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Rat osseous plate alkaline phosphatase: mechanism of action of manganese ions

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Polidocanol-solubilized osseous plate alkaline phosphatase was modulated by manganese ions in a similar way as by zinc ions. For concentrations up to 1.0 nM, the enzyme was stimulated by manganese ions, showing site–site interactions ($n = 2.2$). However, larger concentrations (> 0.1 μM) were inhibitory. Manganese ions could play the role of zinc ions stimulating the enzyme synergistically in the presence of magnesium ions ($K_d = 7.2$ μM; $V = 1005.5$ U mg^{-1}). Manganese ions could also play the role of magnesium ions, stimulating the enzyme synergistically in the presence of zinc ions ($K_d = 2.2$ μM; $V = 1036.7$ U mg^{-1}). However, manganese ions could not substitute for zinc and magnesium at the same time since ion assymetry is necessary for full activity of the enzyme. A steady-state kinetic model for the modulation of enzyme activity by manganese ions is proposed.

Keywords: alkaline phosphatase, manganese ions, osseous plate, Polidocanol, *p*-nitrophenylphosphate

Introduction

Divalent metal ions are essential not only for catalytic activity but also for the stability of several alkaline phosphatases (Harkness 1968, Hiwada & Wachsmuth 1974, Ohkubo *et al.* 1974, Ahlers 1975, Anderson *et al.* 1975, Cathala *et al.* 1975, Ey & Ferber 1977, Coleman *et al.* 1983, Curti *et al.* 1986, Genge *et al.* 1988, Bonet *et al.* 1992a). Alkaline phosphatases from mammalian sources are zinc-metalloenzymes which can be activated by magnesium ions (Harkness 1968, Brunel & Cathala 1973, Hiwada & Wachsmuth 1974, Ohkubo *et al.* 1974, Ahlers 1975, Cathala *et al.* 1975, Ey & Ferber 1977, PetitClerc & Fecteau 1977, Jahan & Butterworth 1986, Ciancaglini *et al.* 1992). However, this stimulation is non-selective and ions such as manganese, cobalt, nickel or calcium can substitute for magnesium ions to give an important stimulation of the enzyme (Brunel & Cathala 1973, Cathala *et al.* 1975; Linden *et al.* 1977, McComb *et al.* 1979, Leone *et al.* 1992).

For cartilage and bone alkaline phosphatases, only a few controversial reports concerning mainly the action of zinc and magnesium ions are available (Fortuna *et al.* 1980, Farley *et al.* 1980, Cyboron *et al.* 1982, Curti *et al.* 1986). Proteolytic effects caused by collagenase used in the preparation of the enzyme, species differences and even unknown factors are claimed to be responsible for such discrepancies (Farley *et al.* 1980, Cyboron *et al.* 1982, Hsu *et al.* 1985).

By using a non-proteolytic method for the preparation of alkaline phosphatase from osseous plate we overcame several of these problems, and showed that it is a membrane-bound metalloenzyme constituted by two apparently identical subunits of M_r 65 000 which require both magnesium and zinc ions for full activity (Pizauro *et al.* 1987, Ciancaglini *et al.* 1989, 1990a). A steady-state kinetic model explaining the synergistic stimulation of the enzyme by zinc and magnesium ions was proposed by us (Ciancaglini *et al.* 1992). Furthermore, we also reported that manganese and cobalt ions also modulate the enzyme activity (Ciancaglini *et al.* 1990b, Leone *et al.* 1992). However, there is no clear-cut information of whether the enzyme molecule has a specific site for metals other than zinc and magnesium.

This paper reports a steady-state kinetic study of the modulation of polyoxyethylene-9-lauryl ether (Polidocanol)-solubilized alkaline phosphatase from rat osseous plate by manganese ions. Our results show that manganese could substitute for zinc or magnesium ions, stimulating the enzyme in the presence of magnesium or zinc ions, respectively. However, manganese ions could not substitute for zinc and magnesium at the same time. A

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steady-state kinetic model for the action of manganese ions has been proposed.

