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The Effect of Mg(II) on the Spectral Properties of Co(II) Alkaline Phosphatase†

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ABSTRACT: Alkaline phosphatase of *Escherichia coli,* isolated by procedures which do not alter its intrinsic metal content, contains 1.3 ± 0.3 g-atom(s) of magnesium and 4.0 ± 0.2 g-atoms of zinc per mol of molecular weight 89 000 (Bosron et al., 1975). Substitution of Co(II) for Zn(II) and/or Mg(II) results in spectral properties which can be correlated with enzymatic activity. Magnesium does not activate the apoenzyme but augments the activity of the 2-Co(II) enzyme almost 3-fold and that of the 4-Co(II) enzyme 1.3-fold. The magnesium-induced increase in activity of the 2-Co(II) enzyme is accompanied by spectral changes which are consistent with an alteration from largely octahedral-like to pentacoordinate-like coordination geometry. Magnesium increases the intensity of the absorption and magnetic circular dichroism (MCD) signals of the 4-Co(II) enzyme but without evidence of changes in coordination geometry. Cobalt when bound to the magnesium sites results in octahedral-like EPR spectra, unperturbed by phosphate which significantly affects cobalt at the pentacoordinate-like sites. In the absence of magnesium, 6 g-atoms of cobalt are required to maximize the spectral properties, but activity does not increase further after the addition of only 4 g-atoms. In the presence of excess magnesium, the enzyme binds only 4 g-atoms of cobalt, while activity is optimal with only 2 g-atoms of cobalt. Hydrogen–tritium exchange measurements indicate that magnesium also stabilizes the dynamic structural properties of the apo- and 2-Co(II) enzymes but has little effect on the structure of 4-Co(II) phosphatase. The response to magnesium of both the spectral properties and enzymatic activities of cobalt alkaline phosphatase demonstrates that magnesium regulates cobalt (and zinc) binding and modulates the activity of the resultant products.

The spectral properties and chemical reactivities of chromophoric metalloenzymes differ greatly from those of the bidentate complex ions of the corresponding metals. Such metal complexes can be characterized by their electronic and magnetic properties as revealed by their absorption, natural and magnetic circular dichroic, electron paramagnetic, and other spectra (Vallee and Williams, 1968a,b).

Zinc metalloenzymes cannot be studied by spectral means, but substitution of chromophoric, paramagnetic metal ions permits examination of the nature of their metal coordination. Cobalt(II) has proved particularly suitable in this regard entailing both enzymatic activity and characteristic spectra to virtually all zinc enzymes examined thus far including alkaline phosphatase (Vallee and Williams, 1968a,b; Vallee, 1974).

Alkaline phosphatase isolated from *E. coli* is a dimer containing 4 g-atoms of zinc per molecular weight of 89 000 (Simpson et al., 1968). One pair of zinc atoms has been postulated to be involved primarily in catalysis while the other is thought to serve in structure stabilization. As isolated, native alkaline phosphatase also contains 1 to 2 g-atoms of magnesium (Plocke et al., 1962; Simpson et al., 1968) whose regulatory role has been recognized only recently (Bosron et al., 1975; Anderson et al., 1975).

The present data demonstrate that magnesium significantly affects the manner in which zinc and cobalt participate in catalysis and structure stabilization of alkaline phosphatase. The characteristics of cobalt and zinc–cobalt hybrid phosphatases have been studied in the presence and absence of magnesium by absorption, magnetic circular dichroic, and electron paramagnetic resonance spectroscopy. In the absence of magne-

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sium, the apoenzyme binds 6 g-atoms of cobalt and it is primarily the second pair of cobalt atoms added that appears to be involved in catalysis. When magnesium is present in excess, the enzyme binds only 4 g-atoms of cobalt but displays 35% higher activity than the enzyme containing solely 6 g-atoms of cobalt. Under these conditions, primarily the first pair of cobalt atoms added appears to be involved in the catalytic process. The increase in activity of cobalt phosphatase observed upon addition of magnesium is accompanied by spectral alterations consistent with changes in the coordination geometry of the cobalt atoms. The responses to magnesium of both the spectral properties and enzymatic activities of cobalt alkaline phosphatase demonstrate that magnesium regulates cobalt (and zinc) binding and modulates activity of the resultant products.

