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ZINC STOICHIOMETRY IN *ESCHERICHIA COLI* ALKALINE PHOSPHATASE

STUDIES BY ^{31}P NMR AND ION-EXCHANGE CHROMATOGRAPHY

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Summary

^{31}P nuclear magnetic resonance spectra and enzymatic activities are compared for alkaline phosphatase (orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1) species with different zinc contents. The enzyme containing two Zn^{2+} per protein dimer exists in two forms; one, prepared by dialysis of native enzyme, has full enzymatic activity and a ^{31}P magnetic resonance spectrum similar to but distinguishable from that of the native enzyme containing four or more Zn^{2+}. The other form, prepared by restoring two Zn^{2+} to apoenzyme, has low enzymatic activity and a ^{31}P magnetic resonance spectrum that indicates stoichiometric binding of phosphate, but otherwise altered properties. Reconstituted enzyme with four Zn^{2+} is similar to but distinguishable from native enzyme with four Zn^{2+}. Chromatography on DEAE-cellulose can separate apoenzyme and enzyme containing two Zn^{2+} and suggests that the binding of a pair of Zn^{2+} is cooperative.

Introduction

Biological phosphate transfer typically requires participation of divalent metal ions, but their mechanistic role has not been well defined. Alkaline phosphatase (orthophosphoric-monoester phosphohydrolase (alkaline optimum) EC 3.1.3.1) from *Escherichia coli*, which has been intensively studied as a

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Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid; E denotes an apo-alkaline phosphatase protein dimer. E-P and E · P denote covalent and non-covalent complexes, respectively, of phosphate with an active alkaline phosphatase molecule (of unspecified zinc content).

model system, requires tightly bound zinc (II) ions for its non-specific catalysis of phosphomonoester hydrolysis. Removal of zinc abolishes all activity, while replacement with cadmium, copper, cobalt, or manganese results in interesting modified properties [1—7]. Various spectroscopic techniques have been applied to the native and metal-substituted enzymes, and X-ray determination of the three-dimensional structure of the metal · protein · substrate complex is in progress [8]. Phosphorus magnetic resonance (^{31}P NMR) studies of phosphate bound to the cobalt enzyme have recently provided the first direct evidence that phosphate binds very close to a metal ion at the active site [9—11].

Although many properties of metallo-alkaline phosphatases have been well documented, some issues have generated a surprising amount of confusion and controversy. Most disturbingly, the rudimentary question of metal-protein stoichiometry has still not been resolved. At least two independent groups have published data indicating that only two metal ions per protein dimer are important for the enzyme's structure and function [1,12,13]. Several other workers have published a figure of four zincs per dimer in native enzyme, and among these, there is additional controversy concerning reconstitution of apo(zinc-free) enzyme. Vallee's laboratory [14] initially reported that adding two zincs to apoenzyme restored 85% of full activity, with a second pair needed to restore 100%. More recent reports from the same laboratory [15] say that two zincs restore only 12% activity, while two more give 80% and the further addition of two magnesiums is required for 100%. Reynolds and Schlesinger [16] simply found a linear increase in activity with up to four zincs per dimer. Lazdunski and co-workers [5] argued that the most tightly bound of four Zn^{2+} induces 10% activity, and the most weakly bound the remaining 90%.

X-ray diffraction at 7.7 Å resolution [8] has shown two metal ions per dimer in the crystalline enzyme. Physical studies in solution have given sharply contradictory results. For example, Simpson and Vallee [14] reported that four cobalts must be added to apoenzyme to give a characteristic optical spectrum, whereas Taylor et al. [4] obtained a similar spectrum with only two cobalts. Various magnetic resonance experiments have been taken as evidence for either two [17—19] or four [20—23] metal ions per dimer.

Csopak and Szajn [12] suggested that all findings of greater than two zincs per dimer were an artifact resulting from contamination with chelating agents, which are used in preparation of apoenzyme and often in the isolation of native enzyme. Vallee's group disputed this assertion, in turn arguing that certain analytical errors have given falsely low zinc stoichiometries [24]. They also argued that magnesium, which they found to have a significant activating effect, was being ignored in other laboratories. However, their own data clearly do not resolve the controversy, since they report a maximum activity for Zn_2E of 54% even in the presence of magnesium [15].

