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ACTIVATION AND INHIBITION PROCESSES OF ALKALINE PHOSPHATASE FROM BOVINE BRAIN BY METAL IONS (Mg^{2+} AND Zn^{2})

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SUMMARY

1. Alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) from bovine brain is strongly activated by Mg^{2+} (5–14-fold). In this respect the enzyme behaves like that of kidney or bone marrow but is different from the intestinal and placental enzymes which are much less activated.

2. The rate of the activation process is pH-dependent. At pH 10.0, the pH optimum of *p*-nitrophenyl phosphate hydrolysis, the activation process is rapid. At lower pH values, it is slow and can be described by an exponential relationship *vs* time. The results suggest that the activation by Mg^{2+} proceeds through a binding of the metal with free enzyme which might be followed by a conformational change, rather than by an action on the substrate.

3. Other metal ions can also bind to the brain enzyme. Mn^{2+}, Co^{2+}, Ni^{2+} with an activating effect, Zn^{2+} with an inhibitory effect. They all bind to the enzyme at the same site and also induce the supposed conformational change. However, Zn^{2+} is able to produce interactions, perhaps with P_i which is one of the products of hydrolysis, which might explain its inhibitory effect.

INTRODUCTION

Mg^{2+} and Zn^{2+} are known to have activating and inhibitory effects, respectively on mammalian alkaline phosphatases (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1), but the question of how the mechanisms of activation and inhibition are accomplished, however, remains unanswered in spite of the various effects which have been described when divalent metal ions are added.

Considering the fact that K_m is not modified by Mg^{2+}, we have briefly discussed the hypothesis according to which the activation of brain alkaline phosphatase operates through a binding of the metal ion with free enzyme and suggested that Mg^{2+} may have mainly a structural role by inducing a conformational change[1]. This is the more attractive hypothesis and perhaps the best conceivable explanation for

all mammalian alkaline phosphatases in spite of the different activating effects usually observed with Mg^{2+}.

One can, in fact, state that the strongly Mg^{2+}-activated enzymes, such as those of bovine brain (5–14-fold)[1], human kidney (2–9-fold)[2], bovine kidney (about 10-fold, our unpublished results)[8]and bone marrow (4–7-fold)[3], are generally heat- and urea-sensitive, which denotes a relative fragility of their tertiary structure. In contrast, the intestinal and placental alkaline phosphatases on which Mg^{2+} has a lower effect (1.2–2-fold)[4–6] are much more resistant. Therefore, if a conformational change actually occurs in the binding of Mg^{2+} to alkaline phosphatases, this should be more pronounced in the brain, kidney and bone marrow enzymes than in those of intestine and placenta; hence the different activating effects of Mg^{2+}.

The present work is an attempt to obtain more information concerning the mechanism of activation of brain phosphatase by Mg^{2+} and to examine if other metal ions, such as Mn^{2+}, Ni^{2+}, Co^{2+} (which have also an activating effect) and mainly Zn^{2+} (which is an inhibitor), act on the brain enzyme at the same site.

METHODS

The purification of alkaline phosphatase from bovine brain has been described previously[7]. In this work, we have only considered the preparations for which the specific activity was greater than 400 μmoles of *p*-nitrophenol released per min per mg of protein, when the assay was carried out with 5 mM *p*-nitrophenyl phosphate in 33 mM $NaHCO_3$–Na_2CO_3 buffer (pH 10.0) and 1 mM $MgCl_2$ at 37 °C. The enzyme preparations were kept in the cold in 0.1 M NaCl and 0.05 M Tris–HCl (pH 8.0), as a result of the last purification step[7].

The enzyme reactions were carried out using *p*-nitrophenyl phosphate as previously described[8]. With other substrates, released P_i was determined according to the method of Delsal and Manhouri[9].

"Mg-enzyme" and "Zn-enzyme" refer to the enzymes incubated with Mg^{2+} and Zn^{2+}, respectively, which were routinely obtained as mentioned earlier[8].

For the Zn-enzyme which shows very little activity, it was convenient to determine the enzymatic activity as follows: The enzyme solution (25–50 μl) was added to the assay medium (final volume 2.5 ml) containing *p*-nitrophenyl phosphate at a chosen concentration, 25 mM borate at a chosen pH and eventually an effector (P_i or arsenate). The reaction was stopped after 1 h by adding 2 ml of 1 M NaOH containing 0.1 M EDTA (it was, of course, verified that the rate of hydrolysis was constant over this period of time). Released *p*-nitrophenol was determined by the reading of $A_{400\ nm}$ on a Beckman DU-2 spectrophotometer. If necessary, the values were corrected for the decrease in activity of the Zn-enzyme during the experiment.

