Two Differentiable Classes of Metal Atoms in Alkaline Phosphatase of *Eschericha coli**

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ABSTRACT: Alkaline phosphatase of *Escherichia coli*, prepared by chromatography on DEAE-cellulose, contains four zinc atoms per mole of protein of mol wt 89,000. Two of the zinc atoms are rapidly removed by 8-hydroxyquinoline-5-sulfonic acid, and concomitantly activity is lost. The other two zinc atoms, apparently not involved in catalytic function, are removed only slowly. Similarly, the results of readdition of zinc to the apoenzyme confirm that only two of the zinc atoms found in the enzyme are required for function. Two classes of metal atoms within the enzyme are also apparent from enzymatic and physicochemical studies of the cobalt enzyme. The first 2 g-atoms of cobalt bound to the apoprotein result in a spectrum similar to that of model octahedral cobalt complexes but without producing enzymatic activity. Addition of a further 2 g-atoms of cobalt generates activity and concomitantly leads to the development of a complex absorption spectrum with maxima at 640 (ϵ 250), 610 sh (210), 555 (350), and 510 mμ (280). This spectrum is dissimilar to those of either octahedral or tetrahedral complexes, and is suggestive of an unusual coordination environment for the metal at the active site of alkaline phosphatase. The circular dichroic properties of cobalt phosphatase lend further support to this hypothesis. Interaction of cobalt phosphatase with phosphate alters the spectral and dichroic properties of the protein yielding spectra more characteristic of octahedral geometry for the metal atoms at the active sites. Titration over the pH range from 8 to 6 abolishes the spectrum associated with the cobalt atoms at the active sites.

The degree of restoration of the spectrum of the "active site cobalt atoms" exactly parallels the enzymatic activity of the cobalt enzyme when studied over the same pH range. The different properties of the metals which are catalytically inert, and those which serve in enzymatic function are consistent with observations on other metalloenzymes and -proteins (Vallee, B. L., and Williams, R. J. P. (1968), *Proc. Natl. Acad. Sci. U. S.* *59*, 498).

Metalloenzymes offer unusual opportunities in the study of the mechanism of action of enzymes. The physical and chemical properties of metal ions are readily differentiated from those of the amino acid side chains of proteins and, thus, can become valuable probes of the active site. Chelating agents, which may inhibit enzymatic activity through formation of a mixed complex or by removing the metal, can assist in defining the role of the metal atom in substrate and cofactor binding, and in the catalytic mechanism. Further, other transition metals can often replace the native metal atom, and result in new metalloenzymes with distinctive catalytic activities and physicochemical properties (*cf.* Vallee and Wacker, 1968).

Zinc, a constituent of alkaline phosphatase of *Escherichia coli*, is a relatively poor probe for such purposes. It is diamagnetic, and its complexes with proteins do not exhibit visible absorption spectra. Cobalt(II), in contrast, is paramagnetic, and its complexes possess characteristic absorption spectra, due to d–d electron transitions. The ionic radii of cobalt and zinc are similar, and these elements share the capacity to accept distorted geometries (Vallee and Williams, 1968a,b). Moreover, cobalt is the only metal which has been found thus far to replace zinc in alkaline phosphatase yielding an enzymatically active species (Plocke and Vallee, 1962).

In evaluating the role of metals in the structure and function of alkaline phosphatase, the present investigation has utilized the catalytic properties of zinc and cobalt phosphatase, the differential reactivity of the zinc atoms of the native protein with a chelating agent, and the physicochemical characteristics of the cobalt enzyme. The data are consistent with the existence of two classes of metals in alkaline phosphatase. The first class appears to be essential for activity and is required for substrate binding. The second binds firmly to the protein at sites apparently distinct from those involved in activity directly. Physicochemical studies have provided information on the geometries of the complexes which the two classes of cobalt atoms form with the enzyme. Interaction with phosphate alters the spectral properties of the enzymatically active cobalt atoms. A brief report of some of these investigations has been presented (Simpson and Vallee, 1968).

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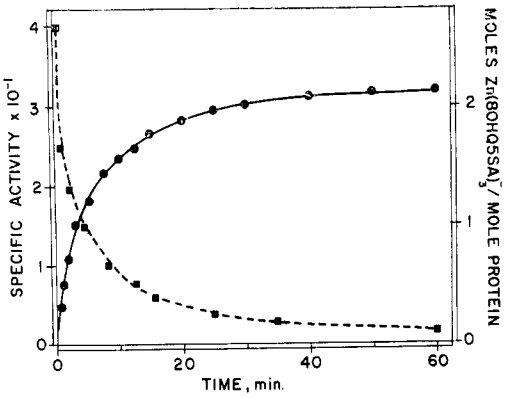

FIGURE 1: Removal of zinc from alkaline phosphatase by 8-hydroxyquinoline-5-sulfonic acid. Enzyme (1.33×10^{-6} M) was incubated with 6.66×10^{-4} M 8-hydroxyquinoline-5-sulfonic acid in 0.1 M Tris-Cl buffer (pH 8.0), 25°. Zinc removal (———) was quantitated by absorbancy at 370 mμ, the maximum for the 3:1 complex of zinc with 8-hydroxyquinoline-5-sulfonic acid (R. T. Simpson and B. L. Vallee, submitted for publication). Activity (- - -) was determined on aliquots as described under Experimental Section, except that 1 M Tris-Cl (pH 8.0) was utilized.