Reid and Wilson [25], in their 1971 review of alkaline phosphatase, proposed a straightforward resolution of the stoichiometry problem: that alkaline phosphatase may have four zinc-binding sites, not all equivalent, and occupancy of these sites may depend on kinetic as well as thermodynamic parameters. Thus Zn_2E could have different properties depending on whether it is prepared from Zn_4E or from apoenzyme, and also depending on the exact conditions of preparation. Furthermore, the Zn_2E species might differ from

each other not only due to occupancies of different types of sites, but also due to existence of slowly-interconverting protein conformational states, which have already been demonstrated in other contexts [13,26,27]. Unfortunately, it has not yet been possible to pinpoint structural differences between Zn_2E species having different activities.

Pioneering work by ourselves [9] and others [10,11] has shown ^{31}P NMR to be a highly useful probe of the active site of alkaline phosphatase. ^{31}P spectra of native alkaline phosphatase in the presence of excess inorganic phosphate were found to have well-resolved peaks corresponding to covalently bound phosphate, non-covalently bound phosphate, and free phosphate. Complete removal of zinc, or substitution with cobalt, cadmium, or manganese, resulted in informative spectral changes [9—11]. Some new perplexing issues were introduced: for example, it was found that the non-covalent complex exists in two slowly-exchanging forms, whose relative amounts vary from sample to sample [10]. In this paper we report additional studies of alkaline phosphatase, particularly of Zn_2E, which demonstrate a striking dependence on the means of preparation of the sample. These experiments shed new light on the thorny issue of metal-protein stoichiometry, and also clarify some of the confusing issues in the earlier NMR works. A very recent paper [28] has demonstrated some similar dependences of alkaline phosphatase ^{31}P NMR spectra on metal content, but did not attempt to compare samples prepared by different methods, nor to correlate the spectra with measurements of catalytic activity. During this work we became interested in the preparation of a homogeneous Zn_1-E to allow additional studies by NMR and other techniques. We were unsuccessful in preparing such a species, finding instead that apo-E reconstituted with one zinc per dimer can be chromatographically resolved into Zn_2E and E. These results, indicating strong cooperativity in the finding of one pair of zinc ions, are included below.

Materials and Methods

General. Tris and HEPES buffers were reagent grade, from Sigma. $ZnCl_2$ was "Ultrapure" from Ventron. Other common chemicals were purchased commercially in reagent-grade form. Water was once distilled, then passed through a mixed-bed ion-exchange resin (Continental), or re-distilled in all glass apparatus. pH measurements were done with a Radiometer model 26 meter using a glass combination electrode.

For metal-free conditions, glassware and plastic-ware were soaked in 3—6 M HNO_3 for several days, then rinsed extensively with deionized water. Buffer solutions were extracted with dithizone (0.01% in CCl_4), and then extracted with CCl_4 to remove dithizone, or else slurried with Chelex 100 resin (Biorad).

Analytical methods. Zinc concentrations were measured using a Perkin-Elmer 360 atomic absorption spectrometer equipped with an HGA-2100 graphite furnace and a deuterium-lamp background corrector. Standards were prepared in dilute HNO_3, from 1000 ppm commercial standards (Fisher). Protein samples were diluted in deionized water. Accuracy to better than 10% was demonstrated by analyzing samples of apoenzyme to which known amounts of zinc were added. Chemical analysis for phosphorus was done by the proce-

dure of Bartlett [29] with a standard prepared from anhydrous potassium phosphate that was dried at 110°C for 12 or more hours.

Alkaline phosphatase was assayed by monitoring release of *p*-nitrophenol (A_{410nm} = 16 200) from 1 mM *p*-nitrophenyl phosphate (Sigma 104 phosphatase substrate) in 1 M Tris · HCl, pH 8.00, at 23°C. 10^{-4} M *trans*-1,2-diaminocyclohexane-*N*,*N*,*N'*,*N'*-tetraacetic acid (CDTA, purchased from Aldrich) was usually included to eliminate effects of extraneous metal ions, but assays carefully performed under metal-free conditions gave the same results. One unit of enzyme activity is defined as the amount causing 1 nmol of substrate hydrolysis per min. Concentration of enzyme was calculated assuming $A^{0.1\%}_{278nm}$ = 0.72 [30], and for calculations of molar concentration a molecular weight of 86 000 was used [26].