RESULTS

Activation process by Mg^{2+}

Evidence for an activity of the Mg^{2+}-free enzyme. The question is to know if Mg^{2+} is an absolute requirement for activity. In the course of our work, it was observed that the Mg^{2+}-free enzyme, *i.e.* the purified enzyme, always exhibited a residual activity. It may be argued perhaps, that sufficient Mg^{2+} or other ions are

present as contaminants in the reagents, or that the enzyme contains in addition to catalytic Zn^{2+}, the presence of which is indubitable in all alkaline phosphatases, another tightly bound metal ion which could not be eliminated by the exhaustive dialyses to which the enzyme is subjected during its preparation. We believe that this is not the case. Indeed, this residual activity has been found with various substrates (*p*-nitrophenyl phosphate, phenyl phosphate, *β*-glycerophosphate and DL-phosphoserine) in different buffer solutions (carbonate–bicarbonate and borate). Besides, for the same enzyme preparation the degree of activation by Mg^{2+} was the same in each case. Another argument is the significant difference in pH optima for *p*-nitrophenyl phosphate hydrolysis: 10.1 with Mg^{2+} added to the assay medium and 10.7 without Mg^{2+} (ref. 1).

Influence of Mg^{2+} on the rate of the reaction and properties of the activation by Mg^{2+}. At pH 10.0, the rate of *p*-nitrophenyl phosphate hydrolysis in the presence of Mg^{2+} reaches a maximal constant value after a short initial period during which the reaction is not linear. If the enzyme is previously incubated with Mg^{2+} and if the assay medium contains Mg^{2+}, the preincubated enzyme exhibits the same activity provided that the initial rate of the reaction is considered, because beyond 2–3 min the enzyme is progressively inactivated.

When the enzyme assay is carried out at lower pH (pH 8.5), the behaviour of the kinetics of *p*-nitrophenyl phosphate hydrolysis in the presence of Mg^{2+} indicates that the enzyme is being activated. However, after an initial period (1–2 min), the activation is a slow process, and it is convenient to consider that the rate of hydrolysis has a constant value corresponding to zero-order kinetics within a period of about 10 min, beyond which the enzyme is progressively activated. If the enzyme is previously incubated in the presence of Mg^{2+} for several hours at 4 °C and if the assay medium contains Mg^{2+}, the rate of hydrolysis reaches immediately a maximal constant value corresponding to the full activation of the enzyme. If the assay medium does not contain Mg^{2+}, the enzyme exhibits the same activity, even if it is diluted, and is not inactivated during the reaction as observed at pH 10.0.

All these data and others previously discussed[1] (*e.g.* the fact that Mg^{2+} does not affect the affinity for the substrate) strongly suggest that Mg^{2+} induces activation of brain alkaline phosphatase through its binding to the free enzyme rather than by an action on the substrate.

At higher pH values, it must be admitted that the enzyme conformation is already propitious to an immediate action of Mg^{2+}, or preferably that Mg^{2+} may induce an almost instantaneous conformational change through its binding. The great heat sensitivity of the brain enzyme at pH 10.0 is consistent with this view. At pH 8.5 or below for which the structure of the enzyme is more stable[5], the activation process is slow and its rate depends upon Mg^{2+} concentration. Mg^{2+} is not liberated upon dilution, but it can be easily eliminated by prolonged dialysis at pH 8.0, which is indicative of a reversible metal–protein association.

The scheme of the activation process as a function of pH has been previously[1] shown, but we think it is necessary to further discuss its interpretation. In Fig. 1, curve A is drawn by considering the activity which is observed when the enzyme has been incubated with Mg^{2+} at pH 8.0, in comparison to the activity which is observed with Mg^{2+}-free enzyme assayed in presence of Mg^{2+}. It can be seen that a higher pH values, the activating effect of Mg^{2+} added to the assay medium approximately