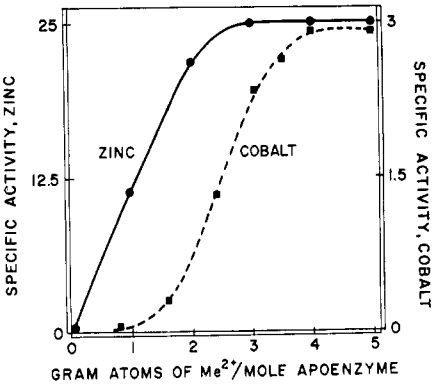

FIGURE 2: Activities of zinc and cobalt phosphatases. Apoenzyme (1×10^{-5} M) was reconstituted by addition of the noted molar excesses of zinc or cobalt and assayed immediately as described under Experimental Section.

Experimental Section

Alkaline phosphatase was prepared by DEAE-cellulose chromatography as previously described (Simpson *et al.*, 1968). This preparation is homogeneous on free-boundary electrophoresis at pH 7.0 and on disc electrophoresis in polyacrylamide gels at pH 9.4. The enzymatic activity of such preparations is 24–28 μmoles of *p*-nitrophenylphosphate hydrolyzed/min per mg of protein, when assayed as described below. The zinc content of preparations employed in this study varied from 3.7 to 4.1 g-atoms per mole of protein, when calculated based on a molecular weight of 89,000 (Simpson *et al.*, 1968; R. T. Simpson, J. L. Bethune, and B. L. Vallee, in preparation).

Apoalkaline phosphatase was prepared by dialysis of solutions containing 10–60 mg of protein/ml against four changes of 100-fold volume excesses of 0.01 M 8-hydroxyquinoline-5-sulfonic acid (pH 8.0), 20°, with a duration of at least 8 hr for each dialysis. The chelating agent was then removed by extensive dialysis against similar volumes of 0.01 M Tris-Cl (pH 8.0). Apoenzyme prepared in this fashion consistently contained less than 0.5% of the original zinc content, and no other transition or group IIb metals could be detected. The apoenzyme exhibited the activity expected from its zinc content. Precautions against contamination by adventitious metal ions were taken as previously described (Thiers, 1957).

Enzymatic activity was determined with *p*-nitrophenyl phosphate as the substrate. Activity was measured by observing the rate of formation of *p*-nitrophenol in a Unicam SP.800 recording spectrophotometer equipped with an external recorder and scale expansion device. Enzyme was added last to a cuvet containing 3 ml of 1×10^{-3} M substrate in 0.01 M Tris-Cl–1 M NaCl (pH 8.0) at 25°. The release of *p*-nitrophenol, as determined by absorption at 400 mμ, was followed for at least 1 min. Activities were based on a molar absorptivity of the product of 1.68×10^4 M^{-1} cm^{-1} under these conditions.

Before use, $^{60}Co^{2+}$ was diluted into a carrier solution of nonradioactive cobalt and measured by counting the isotope in a well-type scintillation counter (Tracerlab Corp.). Zinc was measured by atomic absorption spectrometry (Fuwa and Vallee, 1963; Fuwa *et al.*, 1964).

Protein concentrations were determined at 278 mμ using 0.72 as the absorbancy of the enzyme at a concentration of 1 mg/ml of solution (Plocke *et al.*, 1962). A Zeiss PMQ II spectrophotometer was employed for determination of absorbancy at discrete wavelengths. pH was measured with a Radiometer pH meter equipped with a general-purpose glass electrode.

For reconstitution of the apoenzyme, metal solutions were prepared from the spectrographically pure sulfate salts (Johnson, Matthey Co., Ltd.), dissolved in metal-free distilled water.

The spectra were obtained with water in the reference cuvet. Due to the high protein concentrations necessary (usually about 40 mg/ml), end absorption, due either to the protein itself, or to slight turbidity, was present in the visible region. This end absorption is shown as the featureless curve to the lower left in all figures. Visible absorption spectra were obtained with a Cary Model 15 spectrophotometer equipped with a 0–0.1 absorbance slide wire. Circular dichroism measurements were performed with a Cary Model 60 spectropolarimeter equipped with the Model 6001 circular dichroism accessory.

Results

The metal binding agent, 8-hydroxyquinoline-5-sulfonic acid, removes zinc from alkaline phosphatase of *E. coli*. The kinetics of this removal can be determined by absorbancy at 370 mμ, where the 3:1 zinc complex absorbs maximally (R. T. Simpson and B. L. Vallee, submitted for publication). Figure 1 details the time course of removal of zinc from the enzyme in the presence of a 50-fold molar excess of 8-hydroxyquinoline-5-sulfonic acid. Under these conditions two of the zinc atoms of the native protein are removed with 30 min.