Alkaline phosphatase preparation. Alkaline phosphatase was isolated from *E. coli*, strain CW3747 (generously provided by Dr. Joseph Coleman), grown in "medium B" [31] in a 550 l fermentor at the New England Enzyme Center of Tufts University, Boston, Mass. The procedure of Schlesinger and Olsen [32] was used to extract enzyme from the cells, and purification was achieved by salt gradient elution from DEAE-cellulose [33]. This was sometimes followed by gel filtration on Sephadex G-100. The purified enzyme contained only trace impurities revealed by gel electrophoresis with a dodecyl sulfate discontinuous buffer system and had a specific activity of 50—55 units per mg. The enzyme was concentrated using an Amicon apparatus with PM-10 membranes.

Apoenzyme was prepared by dialyzing native enzyme against 0.1 M Na-EDTA, pH 6.0, at 4°C. Typically a few ml of enzyme, 50—100 mg/ml, were placed in the dialysis chamber of an Amicon 8MC diafiltration apparatus and about 20 volumes of EDTA solution were flowed through over a 2—3 day period. The enzyme activity and zinc content at this point were negligible. In order to insure complete removal of EDTA, the enzyme was then equilibrated with [^{14}C]EDTA for 12—48 h. Dialysis against water or metal-free buffer was then performed until the EDTA concentration, measured by counting protein aliquots before and after dialysis, was less than 0.1 times the protein concentration. Addition of zinc to apoenzyme prepared in this manner restored 80—100% of the original enzyme activity.

NMR measurements. ^{31}P NMR spectra were recorded at 40 MHz in Fourier transform mode, using a JEOL JNM-FT-100 spectrometer interfaced with a Nicolet 1080 data system. The sample was contained in an 8 mm or 10 mm OD glass tube (Wilmad), with an internal capillary containing ^2H_2O for field-frequency locking. Spectra reported here were all taken at ambient probe temperature (23—25°C). 60° pulses at 3.1-s intervals were usually used, 10 000—25 000 being needed per spectrum. Chemical shifts are reported relative to 85% H_3PO_4. Linewidths were calculated from differences in the computer addresses of points at half-height, subtracting the artificial contribution of noise filtering. Area measurements were made by planimeter measurements on spectrum printouts. In order to relate these areas to approximate phosphorus concentrations, 50 mM sodium pyrophosphate, doped with a small amount of $MnCl_2$ to shorten its relaxation time, was included in the internal capillary to serve as an area reference. Its peak area was standardized by running spectra with samples of known phosphate concentration, also metal-doped, in the outer tube. The con-

centration of phosphorus represented by a peak in an arbitrary sample could then be calculated from its peak area relative to the pyrophosphate standard. This calculation is only valid when the spectra are taken under conditions of complete relaxation; otherwise, the signal is partially "saturated" and the calculated concentration is therefore too low. It was found that the total phosphate concentration in enzyme samples, measured by this NMR method, agreed to better than 5% with values obtained by chemical analysis.

Zinc titration experiments. These were performed with samples of apoenzyme, prepared as described above. The apoenzyme was concentrated to 1—2 mM and slightly more than twice this concentration of phosphate was added. An initial ^{31}P NMR spectrum was then taken. Subsequent spectra were taken after the addition of stoichiometric amounts of zinc, in the form of 0.1 M $ZnCl_2$ solution. The enzyme activity was measured before and after each spectrum was taken, and these always agreed to within 10%. The entire experiment required about 5 days of spectrometer time, and the final enzyme activities were 60—90% that of fresh native enzyme.