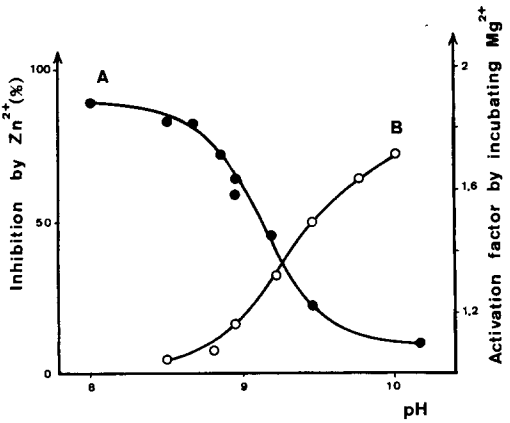


Fig. 1. (A) Activation process by Mg^{2+} as a function of pH. The enzyme was incubated at 4 °C for 1 h with 20 mM $MgCl_2$ in 0.1 M NaCl and 0.05 M Tris–HCl buffer (pH 8.0) or in 25 mM borate buffer (pH 8.0). For reactional pH values between 8.0 and 10.2, the activities of the incubated and the unincubated enzymes were compared (the ratio of the two activities is then an activation factor by "incubating" Mg^{2+}). The substrate was *p*-nitrophenyl phosphate in 25 mM borate and in both cases, the assay medium contained 1 mM $MgCl_2$. As mentioned in the text, it was considered that for the unincubated enzyme, the rate of hydrolysis has a constant value between the second and the tenth min of the assay. (B) Inhibition by Zn^{2+} of the Mg-enzyme as a function of pH. The assay medium was the same as above except that $MgCl_2$ was absent. $[ZnCl_2] = 20\ \mu M$.

corresponds to the maximal activation of the enzyme, whereas this activation is only partial at lower pH values. Our previous interpretation was that the activation follows a titration curve and that the mid-point at pH 9.2 corresponded to the p*K* of an ionizable group of the enzyme. In the light of the results described here, it is more advisable to postulate that this curve reflects the pH-dependent ability of the

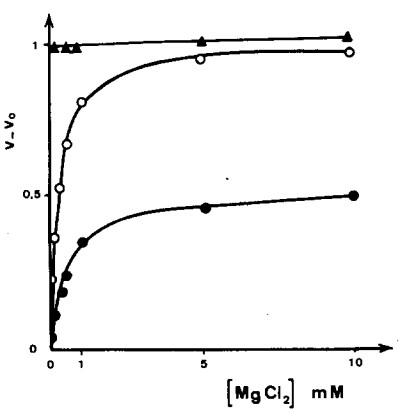


Fig. 2. Variation of the rate of *p*-nitrophenyl phosphate hydrolysis as a function of Mg^{2+} concentration. The substrate was 5 mM in 25 mM borate (pH 8.5). ▲—▲, Mg-enzyme: in this case, the concentration of $MgCl_2$ which was brought to the assay medium with the enzyme was 50 μM; ●—●, purified enzyme (Mg^{2+}-free enzyme): the activity being considered between the second and the tenth min of the assay, the activation which was recorded was only partial; ○—○, purified enzyme assayed with 10 mM glycine added to the assay medium. The value of 1 was given to the maximum activity which was obtained with the incubated enzyme, corrected for activity of the Mg^{2+}-free enzyme (v_0). The units are arbitrary.

enzyme which is to support a slow or a rapid conformational change in the presence of Mg^{2+}.

The variations in the rate of *p*-nitrophenyl phosphate hydrolysis as a function of $MgCl_2$ at pH 8.5 are illustrated in Fig. 2. The experimental points agree with a theoretical Michaelian curve, which means that Mg^{2+} does not operate through cooperative effects. It is possible, however, that the enzyme may be able to bind more Mg^{2+} without affecting its activity.

It should be noted that Mg^{2+} can operate by means of an organic effector, which has no effect in the absence of Mg^{2+}. Earlier studies in this laboratory[1] have demonstrated that at pH 8.5 the addition of an α-amino acid in the assay medium greatly enhances the activation rate by Mg^{2+}.

Kinetic study of the activation process by Mg^{2+}. The experimental conditions were chosen in such a way that the activation process was very slow. Therefore, the brain enzyme was incubated at 0 °C with $MgCl_2$ at a chosen concentration for at least 1 h in 0.1 M NaCl and 0.05 M Tris–HCl buffer (pH 8.0). The enzyme assays were performed at timed intervals with *p*-nitrophenyl phosphate under conditions where $[S] \gg K_m$ at pH 8.5 (25 mM borate). At this pH, the Mg-enzyme is stable and the rate of hydrolysis has a constant value corresponding to zero-order kinetics without Mg^{2+} added to the assay medium. The corresponding rate at zero time of the acti-

