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MILK ALKALINE PHOSPHATASE

STIMULATION BY Mg^{2+} AND PROPERTIES OF THE Mg^{2+} SITE

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Summary

Milk alkaline phosphatase (orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1) is an enzyme which requires two types of metals for maximal activity: zinc, which is essential, and magnesium, which is stimulatory. The main features of the Mg^{2+} stimulation have been analyzed. Mg^{2+} binding to native alkaline phosphatase is characterized by a dissociation constant of 30 μM at pH 8.5, 25°C. The stimulation is pH dependent and is observed mainly between pH 7.8 and 10.2. Both the rate constants of association, k_a, and of dissociation, k_d, have low values and are respectively 9 $M^{-1} \cdot s^{-1}$ and $3.2 \cdot 10^{-4} \cdot s^{-1}$ at pH 8.5, 25°C. Mg^{2+} can be replaced at its specific site by Mn^{2+}, Co^{2+}, Ni^{2+} and Zn^{2+}. Zinc binding to the Mg^{2+} site inhibits the native alkaline phosphatase. The dissociation constant of Zn^{2+} from the Mg^{2+} site is 0.3 μM at pH 8.5, 25°C. Mn^{2+}, Co^{2+}, Cd^{2+} and Ni^{2+} also bind to the Mg^{2+} site with a stimulatory effect which is lower than that of Mg^{2+}. Mn^{2+} is the stimulatory cation which binds most tightly to the Mg^{2+} site; the dissociation constant of the Mn^{2+}milk phosphatase complex is 2 μM at pH 8.5. The stoichiometry of Mn^{2+} binding has been found to be 1 equiv. of Mn^{2+} per mol of dimeric milk phosphatase. The native enzyme displays absolute half-site reactivity for Mn^{2+} binding. The properties of the Mg^{2+} site to milk alkaline phosphatase are very similar to those of bovine kidney phosphatase Mg^{2+} site.

Introduction

The alkaline phosphatase from cow's milk (orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1) is a dimeric enzyme consisting of two identical or similar subunits. Like the bacterial alkaline phospha-

tases [1,2] or the animal phosphatases [3—5] the enzyme from milk contains 4 g atoms of zinc per mol of protein [6]. In addition to zinc, certain alkaline phosphatases are strongly stimulated in the presence of magnesium. This is the case of the enzyme extracted from the rat placenta [5], the bovine brain [7,8] the bovine kidney [9], the pig kidney [10].

The milk alkaline phosphatase belongs to the Mg^{2+}-stimulated enzyme group. Taking account of the research work carried out on the bovine kidney phosphatase, we have studied the effect of magnesium, zinc and other bivalent ions on the enzyme activity.

Materials and Methods

Materials. Pure milk alkaline phosphatase was obtained as described in the previous paper [11]. Metals used were spectrographically pure (Johnson and Mathey, Co, Ltd). Other reagents were of the highest grade commercially available. All the experiments were carried out in demineralized and twide-distilled water.

All solutions were treated on resin Chelex 100 (Biorad) which had previously been equilibrated with buffer at the indicated pH. The solutions were stored on chelex and filtrated before use.

Methods. Phosphatase concentrations were determined from absorbance measurements at 278 nm. The coefficient $E^{1\,\%}_{1\,cm} = 7.7$ was obtained by the refractive index increment method described by Babul and Stellwagen [12].

Hydrolysis of *p*-nitrophenyl phosphate was followed at 410 nm with Beckman recording spectrophotometer equipped with a thermostated cell holder (ϵ_M for *p*-nitrophenolate anion: $1.75 \cdot 10^4\ M^{-1} \cdot cm^{-1}$).

Zinc and magnesium contents from the enzymatic preparations and the solutions treated on Chelex resin were determined with a Varian-Techtron atomic absorption spectrophotometer at an excitation wavelength of 213.8 and 285.2 nm, respectively.

Apophosphatase was obtained by treatment of the native enzyme at 4°C with Chelex 100 in Tris · HCl buffer, pH 8.0, as described by Csopak [13]. Metallophosphatases reconstitution was carried out the method of Cathala et al. [4].

