally determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. In some cases, particularly in the chromatography procedures, the extinction at 280nm was used to estimate the protein concentration.

Derivation of the rate equation for the reaction

The derivation of the rate equation was performed on the basis of a rapid equilibrium reaction mechanism according to models presented by Botts & Morales (1953), Laidler (1956) and Ohlenbusch (1962), and also by using the method reported by Cleland (1970).

Symbols and abbreviations

E = free enzyme

 $[\text{E}]_t$ = total enzyme concentrationS = substrate (sodium β -glycerophosphate)

P = product(s)

 k = reaction rate constant K = dissociation constant k_{+1} , k_{-1} , k_{+2} = rate constants of the following enzyme substrate reaction: k'_{+1} , k'_{-1} = rate constants of the reactions: $k_{+\text{Mg}}$, $k_{-\text{Mg}}$ = rate constants of the reactions $k'_{+\text{Mg}}$, $k'_{-\text{Mg}}$ = rate constants of the reactions $K_m = \frac{k_{-1} + k_{+2}}{k_{+1}}$ = Michaelis constant βk_{+2} = rate constant of the decomposition of the EMgS complex into the end products $K'_m = \frac{k'_{-1} + \beta k_{+2}}{k'_{+1}}$ = modified Michaelis constant $K_s = \frac{k_{-1}}{k_{+1}}$ = dissociation constant of the ES complex $K'_s = \frac{k'_{-1}}{k'_{+1}}$ = dissociation constant for the equilibrium $\text{EMgS} \rightleftharpoons \text{EMg} + \text{S}$ $K_{\text{Mg}} = \frac{k_{-\text{Mg}}}{k_{+\text{Mg}}}$ = dissociation constant of the EMg complex $K'_{\text{Mg}} = \frac{k'_{-\text{Mg}}}{k'_{+\text{Mg}}}$ = dissociation constant for the equilibrium $\text{EMgS} \rightleftharpoons \text{ES} + \text{Mg}^{2+}$ v = reaction rate at any concentration of S and Mg^{2+} V = reaction rate where enzyme is saturated with substrate and activator.

tration ranges for Mg^{2+} that would otherwise not have been usable owing to the normally occurring trace impurities present in the reagents.

In Fig. 1, $1/v$ is plotted against $1/[\text{Mg}^{2+}]$ for various substrate concentrations. Straight lines are obtained with a common point of intersection in the second quadrant close to the abscissa. At low [S] and $[\text{Mg}^{2+}]$ a higher enzyme concentration was used (see the Experimental section), which probably causes the slight deviation from the straight line, as Mg^{2+} ions had been used to stabilize the enzyme during preparation. The linearity of the curves obtained shows in the first place that one Mg^{2+} ion per active centre is bound, and secondly that no residual activity is present and that therefore Mg^{2+} ions are essential for the activity of the alkaline phosphatase from pig kidney. If $1/v$ is plotted against $1/[\text{S}]$ for various Mg^{2+} concentrations (not shown) one also obtains straight lines with a common point of intersection in the second quadrant close to the abscissa.

Scheme (1) for the reaction of E, S and Mg^{2+} describes these results (Ahlers, 1972).

A similar model is discussed by Brunel & Cathala (1973) for the alkaline phosphatase of ox brain. There is one apparent difference, however, in that residual activity in the absence of Mg^{2+} was observed by these workers.

The reaction

Fig. 1. $1/v$ versus $1/[\text{Mg}^{2+}]$ plotted for various β -glycerophosphate concentrations

Experiments were done at 30°C, pH 9.51 in 0.05M-sodium carbonate buffer-2mM- ZnCl_2 -4mM-nitrilotriacetate. The Mg^{2+} concentrations were adjusted with the aid of the nitrilotriacetate buffer according to the calculation carried out in the Appendix. [β -Glycerophosphate]: ■, 0.4mM; ▼, 0.6mM; △, 1.0mM; ●, 2.0mM; □, 4.0mM; ▽, 6.0mM; ○, 10.0mM.

Results and Discussion

In order to obtain insight into the kinetics of activation by Mg^{2+} ions, substrate and Mg^{2+} concentrations were varied. The concentration of free Mg^{2+} was adjusted with the aid of the complexing agent nitrilotriacetate. (For use of complexing agents see the Appendix.) This resulted in reproducible results being obtained and also permitted the use of concen-

is reversible, as are all the other reactions of the above model. However, since initial velocities were measured (see the Experimental section), and thus $[\text{P}] \approx 0$, the reverse reaction does not yet occur to a measurable extent.

