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Alterations in the Structure and Function of *Escherichia coli* Alkaline Phosphatase Due to Zn^{2+} Binding*

Jacqueline A. Reynolds and Milton J. Schlesinger

ABSTRACT: The enzymatic activity of alkaline phosphatase from *Escherichia coli* increases linearly with the mole ratio, Zn^{2+}/86,000 molecular weight of protein. There is one phosphate binding site per molecule of dimer containing from two to four bound zinc ions. The tertiary structure of alkaline phosphatase is altered by metal binding as evidenced by ultraviolet difference spectra and circular dichroism in the wavelength range 2600–3200 Å. In addition, spectrophotometric titration shows four tyrosine residues exposed to solvent in the native dimer containing three Zn^{2+} ions and ten such residues exposed to solvent in the metal-free apoprotein.

A refolded subunit of alkaline phosphatase obtained by neutralization of the acid-dissociated and unfolded protein has optical properties similar to those of the metal-free dimer. This subunit is capable of dimerization in the presence of chelating agent.

Alkaline phosphatase from *Escherichia coli* is a zinc metalloprotein containing identical subunits (Rothman and Byrne, 1963) each of molecular weight 43,000 (Schlesinger and Barrett, 1965). Previous studies from this laboratory (Schlesinger and Barrett, 1965; Schlesinger, 1965; Reynolds and Schlesinger, 1967, 1968) have been carried out with alkaline phosphatase in a dimeric state containing two to three Zn^{2+} per dimer. The protein in this state is globular and can be reversibly dissociated and unfolded by high positive charge or 6 M guanidine hydrochloride. The presence of bound zinc ions is essential for enzymatic activity (Garen and Levinthal, 1960; Plocke *et al.*, 1962), and at least one of the residues to which divalent metal ion is bound has been shown to be an imidazole group (Reynolds and Schlesinger, 1968).

However, the role of bound metal in the formation and maintenance of tertiary and quarternary structure of alkaline phosphatase has not been established. Reynolds and Schlesinger (1967) showed that refolding of the structureless monomer could take place in the absence of metal and that the refolded monomer had an optical rotatory dispersion spectrum identical with that of the native dimer. However, the refolded species had a tertiary structure different from that of the enzymatically active dimer in that 5 ± 1 tyrosine residues/subunit were accessible to solvent in the refolded state as opposed to 2 ± 1 tyrosines/subunit in the native state. At protein concentrations less than 10^{-4} g/ml the refolded state was shown to be monomeric.

The questions to which the present work is directed are as follows: (1) What is the relationship between bound metal ion and the enzyme function of alkaline phosphatase? (2) How does bound metal ion affect the tertiary structure of the protein moiety of alkaline phosphatase? (3) Is the binding of metal ion to the apoprotein necessary for formation and stability of quaternary structure?

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These questions have been partially answered by an investigation of the optical and hydrodynamic properties of the apoprotein in the dimeric state. In addition, the relationships among the mole ratio of bound Zn^{2+}, enzymatic activity, and substrate binding sites have been investigated. During the course of the present investigation, the formation of enzymatically active tetramer (M_n = 172,000) was observed in the presence of excess metal ions and at protein concentrations greater than 1 g/l. The properties of the tetramer of alkaline phosphatase will be dealt with in a separate communication.

Experimental Section

Materials. The preparation and purification of *E. coli* alkaline phosphatase, strain CW 3747, have been described previously (Reynolds and Schlesinger, 1967; Schlesinger and Barrett, 1965). Protein concentrations were determined from the optical density at 2800 Å using $\epsilon^{1\text{ cm}}_{0.1\%}$ 0.77.

EDTA and nitrilotriacetic acid were obtained from Fisher and from Pierce Chemical Co., respectively, and used without further purification.

^{32}P-labeled phosphoric acid was obtained carrier free from Mallinckrodt Chemical Co. and diluted with standard solutions of KH_2PO_4 in glass-distilled water.