Results

Mg^{2+} stimulation. As for the kidney and brain alkaline phosphatases, the milk enzyme is strongly activated by Mg^{2+}. The maximal stimulation at high Mg^{2+} concentrations under the experimental conditions of Fig. 1A is by a factor of 12.5. Mg^{2+} binding to the native enzyme is described by a simple titration curve with a dissociation constant, $K_{Mg^{2+}}$, for the native phosphatase · Mg^{2+} complex of 30 μM at pH 8.5. The Hill plot indicates a Hill coefficient of 1.0 for Mg^{2+} binding to the native phosphatases (Fig. 1B). In addition to this, we observed that Mg^{2+} stimulation of enzymatic activity occurs between pH 7.8 and 10.2 (Fig. 2).

Kinetics of activation by Mg^{2+}. Mg^{2+} binding to native milk phosphatase can

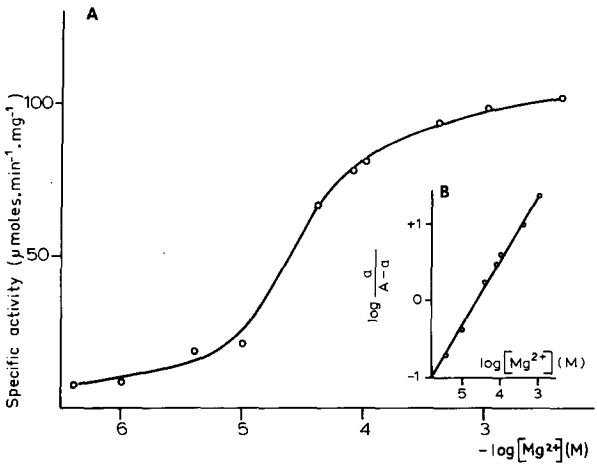

Fig. 1. (A) Mg^{2+} concentration dependence of V for *p*-nitrophenyl phosphate hydrolysis. The enzyme (27 nM) was incubated with increasing concentrations of Mg^{2+} at 25°C in a Tris · HCl buffer (50 mM), pH 8.5, containing 0.4 M NaCl. Activity assays were made with 5 mM *p*-nitrophenylphosphate in the same buffer at 25°C. 1(B) Hill plot indicating a Hill coefficient of 1.0 for Mg^{2+} binding to the native phosphatase. a is the difference between the specific activity measured in the presence of a given concentration of magnesium and the specific activity of the native non-stimulated milk alkaline phosphatase. A is the difference between the specific activity of the fully stimulated Mg^{2+} · phosphatase and the specific activity of the native enzyme.

be represented by:

$$E + Mg^{2+} \underset{k_d}{\overset{k_a}{\rightleftharpoons}} E \cdot Mg^{2+}$$

where E is the native enzyme, E-Mg^{2+} the Mg^{2+}-stimulated enzyme, k_a and k_d the rate constant for the association and dissociation process, respectively. Fig. 3A shows that the association of Mg^{2+} with the native enzyme follows pseudo first-order kinetics. The expression of the rate constant k_{app} is:

$$k_{app} = k_a[Mg^{2+}] + k_d$$

The k_{app} vs. Mg^{2+} plot presented in Fig. 3B is perfectly linear. Values of k_a and k_d evaluated after this representation are 9 $M^{-1} \cdot s^{-1}$ and 3.2 ± 0.2 10^{-4} s^{-1}, respectively at 25°C.

The dissociation constant $K_{Mg^{2+}}$ of the native phosphatase-Mg^{2+} complex can also be calculated from k_a and k_d values: $K_{Mg^{2+}} = k_d/k_a = 35$ μM. This result is in excellent agreement with the direct determination from Fig. 1A.

Effect of other bivalent ions. Several cations can be substituted for Mg^{2+} to give an important stimulation of native milk phosphatase. Mn^{2+}, Co^{2+}, Ni^{2+}, for instance, produce an important activation but smaller than the one found with Mg^{2+} (Table I).