The kinetics of the reaction are pseudo-first order with respect to protein. The remaining two zinc atoms are removed only slowly, over a period of at least 24 hr. Activity is lost to less than 10% of the control native enzyme coincident with the rapid, preferential removal of the first two zinc atoms by the chelating agent (Figure 1). The removal of two of the zinc atoms of the native protein and its correlation with the loss of activity have been confirmed by measurements of zinc content and activity following separation by gel filtration of protein-bound and free zinc.

Phosphate ion, which can serve as a substrate for the enzyme, retards the rate of removal of the two rapidly reacting metal atoms as a function of phosphate concentration (Table I). An apparent dissociation constant for phosphate binding to the enzyme calculated from these data is about 3×10^{-5} M. This value agrees well with the K_I for phosphate inhibition of phosphoester hydrolysis determined kinetically with these same enzyme preparations (M. M. Gottesman, R. T. Simpson, and B. L. Vallee, in preparation).

TABLE I: Effect of Phosphate Ion on Removal of Zinc by 8-Hydroxyquinoline-5-sulfonic Acid.

[Phosphate] (M)	Rate (sec^{-1})[a]
0	116×10^{-4}
2.5×10^{-5}	71×10^{-4}
3.3×10^{-4}	1.8×10^{-4}
3.3×10^{-3}	0.8×10^{-4}

[a] The rates given are the first-order rate constants for the removal of the two rapidly reacting zinc atoms from a protein at a concentration of 1.33×10^{-5} M in 0.1 M Tris-Cl (pH 8.0), 25°, when the ligand is present in a 50-fold molar excess.

Addition of increments of zinc to the apoenzyme further demonstrates the association of only two of the zinc atoms of phosphatase with activity. Addition of 2 g-atoms of zinc/mole of apoprotein restores 85% of the activity of the control (Figure 2). Further addition of 2 g-atoms of zinc to [(P'ase)Zn_2][1] increases this activity only slightly. In contrast, addition of only 2 g-atoms of cobalt/mole of apophosphatase, forming [(P'ase)-Co_2], generates little activity. However, the activity characteristic of cobalt phosphatase is generated when 2 more g-atoms of cobalt/mole of apoenzyme are added, a total of 4 g-atoms/mole, to form [(P'ase)Co_4] (Figure 2).

These kinetic and enzymatic studies suggest the presence of two classes of functionally different metal ions in phosphatase, only one of which is directly involved in enzymatic activity. Physicochemical studies of the cobalt enzyme are consistent with this conclusion. Incremental addition of cobalt to apophosphatase generates the spectra detailed in Figure 3. The first 2 g-atoms of cobalt added per mole of protein generate an absorption band centered at about 500 mμ and of low absorbance (ϵ_{500} 50). In contrast, addition of a further 2 g-atoms of cobalt/mole of protein results in a completely different and complex absorption pattern extending from 450 to 700 mμ. Well-defined maxima are observed at 640, 555, and 510 mμ, with a shoulder at 610 mμ. The molar absorptivities of these bands are $\epsilon_{640} = 250$, $\epsilon_{610} = 210$, $\epsilon_{555} = 350$, and $\epsilon_{510} = 280$. Addition of cobalt in excess of 4 g-atoms/mole of protein does not alter these spectra.

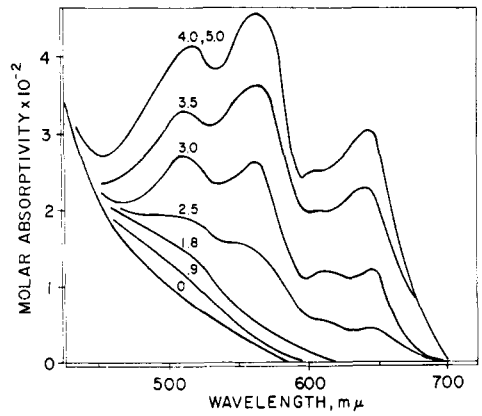

FIGURE 3: Absorption spectra of cobalt phosphatases. Apoenzyme (2×10^{-4} M) in 0.01 M Tris-Cl (pH 8.0) was titrated by addition of the molar excesses of cobalt indicated above the spectral curves. In this and all succeeding spectra, the featureless curve at the lower left represents end absorption due to the protein itself; the spectra were obtained with water as a reference. Absorptivity refers to the molar concentration of protein, not the metal species.

Although these two types of absorption spectra detailed above develop sequentially and differentially, there is no evident discontinuity of the incorporation of cobalt into apophosphatase. When increments of cobalt are added to the apoprotein, and then free metal ions are removed by gel filtration, the amount of cobalt incorporated into the apoprotein increases linearly from 0 to 4 g-atoms of cobalt added per mole of protein (Figure 4). When cobalt in excess of 4 g-atoms/mole is added, no further incorporation is observed.

When 1 g-atom of zinc is added to the apoprotein prior to addition of a fivefold excess of cobalt, the intensity of the spectrum associated with the enzymatically essential cobalt atoms is diminished to half that observed in [(P'ase)Co_4] (Figure 5). This complex spectrum is not seen when cobalt is added to [(P'ase)Zn_2]. Only the spectrum characteristic of [(P'ase)Co_2] is observed in this latter case (Figure 5). Similarly the spectrum of [(P'ase)-Co_2], associated with the catalytically inactive metal atoms, is progressively abolished by the addition of 3 and 4 g-atoms of zinc prior to the addition of cobalt (Figure 5). The sum of zinc and cobalt atoms bound, measured after removal of excess metal of gel filtration, approximates four in all cases (Figure 5, insert).