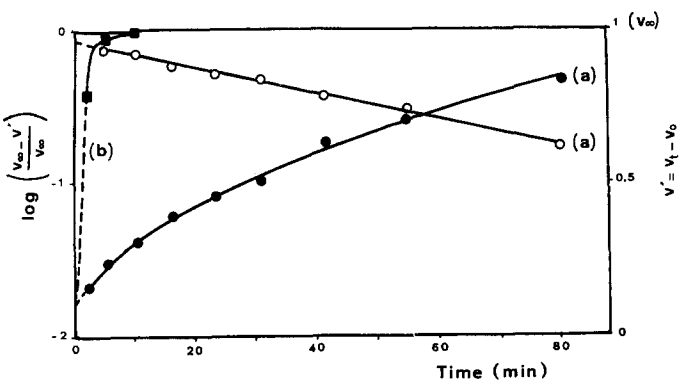


Fig. 3. Kinetics of the enzyme activation by incubation with Mg^{2+} (a) at 0 °C, ●—●, direct plot; ○—○, semilogarithmic plot. (b) at 37 °C, ■—■, direct plot. The incubating $MgCl_2$ concentration was 5 mM at 0 °C and 1 mM at 37 °C. The value of 1 was given to the activity which was obtained after an infinite incubation time and corrected for activity of the Mg^{2+}-free enzyme (v_0). The units are arbitrary.

vation process (v_0) was taken as the activity of the Mg^{2+}-free enzyme assayed in a medium without Mg^{2+}. v_∞ was the rate obtained after an infinite incubation time with $MgCl_2$.

The results show that the activation process can be described by an exponential relationship (Fig. 3a) which is an argument for a conformational change induced by the binding of Mg^{2+} to the enzyme.

However, from the semilogarithmic plot, it can be seen that the curve does not reach the zero value. A possible interpretation is that the binding of Mg^{2+} to the enzyme (it is known that the rate of release of water molecules from Mg^{2+}, $1 \cdot 10^5$ s^{-1}, is the rate-limiting step during the binding of Mg^{2+} to a ligand in aqueous solutions[10])

already produces an immediate activation which might precede the slow conformational change. If this is true, it is possible that Mg^{2+} directly occurs in the catalytic mechanism.

Effect of temperature on the activation process. At 37 °C and pH 8.0, the full activation of the enzyme is reached after an incubation time of about 5 min (Fig. 3b), which is in agreement with an action of Mg^{2+} on the enzyme conformation.

It must be recalled here that the activation of the Mg^{2+}-free enzyme does not reach its maximum value during the assay (at least 10 min) at pH 8.0 or 8.5, although it is performed at 37 °C and in the presence of relatively high concentrations of Mg^{2+} (Fig. 2). It is then likely that the activation process of the enzyme–substrate complex is slower than that of the free enzyme.

Activation by Mg^{2+} after partial heat inactivation of bovine brain alkaline phosphatase. If the binding of Mg^{2+} involves a conformational change of the enzyme, one might expect modifications of this effect after the tertiary structure of the enzyme has been altered by heat denaturation.

The experiment was done with an enzyme preparation which was heated at 55 °C for 60 min, and compared to an unheated sample. In these conditions, the heated enzyme lost 65% of its activity[5]. In each case, we have determined the rate of the activation process by Mg^{2+}. From Fig. 4, it is clear that the enzyme which has been partially denatured by heat is more slowly activated by Mg^{2+} than the unheated enzyme.

Inhibition process by Zn^{2+}

We have observed[5] that, when the alkaline phosphatases are more capable of activation by Mg^{2+}, the inhibitory effect of Zn^{2+} is more powerful, suggesting an identical way of binding of these two metal ions, one having a positive correlation with the catalysis, the other having a negative correlation. Similar results have been described for pyruvate kinase which binds the inhibitory Ca^{2+} at the same site as the activating Mn^{2+} (refs 11 and 12).