Dissociation constants of the ion activator-native phosphatase complex were determined in the same way as previously described in Fig. 1A for the Mg^{2+}-stimulated enzyme. The tightest complex is observed with Mn^{2+} and the weakest one with Ni^{2+}.

The high affinity of the native enzyme containing 4 g atoms of Zn^{2+} per mol

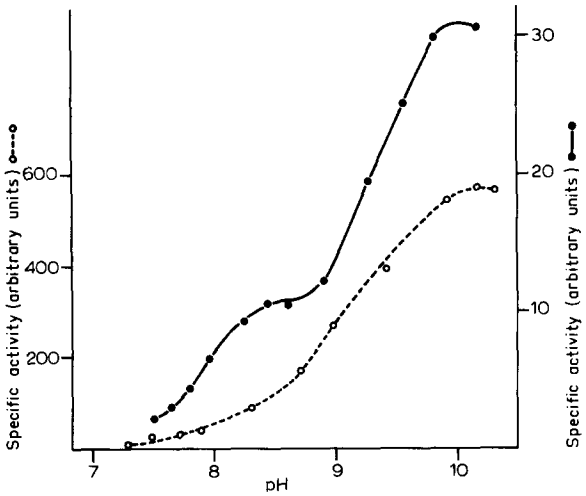

Fig. 2. Activity variation according to pH for the Mg^{2+}-stimulated milk phosphatase (○) and for the native phosphatase (●) at 25°C. The Mg^{2+}-stimulated phosphatase was obtained by incubation of the native phosphatase with 5 mM Mg^{2+} in a Tris · HCl buffer (50 mM, pH 8.5, containing 0.4 M NaCl) at 25°C for 2 h. The specific activities were determined by using *p*-nitrophenyl phosphate (5 mM) in the same buffer. For activity measurements of the Mg^{2+}-stimulated enzyme 1 mM Mg^{2+} was added to buffer.

of protein for Mn^{2+} made it possible to measure the number of metal atoms necessary for the maximal enzyme stimulation. Fig. 4 clearly indicates that the binding of only 1 equiv. of Mn^{2+} per mol of dimeric enzyme is responsible for the stimulation effect.

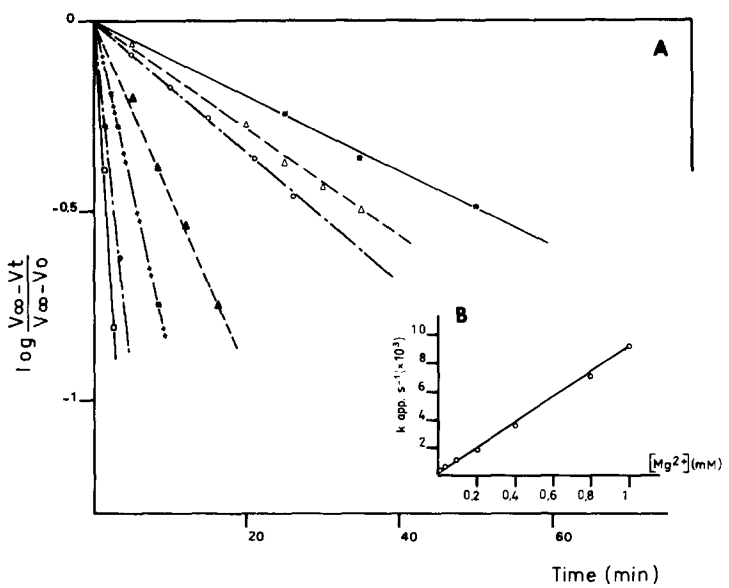

Fig. 3. (A). Kinetics of native enzyme stimulation by incubation with Mg^{2+} at 25°C and pH 8.5. The native enzyme was incubated with Mg^{2+} as described in Fig. 1A. Mg^{2+} concentrations were: 0.01 mM (■———■), 0.04 mM (△- - - - -△), 0.1 mM (○ — · — ○), 0.2 mM (▲- - - - -▲), 0.4 mM (■ — ++ — ■), 0.8 mM (● — · — ●), 1 mM (□———□). V_o is the maximal rate of the native milk phosphatase not stimulated by Mg^{2+}; V_∞ is the value of the maximal rate reached at equilibrium; V_t is the value of the maximal rate attained after incubation during a time t of the native enzyme with Mg^{2+}. 3(B) Variation of the experimental first order rate constant k_{app} with the Mg^{2+} concentration.

