Biochimica et Biophysica Acta, 480 (1977) 143—153

BBA 68004

METAL ION-INDUCED CONFORMATIONAL CHANGES IN *ESCHERICHIA COLI* ALKALINE PHOSPHATASE

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(Received June 29th, 1976)

Summary

Ultraviolet difference spectra are produced by the binding of divalent metal ions to metal-free alkaline phosphatase (EC 3.1.3.1). The interaction of the apoprotein with Zn^{2+}, Mn^{2+}, Co^{2+} and Cd^{2+}, which induce the tight binding of one phosphate ion per dimer, give distinctly different ultraviolet spectra changes from Ni^{2+} and Hg^{2+} which do not induce phosphate binding.

Spectrophotometric titrations at alkaline pH of various metallo-enzymes reveal a smaller number of ionizable tyrosines and a greater stability towards alkaline denaturation in the Zn^{2+}- and Mn^{2+}-enzymes than in the Ni^{2+}-, Hg^{2+}- and apoenzymes. The Zn^{2+}- and Mn^{2+}-enzymes have CD spectra in the region of the aromatic transitions that are different from the CD spectra of the Ni^{2+}-, Hg^{2+}- and apoenzymes.

Modifications of arginines with 2,3-butanedione show that a smaller number of arginine residues are modified in the Zn^{2+}-enzyme than in the Hg^{2+}-enzyme.

The presented data indicate that alkaline phosphatase from *Escherichia coli* must have a well-defined conformation in order to bind phosphate. Some metal ions (i.e. Zn^{2+}, Co^{2+}, Mn^{2+} and Cd^{2+}), when interacting with the apoenzyme, alter the conformation of the protein molecule in such a way that it is able to interact with substrate molecules, while other metal ions (i.e. Ni^{2+} and Hg^{2+}) are incapable of inducing the appropriate conformational change of the apoenzyme. These findings suggest an important structural function of the first two tightly bound metal ions in enzyme.

Introduction

Alkaline phosphatase from *Escherichia coli* (orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1) is a dimeric metalloenzyme. The zinc ions can be removed from the enzyme and replaced by various other divalent metal ions. The apoenzyme is inactive and cannot bind phosphate

[1—4]. It has been shown that Co^{2+}, Mn^{2+}, Cd^{2+}, Ni^{2+} and Hg^{2+} compete with Zn^{2+} for the specific metal binding sites of the enzyme [4—6]. Among these metal ions only Zn^{2+} and Co^{2+} yield significant enzyme activity.

The mechanism of alkaline phosphatase catalysis is dominated by the fact that phosphate transfer proceeds through a covalent phosphoryl intermediate. It has been shown that the binding of phosphate to alkaline phosphatase, and the phosphorylation and dephosphorylation of the enzyme, are metal ion dependent [4]. The changes in the rates of phosphorylation and dephosphorylation induced by different metal ions may relate either to intrinsic chemical properties of the metal or to differences in the conformational changes induced by different metal ions.

The present investigation was undertaken in an attempt to determine whether the role of metal ions in phosphate binding could be explained in terms of metal-induced conformational changes.

Materials and Methods

Chemicals. All reagents were analytic grade. Buffer solutions were prepared free of metal ion impurities as previously described [7]. Glassware was treated according to Thiers [8]. Chelex 100 (200—400 mesh) was from Bio-Rad Laboratories Richmond, Ca., U.S.A.; DEA A23 cellulose from Whatman, Kent, U.K.; Sephadex G-100 from Pharmacia, Sweden; 2-(*N*-morpholino)-ethanesulphonic acid (Mes), 2-(*N*-hydroxyethylpiperazine) *N'*-2-ethanesulfonic acid (Hepes) and *p*-nitrophenyl phosphate (Sigma 104) from Sigma Chemical Company, St. Louis, Mo., U.S.A.; and 2,3-butanedione from Aldrich.