Results

^{31}P NMR of native enzyme at alkaline pH

Fig. 1 shows ^{31}P NMR spectra of native alkaline phosphatase at alkaline pH, where enzyme activity is maximal and phosphate binds predominantly as a non-covalent ($\underline{E} \cdot P$) [1]. The sample whose spectrum is shown in Fig. 1a was prepared without special precautions to minimize metal content, and its measured zinc/enzyme ratio was 4.9. Two overlapping peaks were observed, centered at —3.5 and —4.0 ppm, both representing bound phosphate. (There is also a small shoulder at —2.5 ppm, representing P_i.) When additional P_i was

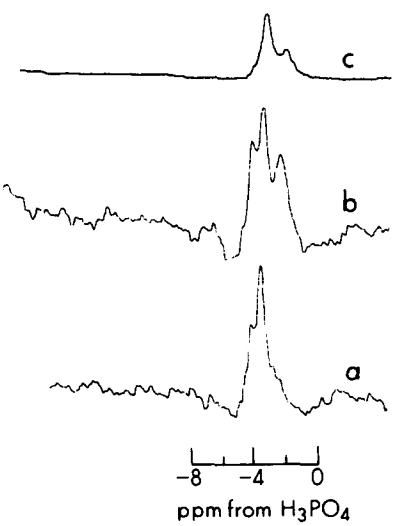

Fig. 1. ^{31}P NMR spectra of alkaline phosphatase-phosphate complexes. (A) 2.0 mM phosphatase dialyzed against 20 mM Na-HEPES/0.1 mM KH_2PO_4. pH of sample = 7.8, Zn/E ratio measured as 4.9. (B) Same sample after addition of 2.5 mM KH_2PO_4 at same pH. (C) 1.8 mM phosphatase dialyzed against 20 mM Na-HEPES/2.0 mM KH_2PO_4. pH of sample = 7.8, Zn/E ratio measured as 2.4.

added (Fig. 1b), the relative intensities of these peaks changed slightly, and a third peak at the correct P_i position at this pH became prominent. Fig. 1c shows a spectrum of a different sample, which was dialyzed for 2 days against chelex-treated HEPES buffer and measured to have a zinc/enzyme ratio of only 2.4. Its specific activity was 53 units per mg, no lower than that of samples containing greater amounts of zinc. Its spectrum differs significantly from that of the previous sample, in that the peak at −4.0 ppm is absent, with only a peak at −3.5 ppm representing bound phosphate. (The smaller peak at higher field represents P_i.) This phenomenon was reproducible in comparing spectra of native enzyme with 2—3 Zn/E to samples with ⩾4 Zn/E. Analysis of peak areas showed that this latter sample contained 1.3 bound phosphates per enzyme, while the first sample contained 1.4 bound phosphates per enzyme.