Reaction (1) proceeds via at least one intermediate product E-P (Schwartz, 1963; Engström, 1964). Since these intermediate products are not detectable by the kinetic measurements used, the simplified expression was chosen. Provided that reaction (1) is the rate-determining step, complete thermodynamic equilibrium may exist between E, ES, EMg and EMgS (Ohlenbusch, 1962).

The linearity of the $1/v$ versus $1/[\text{S}]$ and $1/v$ versus $1/[\text{Mg}^{2+}]$ plots is evidence for this assumption, since in the case of equilibrium, the steady-state equations, which are generally non-linear, can be simplified to linear reciprocal initial-rate equations.

On the basis of microscopic reversibility (Ohlenbusch, 1962), it follows from these assumptions that:

$$K_s \cdot K_{\text{Mg}} = K'_s \cdot K'_{\text{Mg}} \quad (2)$$

For the derivation of the reaction velocity equation, one equilibrium (for example: $\text{ES} + \text{Mg}^{2+} \rightleftharpoons \text{ESMg}$) can be disregarded in this case, since according to eqn. (2) the remaining dissociation constant results from a combination of the other ones.

Thus for Scheme (1) the initial rate equation is:

$$v = \frac{V \cdot [\text{S}]}{K'_m \left(1 + \frac{K_{\text{Mg}}}{[\text{Mg}^{2+}]}\right) + [\text{S}] \left(1 + \frac{K'_m \cdot K_{\text{Mg}}}{K_s \cdot [\text{Mg}^{2+}]}\right)} \quad (3)$$

From the reciprocal form:

$$\frac{1}{v} = \frac{1}{V} + \frac{K'_m \cdot K_{\text{Mg}}}{V} \left(\frac{1}{[\text{S}]} + \frac{1}{K_s} \right) + \frac{K'_m}{V} \cdot \frac{1}{[\text{S}]} \quad (4)$$

it can be seen that the slopes of the straight lines in Fig. 1 are equal to

$$\frac{K'_m \cdot K_{\text{Mg}}}{V} \left(\frac{1}{[\text{S}]} + \frac{1}{K_s} \right)$$

Fig. 2. Secondary plots of the intercepts and slopes of Fig. 1 versus the reciprocal β -glycerophosphate concentration

$$\bullet, \text{Intercept} \left(\frac{1}{V} \right); \square, \text{slope} \left(\frac{d(1/v)}{d(1/[\text{Mg}^{2+}])} \right)$$

and the intercepts are equal to

$$\frac{1}{V} + \frac{K'_m}{V} \cdot \frac{1}{[\text{S}]}$$

In Fig. 2 slopes and intercepts of each of the straight lines from Fig. 1 are plotted against the reciprocal substrate concentration. In both cases straight lines are obtained. From the slopes and intercepts of Fig. 2 the kinetic constants were calculated:

K'_m , $3.2 \times 10^{-3} \text{ M}$; K_s , $5.9 \times 10^{-3} \text{ M}$; K_{Mg} , $9.8 \times 10^{-7} \text{ M}$; V , $16 \mu\text{mol/min per mg of protein}$.

Scheme 1 represents the most likely model that agrees with the experimental results. If the enzyme first reacted with the activator and could only bind and convert the substrate molecule into this activated form by the mechanism

then one could obtain straight lines with a common point of intersection on the ordinate in the Lineweaver-Burk plot (Ahlers *et al.*, 1974).

A mechanism in which the substrate molecule first reacts with the free enzyme and then subsequently the ES complex accepts the activator

would yield straight lines with common points of intersection in the second quadrant with both plotting methods. On the basis of initial-rate data one cannot exclude this mechanism. Direct binding studies would be necessary to do this. However as the

straight lines in the reciprocal plots have a common point of intersection with an ordinate value of

$$\frac{1}{V} [1 - (k_{+2}/k_{-1})]$$

only when $k_{+2} \approx k_{-1}$ is this point near the abscissa. k_{+2} is the rate constant of reactions that include the breaking and formation of covalent bonds whereas k_{-1} is the rate constant of a reaction that involves formation and breaking of ionic bonds. Thus it seems to be more likely that k_{+2} is smaller than k_{-1} and so this mechanism is less likely.