Enzyme. Bacterial growth and preparation of alkaline phosphatase including heat treatment were carried out as previously described [9,10]. The protein had a specific activity of 3000 ± 200 μmol/h per mg at 25°C when assayed under standard conditions [10]. Apoenzyme was prepared by treating the enzyme with Chelex-100 (200—400 mesh) as previously described [7]. Enzyme containing metal ions other than zinc was prepared from the apoenzyme by the addition of slightly more than 3 equivalents of the appropriate metal ion. For concentration calculations a molecular weight of 80 000 per dimer was used [2]. A value of 0.72 was employed as the absorbance at 278 nm of a 1 mg/ml solution of the enzyme [10].

Spectral measurements. Ultraviolet absorption difference spectra were obtained with a Gilford single beam spectrophotometer model 240 or a Beckman double beam spectrophotometer model Acta III. Solution preparations and spectral measurements were performed essentially as described by Donovan [11]. Protein concentrations were maintained at $3.5—4.0 \cdot 10^{-5}$ M in most experiments. The temperature was maintained at 27°C by means of Colora Ultra Thermostat.

When the measurements were made with the single beam spectrophotometer apoenzyme in buffer was placed in both parts of the cell (1 cm light path) and the baseline was recorded. Then successive additions of metal ions solutions to the sample cell were made. Volumes in the reference part were correspondingly increased by the addition of the same volume of the buffer. After each addition the spectrum was scanned. With the double beam spectrophotometer the procedure was essentially the same.

Spectrophotometric titrations at alkaline pH. A Zeiss PMQII spectrophotometer was used at single wavelengths and a Gilford 240 spectrophotometer for recording spectra. pH was measured with a radiometer 26 pH-meter equipped with an expanded scale; a glass electrode GK2322C was employed. The following buffer systems were used: glycine/NaOH, pH 8.4—10.5; lysine/NaOH, pH 10.6—11.2; and NaOH, pH 11.2—13.7. The ionic strength was adjusted with KCl to 0.1 M. Alkaline phosphatase from stock solutions was added to the different buffers to get a final enzyme concentration of 0.5 mg/ml. All readings were made at 25°C and extrapolated to zero time. The pH of the solution was measured after each run. The number of ionizable tyrosines was determined at 295 nm ($\Delta\epsilon_{295}$ = 2100 $M^{-1} \cdot cm^{-1}$ per tyrosine residue) and corrected for the absorption at neutral pH.

Circular dichroism. CD spectra were obtained with a Cary Model 60 recording spectropolarimeter equipped with a Cary 6002 circular dichroism attachment. The results are expressed in terms of the molar ellipticity $[\theta]$ in deg cm^2dmol^{-1}, defined as $[\theta] = M \times \theta_{obs}/10 \times l \times c$, where M is the mean residue weight (calculated to be 97,5 from the amino acid composition [10]), θ_{obs} is the observed ellipticity in degrees, l is the optical pathlength in cm and c is the protein concentration in g/ml determined as above. CD measurements between 320 and 250 nm were performed in 1 cm quartz cell with an enzyme concentration of about 2 mg/ml.

Arginine modification. Modification reactions with 2,3-butanedione (borate buffer, pH 7.5) of Zn^{2+}- and Hg^{2+}-enzymes were carried out as described by Daemen and Riordan [12] except that the excess of butanedione was removed by using a Minicon A-25 device instead of gel filtration over a Bio-Gel P-4 column. The modification of arginine was determined according to ref. 13 by the Sakaguchi procedure. Only after prolonged reaction with butanedione (for 7 days) did the enzyme activity fall to about 1% of that of the untreated enzyme. After 22 h of reaction the enzyme still showed 25% activity.