[1] The enzymes formed by addition of increments of metal to the apoprotein will be designated as [(P'ase)Me_x], where x = 1, 2, 3, or 4 g-atoms of metal added per mole of protein.

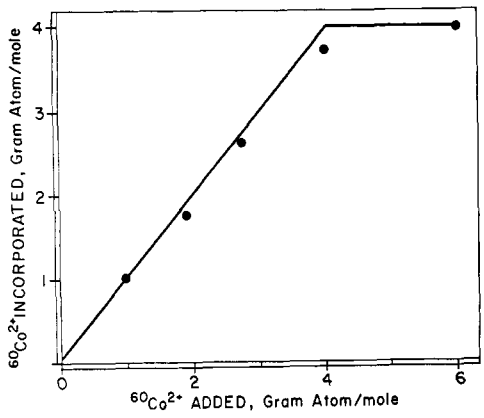

FIGURE 4: Cobalt incorporation into phosphatase. $^{60}Co^{2+}$ was added to apophosphatase (2×10^{-4} M) in 0.01 M Tris-Cl (pH 8.0) in the indicated molar excesses. An aliquot of the solution was passed over a Bio-Gel P-6 column (0.9 × 20 cm) in 0.01 M Tris-Cl (pH 8.0), and $^{60}Co^{2+}$ was determined in the protein-containing fractions.

Potentiometric titration of [(P'ase)Co_4] leads to significant alterations in the environment of the cobalt ions which are presumably located at the active site. When the enzyme is acidified to pH 6, the distinctive spectrum associated with cobalt ions at the active site is abolished, and a featureless spectrum resembling that of [(P'ase)-Co_2] is observed. At pH values from 6 to 8, the general qualitative features of the spectrum of [(P'ase)Co_4] are retained, although the intensity of the spectrum increases with increasing pH. The development of this spectrum as a function of pH is illustrated by the absorbancy at 640 mμ, a characteristic of the spectrum of the cobalt atoms at the active sites (Figure 6). The apparent pK for the generation of the spectrum is 7.0 and its appearance exactly parallels the pH dependence of the enzymatic activity

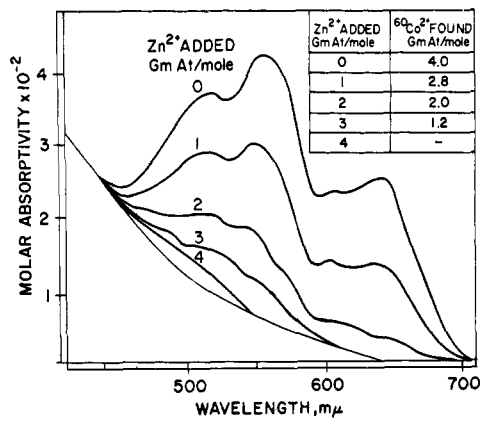

FIGURE 5: Competition of zinc and cobalt for the metal binding sites of phosphatase. A fivefold molar excess of $^{60}Co^{2+}$ was added to apoenzyme (1.5×10^{-4} M) in 0.01 M Tris-Cl (pH 8.0) following addition of the molar excesses of zinc indicated by the numbers above the spectral curves. After the spectra were obtained, an aliquot of the solution was passed over a Bio-Gel P-6 column (0.9 × 20 cm) in 0.1 M Tris-Cl (pH 8.0) and zinc and cobalt bound to the protein were determined by atomic absorption spectrometry and isotope counting, respectively, as under Experimental Section. The insert presents the results of these latter determinations. In all cases, the amount of zinc bound was within 0.1 g-atom/mole of protein of the amount added.

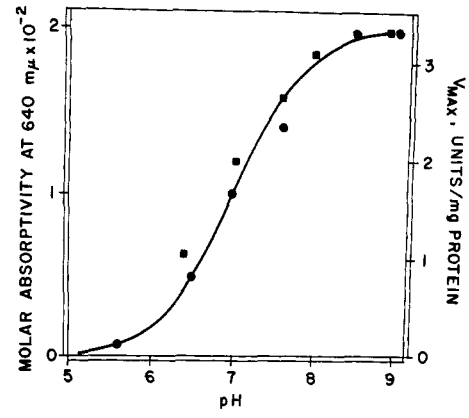

FIGURE 6: pH dependence of enzymatic activity and absorptivity at 640 mμ of cobalt phosphatase. Apoenzyme (1×10^{-4} M) in water was adjusted to pH 6 after addition of a fivefold molar excess of cobalt. The solution was titrated with dilute NaOH and A_{640} (●) determined as characteristic of the presence of the spectrum associated with enzymatically active cobalt (see Figure 3). Enzymatic activity of cobalt phosphatase is the V_{max} (■) for hydrolytic activity determined as in the Experimental Section except that 0.01 M Tris-acetate was used as buffer.

of the cobalt enzyme (Figure 6) (M. M. Gottesman, R. T Simpson, and B. L. Vallee, in preparation).