Materials and Methods

Alkaline phosphatase was prepared by DEAE[1]-cellulose chromatography (Simpson et al., 1968) except that Tris-HCl, pH 7.5, was substituted for imidazole chloride. All enzymatic activities were determined in both 20 mM Veronal–0.4 M NaCl, and 1 M Tris-HCl, pH 8.0, 25 °C, with *p*-nitrophenyl phosphate (1 mM) as substrate. A unit of activity is defined as the μmol of substrate hydrolyzed per min per mg of protein, using a molar absorptivity of 1.68×10^4 for the *p*-nitrophenolate ion at 400 nm. The activity of the cobalt enzyme, which does not exhibit transferase activity, is higher when assayed in Veronal than in 1 M Tris (Tait and Vallee, 1966), and the activity of the zinc enzyme is greater in Tris. Tris serves as an acceptor for transferase activity (Wilson et al., 1964). Hence, in the presence of 1 M Tris, the assay encompasses both hydrolase and transferase activities of zinc phosphatase. Buffers, glassware, and substrate were rendered metal free as described (Thiers, 1957). After metal extraction with dithizone, buffers were stored in the presence of Chelex 100, 10% v/v, to remove extraneous metals (Anderson and Vallee, 1975). Metal content was determined by atomic absorption (Fuwa et al., 1964; Wacker et al., 1965) or emission spectrography (Vallee, 1955). Apoalkaline phosphatase was prepared using 8-hydroxyquinoline-5-sulfonic acid (Simpson and Vallee, 1968); the apoenzyme contained less than 0.03 g-atom of zinc, 0.01 g-atom of magnesium, and no other metals.

Metal solutions prepared from spectrographically pure sulfate salts (Johnson, Matthey & Co.) dissolved in metal-free distilled water were used to reconstitute the apoenzyme. The metal content of reconstituted enzymes was determined as described (Anderson and Vallee, 1975) and calculated based on a molecular weight of alkaline phosphatase of 89 000 and using $E_{278}^{1\%} = 7.2$ (Plocke et al., 1962).

Visible absorption spectra were obtained with a Cary Model 14 spectrometer equipped with a 0–0.1 absorbance slide wire and magnetic circular dichroism (MCD) spectra with a Cary Model 61 spectropolarimeter at a magnetic field of 40 kG, all at room temperature. Since MCD and circular dichroism (CD) spectra are additive, all MCD spectra were corrected for the CD component. Molar absorptivities, ϵ, are in units of M^{-1} cm^{-1} and molecular magnetic ellipticities, $[\theta]_m$, are given in deg cm^2 $dmol^{-1}$ kG^{-1}. We have followed the suggestion of McCaffrey et al. (1967) to define the Verdet constant of water as negative to serve as a basis of the sign convention for $[\theta]_m$.

[1] Abbreviations used: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EPR electron paramagnetic resonance; MCD, magnetic circular dichroism; DEAE, diethylaminoethyl.

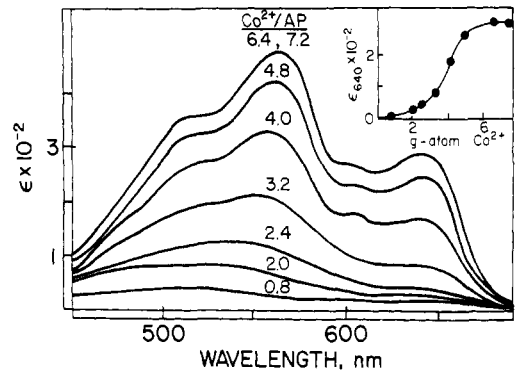

FIGURE 1: Absorption spectra of cobalt phosphatases. Apoenzyme (0.162 mM) in 20 mM Hepes, pH 8.0, 23 °C, was titrated with molar excesses of cobalt sulfate as indicated. In this and all succeeding figures, all absorption and MCD spectra have been corrected by subtracting the contribution of the apoenzyme. Absorptivity refers to the molar concentration of the protein and not of the metal. In succeeding figures conditions are the same unless stated otherwise.

Electron paramagnetic resonance measurements were performed at 5 K with a Varian E-9 spectrometer (Kennedy et al., 1972). To avoid time-dependent side reactions observed in Tris-HCl, absorption and MCD spectra were determined on samples in 20 mM Hepes, pH 8.0. However, all electron paramagnetic resonance (EPR) measurements were performed on samples in Tris where the definition of the spectra is superior to that in Hepes buffer.

The conditions and procedural details of the hydrogen–tritium exchange experiments have been described (Brown et al., 1974).