Materials and methods

All solutions were made up with glass-distilled deionized water. Analytical grade salts were used without further purification as the source of metal ions. Chelex-100, 200–400 mesh was from BioRad (Richmond, CA); Tris, Polidocanol and 2-amino-2-methyl-1-propanol were from Sigma (St Louis, MO) and *p*-nitrophenyl-phosphate (PNPP), disodium salt was from Merck (Darmstadt, Germany).

Rat osseous plate alkaline phosphatase was prepared according to the procedure described by Curti *et al.* (1986). Solubilization was performed with 1% (v/v) Polidocanol and the detergent-solubilized enzyme was purified on a Sephacryl S-300 column (130 × 1.7 cm) according to Ciancaglini *et al.* (1990b). Chelex-treated detergent-solubilized enzyme was prepared according to Ciancaglini *et al.* (1992). Nitrophenyl-phosphatase (PNPPase) activity was estimated discontinuously in a DMS-80 Varian spectrophotometer as described elsewhere (Ciancaglini *et al.* 1992). Standard assay conditions were 50 mM 2-amino-2-methyl-1-propanol (AMPOL) buffer, pH 9.4, containing 1 mM PNPP in a final volume of 1.0 ml. The reaction was initiated by the addition of the enzyme and stopped by the addition of 1.0 ml of 1 M NaOH. Determinations were carried out in duplicate and the velocities were constant at least for 90 min, provided that less than 5% of substrate was hydrolyzed. Controls without added enzyme were included in each experiment to allow for the non-enzymic hydrolysis of the substrate. One enzyme unit (U) was defined as the amount of detergent-solubilized enzyme hydrolyzing 1.0 nmol of substrate per minute at 37 °C.

Magnesium and zinc ions were determined in an Atom Spek atomic absorption spectrometer using as standard 0.2 p.p.m. zinc chloride and 0.4 p.p.m. magnesium chloride solutions previously standardized against EDTA. Protein concentrations were determined according to Hartree (1972) and Cadman *et al.* (1979) for the membrane-bound and detergent-solubilized enzyme, respectively. In both cases, bovine serum albumin was used as standard. Kinetic parameters obtained from substrate hydrolysis were fitted on an IBM PC microcomputer according to the procedure described by Leone *et al.* (1992). V, K_d, n and v, which appear in this paper as computed values, stand for maximal velocity, apparent dissociation constant, Hill coefficient and initial velocity, respectively. Data are reported as the mean of duplicate determinations which differed by less than 5%.

Results

Manganese ions showed striking effects on the modulation of PNPPase activity of Chelex-treated detergent-solubilized alkaline phosphatase from osseous plate. For instance, in a metal-free system, increasing concentrations of manganese ions up to 0.1 μM stimulated the activity of the solubilized enzyme 3-fold (Figure 1A). Under such conditions the enzyme showed site–site interactions ($n = 2.2$) and a single family of metal binding sites with $K_d = 0.04$ μM. However, concentrations of manganese ions above 0.1 μM were inhibitory (Figure 1A). In the presence of zinc ions (0.1 and 10 μM), manganese ions stimulated the activity of the enzyme synergistically (Figure 1B and 1C). It should be noted that the maximum effect occurred with a 55-fold increase in K_d values (K_d increased from 0.04 to 2.2 μM when zinc ions varied from 0 to 10 μM in the system). Maximal synergistic stimulation ($V = 1036.7$ U mg^{-1}) was observed for zinc concentrations of about 10 μM (Table 1), which was very close to that of detergent-solubilized enzyme (about 1058 U mg^{-1}).