Addition of phosphate does not alter the absorption spectrum of [(P'ase)Co_2]. In striking contrast, incremental addition of phosphate to [(P'ase)Co_4] abolishes the maxima at 640, 610, 555, and 510 mμ and generates a simpler spectrum with maxima at 475 (ϵ_{475} 175) mμ and 550 mμ (ϵ_{550} 280) (Figure 7). Phosphate ion (2 moles) interacts with each mole of cobalt phosphatase presumably containing two atoms of enzymatically active cobalt as is apparent from titration of the enzyme with increasing concentrations of this substrate (Figure 7, insert). In contrast to the striking effect of phosphate on the cobalt phosphatase spectrum, the addition of stoichiometric amounts of a chelating agent such as cyanide does not affect it.

[(P'ase)Co_4] exhibits two negative and two positive ellipticity bands within the region of the two shorter wavelength absorption bands of this protein (Figure 8). In the presence of stoichiometric amounts of phosphate ion, these four bands are replaced by two ellipticity bands located within the region of the 550-mμ absorption peak of the [(P'ase)Co_4] phosphate complex (Figure 8). When compared with the cobalt enzyme alone, the sign of the major ellipticity band is reversed in the E·S complex.

Discussion

Complexation or removal of the native metal at the active sites of metalloenzymes, or its replacement by other transition metals, has proven useful in the study of enzymatic mechanisms, in the examination of the metal binding groups of proteins, in investigation of the geometry of metal binding, and of alterations in this geometry on binding inhibitors and substrates. Thus, the stability constants of different metallocarboxypeptidases have been employed to indicate the nature of the metal binding sites of this enzyme (Coleman and Vallee,

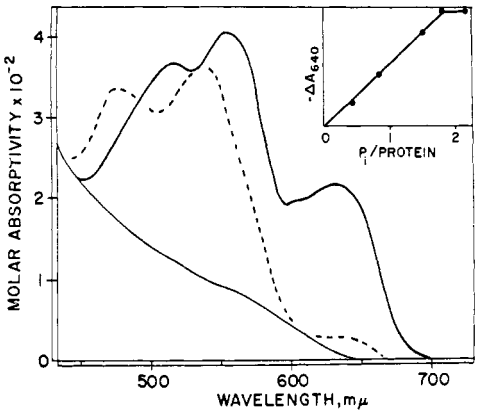

FIGURE 7: Effect of phosphate on the spectrum of cobalt phosphatase. Apoenzyme (1.5×10^{-4} M) was reconstituted with a fivefold molar excess of cobalt (———) and then titrated with increments of phosphate ion to a final molar excess of 2.5 moles of phosphate/mole of enzyme (- - -). The insert presents the loss of absorptivity at 640 mμ as a function of the molar excess of phosphate added to the cobalt protein.

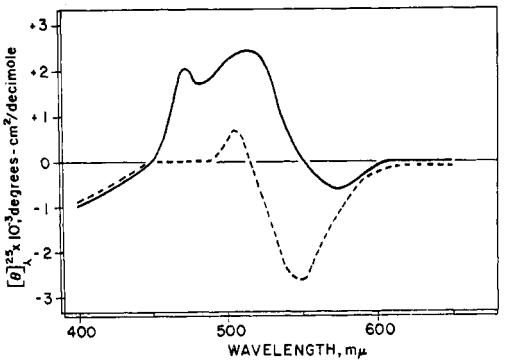

FIGURE 8: Circular dichroism of cobalt phosphatase and the phosphate cobalt phosphatase complex. The circular dichroism of apophosphatase reconstituted with a fivefold molar excess of cobalt (———) and the same sample plus a threefold molar excess of phosphate (- - -) were determined as under the Experimental Section. Protein concentration was 2×10^{-4} M in a 2-cm path-length cell, 0.01 M Tris-Cl (pH 8.0).

1961; Vallee *et al.*, 1961; Piras and Vallee, 1967). The spectrum of cobalt carboxypeptidase has given indications concerning the nature of the ligands and geometry of the metal at the active site (Coleman and Vallee, 1960). Similarly, spectral studies of cobalt carbonic anhydrase have elucidated features of the role which the metal atom may play in the mechanism of action of this metalloenzyme (Lindskog and Nyman, 1964; Coleman, 1965). The present investigations of zinc and cobalt alkaline phosphatase, proteins which are structurally complex relative to the others previously studied, pertains to the understanding of the role of metal atoms both in structure and in function of this enzyme.

Two different experimental techniques, used to investigate the role of metal atoms in the structure and function of the alkaline phosphatase of *E. coli*, have indicated the presence of two classes of firmly bound metal atoms in this enzyme; one class of ions is concerned with enzymatic activity while the other, albeit firmly bound to the protein, has no apparent direct effect on function. The two approaches comprise interaction of the zinc atoms of the native protein with chelating agents, on the one hand, and delineation of the physicochemical characteristics of the cobalt enzyme, on the other.