^{31}P NMR of reconstituted apoenzyme

Fig. 2 shows spectra of a sample at acidic pH prepared by adding 6 Zn/E to

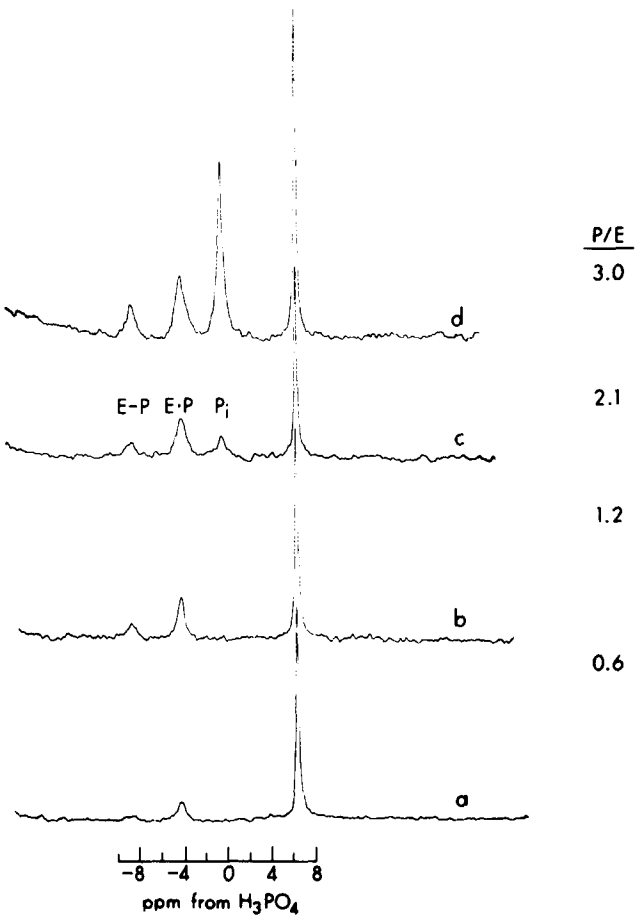

Fig. 2. ^{31}P NMR spectra of alkaline phosphatase, 2.1 mM in H_2O, pH 5.85, titrated with inorganic phosphate. Sample was prepared by adding 6 Zn/E to apoenzyme. Phosphate was added as 0.100 M KH_2PO_4 at the same pH as the sample. Peak at +7 ppm in each spectrum arises from pyrophosphate in the concentric capillary tube.

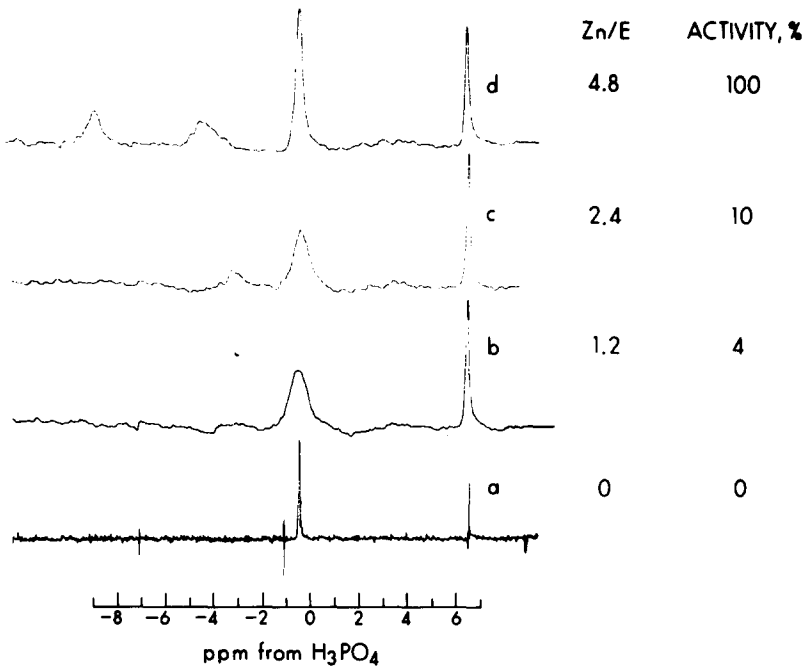

Fig. 3. Zinc titration of apoalkaline phosphatase, 2.1 mM in 0.1 M sodium succinate, pH 5.65, in the presence of 6.2 mM phosphate. Zinc was added as 1.00 M $ZnCl_2$. Activities, measured after each spectrum was taken, are relative to a maximum activity of 40 units/mg obtained when excess zinc was added to the sample after the last spectrum was taken. The vertical scales in B, C, and D are about two times greater than in A. Exponential noise filtering was used only for B, C, and D, and the spikes at −1 and −7 ppm in A are instrumental artifacts.

apoenzyme, which restored a specific activity of 50 units/mg, and then titrating with inorganic phosphate. (The sample was prepared from apoenzyme so that no bound phosphate could be present initially). The first approx. 1.5 phosphates per enzyme bound tightly, giving rise, at this pH, to a peak at −8.5 ppm representing phosphoenzyme (E-P) and a peak at higher field representing non-covalent phosphate (E · P) [9—11]. Excess phosphate gave rise to a peak at the correct P_i position. In these, and other spectra of samples reconstituted with excess zinc, the E · P resonance was always a single peak somewhere between −4.0 and −4.2 ppm. Thus, although the E · P complex of native enzyme typically gives rise to peaks at both −3.5 and −4.0 ppm [9,10], it is possible to prepare samples showing only the upfield (Fig. 1c) or downfield (Fig. 2) component. The significance of these components remains unclear.