In the presence of magnesium ions high K_d values were

Table 1. Kinetic parameters for the stimulation of PNPPase activity of Chelex-treated Polidocanol-solubilized alkaline phosphatase by manganese ions in the presence of fixed concentration of zinc or magnesium ions, at pH 9.4

Metal	V(U mg^{-1})	K_d(μM)	n	Stimulation
Zn^{2+} alone	230.5	0.009	1.5	1.9
Mn^{2+} alone	308.7	0.040	2.2	2.9
Mn^{2+} + 0.1 μM Zn^{2+}	1,000.6	1.6	2.4	5.4
Mn^{2+} + 10 μM Zn^{2+}	1,036.7	2.2	1.5	20.7
Mn^{2+} + 10 μM Mg^{2+}	619.5	1.6	1.7	1.9

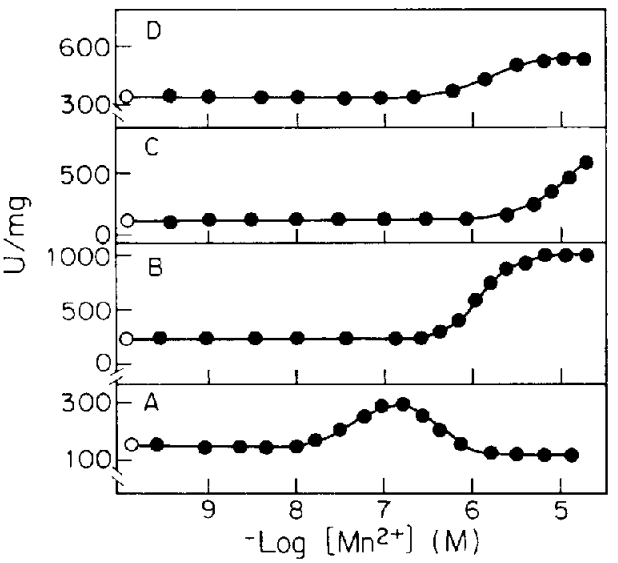

Figure 1. Stimulation of PNPPase activity of Chelex-treated Polidocanol-solubilized enzyme by: (**A**) manganese ions, (**B**) manganese ions in the presence of 0.1 μM zinc ions, (**C**) manganese ions in the presence of 10 μM zinc ions and (**D**) manganese ions in the presence of 10 μM magnesium ions. PNPPase activity was determined in 50 mM AMPOL buffer, pH 9.4, containing 1 mM PNPP as described in Materials and methods. Protein concentration used was 3 μg. Open symbols represent the activity of Chelex-treated enzyme in the absence of manganese ions.

also obtained, but manganese ions did not stimulate the enzyme to the same extent as in the presence of zinc ions (Figure 1D). Furthermore, manganese ions had no effect on enzyme activity when the concentration of magnesium ions was higher than 1.0 mM (not shown).

The high K_d values, obtained for the stimulation of the enzyme by manganese ions in the presence of magnesium or zinc ions when compared to those obtained for manganese ions alone, suggest a competition between these three ions for metal binding sites on the enzyme molecule (Table 1).

Figure 2 shows the effect of increasing concentrations of zinc ions on the activity of Chelex-treated detergent-solubilized enzyme in the presence of a fixed concentration of manganese ions. In a manganese-free system, a 2-fold stimulation of enzyme activity was observed, for increasing concentrations of zinc ions, and the calculated value for K_d was 9.0 nM. Similarly, as observed for manganese ions, concentrations of zinc ions above 0.1 μM were also inhibitory, independent of the presence of manganese ions. Another interesting observation is that zinc ions showed no synergistic effects in the presence of manganese ions. Taken together these results suggest that manganese and zinc ions bind to the same site.

In a manganese-free system, increasing concentrations of magnesium ions stimulated the activity of detergent-solubilized enzyme 3-fold (Figure 3A). In this case, K_d was calculated as 3.8 μM (Table 2) and negative cooperative effects ($n = 0.5$) were observed. As observed for zinc ions, at a fixed concentration of manganese ions, a synergistic stimulation of the enzyme occurred with increasing concentration of magnesium ions (Figure 3B and 3C and Table 2). These results indicated that the two ions bind at different sites.