Two of the four zinc atoms of phosphatase are rapidly removed by 8-hydroxyquinoline-5-sulfonic acid, and concomitantly, enzymatic activity is lost (Figure 1).[2] Phosphate ion, the product of the energetically favored hydrolysis of the phosphomonoester substrates of the enzyme, slows the rate of removal of the rapidly reacting pair of zinc atoms, consistent with the supposition that these two zinc atoms are those directly involved in activity.

Apoenzyme to which only 2 g-atoms of zinc have been added is nearly as active enzymatically as is the native enzyme, further supporting the idea that only two of the zinc ions are required directly for activity (Figure 2). The two zinc atoms at the active site appear to bind most readily to the enzyme, and yet, these same two enzymatically essential metal ions are removed most rapidly by the chelating agent. Such data would suggest that the location of the functionally active zinc atoms makes them readily accessible to the ambient environment, *i.e.*, that they might be located at the surface of the molecule. The two zinc atoms which are removed more slowly might be buried in the interior of this multichain enzyme, and hence, be much less readily accessible to chelating agents.[3] As in equine liver alcohol dehydrogenase (Drum *et al.*, 1967) the second pair of zinc atoms might serve in stabilization of the quaternary structure of the alkaline phosphatase molecule. Evidence supporting this supposition will be presented elsewhere (R. T. Simpson, J. L. Bethune, and B. L. Vallee, in preparation).

Again, studies of the cobalt derivative of alkaline phosphatase support the supposition that the four metal atoms present in this enzyme may be divided into two pairs: one pair involved directly in catalysis, and a second, firmly bound to the protein, but apparently not affecting function directly.

The first two cobalt atoms to associate with the apoprotein generate a simple absorption spectrum with a maximum near 500 mμ and an absorptivity of 50 M^{-1} cm^{-1} (Figure 3). In contrast, addition of the second pair of cobalt ions to [(P'ase)Co_2] produces a complex spectrum, with four absorption bands of relatively high intensity located within the wavelength range from 450 to 700 mμ (Figure 3). To the best of our knowledge, the

[2] Although a number of chelating agents, such as α,α-bipyridyl, 1,10-phenanthroline, EDTA, etc., remove zinc from phosphatase, and thereby inactivate the protein (Plocke *et al.*, 1962), 8-hydroxyquinoline-5-sulfonic acid presents certain operational advantages for the present study and removes zinc in a biphasic manner, illustrated in Figure 1. It interacts rapidly with the protein and forms a spectrophotometrically measurable zinc complex. It is not known whether or not other chelating agents might act in a similar fashion.

[3] This does not imply necessarily that the formation constants associated with the different classes differ.

generation of at least two different types of absorption spectra on addition of cobalt to the apoprotein is without precedent in enzyme chemistry, thus precluding comparison with analogous systems studied previously. The occurrence of two types of spectra is all the more intriguing since functional capacity seems to be associated only with the second pair of cobalt ions bound to the protein. Thus, the correlation of activity with the binding of the second pair of cobalt ions (Figure 2), their preferential displacement by zinc (Figure 5), and the effects of phosphate ions on their characteristic spectrum (Figure 7) suggest that the cobalt ions giving rise to it are at the active site of the enzyme.

In contrast the first pair of cobalt atoms bound do not appear to relate directly to activity but, instead, seem to stabilize quaternary structure. Their absorption spectrum resembles that associated with cobalt(II) in an octahedral environment of nitrogen and/or oxygen donor groups in simple ligands, with maxima at about 500 mμ, and absorptivities of 5–20 M^{-1} cm^{-1} (Dennard and Williams, 1966). On the assumption that such cobalt–protein spectra can be compared directly with cobalt complex ions, the cobalt atoms which give rise to these spectra in alkaline–phosphatase may occupy an octahedral coordination environment.[4]

While the spectrum generated by the second pair of cobalt ions invites comparisons with those of cobalt complex ions, it would seem much more difficult to arrive at similar analogies in this instance. The absorptivity of the spectrum associated with the catalytically active cobalt ions in alkaline phosphatase is significantly higher than that of known octahedral cobalt complexes but well below that of tetrahedral cobalt complexes previously studied (ϵ 800). The location of the maxima corresponds neither to that of simple octahedral nor of simple tetrahedral cobalt complexes (Vallee and Williams, 1968a,b).

It might be considered that the spectrum generated by the second pair of cobalt atoms is the complex summation of two simpler spectra, each generated by a single cobalt atom occupying different sites with approximately equal binding constants. In that case, interaction with inhibitors or substrate might alter each of these two hypothetical spectra in unique fashion. In fact, the spectrum associated with the catalytically essential cobalt ions behaves as a single entity, both on titration with H^+ and with phosphate ion (Figures 6 and 7). These facts militate against this view, but do not rule it out. It may be recalled, however, that cobalt carbonic anhydrase exhibits a spectrum closely similar to that of the catalytically active cobalt atoms of alkaline phosphatase, though cobalt carbonic anhydrase contains only one cobalt atom which could give rise to such spectra (Lindskog and Nyman, 1964).