Results

Ultraviolet difference spectra of various metallo-enzymes vs. metal-free alkaline phosphatase

The effect of metal ion binding to the apoenzyme was examined with ultraviolet absorption difference spectra. Fig. 1 shows the difference spectra produced on addition of Zn^{2+}, Co^{2+}, Mn^{2+} and Cd^{2+} to metal-free enzyme at pH 8.0. Addition of these metals caused the appearance of difference spectra with peaks at 280, 286—7 and 297 nm. Difference spectra in the presence of Zn^{2+} are qualitatively similar to those obtained when Co^{2+}, Mn^{2+} and Cd^{2+} were added to apoenzyme. The same spectral pattern was obtained for Zn^{2+}-, Co^{2+}-, Cd^{2+}- and Mn^{2+}-enzymes at both pH 6.5 and 7.2.

On the other hand, addition Ni^{2+} or Hg^{2+} to the apoenzyme at pH 8.0 resulted in difference spectra which differed from those of the Zn^{2+}-, Mn^{2+}-, Co^{2+}- and Cd^{2+}-phosphatases (Fig. 2). At pH 7.2 the spectra for Ni^{2+}-enzyme were the same as for Zn^{2+}-enzyme, but Hg^{2+} does not induce spectra characteristic for the Zn^{2+}-enzyme either at pH 6.5 or 7.2.

Since the properties of Cu^{2+}-enzyme have been extensively studied by EPR

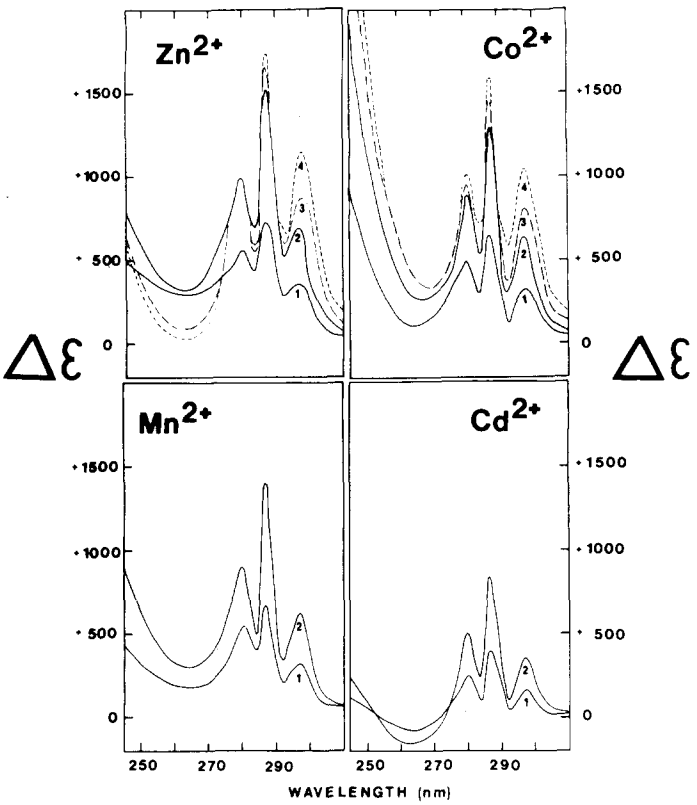

Fig. 1. Ultraviolet difference spectra of different Me^{2+}-alkaline phosphatase vs. apoenzyme. The conditions were as follows: 3.5—4.0 · 10^{-5} M apophosphatase, 0.1 M Hepes buffer pH 8.0, 27°C, 1 cm path light. The molar ratio of metal ion to enzyme is indicated.