Finally, it should be noted in Table 1 that maximal specific activity of the enzyme in the presence of manganese and magnesium ions ($V = 1005.5\ U\,mg^{-1}$) was quite similar to that obtained for manganese and zinc ions ($V = 1036.7\ U\,mg^{-1}$). These data, quite similar to those obtained for detergent-solubilized enzyme, of about 1058 U mg^{-1} (not shown) indicate that manganese ions could reconstitute the enzyme in the presence of zinc or magnesium ions.

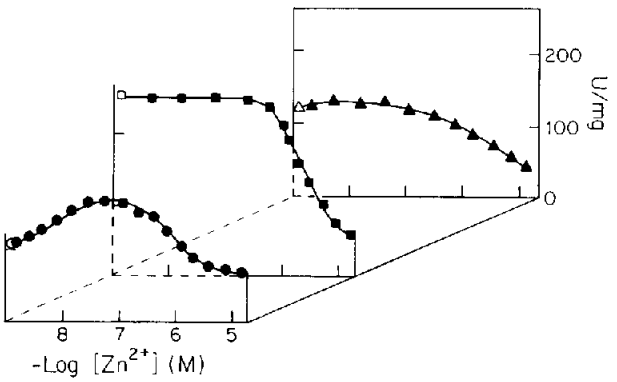

Figure 2. Stimulation of PNPPase activity of Chelex-treated Polidocanol-solubilized enzyme by: (●) zinc ions, (■) zinc ions in the presence of 0.1 μM manganese ions and (▲) zinc ions in the presence of 1.0 μM manganese ions. PNPPase activity was determined in 50 mM AMPOL buffer, pH 9.4, containing 1 mM PNPP, as described in Materials and methods. Protein concentration used was 3 μg. Open symbols represent the activity of Chelex-treated enzyme in the absence of zinc ions.

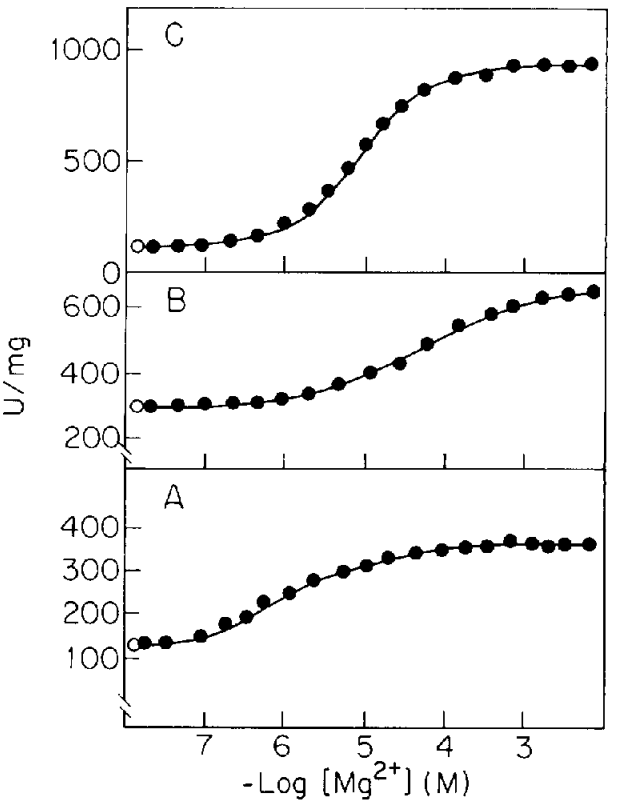

Figure 3. Stimulation of PNPPase activity of Chelex-treated Polidocanol-solubilized enzyme by: (**A**) magnesium ions, (**B**) magnesium ions in the presence of 0.1 μM manganese ions and (**C**) magnesium ions in the presence of 1.0 μM manganese ions. PNPPase activity was determined in 50 mM AMPOL buffer, pH 9.4, containing 1 mM PNPP as described in Materials and methods. Protein concentration used was 3 μg. Open symbols represent the activity of Chelex-treated enzyme in the absence of magnesium ions.