In the absence of any unusual features in the geometry of binding of the cobalt atoms, this type of spectrum might conceivably arise from the diversity of ligand species present in proteins. Alternatively, it has been suggested that such unusual spectra associated with enzymatically active cobalt enzymes might be the consequence of unusual geometries of metal binding (Vallee and Williams, 1968a,b), an interpretation consistent with recent studies of pentacoordinate cobalt(II) complexes.[5]

Irrespective of its origin, the presence of the unusual spectrum associated with the catalytically active cobalt atoms in phosphatase generates a "probe" at the active site, and allows study of interactions of the enzyme with inhibitors and substrates. While at pH 6 the intensity is markedly reduced, the general characteristics of the spectrum of [(P′ ase)Co_4] are the same at all pH values between pH 6 and 9.6. In contrast to the pH-dependent spectral transitions of cobalt carbonic anhydrase (Lindskog and Nyman, 1964), in phosphatase, spectral intensity increases with increasing pH. The spectral changes at lower pH values are not due to loss of the metal since 4 g-atoms of cobalt are bound to the protein at pH values from 6.3 to 8.0 under the conditions of these titrations, *i.e.*, a fivefold molar excess of cobalt over protein. The appearance of the spectrum associated with the cobalt atoms at the active site is related directly to the pH dependence of enzymatic activity of the cobalt enzyme, *i.e.*, the absorbancy of the cobalt enzyme at 640 mμ, and V_{max} for hydrolytic activity, correlate exactly as a function of pH (M. M. Gottesman, R. T. Simpson, and B. L. Vallee, in preparation) (Figure 6).

Stoichiometric concentrations of metal-binding inhibitors, such as CN^-, do not alter the spectrum of cobalt phosphatase, although higher concentrations inhibit the enzyme by removal of metal (Plocke and

[4] The fact that zinc apparently binds preferentially at the active sites, while cobalt preferentially occupies sites distinct from those involved in function (Figure 2), is of some interest. In octahedral complex ions, the stabilities of the cobalt complexes are greater than or equal to those of zinc (Sillen and Martell, 1964). In contrast, for tetrahedral geometries, the stability of zinc complexes is generally greater than that of the cobalt complexes (Sillen and Martell, 1964). Thus, zinc preferentially occupies ligand configurations in the order tetrahedral > octahedral; while for cobalt the ordering is octahedral > tetrahedral (Dennard and Williams, 1966). Both these metals, however, share the ready capacity to occupy irregular and distorted geometries, a factor thought to be of import in the biological activity of metalloenzymes formed with zinc or cobalt (Vallee and Williams, 1968a,b). The present investigations have not been shown to reflect equilibrium conditions, and hence, the preferential binding of zinc and cobalt to the two different groups of metal binding sites cannot be equated with differences in stability constants. Nevertheless, the order of binding observed for the metals interacting with the protein would be consistent with the equilibrium stabilities predicted from model systems.

[5] This type of visible absorption spectrum has recently been observed for a group of five-coordinate cobalt complexes with ligands of the type bis-(R_2-aminoethyl)-ZX_2, where R is methyl or ethyl, X is one of the halides, and Z is >O, >NH, or >NR (Ciampolini and Nardi, 1966; Ciampolini and Speroni, 1966; Dori and Gray, 1966; Ciampolini and Nardi, 1967). The extinctions and the visible band positions of the models and the protein are quite similar, although the spectrum of cobalt phosphatase reveals a higher degree of splitting within the absorption envelope, as would be expected if the symmetry of the metal binding groups were reduced. Such a reduction in symmetry might be expected on progressing from a simple organic ligand to the more complex field likely to be generated by the metal binding site of a protein. The geometry of these model complexes with several transition metal ions has been shown to be near that of a trigonal bipyramid.

Vallee, 1962). In contrast, stoichiometric amounts of phosphate markedly affect the spectrum of the catalytically essential cobalt atoms of this enzyme. Phosphate, the product of the energetically favored hydrolytic reaction, interacts with the enzyme to form a phosphoseryl intermediate (Schwartz and Lipmann, 1961), exchanges labeled oxygen with water in the presence of the enzyme (Stein and Koshland, 1952) and can also serve as a substrate for the formation of phosphoesters with suitable alcoholic acceptors (Meyerhoff and Green, 1949), and thus may be considered as a substrate for the enzyme.

The changes in absorption on addition of phosphate indicate that 2 moles interact with each mole of cobalt alkaline phosphatase (Figure 7, insert). These data do not reveal how firmly either of these phosphate ions may be bound. In contrast with earlier conclusions (Levinthal *et al.*, 1962) equilibrium dialysis of phosphate binding studies to the native enzyme has indicated one phosphate binding site per mole (Reynolds and Schlesinger, 1969).[6] Clearly additional work is required to evaluate the effect of differences in the materials and methods employed on these results.

The absorption spectrum of the phosphate [(P'ase)-Co_4] complex (Figure 7) is much simpler than that of the parent cobalt protein, with maxima at 550 and 475 mμ. The positions of these maxima correspond more nearly to those expected for an octahedral environment, rather than for a tetrahedral structure. The absorptivity of the complex is, however, much greater than that of known model octahedral complexes. It should be recalled that phosphate does not alter the [(P'ase)Co_2] spectrum (*vide supra*).