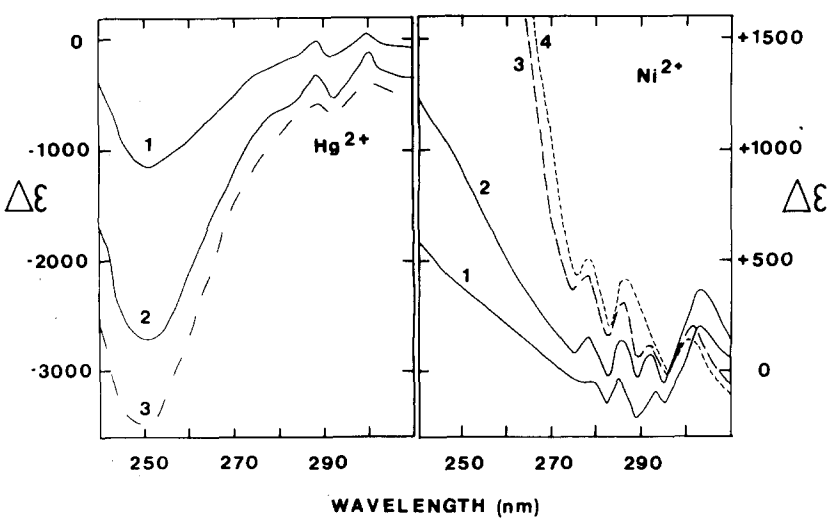

Fig. 2. Ultraviolet different spectra of Ni^{2+}- and Hg^{2+}-alkaline phosphatases vs. apoenzyme. Conditions as in Fig. 1.

in our laboratory, we investigated also the ultraviolet difference spectra produced by the addition of Cu^{2+} to apoenzyme. Unfortunately these spectra were difficult to reproduce and interpret, probably because binding equilibrium had not been attained during the time of the experiment.

It has recently been reported that 1.3 equivalents of Mg^{2+} per dimer are bound tightly to apoenzyme [14,15]. Our observations show that no change in the apo- or Zn^{2+}-enzyme spectra are caused by Mg^{2+}.

Spectral titrations

The changes in extinction coefficient at 286—7 and 297 nm, $\Delta\epsilon_{286-7}$ and $\Delta\epsilon_{297}$ observed upon metal binding were used to investigate the binding stoichiometry of the metal ions. Values of $\Delta\epsilon_{286-7}$ and $\Delta\epsilon_{297}$ were obtained as in Fig. 1 from titrations with Zn^{2+}, Co^{2+}, Mn^{2+} and Cd^{2+}. Fig. 3 shows plots of $\Delta\epsilon_{286-7}$ and $\Delta\epsilon_{297}$ against the metal to enzyme ratio. The sharp break which occurs at a metal to enzyme ratio of 2 : 1 indicates that the binding of two metal ions produces similar spectral changes. It is noticeable, however, that the $\Delta\epsilon$ values observed for Cd^{2+}-enzyme are smaller than these of the Zn^{2+}-, Co^{2+}- and Mn^{2+}-enzymes.

The titrations carried out with Zn^{2+} or Co^{2+} showed that on the addition of the third and fourth metal ion per apoenzyme the absorption continued to increase. However, as shown in Fig. 3, the slope of these increases in absorption were different from the slope of the linear increase in absorption produced by the addition of the two first metal ions per mole of alkaline phosphatase. This indicates that the nature of the binding of the third and the fourth Zn^{2+} or Co^{2+} ions was different from that observed for the two first metal ions and different from each other. When the Zn^{2+} titration was made in 0.1 M Tris ·