From Scheme (1) a common point of intersection near or on the abscissa can easily be explained. Since the ordinate value of this point is equal to

$$\frac{1}{V} [1 - (K'_m/K_s)],$$

it follows that $K'_m \approx K_s$. Provided that $\beta \cdot k_{+2}$ is small compared with the other rate constants, this would mean that $K_s \approx K'_s$. That is, the substrate binding is practically independent of whether the activator is or is not bound to the enzyme (Ahlers, 1972). From eqn. (2) it can be further deduced that $K_{\text{Mg}} \approx K'_{\text{Mg}}$ and that the activator binding is thus not influenced by the substrate binding. As predicted from this, plots of $1/v$ against $1/[\text{Mg}^{2+}]$ at different concentrations of β -glycerophosphate intersected on the horizontal axis.

From these results it is apparent that alkaline phosphatase from pig kidney has independent sites for the binding of Mg^{2+} and substrate. The Mg^{2+} ions are not involved in the substrate binding. The binding of Mg^{2+} ions to the enzyme is, however, essential for the further conversion of the substrate, since no residual activity was observed. It is possible that the Mg^{2+} ions play a direct role here in the reaction as part of an intermediary. It is, however, also possible that they react with a regulatory site of the enzyme and effect a conformational change.

These results are in agreement with those of Brunel & Cathala (1973) for the alkaline phosphatase from ox brain. However, they do not agree with those for the placenta enzyme (Fernley, 1971), where the Mg^{2+} ions are thought to bring about a redistribution of the Zn^{2+} ions. To obtain more insight into the molecular mechanism of substrate conversion by alkaline phosphatase from pig kidney as well as into the structure of the sites at which the substrate binding and conversion take place and where the activator binding occurs, it is necessary to investigate the functional groups in these sites with the aid of pH variations and through the use of group-specific inhibitors.

The appearance of residual activity (Brunel & Cathala, 1973; Fishman *et al.*, 1962; Albers *et al.*, 1962) is apparently due to the high affinity of the alkaline phosphatase for the Mg^{2+} ions. It may be

caused by Mg^{2+} contamination in the preparation and in the reagents used. Therefore it is absolutely necessary to adjust the metal-ion concentration with the aid of complexing agents.

I thank Dr. H. U. Wolf for helpful suggestions and valuable discussion, Dr. B. P. Ackermann for his co-operation and Miss H. Köth for technical assistance. The English text was prepared by Mrs. I. Hendrick.

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APPENDIX

(1) *Working with complexing agents*

In order to obtain reproducible results and also to be able to work in the ranges of concentration which are otherwise not accessible because of metal-ion contamination in the reagents used, kinetic measurements were made in the presence of metal-ion buffers.

One has to distinguish between two different cases. In one case, measurements can be made with a constant Mg^{2+} concentration at the optimum. In this case, the chelating agent is present at a limiting concentration and has the function of taking up heavy metal contaminants and maintaining a constant Zn^{2+} concentration. Such buffer systems are advantageous in that they show a very low pH-dependence with respect to metal-ion concentration and buffering capacity at $pH \geq 6$ (Wolf, 1973). These buffer systems are of particular value when kinetic measurements are to be made on an enzyme such as alkaline phosphatase, whose activity is dependent on two separate metal ions. Thus kinetic measurements can be made at various pH values while keeping the metal-ion concentrations constant.

However, the use of this buffer system is limited to the case where the metal ion, whose concentration is to be controlled with the aid of the buffer, is bound by the complexing agent at least 10–100 times more firmly than the ion that is present in excess (Wolf, 1973). Applied to alkaline phosphatase, this means that one can adjust the Zn^{2+} concentration and keep it constant very simply by adding an excess of Mg^{2+} ions to the reaction mixture and having Zn^{2+} ions present in limiting amounts with respect to the concentration of the complexing agent.

In the present work, however, the metal buffer was supposed to keep the concentration of Zn^{2+} ions constant while adjusting that of the Mg^{2+} ions (which are bound by the complexing agent much less tightly) in the range of $10 \mu M$ or less. These requirements can only be realized with a buffer system in which the total metal-ion concentration is smaller than the concentration of the complexing agent. This case will now be considered.