The apoprotein of alkaline phosphatase was obtained by exhaustive dialysis against EDTA or nitrilotriacetic acid at 4°. Analysis by atomic absorption spectroscopy showed <0.01 Zn^{2+} bound to protein with zero enzymatic activity.

Methods. Ultraviolet difference spectra were obtained using a Cary 14 spectrophotometer equipped with a thermostatted cell compartment and tandem cells of 1-cm path length. In all experiments reagents added to the sample cells were compensated in the reference beam. The validity of Beer's law was established by determining the difference spectra at a number of protein concentrations.

Enzyme assays were carried out in 1 M Tris-HCl (pH 8.0) at 37°. *p*-Nitrophenyl phosphate (0.2 g/l.) was used as a substrate and the absorbance at 4100 Å of the product, *p*-nitrophenol ($\epsilon^{1\text{ cm}}_{1\text{ M}}$ 16,200), determined in a Gilford spectrophotometer with a 1-cm path-length cell. Activities are reported as micromoles of *p*-nitrophenol per minute per milligram of protein. Initial rates of partially inactivated enzyme were determined to assure that regeneration of activity did not occur in the assay solution.

Spectrophotometric titrations were carried out by measuring $\Delta\epsilon$ at 2950 Å on a Cary 14 spectrophotometer using a protein reference solution at pH 6.3, μ = 0.1 KCl. Additions of base were made to the sample cell in small enough aliquots that the total volume change at the end point of the titration was <1.0%. No time-dependent change in $\Delta\epsilon$ was noted up to 30 min. The relationship $\Delta\epsilon$ = 2450/tyrosine ionized was used to calculate the number of tyrosine residues titrated. The cell compartment was thermostated to 10 ± 0.1°. pH was determined using Leeds and Northrup electrode couple no. 124138 and a Radiometer pH meter.

Optical rotatory dispersion and circular dichroism were measured with a Cary 60 recording spectropolarimeter in a 1-cm thermostatted cell. Protein concentrations were adjusted to give suitable rotations or ellipticities over the wavelength range studied. A constant band width of 15 Å was used over the entire wavelength range.

Equilibrium dialysis of alkaline phosphatase with HPO_4^{2-} was carried out at 18° in 0.1 M KCl–0.01 M Tris-HCl (pH 9.0). Equilibrium was always reached within 24 hr as determined by the identity of phosphate binding when all the phosphate was placed with the protein and alternatively when all the phosphate was placed in the outside solution.

Concentrations of HPO_4^{2-} were determined for solutions on both sides of the membrane using a Packard scintillation counter and 0.1-ml samples in 10 ml of Bray's scintillation solution (Bray, 1960). Differences in counts per unit volume on the two sides of the membrane were converted into concentration differences and to the average mole ratio of HPO_4^{2-} dimer. The total recovery of radioactivity was always within 1% of that initially present.

Sedimentation velocity was determined in a Spinco Model E ultracentrifuge at a rotor speed of 52,640 rpm. The ultracentrifuge was equipped with a schlieren optical system and photographs were taken at 4-min intervals after reaching constant speed.

Viscosity measurements were made in Canon-Manning semimicro viscometers in a constant-temperature bath thermostatted to ±0.005°. Flow times varied from 300–600 sec and were reproducible to ±0.2 sec.

Zinc analysis was carried out on a Perkin-Elmer 303 atomic absorption spectrophotometer.

Results

Relationship between the Molal Binding Ratio, Zn^{2+}/Dimer, and Enzymatic Activity. Figure 1 shows the enzymatic activity of alkaline phosphatase in micromoles of substrate cleaved per minute per milligram of protein as a function of the number of moles of Zn^{2+} bound per mole of protein (dimer molecular weight = 86,000). The points designated by open circles were obtained from purified preparations of enzyme dialyzed against distilled water. The closed circles represent solutions of native alkaline phosphatase prepared by dialysis against various concentrations of EDTA or nitrilotriacetic acid. Correction for free Zn^{2+} was negligible since all dialysates were analyzed for Zn^{2+} and found to contain <1.0% of the total bound Zn^{2+}.