Few details are available on the kinetics of Zn^{2+} inhibition of alkaline phos-

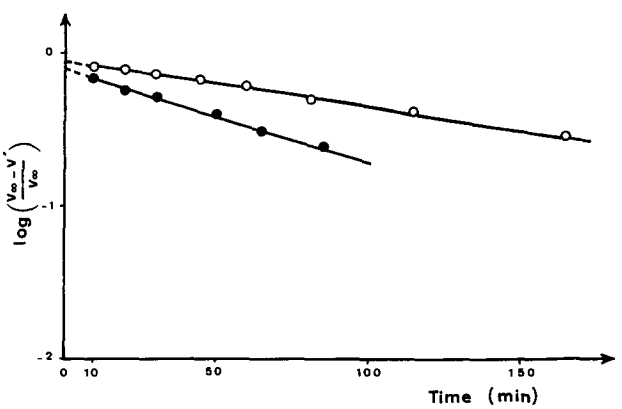


Fig. 4. Kinetics of enzyme activation by Mg^{2+} after the structure of the enzyme had been altered by heat denaturation: ●—●, unheated enzyme; ○—○, heated enzyme. The enzymes were incubated with 5 mM $MgCl_2$. (see Fig. 3 and text under Kinetic study of the activation by Mg^{2+}).

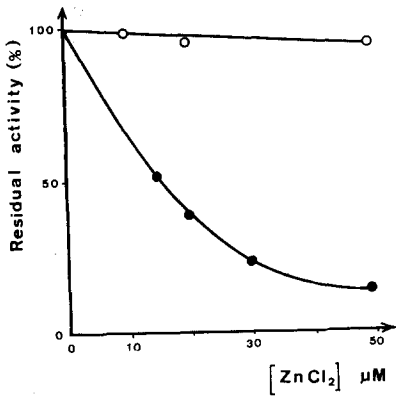


Fig. 5. Inhibition of the Mg-enzyme by Zn^{2+}. ○—○, pH 8.5; ●—●, pH 10.2. The assay medium contained 5 mM *p*-nitrophenyl phosphate, 1 mM $MgCl_2$ in 25 mM borate.

phatases. One report on the intestinal enzyme[13] states that it is of non competitive or of mixed type. We have considered here the two following cases:

(a) The brain enzyme was incubated with Mg^{2+} as mentioned above and the influence of Zn^{2+} added to the assay medium was examined as a function of pH. At pH 8.5, there is no effect even if the concentration of Zn^{2+} approaches the solubility limit. At pH 10.0 the inhibition increases rapidly with the concentration of Zn^{2+} (Fig. 5). At a given value of $[Zn^{2+}]$ the inhibition can be illustrated by a curve (Fig. 1, Curve B) which is the opposite curve from that showing the activating effect by Mg^{2+}. It is then likely that Mg^{2+} and Zn^{2+} bind to the enzyme at the same site.

At higher pH values, Mg^{2+} is loosely bound to the enzyme. It seems therefore normal that Zn^{2+}, which is known to be more able than Mg^{2+} to form stable complex with proteins, displaces Mg^{2+} from the enzyme. At lower pH values, Mg^{2+} is more tightly bound to the enzyme and Zn^{2+} does not displace Mg^{2+}.

Due to its binding it is possible: (i) Zn^{2+} induces a different and unfavourable conformational change from that which is induced by Mg^{2+}. In order to know that, it would be necessary to attempt a conformational study of the enzyme. (ii) The newly bound Zn^{2+} produces interactions either with the substrate or with the products of the hydrolysis. The two situations are not, of course, mutually exclusive.

(b) The purified enzyme was subjected to an exhaustive dialysis, in order to eliminate the possible traces of the unbound metal ions, and incubated with Zn^{2+}. As expected, the Zn-enzyme shows very little activity, less than the purified enzyme and 50–60-fold less than the Mg-enzyme. It is unstable and, whatever the pH, insensitive to Mg^{2+} at the concentrations that usually produce maximum activation. If the enzyme is first incubated with Mg^{2+} and then with Zn^{2+} at pH 8.0, the resulting enzymatic activity is intermediate between that of the Zn-enzyme and that of the Mg-enzyme (Table I). which means that Zn^{2+} can displace Mg^{2+}, in contrast to our observations in the preceding case.

Properties of the Zn-enzyme. In a preceding paper[8], we have shown that the brain enzyme, and more generally the alkaline phosphatases which are not very sensitive to L-phenylalanine, are strongly inhibited by imidazole. This inhibition is apparently of uncompetitive type when the purified enzyme or the Mg-enzyme are

TABLE I

EFFECT OF METAL IONS ON THE ACTIVITY OF ALKALINE PHOSPHATASE FROM BOVINE BRAIN

The substrate was *p*-nitrophenylphosphate under conditions where $[S] \gg K_m$ ($[S]$ = 5 mM; K_m = 0.17 mM) in 25 mM borate (pH 8.5). The units are arbitrary.