Figs. 3 and 4 show experiments in which approximately three equivalents of P_i were added to apoenzyme, and the samples were then titrated with zinc. In Fig. 2 the experiment was performed at pH 5.65. The initial ^{31}P NMR spectrum, taken before any zinc was added, showed a single, sharp ($\Delta\nu_{1/2}$ = 3 Hz) peak at the P_i position. When 1.2 equivalents of zinc were added the P_i peak broadened to 28 Hz, and a very broad peak centered at about −3 ppm seemed to appear. A distinct peak at −3.5 ppm was visible after a second equivalent of zinc was added. The specific activity of the enzyme at this point was only 4

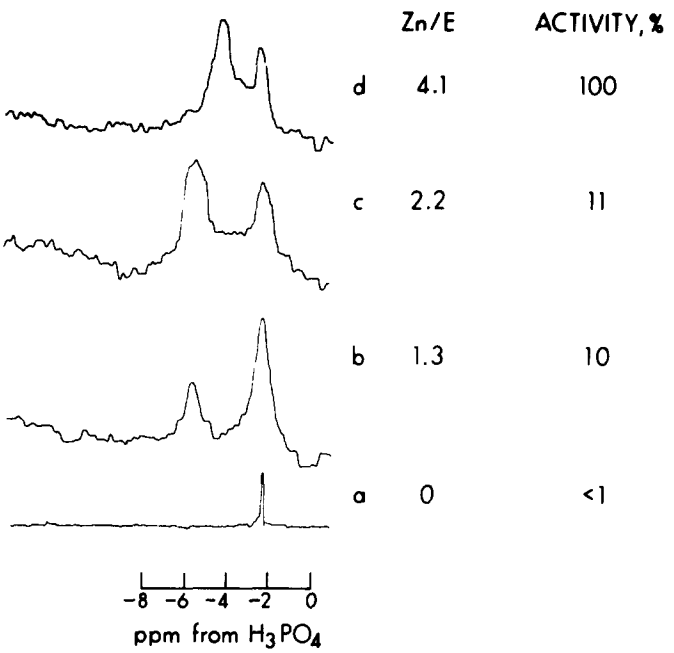

Fig. 4. Zinc titration of apoalkaline phosphatase, 1.1 mM in 0.1 M Na-HEPES, pH 7.5, in the presence of 2.7 mM phosphate. Zinc was added as 0.100 M $ZnCl_2$. Activities, measured after each spectrum was taken, are relative to a maximum of 32 units/mg achieved when excess zinc eas added to the sample after the last spectrum was taken. Vertical scales in B, C, and D are about nine times greater than in A.

units/mg. When two more equivalents of zinc were added, the P_i peak narrowed to 5 Hz, the other peak shifted downfield to —4.2 ppm, and a peak at —8.5 ppm appeared. The spectrum was then virtually the same as that of native enzyme at this pH (except that the $\underline{E} \cdot P$ peak of native enzyme generally has two components), and the specific activity of 40 units/mg was also nearly the same (80%). The specific activity did not increase with further addition of zinc, possibly because some enzyme denatured during the course of the experiment.

When the titration experiment was performed at pH 7.5 in HEPES buffer (Fig. 4), the first two zincs again caused broadening of the P_i peak (though less than in the previous experiment). They also caused a peak to appear at —6.0 ppm, which is well downfield from any peak ever observed with native enzyme at this pH. Again the specific activity at this point was only 10% maximum. Again adding a second pair of zincs restored the properties of native enzyme except that the activity was somewhat lower and the $\underline{E} \cdot P$ resonance was a single peak at —4.2 ppm.

Repeated zinc titrations at both low and high pH gave results similar to described above.

We have always found that four zincs per dimer must be added to apoenzyme in order to reconstitute full enzyme activity. This is true regardless of whether apoenzyme was prepared by our usual EDTA method, by treatment with chelex resin [34], or simply by dialysis at low pH. The activity of Zn_2E prepared from apoenzyme was variable, but was no greater than 50% and

usually about 10% maximal. The only data in the literature directly contradictory to these findings is that of Csopak and Szajn [12], who reported that 2 Zn/E restore full activity to apoenzyme in linear fashion. It should be noted that our activity and NMR measurements are carried out at protein concentrations differing by a factor of $\approx 10^5$ and other features might become apparent if these measurements could be done with similar protein concentrations.