If the enzymatic assay is carried out in 0.1 N KCl–0.01 M Tris-HCl (pH 8.0) instead of 1.0 M Tris-HCl (pH 8.0) a lower specific activity is observed but the activity is still a linear function of the Zn^{2+}/dimer ratio.

A recent communication from Simpson and Vallee[1] (1968) in which 8-hydroxyquinoline-5-sulfonic acid was used as a chelating agent shows preferential, rapid removal of two out of four bound Zn^{2+} ions from alkaline phosphatase and the concomittant loss of most of the

[1] The authors are grateful to Dr. R. T. Simpson and Dr. B. L. Vallee for providing a copy of their manuscript prior to publication.

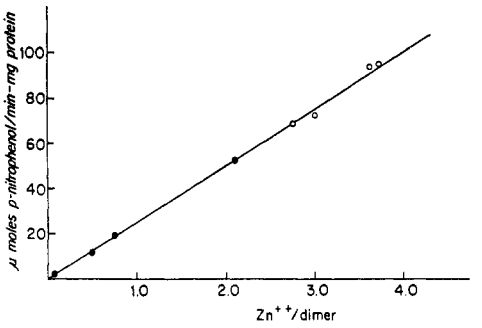


FIGURE 1: μmoles of *p*-nitrophenol/(mg min) *vs.* Zn^{2+}/dimer. (○) Enzyme preparations; (●) EDTA or nitrilotriacetic acid treated enzyme.

enzymatic activity. Since EDTA and nitrilotriacetic acid have higher association constants for Zn^{2+} than does 8-hydroxyquinoline-5-sulfonic acid, the bound metal ions may be removed randomly by the former chelating agents leaving a random distribution of protein molecules containing varying amounts of Zn^{2+}. It is not possible to postulate from the data presented in Figure 1 whether each macromolecule has partial activity directly related to the amount of bound Zn^{2+} or alternatively all molecules not containing two essential Zn^{2+} ions have zero activity. However, the data of Figure 1 are consistent with the hypothesis of Simpson and Vallee that only two of the four bound Zn^{2+} ions are essential for enzymatic activity.

Enzymatic activities up to 188 μmoles/(min mg) have been observed in this laboratory when free Zn^{2+} concentrations exceed 10^{-4} M. However, at this high Zn^{2+} concentration both sedimentation velocity and osmotic pressure measurements show the formation of tetramers. These results will be subject of a separate communication and do not bear directly on the present investigation.

Relationship between the Molal Binding Ratio, Zn^{2+}/Dimer, and the Number of Substrate Binding Sites. P_i is a competitive inhibitor of alkaline phosphatase activity (Garen and Levinthal, 1960). The binding of HPO_4^{2-} to native alkaline phosphatase was therefore measured as a function of the Zn^{2+}/dimer ration. Figure 2 shows $1/\bar{\nu}$ *vs.* $1/C$ in 0.1 M KCl–0.01 M Tris-HCl (pH 9.0), where $\bar{\nu}$ = mole binding ratio, HPO_4^{2-}/dimer and c = equilibrium concentration of phosphate. (At pH 9.0 the P_i is in the form HPO_4^{2-}. The enzymatic activity at pH 9.0 is identical with that at pH 8.0 in the same solvent system.) Table I lists the values of n, number of phosphate binding sites, and K, the association constant, obtained from Figure 2. These values result from the application of the law of mass action to the binding data using the following linearized equation $1/\bar{\nu} = (1/nkc) + (1/n)$. Within experimental error, the value of n is the same for dimer containing 3.3 and 3.8 Zn^{2+} per mole of protein. Included in Table I for comparison are the data of Ko and Kézdy (1967) obtained by stop-flow techniques on a preparation of alkaline phosphatase having 41.5 μmoles/(min mg) of activity at 27°. From this value for enzymatic activity (corrected to 37° by the temperature-dependence relationship given by Garen and Levinthal, 1960) and Figure 1, we estimate their enzyme preparation had approximately 2.7 Zn^{2+}/dimer.