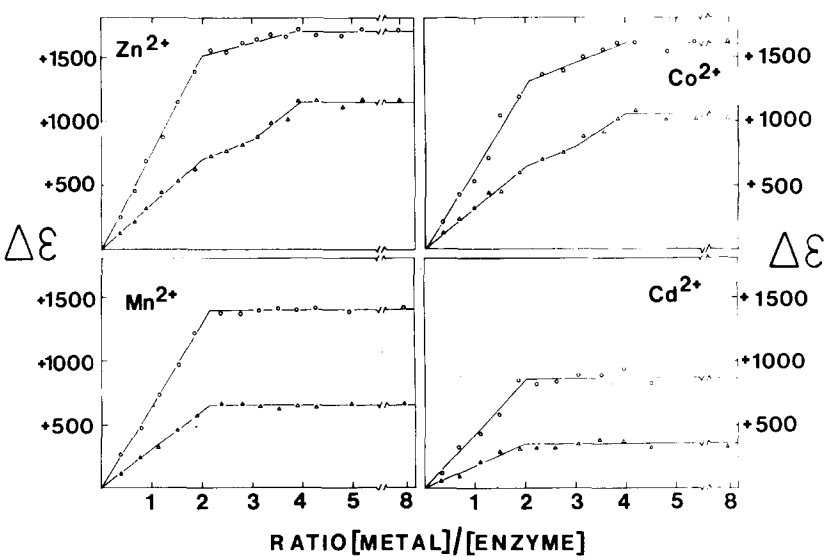

Fig. 3. Spectral titrations of apoenzyme with Zn^{2+}, Co^{2+}, Cd^{2+}, and Mn^{2+}. Plot of changes in molar extinction coefficient at given wavelength against metal-enzyme ratio. △, 297 nm; ○, 286—7 nm. Conditions as in Fig. 1.

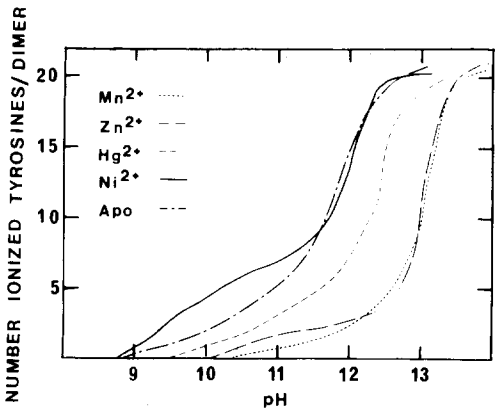

Fig. 4. The spectrophotometric titration curves of different Me^{2+}-alkaline phosphatases as function of pH. Conditions as in Materials and Methods.

HCl buffer at pH 8.0 instead of 0.1 M Hepes buffer at pH 8.0 no further changes were observed when the fourth Zn^{2+} ion was added to the apoenzyme.

In the case of Cd^{2+} and Mn^{2+} the addition of more than two metal ions per apoenzyme did not have any further effect on the absorbance.

Spectrophotometric titration at alkaline pH of Ni^{2+}-, Hg^{2+}-, Mn^{2+}-, Zn^{2+}- and apoenzymes

Spectrophotometric titrations at alkaline pH of Zn^{2+}-, Mn^{2+}-, Ni^{2+}-, Hg^{2+}- and apoenzymes are shown in Fig. 4. The titrations of different Me^{2+}-alkaline phosphatases show that the Mn^{2+}- and Zn^{2+}-enzymes have similar titration curves. These are different from those of the apo-, Hg^{2+}- and especially Ni^{2+}-enzymes. Tyrosyl residues appear to be more readily ionizable in Ni^{2+}-, Hg^{2+}- and apoenzymes than in Zn^{2+}- and Mn^{2+}-enzymes. Furthermore, Zn^{2+}- and Mn^{2+}-enzymes show greater stability towards alkaline denaturation, which occurs at higher pH than for Ni^{2+}-, Hg^{2+}- and apoenzymes.

The native enzyme (where Zn^{2+} and Mg^{2+} have not been removed) and alkaline phosphatase reconstituted by the addition of slightly more than three equivalents of zinc (without Mg^{2+}) give the same titration curves. The number of ionized tyrosines was calculated using the relationship $\Delta\epsilon_{295} = 2100\ M^{-1} \cdot cm^{-1}$ per tyrosine residue, assuming 20 tyrosines per enzyme molecule [10]. This unusually low value of $\Delta\epsilon_{295}$ per tyrosine suggests interference of tryptophan residues as in the case of human carbonic anhydrase B [16].

Near ultraviolet CD spectra of different metalloenzymes

The examination of CD spectra in the region of aromatic transitions of the enzyme reveal that the spectra of the Hg^{2+}-, Ni^{2+}- and apoenzymes are different from those of the Zn^{2+}- and Mn^{2+}-enzymes. While the general positions of the bands are maintained, there are differences in the band intensities. For the Zn^{2+}-enzyme a positive band is present at 295 nm, $[\theta] = 7$ and a negative band at 277 nm, $[\theta] = -36$. A similar spectrum was observed for the Mn^{2+}-enzyme. However, Ni^{2+}-, Hg^{2+}- and apoenzymes show these bands with different magnitudes (Fig. 5).