Table 2. Kinetic parameters for the stimulation of PNPPase activity of Chelex-treated Polidocanol-solubilized alkaline phosphatase by magnesium ions in the presence of fixed concentration of manganese ions, at pH 9.4

Metal	V(U mg^{-1})	K_d(μM)	n	Stimulation
Mg^{2+} alone	335.7	3.8	0.5	3.0
Mg^{2+} + 0.1 μM Mn^{2+}	650.4	52.0	0.6	2.2
Mg^{2+} + 1.0 μM Mn^{2+}	1,005.5	7.2	0.9	9.0

Discussion

Rat osseous plate alkaline phosphatase shares a great number of properties with other mammalian alkaline

phosphatases. It is a dimeric glycoprotein constituted by two apparently identical subunits of 65 000. The enzyme belongs to the category of alkaline phosphatases which need not only zinc but also magnesium ions for maximal activity (Ciancaglini *et al.* 1992). The enzyme has three metal binding sites: two for zinc ions and one for magnesium ions. Zinc ions bind to sites I and III, while magnesium ions bind to site II in the full active enzyme. Saturation with zinc of site I stimulates the enzyme, but the simultaneous saturation of sites I and II causes inhibition. Alternatively, in a zinc-free system, the binding of magnesium ions on sites I and II also stimulates the enzyme. Taking in account this model it can be suggested that zinc inhibits the enzyme by displacing magnesium ions from site II (Ciancaglini *et al.* 1992). This interpretation is different from that reported by Jahan & Butterworth (1986) and Curti *et al.* (1986).

Despite the existence of a few controversial reports, the mechanism of action of manganese ions in the process of substrate conversion as well as the nature of their binding sites are still unclear. According to Linden *et al.* (1977), manganese is a stimulatory cation that binds tightly to the magnesium site ($K_d = 2\ \mu M$). For bovine matrix-vesicle alkaline phosphatase, manganese showed neither stimulatory nor inhibitory effects, while a slight stimulation was reported for bovine chondrocytic enzyme (Fortuna *et al.* 1979, 1980). Manganese ions have also been reported to stimulate kidney alkaline phosphatase when replacing magnesium ions, but the substitution of zinc by manganese ions, inhibited the enzyme (Cathala *et al.* 1975).

Of particular interest are the results reported for alkaline phosphatase from *Halobacterium halobium* suggesting that the mechanism of substrate hydrolysis changes in the presence of manganese ions. The binding of such ions unmasks thiol groups, normally buried in the protein, which are responsible for fast dephosphorylation of the enzyme (Bonet *et al.* 1992b).

The effects of manganese ions on PNPPase activity of rat osseous plate alkaline phosphatase were similar to those observed for zinc ions (Ciancaglini *et al.* 1992). In a zinc-free system (Figure 1A), the stimulatory effect observed for concentrations up to 0.1 μM manganese ions could be interpreted as a consequence of the ion binding to site I. The inhibition occurring at concentrations above 0.1 μM manganese ions was due to the binding of such ions to site II. Furthermore, the synergistic effect of manganese ions, stimulating the enzyme 5.4-fold when in the presence of 0.1 μM zinc ions, was consistent with the suggestion that the two ions are bound to different sites, as reported elsewhere for zinc and magnesium ions (Ciancaglini *et al.* 1992).

The increase of K_d from 40 nM (in the absence of zinc ions) to 2.2 μM (in the presence of 10 μM zinc ions) apparently indicates that zinc ions were displaced by manganese from site II. The result shown in Figure 1(D) was consistent with this interpretation since in the presence of 10 μM magnesium ions, manganese also stimulated synergistically the enzyme in a similar way to that observed for zinc ions. In both cases, K_d and the specific activity of the enzyme were very similar (Table 1).