Simpson and Vallee[1] (1968) have suggested on the basis of spectral perturbations that alkaline phosphatase containing four Co^{2+} instead of four Zn^{2+} has two binding sites per dimer for P_i. The thermodynamic data presented here shows that the Zn^{2+}–enzyme preparation used in these studies has only one binding site and that increasing the Zn^{2+}/dimer ratio from 2 to 4 does not increase the number of substrate binding sites on alkaline phosphatase.

Characterization of the Apoprotein. Total removal of metal ions at pH 6.3 (isoionic point, Reynolds and Schlesinger, 1968) from native alkaline phosphatase pro-

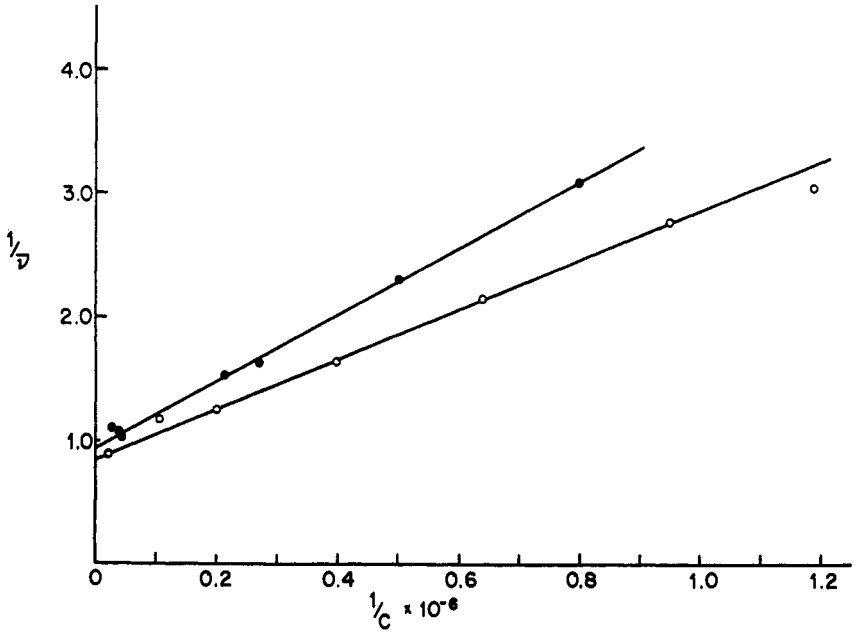


FIGURE 2: Binding of HPO_4^{2-} to alkaline phosphatase, μ = 0.1 KCl, 0.1 M Tris-Cl, pH 9.0, T = 18°, (○) 3.8 Zn^{2+}/dimer; (●) 3.3 Zn^{2+}/dimer.

TABLE I: Binding of HPO_4^{2-} to Alkaline Phosphatase.

n	K	Zn/Dimer	μ	pH	I (deg)
1.05 ± 0.10	3.5×10^5	3.3	0.1 KCl	9.0	18
1.18 ± 0.10	4.2×10^5	3.8	0.1 KCl	9.0	18
0.9 ± 0.10[a]	4.2×10^{5}[a]	2.7	0.1	5.7	25

[a] Ko and Kézdy (1967).

duces an inactive dimer with hydrodynamic properties identical with the native enzyme (Table II). Sedimentation velocity measurements at protein concentrations from 1 to 5 mg per ml in 0.1 M KCl gave a single symmetrical peak for both native and apoprotein. s^0 did not change significantly with pH for either species, but $[\eta]$ does increase slightly at pH 9.6 for the native enzyme and at pH 8.0 for the apoprotein. It has been reported previously (Reynolds and Schlesinger, 1967) that the electrostatic interaction factor, w, in the Linderstrøm-Lang equation, for the lysine and tyrosine ionization of native alkaline phosphatase and the histidine ionization of the apoenzyme, was anomalously low. This information together with the $[\eta]$ values of Table II suggests a small expansion of both species with increasing negative charge. The inactive apoprotein dimer appeared to be less stable to electrostatic repulsion that the native enzyme.