Cation incubated with enzyme	*$[MeCl_2]$ (mM)**	*$[MeCl_2]$ (μM)***	*Activity in the absence of Mg^{2+}*	*Activity in the presence of Mg^{2+}*
o	—	—	0.12	0.42***
Mg^{2+}	5	30	1.18	1.21
Mn^{2+}	5	30	0.69	0.66
Ni^{2+}	5	30	0.69	0.69
Co^{2+}	5	30	0.57	0.61
Zn^{2+}	1.6	9.5	0.02	0.03
Zn^{2+}	16 μM	0.95	0.02	0.03
Mg^{2+} then Zn^{2+}	5 1.6	30 9.5	0.08	—
Zn^{2+} then Mg^{2+}	1.6 5	9.5 30	0.02	—
Ca^{2+}	5	30	0.13	0.52***
Sr^{2+}	5	30	0.10	0.54***

* In the incubating medium.
** Brought in the assay medium with the enzyme solution.
*** Activity determined as in Fig. 1 between the second and the tenth min of the assay.

considered. With the Zn-enzyme, the type of inhibition by imidazole is changed to noncompetitive, suggesting that the newly bound Zn^{2+} is able to produce interactions which do not seem to be produced by Mg^{2+}.

In this study, an attempt was made in order to know if other effectors might act on the newly bound Zn^{2+}. An important inhibitor of alkaline phosphatases is P_i, normally a very effective competitor with an affinity comparable to that of several substrates. From Fig. 6B, it can be seen that the type of inhibition by P_i on the Zn-enzyme is modified. The inhibition is noncompetitive which means that the newly bound Zn^{2+} is able to bind P_i added to the assay medium. This action, which is

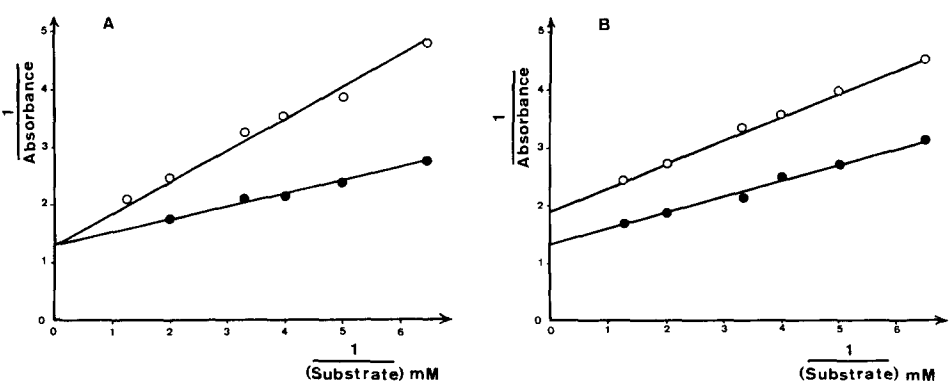


Fig. 6. Inhibition by P_i. Lineweaver–Burk plot (pH 8.5) of Mg-enzyme (A) and Zn-enzyme (B). ●—●, without P_i; ○—○, with 1 mM P_i. The substrate concentrations (*p*-nitrophenyl phosphate) varied from 0.1 to 0.8 mM in 25 mM borate.

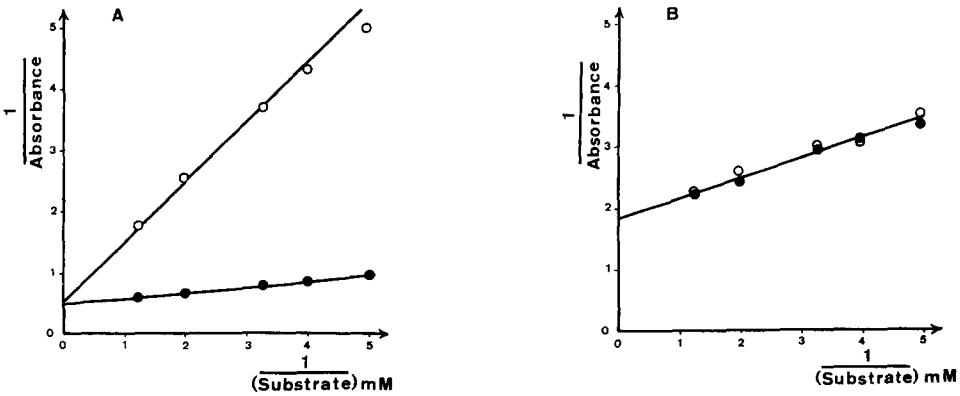


Fig. 7. Inhibition by arsenate. Lineweaver–Burk plot (pH 8.5). (A) Mg-enzyme; (B) Zn-enzyme; ●—●, without arsenate; ○—○, with 0.5 mM arsenate.

qualitatively comparable to that of imidazole on the Zn-enzyme[8], is in agreement with the observation of Engstrom[14] on calf intestinal alkaline phosphatase, who noted that Zn^{2+} decreased the incorporation of $^{32}P_i$ to the active site of the enzyme.