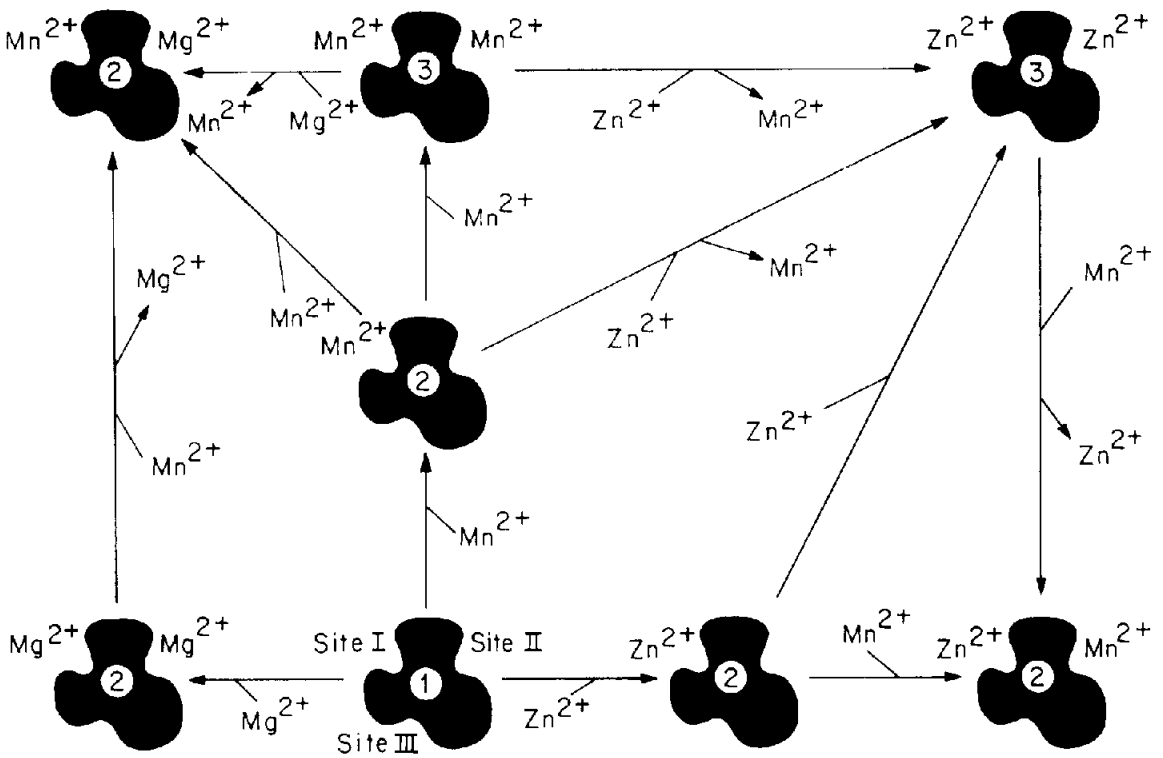

Figure 4. Proposed model for the effects of manganese, zinc and magnesium ions on PNPPase activity of Polidocanol-solubilized alkaline phosphatase. (1) Chelex-treated enzyme, (2) active reconstituted enzyme and (3) inactive reconstituted enzyme.

The addition of zinc ions in a manganese-containing system (Figure 2) confirmed that manganese ions were displaced from site II. The similarities between K_d values, i.e. the inhibition observed for concentrations of zinc ions above 1 μM and the close values calculated for specific activities of magnesium–manganese–enzyme ($V = 1005.5\ U\,mg^{-1}$) and zinc–manganese–enzyme ($V = 1036.7\ U\,mg^{-1}$), suggested that site II was indeed occupied by zinc ions.

Finally, data from Figure 3 confirmed that manganese ions could be displaced from site II by magnesium ions. Synergistic effects of manganese and magnesium ions give rise to a fully active enzyme with minor changes of K_d values.

In conclusion, these results suggested that manganese could play the role of zinc ions stimulating the enzyme in the presence of magnesium ions. Furthermore, manganese ions could also play the role of magnesium ions, stimulating the enzyme in the presence of zinc ions. However, manganese ions could not play the role of these two ions at the same time since ion assymetry is necessary for full activity of the enzyme.

The results obtained by steady-state kinetics allow us to propose a model which explains the mechanism of action of manganese during the catalytic cycle of rat osseous plate alkaline phosphatase (Figure 4).

Acknowledgments

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