Optical rotatory dispersion spectra between 2500 and 2000 Å are shown in Figure 3. Native and Zn^{2+}-free alkaline phosphatase were nearly identical within the experimental error of ±200° ml/g dm between pH 6.3 and 11.2. Unfolding of some ordered structure is noted above pH 11.2 for the inactive dimer and above pH 12.0 for the native protein. The decrease in positive rotation at 2110 Å which accompanies unfolding was also observed in the acid unfolded subunit (Reynolds and Schlesinger, 1967).

Circular dichroism of native and apoprotein between 3200 and 2550 Å is shown in Figure 4. There was a reduction in the negative troughs at 2775 and 2850 Å together with a small shift in the 2775-Å band when metal was removed from the enzyme. Unfolding of native alkaline phosphatase at pH 2 led to total loss of this Cotton effect in Figure 4.

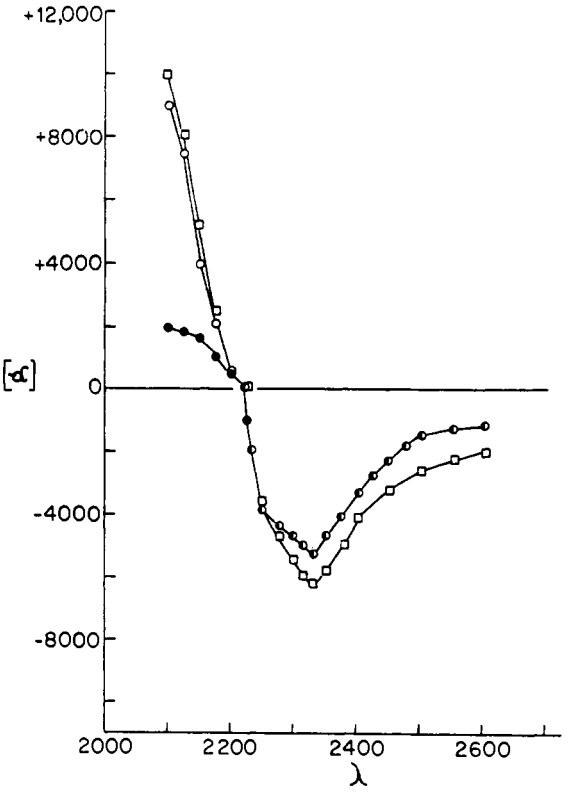


FIGURE 3: Optical rotatory dispersion spectra, μ = 0.1 KCl, T = 10°. (□) Native alkaline phosphatase, pH 6.3–11.9; (○) apoprotein, pH 6.3–11.1; (●) native alkaline phosphatase, pH 12.0; and apoprotein, pH 11.2.

TABLE II: Hydrodynamic Properties of Alkaline Phosphatase, μ = 0.1 KCl, 0.01 M Tris-Cl.

Apoprotein				Native	
pH	Temp (°C)	s^0	$[\eta]$	s^0	$[\eta]$
6.3	22	6.3 ± 0.1	3.4	6.3 ± 0.1	3.4
	10	5.0 ± 0.1	3.4	5.0 ± 0.1	3.4
8.0	22	6.3 ± 0.1	4.3	6.3 ± 0.1	3.4
	10	5.0 ± 0.1	5.7	5.0 ± 0.1	3.4
9.0	22	6.6 ± 0.1	4.3	6.3 ± 0.1	3.4
	10		5.7		
9.6	10				5.7

Spectrophotometric titration of the tyrosine residues in the apoprotein is shown in Figure 5 together with that of native dimer (Reynolds and Schlesinger, 1968). Between five and six additional tyrosines per dimer were titrated below pH 11.2 in the inactive Zn^{2+} free dimer as compared with the native macromolecule.