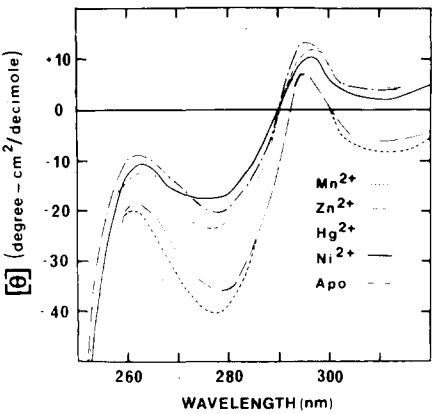

Fig. 5. Near ultraviolet CD spectra of different Me^{2+}-alkaline phosphatases. Conditions as in Materials and Methods.

Modification of arginines by butanedione in Zn^{2+}- and Hg^{2+}- alkaline phosphatases

The effect on arginine modification by substituting Zn^{2+} by Hg^{2+} was examined according to the procedure of Daemen and Riordan [12]. This method was used as a tool to detect different enzyme conformations. The results shown in Table I indicate that the number of modified arginines in Hg^{2+}-enzyme is higher than in the case of the Zn^{2+}-enzyme. However, the number of modified arginines obtained for Zn^{2+}-alkaline phosphatase differs from those reported by Daemen and Riordan [12].

Discussion

The effect of metal ions on enzymatic activity of alkaline phosphatase from *E. coli* is well known [1—4,6,17—24]. The metal ions can be removed and the apoenzyme is incapable of binding phosphate. The dimeric structure, however, is unchanged and the gross conformational features in the apo- and holoenzyme are apparently similar [2].

All metallo-phosphatases can be divided into two groups: one which is capable of binding tightly one phosphate ion per dimer molecule, and the other

TABLE I

Reaction time	Number of modified arginine residues (mol/mol of enzyme)	
	Zn^{2+}-enzyme *	Hg^{2+}-enzyme
15 min	0.9 (75%)	2.5
3 h	4.4 (50%)	5.3
22 h	6.2 (25%)	7.6
10 days	11.1 (0%)	12.4

* The figures given within parentheses are the remaining enzyme activities as compared to the untreated Zn^{2+}-enzyme

which is ineffective in binding phosphate. Applebury et al. [4] demonstrated that besides Zn^{2+}- and Co^{2+}-alkaline phosphatases, which are enzymatically active, the inactive Mn^{2+}- and Cd^{2+}-derivatives also induce the binding of phosphate. Ni^{2+}- and Hg^{2+}-enzymes at pH 8.0 are inactive and do not bind phosphate significantly (cf. Table II containing data from ref. 4).

Our spectral observations show that all the metals which induce the tight binding of one phosphate (i.e. Zn^{2+}, Co^{2+}, Cd^{2+} and Mn^{2+}) produce similar ultraviolet difference spectra (Fig. 1). The spectral changes induced by the addition of Hg^{2+} and Ni^{2+} to the apoenzyme are different (see Fig. 2).

However, at pH 7.2 the spectra for Ni^{2+}-enzyme were the same as for those metal ions which induce one tight phosphate binding. Probably Ni^{2+} forms different types of complexes with the apoenzyme at pH 8.0 and pH 7.2, and at lower pH values the Ni^{2+}-enzyme perhaps is capable of binding phosphate.

Since all spectral studies have been performed under similar conditions as those reported for phosphate binding studies [4], both kinds of experiments are directly comparable.