Results

Titration of Apoalkaline Phosphatase with Cobalt. Titration with cobalt sulfate of metal-free apoalkaline phosphatase, 0.162 mM, in Hepes buffer, 20 mM, pH 8.0, 23 °C, generates a well-defined absorption spectrum between 450 and 700 nm increasing in intensity until 6 g-atoms of cobalt have been added (Figure 1). The first 2 g-atoms of cobalt generate a broad, weak absorption band which extends from 450 to 700 nm with the highest molar absorptivity near 500 nm. Another 2 g-atoms of cobalt—a total of 4 g-atoms—results in a well-defined spectrum with maxima at 510 nm (ϵ 275), 555 (335), 605 (180), and 640 (170). An increase to 6 g-atoms of cobalt largely intensifies the existent bands and results in maximal molar absorptivities of ϵ_{510} 365, ϵ_{555} 480, ϵ_{605} 270, and ϵ_{640} 295. The insert of Figure 1 shows the absorbance at 640 nm as a function of the number of gram-atoms of cobalt added to the apoenzyme. Between 0 and 2 g-atoms, the intensity is approximately 15% of maximum; it rises linearly only after addition of 3 g-atoms and becomes maximal between 5 and 6 g-atoms but does not change further thereafter.

The features of the MCD spectra correspond closely to those of the absorption spectra. When less than 2 g-atoms of cobalt are present, the magnetically induced ellipticity above 600 nm is minimal (Figure 2), but further additions up to a total of 6 g-atoms of cobalt generate negative extrema at 555 ($[\theta]_M^{23} = -260$), 605 ($[\theta]_M^{23} = -245$) and 640 nm ($[\theta]_M^{23} = -210$) and a positive extremum at 475 nm ($[\theta]_M^{23} = +45$) (Figure 2).

Figure 3 shows the cobalt EPR titration of apoalkaline phosphatase, 0.162 mM, 10 mM Tris-HCl, pH 8.0. Addition of 2 g-atoms of cobalt to apophosphatase generates hyperfine structure in the region of $g_1 \cong 6.0$; overall the EPR spectra are

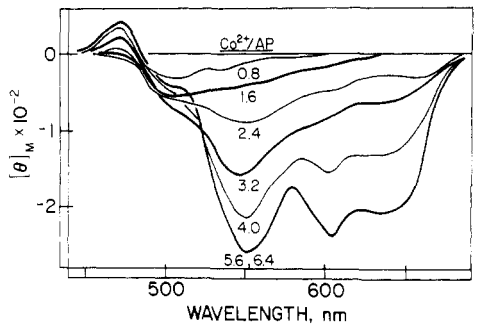

FIGURE 2: MCD spectra of cobalt phosphatases. Molecular magnetic ellipticities, $[\theta]_M$, are given in deg cm^2 dmol^{-1} kG^{-1} with reference to the molar concentration of protein. All MCD spectra are corrected for the CD component.

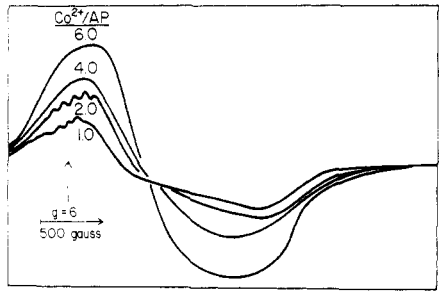

FIGURE 3: EPR spectra of cobalt phosphatases. Apoenzyme, 0.162 mM in 10 mM Tris-HCl, pH 8.0; microwave power, 10 mW, modulation frequency 100 kHz, microwave frequency 9.39 kHz, 5 K.

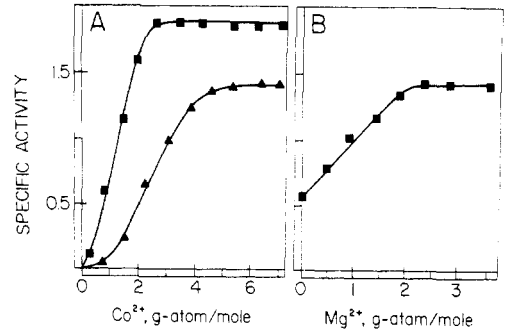

FIGURE 4: (A) Hydrolase activities of cobalt phosphatases ± magnesium. Aliquots of enzymes in Figure 1 were assayed in 20 mM Veronal-400 mM NaCl, pH 8.0, 25 °C, minus magnesium (▲) and plus magnesium (■); all assays were performed 5 min after adding cobalt. Conditions for the cobalt enzymes plus magnesium are described in Figure 5. (B) Effect of magnesium on activity of 2-Co(II) phosphatase. 2-Co(II) phosphatase in 20 mM Hepes, pH 8.0, 23 °C, was titrated with the designated molar excesses of magnesium and assayed after 15 min as described in A.