A previous publication from this laboratory (Reynolds and Schlesinger, 1967) stated that no difference spectra were observed when Zn^{2+} was removed from native alkaline phosphatase by EDTA. In the earlier work very small concentrations of protein ($\leq 10^{-6}$ M) were used to facilitate the removal of Zn^{2+} by the chelating agent. A reexamination of the difference spectra at 10^{-5} M protein in the present study showed significant tyrosine perturbation when metal was removed from native enzyme. In addition there was a very small difference spectra attributable to tryptophan residues (see Figure 6).

Figure 7 shows $\Delta\epsilon_{2860}$ as a function of enzymatic ac-

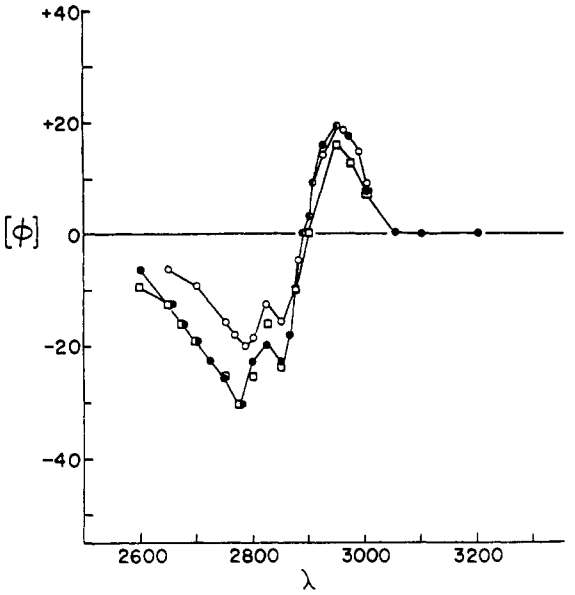


FIGURE 4: Circular dichroism spectra, μ = 0.1 KCl, T = 20°. (□) Native alkaline phosphatase, pH 6.3, 3 Zn^{2+}/dimer; (●) native alkaline phosphatase, pH 6.3, 2 Zn^{2+}/dimer; (○) Apoprotein, pH 6.3.

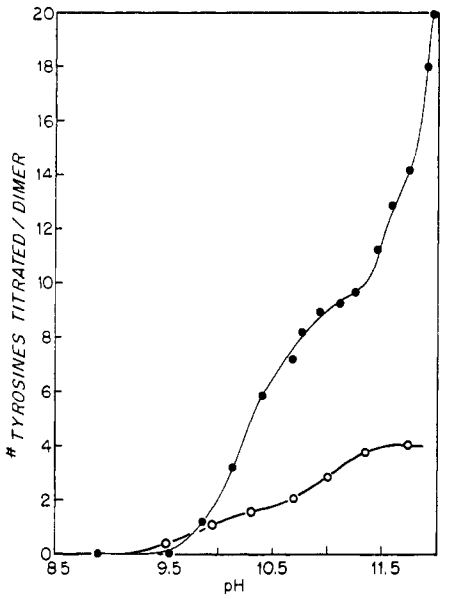


FIGURE 5: Spectrophotometric titration, μ = 0.1 KCl, T = 10°. (●) Apoprotein; (○) native alkaline phosphatase.

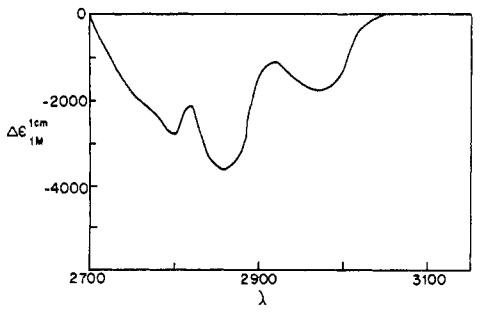


FIGURE 6: Difference spectra–apoprotein 0.8 Zn^{2+}/dimer *vs.* native alkaline phosphatase, 3.0 Zn^{2+}/dimer, μ = 0.1 KCl, pH 7.4, T = 10°.