The metal-induced difference spectra involve both tyrosine and tryptophan residues, indicating that the environment of these residues has been affected. The difference spectra due to Zn^{2+}, Co^{2+}, Mn^{2+} and Cd^{2+} resemble those which are seen when tyrosine and tryptophan in 20% (v/v) ethylene glycol are compared with the same compounds in water [11], suggesting that the environment of the tyrosines and tryptophanes becomes more non-polar as a result of metal binding. The magnitude of the ultraviolet spectral changes caused by the first and second metal ions (for Zn^{2+} $\Delta\epsilon_{286-7} \approx 1500\ M^{-1} \cdot cm^{-1}$) suggests that, according to conventional methods, there is a net movement of about two tyrosine residues from a polar to a hydrophobic environment, if one assumes a $\Delta\epsilon_{286-7} \approx 700\ M^{-1} \cdot cm^{-1}$ for such a transfer [11]. This transfer reflects a conformational change of the apoenzyme as a result of metal binding. However, direct binding of the metal ions to a tyrosine cannot be ruled out. On the other hand, the ^{19}F NMR data on fluorotyrosine alkaline phosphatase indicate that there are 7 tyrosines per subunit experiencing changes in environment upon

TABLE II

PHOSPHATE BINDING AND ACTIVITY OF APO- AND METALLOALKALINE PHOSPHATASES AT pH 8.0

Contains data from ref. 4.

Enzyme	$^{32}P_i$ bound (mol/mol dimer)	K_1 ($\times 10^6$ M) *	Phosphatase activity (μmol/h per mg protein)
Zn^{2+}-enzyme	1.36 ± 0.19	1.2 ± 0.2	3000
Co^{2+}-enzyme	1.25 ± 0.19	0.5—1.0	600
Mn^{2+}-enzyme	1.23 ± 0.06		200
Cd^{2+}-enzyme	1.31 ± 0.07	0.5—1.0	100
Apoenzyme	0.22 ± 0.15		100
Ni^{2+}-enzyme	0.30 ± 0.12		100
Hg^{2+}-enzyme	0.08 ± 0.04		100

* Conditions: 0.01 M Tris, pH 8.0, 4°C.

removal of zinc [25]. These results are not in disagreement with our data, because in ultraviolet spectroscopy one looks at the total response of all tyrosines without resolution of individual sites.

Ultraviolet spectral titrations (Fig. 3) show that the addition of the first and second Zn^{2+} ions to the apoprotein induce equivalent spectral changes. It is well established that the two first Zn^{2+} ions are required for enzyme activity. These two Zn^{2+} ions have dissociations constants which differ only by the statistical factor of four: $pK_1 = 11.8$ and $pK_2 = 11.2$ (pH 8.5, 25°C) [7]. The spectral alterations induced by the third and fourth Zn^{2+} ions are different from, and smaller than the ones produced by the first and second Zn^{2+} ions. They also differ from each other (Figs. 1 and 3). This is consistent with the observation that the third and fourth Zn^{2+} ions bind with different stability constants, which are smaller than those of the first two Zn^{2+} ions [9].

Our ultraviolet difference spectra of Zn^{2+}-enzyme are not entirely consistent with the results published by Reynolds and Schlesinger [26]. These authors showed that $\Delta\epsilon_{286} = -5000\ M^{-1} \cdot cm^{-1}$ when three Zn^{2+} ions, equivalently bound, are removed from the holoenzyme. In the same study it was shown that enzyme activity increased linearly up to four Zn^{2+}/apoenzyme. These differences may be attributed to different methods of apoenzyme preparations.

The addition of more than two equivalents of Mn^{2+} and Cd^{2+} per apoenzyme molecule produce no further changes of the absorption spectra. Lack of further spectral changes with an excess of metal ions does not exclude, of course, the possibility that more metal ions may bind to the enzyme without inducing a change in absorption (ref. 2 and Norne, J.E., Csopak, H. and Lindman, B., unpublished results).

Lazdunski et al. using immunological methods found that there are only two antidimer antigenic determinants exposed in Cd^{2+}- and Mn^{2+}-enzymes compared to four in Zn^{2+}-enzyme. Furthermore, according to their data the antigenic structure of these Me^{2+}-enzymes is nearly identical with that of the apoenzyme [27]. However, our data show distinct conformational differences between Cd^{2+}- and Mn^{2+}-enzymes on the one hand and apoalkaline phosphatase on the other hand.