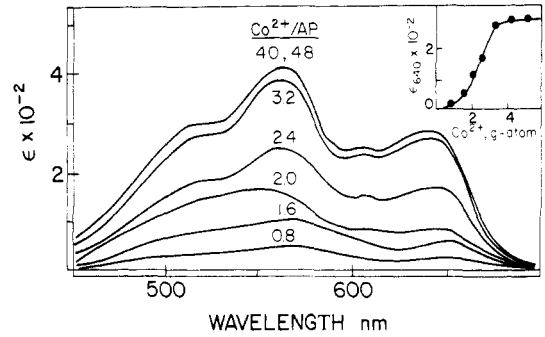

FIGURE 5: Absorption spectra of cobalt phosphatases plus magnesium. The apoenzyme (0.162 mM) in 20 mM Hepes, pH 8.0, 23 °C, was incubated with a 5.7 molar excess of magnesium for at least 3 h before adding cobalt.

consistent with cobalt atoms predominantly in octahedral coordination geometry (Kennedy et al., 1972). The EPR spectrum of 4-Co alkaline phosphatase[2] is more complex and unsuitable for interpretation in terms of three g values. It resembles that of a magnetically concentrated sample and could reflect intramolecular magnetic interactions. Further addition of cobalt up to 6 g-atoms increases the amplitude of the EPR signal but does not alter it qualitatively (Figure 3).

Cobalt atoms in octahedral-like coordination geometry have been thought to reflect structural ions which stabilize and, hence, indirectly affect the function of alkaline phosphatase. Based on the octahedral-like spectra of 1-Co phosphatase (Figures 1-3), the activity of this species would be expected to be minimal, in accord with the data (Figure 4A, lower curve). However, 2-Co phosphatase exhibits 0.5 unit of activity and the 4-Co enzyme, 1.4 units, unaltered by further addition of cobalt (Figure 4A, lower curve). Thus, once 4 g-atoms of cobalt have been added, enzymatic activity does not change, although the spectral manifestations become constant only after addition of 6 g-atoms.

Titration of Magnesium Phosphatase with Cobalt. Magnesium itself does not induce activity, but it does alter the activity of zinc and cobalt alkaline phosphatase (Plocke and Vallee, 1962; Anderson et al., 1975; Bosron et al., 1976). Hence, we have sought to correlate the effect of magnesium on both the functional and spectral properties of cobalt phosphatase.

Extensive kinetic and thermodynamic studies demonstrate that magnesium binds to only two sites of alkaline phosphatase, but not to its primary zinc binding sites (Bosron et al., 1976). Therefore, to ensure occupancy of the magnesium sites[3] apophosphatase (0.162 mM) was incubated for 5 h with magnesium (0.92 mM) before addition of cobalt. Titration of this magnesium phosphatase with cobalt sulfate generates a well-defined absorption spectrum between 450 and 700 nm whose intensity increases until 4 g-atoms of cobalt have been added (Figure 5). In the presence of magnesium the molar absorptivities for the 4-Co enzyme are ϵ_{510} 310, ϵ_{555} 425, ϵ_{605} 260, and ϵ_{640} 295. The cobalt absorption titration curve at 640 nm rises linearly after addition of 1.5 g-atoms of cobalt and becomes maximal at 4 g-atoms, a significant shift to lower cobalt concentrations compared with the titration curve in the absence of magnesium (Figure 1, insert). Thus, in the absence of magnesium it requires 6 g-atoms of cobalt to achieve maximal absorptivity (Figure 1), but 4 g-atoms of cobalt per mol of enzyme suffice in its presence (Figure 5).

The characteristics of the cobalt MCD titration of magnesium phosphatase correspond to those of its absorption titrations, with well-defined extrema on addition of less than 2 g-atoms of cobalt and maximal ellipticity on addition of 4 g-atoms (Figure 6). Increments between 2 and 4 g-atoms further resolve and increase the ellipticities; there are negative extrema at 555 ($[\theta]_M^{23} = -180$), 605 ($[\theta]_M^{23} = -195$), and 640

[2] The number of metal atoms added to alkaline phosphatase is identified by an Arabic number preceding the metal symbol, and Roman numerals after the metal symbol indicate the presumed oxidation state of the metal; e.g., 2-Co(II)-alkaline phosphatase and 2-Co(III) phosphatase identify the enzyme containing 2 g-atoms of cobalt per mol of enzyme in the reduced and oxidized states, respectively.