Chromatography of partially reconstituted apoenzyme

Evidence from our own and other work indicates that when two equivalents of zinc are added to apoenzyme, a homogeneous population of Zn_2E molecules results. It has been less clear what happens when only equivalent of zinc is added. Is binding of zinc sequential, so that a homogeneous Zn_1E population results, is it fully cooperative, so that an equimolar mixture of Zn_2E and apoenzyme results, or is it random so that a mixture of all three species results? Our titration experiments suggested that Zn_1E, if such a species is stable, should have qualitatively similar properties to Zn_2E. In order to test for the presence of Zn_1E in samples of partially reconstituted apoenzyme, we looked for a chromatographic method for separating alkaline phosphatase molecules based on their zinc content. We found that salt gradient elution from DEAE-cellulose, as is used in purification of native enzyme, is a suitable method.

Fig. 5a shows that apoenzyme applied to DEAE-cellulose elutes as a single peak. Activity of the eluted protein was restored by addition of zinc. When

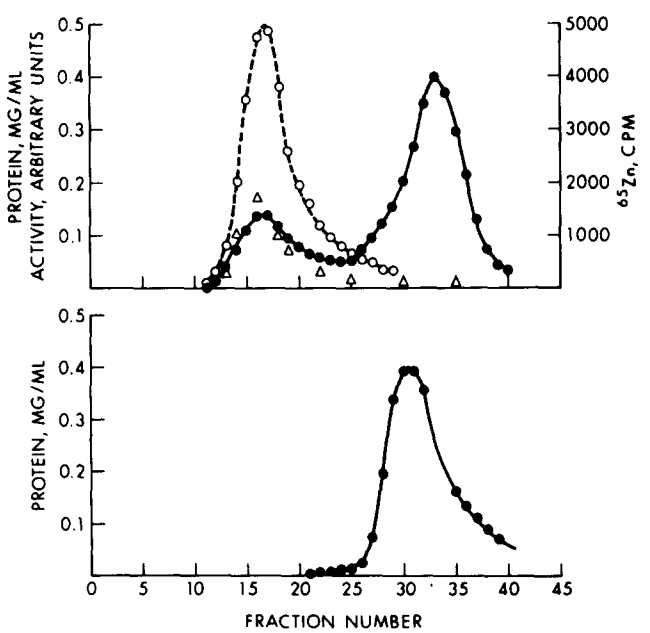

Fig. 5. Separation of apo- and Zn-alkaline phosphatase by ion-exchange chromatography. A 1 X 5 cm column of DEAE-cellulose was equilibrated with 0.01 M Tris · HCl, pH 7.5, and eluted with a 40 ml gradient of 0—0.1 M NaCl in the same buffer. 1-ml fractions were collected. Lower figure: 50 nmol of apoenzyme was applied; Upper figure: 29 nmol of apoenzyme was equilibrated with 20 nmol $^{65}ZnCl_2$ (Zn/E = 0.7) and then applied. Symbols: ●, protein; ○, ^{65}Zn; △, enzyme activity.

approx. one equivalent of zinc was added to apoenzyme, and allowed to equilibrate for ≈30 min before application to the column, two fairly well separated protein peaks were always eluted, the first of which coincided with a peak of zinc and a peak of enzymatic activity. A typical profile is shown in Fig. 5b. The second peak eluted at the same ionic strength as the apoenzyme in Fig. 5a. The first peak, near its center, had a measured Zn/E ratio of 1.7, and a specific activity which roughly doubled on addition of excess zinc. When more zinc was present in the original sample, the area of the first peak was proportionately greater. The results of these experiments were most consistent with the exclusive presence of Zn_2E and apoenzyme in the samples.