Spectrophotometric titrations at alkaline pH reveal differences between various metallo-alkaline phosphatases. The difference in the number of ionizable tyrosines reflected in these results correlates with the ability to bind phosphate. Metallo-enzymes which induce phosphate binding have rather similar titration curves, while metallo-alkaline phosphatases which do not preserve the capability for phosphate binding have titration curves which more resemble that of the apoenzyme (Fig. 4). The observed differences in tyrosine ionisation for the various metallo-alkaline phosphatases can be explained in terms of different conformations. However, the conformation of the protein at alkaline pH may be different from the conformation at neutral pH. Moreover, Ni^{2+}- and Hg^{2+}-enzymes are less stable towards alkaline denaturation and their tyrosines ionize more readily at lower pH. Nevertheless, the results support the conclusion that there are differences in conformation and in stability between Me^{2+}-alkaline phosphatases which can induce one tight phosphate binding site (such as Zn^{2+} and Mn^{2+}) and the other ones which cannot (Ni^{2+} and Hg^{2+}).

Daemen and Riordan studied the function of arginines in *E. coli* alkaline

phosphatase using the modification procedure and concluded that two arginyl residues are essential for the enzymatic activity [12]. By use of the same modification method we examined the possible effects of metal ions on the alkaline phosphatase conformation. The data presented here (Table I) show that the number of modifiable arginines is higher in Hg^{2+}-enzyme than in Zn^{2+}-enzyme, indicating a conformational difference between these two derivatives. This is consistent with the other data which demonstrate the difference between the native enzyme and those which are unable to induce phosphate binding.

CD investigations in the near ultraviolet revealed that the substitution of Zn^{2+} by Mn^{2+} gives a spectrum very similar to that of the native enzyme. On the other hand, CD spectra of the Ni^{2+}- and Hg^{2+}-enzymes are almost identical to that of the apoenzyme, and while maintaining the same band positions as the native protein they all show an alteration in band intensities. The CD spectra of metal-free and Zn^{2+}-enzymes shown in Fig. 5 agree well with the ones published in ref. 26.

According to a recent report [14,15] Mg^{2+} affects the properties of *E. coli* alkaline phosphatase. However, in our studies, the ultraviolet difference spectra, titration at alkaline pH, CD spectra and modification of arginines were the same whether only zinc or both zinc and magnesium had been added to the apoenzyme to produce the active enzyme.

The results we have presented here show that there are structural differences between apo- and various metalloalkaline phosphatases. The correlation between ultraviolet spectral changes produced by the different metal ions and their effectiveness in inducing phosphate binding suggests a very important structural role for the metal ions. Our data indicate that the role of metal ions in the mechanism of alkaline phosphatase might be explained in terms of a metal-induced conformational change. The main function of the two tightly bound metal ions could be to induce and maintain the protein in an active conformation.

This interpretation is supported by a report on tritium exchange [28], by recent data on immunological studies of the alkaline phosphatase [27] and by ^{19}F NMR studies on fluorotyrosines [25]. However, our data do not enable us to rule out the possibility that the metal ions can participate directly in the catalytic process. In fact, the two first bound metal ions may play a dual role in the alkaline phosphatase, serving both as catalytic entities and as modifiers of the enzyme structure.

Acknowledgements

The authors thank Professor Bo G. Malmström for his kind interest in this work. We are deeply indebted to Dr. Sven Lindskog for stimulating discussions and helpful criticism. The excellent technical assistance provided by Mrs. A. Arvidsson in preparing the enzyme is greatly appreciated. Drs R. Faiman, G. Folsch and W.E. Hull are thanked for valuable comments and for revision of the English. This investigation has been supported by research grant from the Swedish Natural Research Council (K 3608-002).

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