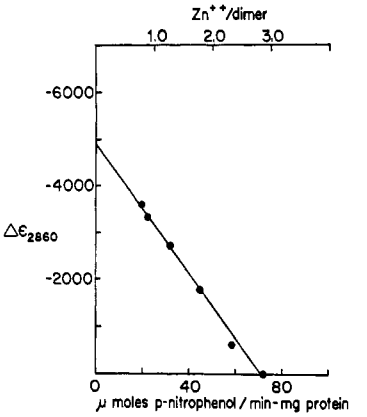


FIGURE 7: $\Delta\epsilon_{2860}$ *vs.* Zn^{2+}/dimer, μ = 0.1 KCl, pH 7.4, T = 10°.

tivity (lower axis) or Zn^{2+}/dimer (upper axis). In this experiment EDTA was added to the protein in the sample chamber and the difference spectra followed with time. Inactivation to 15.4 μmoles/(min mg) (0.6 Zn^{2+}/dimer) required 5 days at 10°. Identical results were obtained when nitrilotriacetic acid was used as a chelating agent with the exception that the time required for inactivation was only 2 days using the same molar concentrations of protein and chelating agent. The alkaline phosphatase used in this experiment contained 3.0 Zn^{2+}/dimer initially. The extrapolated value of $\Delta\epsilon_{2860}$ in Figure 7 is 5000 which corresponds to 5 tyrosines exposed on total metal removal (Wetlaufer, 1962).

Each bound Zn^{2+} has two protein binding sites one of which is a histidine residue (Reynolds and Schlesinger, 1968). If the second site is a tyrosine group 3 of the 5 ± 1 additional tyrosines in the apoprotein could be accounted for and 2 ± 1 residues would be exposed to solvent as the result of some small conformational change.

A solution containing 2×10^{-3} g/ml of the apoprotein (Zn^{2+}-free dimer) in 10^{-3} M EDTA–0.1 M KCl, T = 0°, was diluted to 5×10^{-5} g/ml and 3×10^{-3} M Zn^{2+} was added. The enzymatic activity returned to a value corresponding to 2.5 Zn^{2+}/dimer in less than 30 sec. Under identical experimental conditions, the refolded subunit obtained by acidifying native alkaline phosphatase, neutralizing on ice in the presence of EDTA, warming to room temperature and adding 3×10^{-3} M Zn^{2+} requires 2 hr to regain an activity corresponding to 0.1 Zn^{2+}/dimer. The difference in the kinetics of reactivation of these two species strongly suggests that the apoprotein does not dissociate to subunits on dilution.

Dimerization of the Refolded Subunit in the Presence of EDTA. On the other hand, the refolded subunit obtained as described above is capable of dimerizing at high protein concentrations even in the presence of chelating agents. At protein concentrations $<10^{-4}$ g/ml, the refolded subunit does not dimerize in the presence of EDTA (Reynolds and Schlesinger, 1967). However, at protein concentrations between 1 and 5 mg/ml in the presence of 10^{-3} M EDTA, a solution of the refolded subunit was observed in the ultracentrifuge to contain di-