TABLE I

STIMULATION OF NATIVE MILK ALKALINE PHOSPHATASE BY BIVALENT CATIONS

Maximal specific activities and $K_{Me^{2+}}$ values were obtained as described in Fig. 1A, in 50 mM Tris · HCl, pH 8.5, 0.4 M NaCl, 25°C.

Me^{2+}	$K_{Me^{2+}}$	Specific activities ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)	Activity ratio to native enzyme
Mg^{2+}	30 μM	102	12.5
Mn^{2+}	2 μM	61	7.6
Co^{2+}	70 μM	61	7.6
Cd^{2+}	0.15 mM	36	4.5
Ni^{2+}	0.65 mM	30	3.7

In addition, we did not observe any activation effect on native enzyme in presence of Ca^{2+} (10^{-2} M) or Cu^{2+} (10^{-3} M).

Action of Zn^{2+} on Mg^{2+}-stimulated enzyme. Zn^{2+} also binds to the Mg^{2+} site and produces from a concentration of 10^{-5} M a strong inhibition of the Mg^{2+}-stimulated enzyme (Fig. 5A).

The expression of the dissociation constant K_{app} of Zn^{2+} can be evaluated by:

$$K_{app}\ Zn^{2+} = K_{Zn^{2+}}\left(1 + \frac{[Mg^{2+}]}{K_{Mg^{2+}}}\right)$$

$K_{app\ Zn^{2+}} = 10^{-4}$ M; $Mg^{2+} = 10^{-2}$ M; $K_{mg^{2+}} = 30\ \mu$M; $K_{Zn^{2+}} = 0.3\ \mu$M

The Zn^{2+}-inhibited enzyme is slowly reactivated by Mg^{2+}, Co^{2+}, Mn^{2+} . . . Fig. 5B indicates that the rate of Mg^{2+} activation of the Zn^{2+}-inhibited enzyme is about 25 times slower than that observed for Mg^{2+} activation of the native enzyme.

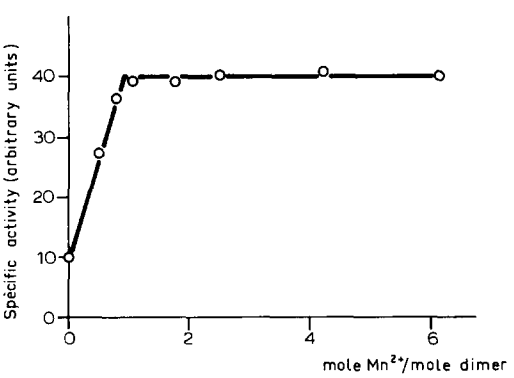

Fig. 4. Mn^{2+} stimulation of native milk alkaline phosphatase. The native enzyme at a concentration of 50 μM, i.e. 25 times higher than $K_{Mn^{2+}}$ was incubated during 20 min by adding increasing Mn^{2+} concentrations in Tris · HCl (50 mM) containing 0.4 M NaCl at pH 8.5. Activity was measured in the same buffer at 25°C with 5 mM *p*-nitrophenyl phosphate. Incubation of 60 min instead of 20 min gave the same stoichiometry: i.e. a plateau value in activity was attained for 1 mol Mn^{2+} per mol of dimeric milk phosphatase.