Measurements of circular dichroism of the cobalt enzyme and of the [(P'ase)Co_4] phosphate complex support the thought that the geometry of the cobalt atoms at the active site is altered upon interaction with this substrate (Vallee and Williams, 1968a,b). Dissymmetric metal complexes generally exhibit low-energy absorption bands; however, due to magnetic transition dipoles they may manifest relatively large circular dichroic absorption. In the cobalt enzyme, optical activity appears to be associated only with the bands with maxima at 555 and 510 mμ. Apparently the other d–d transitions resulting in absorption are not dissymmetric. Similarly, the [(P'ase)Co_4] phosphate complex exhibits optical activity associated only with the band at 550 mμ and not with that at 475 mμ. In this case, however, the sign of the major dichroic band of the complex is reversed when compared with that of the cobalt enzyme alone (Figure 8). Further interpretation of these results requires additional information about the geometry and magnetic properties of the cobalt substituents.

These spectral changes on binding of phosphate might reflect a transition from five-coordinate to six-coordinate geometry of the catalytically active metal atoms and would suggest that phosphate might be bound directly to them. Although it is difficult to examine this hypothesis by spectral studies alone, such information is critical to the discernment of the role of metal ions in the mechanism of alkaline phosphatase catalysis and constitutes a problem currently under investigation by other approaches.

The characteristics and presumable implications of chromophoric metals at the active centers of enzymes have recently been discussed in relation to the basis of biological activity of metalloenzymes (Vallee and Williams, 1968a,b). The lack of similarity between the spectral properties of catalytically active metal atoms of metalloenzymes and those of the same metals bound to biologically inactive metalloproteins or in simple model complex ions has been noted. This dissimilarity has been thought to imply a state of "entasis," *i.e.*, an irregular or strained geometry of metal binding in the enzymatically functional systems. It has been conjectured that this irregularity of geometry might reflect a localized state energetically favorable to catalysis, a potential factor in the biological activity of such enzymes.

Alkaline phosphatase of *E. coli* has provided a most interesting system for the examination of this hypothesis, since the protein apparently contains two classes of metal atoms, both of which can be replaced by cobalt. The present investigations indicate that those metal atoms which do not participate directly in enzymatic function have spectral properties similar to those of simple complex ions. In contrast, the spectral properties of the catalytically essential metal atoms do not resemble those of simple model systems. While these present data are thus consistent with the entatic site hypothesis, definition of the chemical basis of the spectra, attributed to cobalt atoms involved in catalytic activity, must await further knowledge of the electronic and magnetic spectral properties of this metalloprotein.

The presence of cobalt, serving as a probe with distinctive spectral and magnetic properties, should afford a broad range of physicochemical and enzymatic approaches to assist in the elucidation of the mechanism of action of this enzyme.

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Conformational Studies on Polyriboadenylic Acid in Ethylene Glycol*

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ABSTRACT: The conformation of polyriboadenylic acid in ethylene glycol in the presence and the absence of a 4- to 12-fold ratio of protons to adenine has been examined by spectral and optical rotatory techniques. When the glycol solvent is slightly basic, the stacking of the adenine residues in polyriboadenylic acid is completely abolished. In the presence of a small excess of hydrogen ions, however, a base-stacked helical conformation is preserved at glycol concentrations as high as 97–99.7%.

The optical properties as well as the thermal stability of this conformation in this nonaqueous solvent are very similar to those exhibited by the conformation of polyriboadenylic acid in acidified aqueous solvents at low ionic strength. The form present in the acid glycol solvent can be converted into the base-unstacked conformation by the addition of approximately 1 mole of hydroxide ion/mole residue of polymer. Despite the fact that glycol is normally a polynucleotide denaturing solvent, the conformation of polyriboadenylic acid present in the acidified glycol is similar to if not identical with the structures formed in acidified aqueous solvents under comparable conditions of ionic strength and acid concentration. The latter is presumably a subtle variant of the structure proposed by Rich *et al.* (Rich, A., Davies, D. R., Crick, F. H. C., and Watson, J. D. (1961), *J. Mol. Biol. 3*, 71). The stability of such a structure in a solvent which normally destroys base-stacked polynucleotide conformations can be ascribed to the effect of the lower dielectric constant of ethylene glycol on the strength of the internal ion pair which maintains this conformation.

A convenient index of the stability of an organized polynucleotide conformation is the standard free-energy change, ΔG°, for the helix–coil transition

$$\left[\begin{array}{l}\text{organized,}\\ \text{base stacked,}\\ \text{helical form}\end{array}\right] \rightleftarrows \text{[random coil]} \qquad (1)$$

As information about polynucleotide conformations in both solution and the solid state has accumulated, it has become apparent that a unique conformation reflects a balance of many types of interactions of the constituent parts of the polymer both with each other and with the solvent. Thus, ΔG° for reaction 1 represents the difference in contribution of these constituent interactions between the helix and the coil form.

There are three general categories of self-interactions which make major contributions to the free-energy difference between the helix and the coil forms of poly-

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