meric material. In a typical experiment a solution containing 5 mg/ml of alkaline phosphatase was dialyzed against 10^{-2} M H^+, 0.1 M KCl, and 10^{-3} M EDTA at 0° overnight. The resulting monomer unit had an $s_{20,w}$ value of 2.3 S in agreement with an earlier value (Schlesinger, 1965). The solution was divided into two parts. One portion was dialyzed overnight against 0.1 M KCl–0.01 M Tris–HCl (pH 8.5)–10^{-3} M EDTA and the second overnight against 0.1 M KCl–0.01 M Tris-HCl (pH 6.5)–10^{-3} M EDTA. The neutralized solutions were then sedimented in a Model E ultracentrifuge and the sedimentation coefficients were determined. At pH 8.5 a mixture of dimer and refolded monomer was observed. The former had an $s_{20,w}$ value of 6.0 S and the latter of 3.4 S. At pH 6.5 only dimer was observed with an $s_{20,w}$ value of 6.0 S. No Zn^{2+} was detected bound to the protein when the solutions were analyzed by means of atomic absorption spectroscopy. In view of the long period of time (approximately 5 days) required to remove zinc from the native enzyme by dialysis under identical conditions and concentrations of protein and EDTA, it is unlikely that metal was bound by the refolded subunit prior to dimerization and then lost by chelation with EDTA after dimerization had taken place.

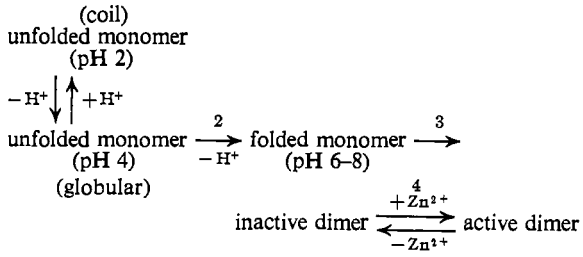
Since the inactive dimer and refolded subunit could be separated by sedimentation in the ultracentrifuge, there is not a rapid equilibrium between these species under the experimental conditions employed here.

Discussion

The removal of metal ions from native alkaline phosphatase leads not only to a loss in enzymatic activity, but also to the exposure to solvent of an additional 5 ± 1 tyrosine residues. However, the apoprotein remains in the dimeric state and no experimentally significant changes in optical rotatory dispersion are observed. Thus, bound Zn^{2+} in this protein appears to stabilize tertiary structure, in addition to its role in the catalytically active site, a conclusion further supported by the fact that the apoprotein is more easily disrupted by charge effects than the native enzyme.

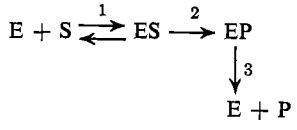
The inactive dimer does not dissociate into subunits at concentrations less than 10^{-4} g/ml at neutral pH. On the other hand, the refolded subunit associates to an inactive dimer containing no Zn^{2+} at concentrations as low as 10^{-3} g/ml. (The lowest concentration at which molecular weights can be determined.) The refolded subunit and inactive dimer have similar optical properties in that 6 ± 1 tyrosines/monomer in the former and 5 ± 1 tyrosines/monomer in the latter are exposed to solvent. Within experimental error no tyrosine residues appear to be perturbed in the process of association.

The original pathway proposed by Reynolds and Schlesinger (1967) for the formation of active enzyme from unfolded subunits should be revised to the following at protein concentrations $\geq 10^{-3}$ g/ml.



The association step (3) does not appear to involve a conformational change, but the binding step (4) results in the removal of 3 ± 1 tyrosines/monomer from contact with the solvent.

It has been shown that the number of P_i binding sites on the native enzyme does not change with the mole ratio, Zn^{2+}/dimer. The following steps have been proposed to describe the reaction of alkaline phosphatase with appropriate substrates (see, for example, Trentham and Gutfreund, 1968).



Trentham and Gutfreund (1968) have recently suggested that the rate-controlling step under the assay conditions employed here (1 M Tris-Cl, pH 8.0) occurs between the formation of ES and the formation of EP and may be a protein conformational change. If this proposal is correct, the effect of increasing the Zn^{2+}/dimer ratio may be to lower the energy barrier for this conformational change thus increasing the enzymatic activity. Support for this view comes from the observation that the tertiary structure of native alkaline phosphatase is altered as the number of bound metal ions is increased.

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