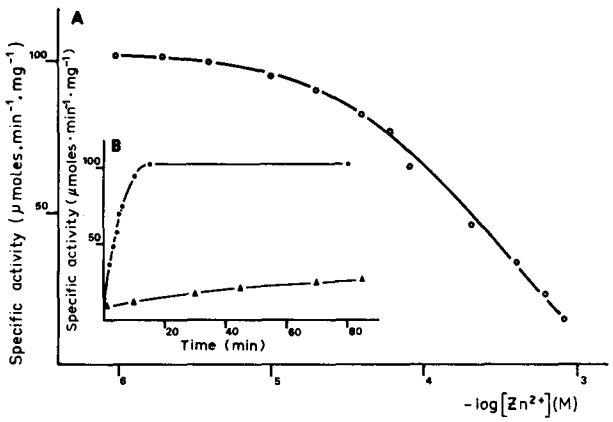

Fig. 5. (A) Zn^{2+} inhibition of Mg^{2+} (10^{-2} M)-stimulated milk alkaline phosphatase. The activity was measured at 25°C in a Tris · HCl buffer (50 mM) containing 0.4 M NaCl at pH 8.5 with 5 mM *p*-nitrophenyl phosphate. (B) Kinetics of Mg^{2+} stimulation (10^{-2} M) of the native milk alkaline phosphatase (•) and of the enzyme inhibited by Zn^{2+} ($6 \cdot 10^{-4}$ M) (▲).

Discussion

Milk alkaline phosphatase shares a great number of properties with other eukaryotic alkaline phosphatases. The enzyme has a dimeric structure and, Zn^{2+} and Mg^{2+} are important for catalytic activity. Zn^{2+} is essential for the activity and Mg^{2+} stimulates the activity of the native Zn^{2+} enzyme. The milk phosphatase is therefore a good model of an enzyme which requires multiple metal species for its activity.

Mg^{2+} shows an affinity for the native phosphatase that is comparable to those determined for the enzyme of bovine kidney [9] and rat placenta [5]. We previously pointed out that the only apparent catalytic effect of Mg^{2+} was an increase of V, the maximal rate, the K_m values for different substrates being unaffected [6]. A similar result was found in the case of other phosphatases [5,8,9]. This observation added to the demonstration that the presence of inorganic phosphate does not affect the association or dissociation rate of Mg^{2+} on enzyme [5,9] indicates that Mg^{2+} stimulates by binding to a site distinct from the catalytic one.

Other metals can bind to the Mg^{2+} site. They are Ni^{2+}, Cd^{2+}, Co^{2+} and Mn^{2+}. Those different metals bind to the Mg^{2+} site with different affinities and the stimulation factor they give seems to decrease with the affinity they have for the enzyme except for Mn^{2+}. Zn^{2+} presents a similar affinity to Mg^{2+} for the Mg^{2+} site but strongly inhibits the enzyme.

Kinetic studies of Mg^{2+} activation show that: (i) The binding is abnormally slow at pH 8.5. The apparent rate constant of association is only of 9 $M^{-1} \cdot s$,1 whereas association rate constants of the order of 10^8 $M^{-1} \cdot s^{-1}$ should be expected for a diffusion-controlled process [9]. Such an abnormally low rate of association indicates that the diffusion-controlled initial binding of Mg^{2+} to the enzyme is followed by a slow conformational rearrangement of the Mg^{2+}-phosphatase complex essential to produce a Mg^{2+}-stabilized high activity

state of the enzyme. (ii) The reactivation by Mg^{2+} of the milk phosphatase inhibited by Zn^{2+} is 25 times slower than Mg^{2+} activation of the native enzyme. The rate-limiting step of the reactivation process seems to be the slow dissociation of Zn^{2+} from the Mg^{2+} site. The dissociation rate would be $4.6 \cdot 10^{-4}$ s^{-1} at pH 8.5, 25°C.

It has been demonstrated that only 1 equiv. of Mn^{2+}, i.e. probably 1 equiv. of any stimulating cation, was necessary for the stimulation of the milk phosphatase. The same observation has already been made for the bovine kidney alkaline phosphatase [9]. The stoichiometry of Mn^{2+} binding per dimer of kidney phosphatase shows that the enzyme presents a half-site reactivity for the activator ion. Half-site reactivity for the activator ion is most probably connected with the half-site reactivity for the substrate transformation observed in the case of *Escherichia coli*, intestine and kidney phosphatase [14,15,9]. It seems likely that Mg^{2+} (or Mn^{2+}) which binds to a site distinct from the active one induces or stabilizes an asymmetry necessary for the catalytic activity of the enzyme.

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