By contrast, arsenate which is also a competitive inhibitor of alkaline phosphatases actually behaves towards the purified enzyme and the Mg-enzyme as a competitive inhibitor, but has no significant effect on the Zn-enzyme (Figs 7A and 7B).

Other metal ions

It is clear from Table I that other divalent cations act on the brain enzyme. They all produce an activating effect, the extent of which is depending on the ion considered. Mn^{2+} and Ni^{2+} are the most effective in producing the activation, which is, however, weaker than that produced by Mg^{2+}. As in the case of Zn^{2+}, the enzymes incubated with Mn^{2+}, Co^{2+} and Ni^{2+} are insensitive to Mg^{2+} at the concentrations

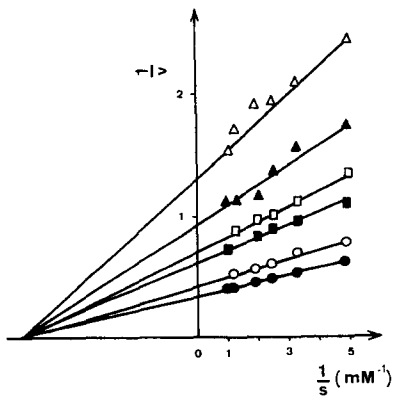


Fig. 8. Lineweaver–Burk plots of metal-incubated alkaline phosphatase from bovine brain. ●—●, Mg-enzyme; ○—○, Mn-enzyme; ■—■, Ni-enzyme; □—□, Co-enzyme; ▲—▲, Zn-enzyme; △—△, Mg^{2+}-free enzyme. The substrate concentrations (*p*-nitrophenyl phosphate) varied from 0.15 to 1 mM in 25 mM borate (pH 8.5). In order to facilitate the reading of the diagram, the scale $1/v$ is different for each incubated enzyme. The units are arbitrary.

that usually produce maximum activation, which confirms that all metal ions which are able to act on the enzyme bind at the same site. Ca^{2+} and Sr^{2+} have no effect, and the enzyme retains its sensitivity to Mg^{2+}.

As in the case of Mg^{2+} and Zn^{2+}, K_m is not modified after the binding of one of these ions (Fig. 8). P_i and arsenate are also competitive inhibitors.

Ni^{2+} and above all Co^{2+} seem to bind more loosely to the enzyme than the other ions, as judged by the rapid inactivation observed during the assays, even at pH 8.5 for which it has been shown that the structure of the enzyme is very stable. This apparent inactivation is in part suppressed by addition of Mg^{2+}.

DISCUSSION

Two principal mechanisms are generally proposed to explain the role of metal ions in metal-activated enzyme systems: the metal ion can act as a bridge between the enzyme and its substrate, or it can induce conformational changes and thereby convert an inactive or partially active form of an enzyme into a catalytically active or more active form.

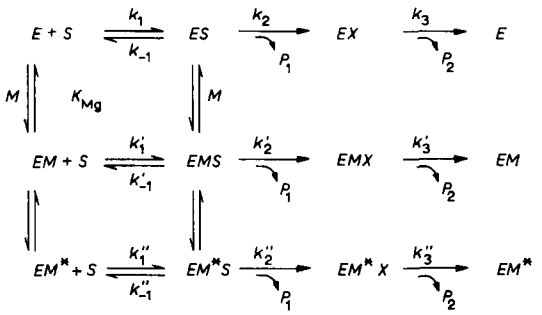
With regard to alkaline phosphatases, it is necessary to take into account the constitutive zinc atom(s) which is known to be implied in the formation of the Michaelis complex. In order to explain the activating effect of Mg^{2+}, different interpretations have been discussed by Clark and Porteous[15] who concluded that Mg^{2+} has an exclusively catalytical role. This is in agreement with generally accepted views, although some workers such as Dabich and Neuhaus[16] propose the hypothesis of a double role, both catalytic and structural. One of the more recent interpretations for placental enzyme is that of Fernley[17] who states that "Mg^{2+} may act by increasing the number of active sites rather than enhancing a particular rate" or else "Mg^{2+} (and certain other cations) facilitate a redistribution of Zn^{2+} either by restoring it to an essential site or perhaps by removing it from an inhibitory site". These last possibilities are not conceivable for brain alkaline phosphatase, the activity of which can be increased up to 14-fold in the presence of Mg^{2+}.