Discussion

The properties of Zn_2-alkaline phosphatase have been a matter of great controversy in the literature over the past several years, with measurements of its hydrolytic activity ranging from 10% [5,15] to "unstable" [35] to 100% [12] of maximum. In the work described here, we have prepared two distinct forms of Zn_2-alkaline phosphatase, one having 100% and the other having 10% activity. The fully active form was prepared by dialyzing purified native enzyme against metal-free buffer, then determining by careful atomic absorption measurements that 2.4 Zn/E were present. The phosphate complex of this species was distinguishable by ^{31}P NMR from the phosphate complex of native enzyme with 4.9 Zn/E (Fig. 1). The less active form was obtained when 2 Zn/E was added to apoenzyme. ^{31}P NMR studies with this species showed that it bound about the same amount of phosphate as native enzyme or reconstituted Zn_4E, but its phosphate complex clearly had altered properties (Figs. 3 and 4).

The only apparent difference between these two forms of Zn_2E is in the history of the species, though other differences could be present. For example, the two forms could differ in their content of magnesium, which has been shown to modulate catalytic activity [15]. However, magnesium content probably cannot explain the differences between these species, since Anderson et al. [15] showed, and we have confirmed, that reconstituted Zn_2E has no more than 50% activity even in the presence of magnesium. Also, our results cannot be attributed to problems with contamination by chelating agents, not only because we were scrupulous in removing EDTA from our apoenzyme, but also because the NMR spectra of Figs. 3 and 4 clearly show that two pairs of zincs had effects on phosphate binding, and therefore neither could be bound to spurious sites such as contaminating chelator molecules.

We, therefore, tentatively conclude that the binding of metal ions to alkaline phosphatase is characterized by hysteresis effects, due to the existence of a metastable form of Zn_2E (where "metastable" in this context refers to a time scale of several hours to a few days.) A simple model for such a situation would postulate two different pairs of metal sites per protein dimer, which we may designate type "A" and type "B", If "A" sites had higher affinity but were less accessible to solvent than "B" sites, and if exchange between sites was slow, then we would expect "native" Zn_2E to have its A sites occupied while reconstituted Zn_2E would have its B sites occupied. More probably, however, since metal binding is known to cause conformational alterations in alkaline phos-

phatase [13,16,26], hysteresis could arise because of metastable protein structures. In fact, long-lived metastable conformations have already been demonstrated by hysteresis effects in pH-titration behavior [13,26], and, most recently, by differential scanning calorimetry [27].

Given the unusual properties of Zn_2E, we also wished to study the properties of Zn_1E, if such a species were stable and could be purified. There was already some reason to believe that Zn_1E was not stable relative to a mixture of Zn_2E and E. Applebury et al. [1] published Scatchard plots showing that apoenzyme reconstituted with a single equivalent of zinc had 0.5 apparent phosphate-binding sites per dimer, implying that the enzyme was a 1 : 1 mixture of Zn_2E and E (at least in the presence of phosphate). Very recently, differential scanning calorimetry has also provided evidence for strong cooperativity in the binding of one pair of zinc ions per dimer [27].

We found that salt-gradient elution from DEAE-cellulose, which has generally been used for purifying alkaline phosphatase [33] and has also been used to purify biosynthesized apoenzyme [36], to separate isozymes [37], and to separate species with 0, 1, and 2 bound phosphates [38], can also separate species with different zinc content. As illustrated in Fig. 5, elution profiles obtained with apoenzyme plus one equivalent of zinc were most consistent with cooperative binding of a pair of Zn^{2+}. We never saw evidence of a Zn_1E species, which, if binding were not cooperative, should be the dominant species in such an enzyme sample. Unless binding to DEAE-cellulose somehow alters the zinc-binding properties of the enzyme, it may be concluded that binding of a pair of Zn^{2+}, even in the absence of phosphate and at relatively low protein concentration, is a cooperative process.

We add a final note regarding the phosphate-binding stoichiometry of alkaline phosphatase which has also been controversial in the past. Our measurement, both by NMR and chemical analysis, consistently indicate binding of 1.4 ± 0.2 phosphates per dimer. The non-integral stoichiometry remains puzzling, and may reflect inactivity of some active sites [39] or some more complex situation.

Acknowledgements

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