(2) *Selecting a suitable complexing agent*

Nitrilotriacetate was used as a chelator, because it meets the necessary condition of binding Zn^{2+} less strongly than the enzyme, so that no essential zinc is removed from the alkaline phosphatase. An additional advantage is that the binding constant of nitrilotriacetate- Mg^{2+} complex approximates to the binding constant of the enzyme for Mg^{2+}

($pK_{\text{Nitrilotriacetate-Zn}^{2+}}$, 10.7, $pK_{\text{Nitrilotriacetate-Mg}^{2+}}$, 5.6; Sillén & Martell, 1964).

Nitrilotriacetate has the following dissociation constants for H^+ : K_1 , $10^{-9.73} M$, K_2 , $10^{-2.5} M$; K_3 , $10^{-1.89} M$ (Sillén & Martell, 1964); so that the resultant concentration of the free metal ions in the range of measurement is simply a function of K_1 .

(3) *Calculation of the concentration of free metal ions in a metal buffer system with various metal ions: the equations and FORTRAN IV program*

Symbols: MGT, CAT, total concentration of the Mg^{2+} or Ca^{2+} ions; MG, CA, concentration of the free Mg^{2+} , or Ca^{2+} ions present; LT, total concentration of the complexing agent L; FL, concentration of the free complexing agent present; H, concentration of H^+ ions.

In the following, a system will be described which actually goes beyond the requirements of the present work. Here it will be shown that by using the complexing agent the concentrations of the Zn^{2+} and Mg^{2+} ions can be adjusted, the concentration of an additional metal ion, for example Ca^{2+} , can be adjusted and the pH dependence of the system can also be taken into account. The system can be simplified to the problem treated in the main paper by setting $[Ca^{2+}]$ equal to zero and holding $[H^+]$ constant.

In general, the following equilibrium obtains under conditions where the complexing agent is present in excess.

Since Zn^{2+} ions are added in deficient amount as LZn complex, their concentration does not influence the system detectably because of the high binding constants. The concentration of free Zn^{2+} ions is in the range 10–20 pM for all measurements, and thus it has no detectable influence on the concentration of the free complexing agent. Thus the following simplification is possible:

On the basis of the law of conservation FL is calculated as:

$$\text{FL} = \text{LT} - (\text{LMG} + \text{LCA} + \text{LH}) \quad (1)$$

where

$$\text{LMG} = \frac{\text{MG} \cdot \text{FL}}{K_{\text{MgL}}}; \text{LCA} = \frac{\text{CA} \cdot \text{FL}}{K_{\text{CaL}}}; \text{LH} = \frac{\text{H} \cdot \text{FL}}{K_{\text{HL}}} \quad (2)$$

H/K_{HL} is constant and has the value 1.35 at pH 9.6.

It follows that

$$\text{LH} = 1.35 \cdot \text{FL} \quad (3)$$

Further, in analogy to eqn. (1)

$$\text{MG} = \text{MGT} - \text{MGL}; \text{CA} = \text{CAT} - \text{CAL} \quad (4)$$

and when eqn. (2) is taken into consideration

$$\text{MG} = \frac{\text{MGT}}{1 + \text{FL}/K_{\text{MgL}}}; \text{CA} = \frac{\text{CAT}}{1 + \text{FL}/K_{\text{CaL}}} \quad (5)$$

By combining eqns. (1), (4) and (5) one obtains:

$$\text{FL} = \text{LT} - \text{MGT} - \text{CAT} - (1.35 \cdot \text{FL}) + \frac{\text{MGT}}{1 + \text{FL}/K_{\text{MgL}}} + \frac{\text{CAT}}{1 + \text{FL}/K_{\text{CaL}}} \quad (6)$$

Since all factors are known, FL can be calculated and by inserting it into eqn. (5), the concentration of the resultant free Mg^{2+} and Ca^{2+} ions can be obtained.

When $\text{FL} \geq K_{\text{MgL}}$ and $\text{FL} \geq K_{\text{CaL}}$ then eqn. (6) simplifies to

$$\text{FL} \approx \text{LT} - \text{MGT} - \text{CAT} - (1.35 \cdot \text{FL})$$

$$\text{FL} \approx (\text{LT} - \text{MGT} - \text{CAT})/2.35 \quad (7)$$

A FORTRAN IV program has been prepared to calculate the concentrations of free metal ions by using eqn. (5) and eqn. (7). The results are given in tables and graphs. Copies of the program are available on request from the author or from the British Library (Lending Division) (formerly the National Lending Library for Science and Technology), Boston Spa, Yorks LS23 7BQ, U.K.

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