The results that we describe here principally suggest that Mg^{2+} induces activation of brain alkaline phosphatase through its binding to the enzyme. There is no evidence for an action on the substrate. As a function of pH, the activation is a rapid or a slow process. Therefore it has been possible to study it kinetically by choosing the experimental conditions. The binding of Mg^{2+} probably produces an immediate but weak activation of the enzyme. This is suggested by the analysis of the activation process using a semilogarithmic plot in which the curve does not reach the zero value. The second step probably involves a conformational change of the enzyme as judged by the following criteria: (a) The fact that the activation can be described by an exponential relationship. (b) The enhancement of the rate of the activation by increasing the temperature. (c) The decrease of the rate of the activation after the structure of the enzyme has been altered by heat denaturation. However, our results indicate but do not prove that a change in conformation takes place. Physicochemical experiments are in progress in this laboratory.

As we have indicated above, an action of Mg^{2+} on the quaternary structure must be discarded. Besides, there is still no evidence for a polymeric structure of brain alkaline phosphatase. To our knowledge, among mammalian alkaline phosphatases

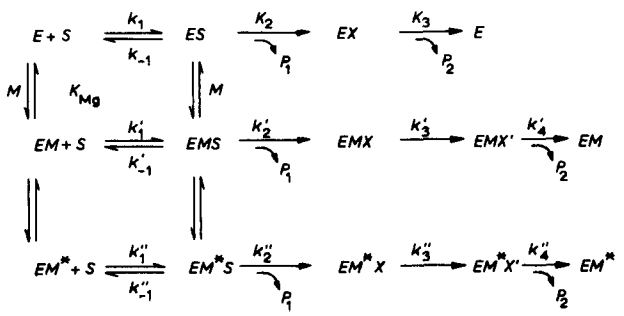
only the placental enzyme has been shown to have a quaternary structure[18], but it is also known that the placental enzyme differs in several properties from brain enzyme and some others, like kidney, bone and liver enzymes (*e.g.* molecular weight, heat and urea sensitivity, L-phenylalanine sensitivity, inhibition by imidazole, *etc.*)[5].

Our kinetics results and conclusions are similar to those theoretically discussed by Yon and co-workers[19] in a recent work on the activation process of β-galactosidase from *Escherichia coli* by Mg^{2+}. It is then by analogy that we have written in Scheme I



a reaction sequence of alkaline phosphatases. In this scheme, E is the free enzyme, EM the Mg-enzyme, EM^* the Mg-enzyme which would have supported the conformational change, S the monoester substrate, ES the enzyme–substrate complex, EX the phosphorylenzyme intermediate, P_1 the non-phosphate product and P_2 the inorganic phosphate.

However, another mechanism taking into account both the conformational change and an eventual catalytical effect of bound Mg^{2+}, could be proposed. Peck and Ray[20–22] have shown that a double action of Mg^{2+} is conceivable for phosphoglucomutase. This eventual catalytical role of Mg^{2+} is indirectly suggested by the inhibitory effect of Zn^{2+}, which acts on the enzyme at the same site as Mg^{2+}. Moreover, we have shown that the inhibition of the Zn-enzyme by P_i is of noncompetitive type, suggesting that P_i reacts with the Zn^{2+} newly bound to the enzyme. It is then possible that P_i released by the breakdown of the phosphorylenzyme intermediate may be attracted by this bound Zn^{2+}, which could result in a decrease in enzymatic activity, itself dependent on the rate of breakdown of this new phosphoenzyme intermediate. By analogy with the Zn-enzyme it is not unreasonable to think that



this intermediate is formed also in the case of the Mg-enzyme, but its rate of breakdown is very high. This might then support the idea of a catalytical role for Mg^{2+}.

In Scheme II we have rewritten a reaction sequence taking into account the possible existence of this phosphoenzyme intermediate (EMX' and EM^*X'). This is, of course, a hypothesis, but the data which are known for mammalian alkaline phosphatases are not in disagreement with this view.

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