[3] The terms "zinc sites" or "magnesium sites" identify those sites which bind zinc or magnesium in the native enzyme.

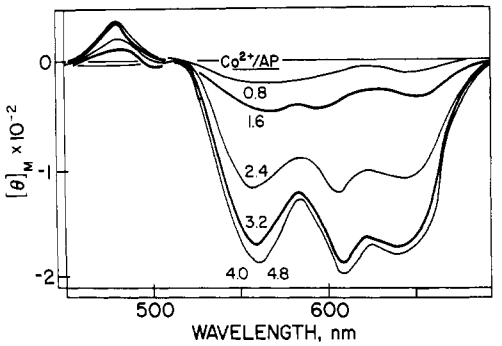

FIGURE 6: MCD spectra of cobalt phosphatases plus magnesium. Incubation as in Figure 5; see also Figure 2.

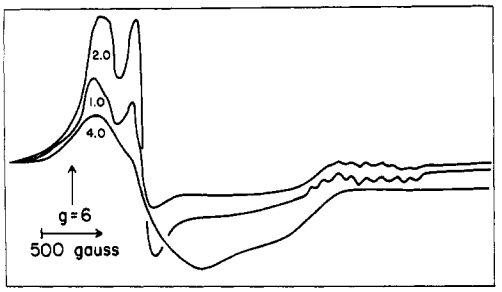

FIGURE 7: EPR spectra of cobalt phosphatases plus magnesium. Apoenzyme (0.162 mM) in 10 mM Tris-HCl, pH 8.0, was incubated with 5.7 molar excess of magnesium for at least 3 h prior to adding cobalt. Spectra were measured at 5 K.

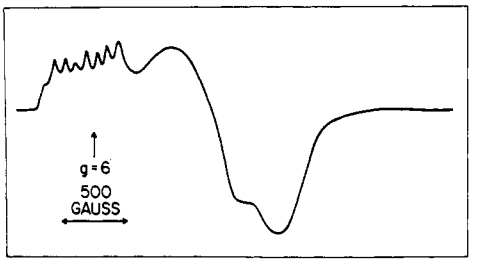

FIGURE 8: EPR spectrum of 4-Zn 2-Co alkaline phosphatase. Four gram-atoms of zinc were added to apophosphatase followed by 2 g-atoms of cobalt (other conditions as in Figure 3).

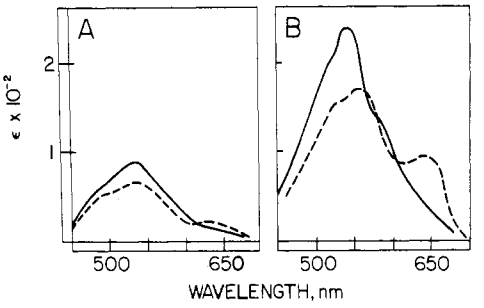

FIGURE 9: Effect of phosphate on the absorption spectra of 2-Co(II) phosphatase ± magnesium. (A) Phosphate (0.48 mM) was added to 2-Co(II) phosphatase (0.162 mM) in 20 mM Hepes, pH 8.0, 23 °C, and the spectra were recorded. (B) Same as A, except the apoenzyme was preincubated for 3 h with magnesium before adding cobalt.

($[\theta]_M^{23} = -175$) and a positive extremum at 475 nm ($[\theta]_M^{23} = +45$).

Accompanying the magnesium-dependent increases in absorption (Figure 5) and magnetically induced ellipticities (Figure 6), enzymatic activity increases significantly, more markedly at lower molar ratios of cobalt to enzyme, e.g., magnesium enhances the activity of the 2-Co enzyme by more than 225% but that of the 4-Co enzyme increases only 35% (Figure 4). Moreover, in the absence of magnesium, addition of 4 g-atoms of cobalt generates 1.4 units of activity, unaltered by further cobalt additions (vide supra), while in its presence, addition of only 2.2 g-atoms of cobalt generates 1.9 units of activity which also remains constant thereafter.

The changes in the absorption and MCD spectra also reflect in the EPR spectra. The EPR spectra of 1 and 2 cobalt magnesium phosphatases are well resolved with three distinct spectral g values, $g_1 = 4.95$, $g_2 = 3.98$, and $g_3 = 1.95$ and an average g value of 3.62, consistent with that of cobalt in a distorted 4 or 5 coordinate geometry (Figure 7) (Cotton et al., 1963; Carlin, 1965; Kennedy et al., 1972). The 2-Co 1.6-Mg enzyme[4] exhibits hyperfine splitting in g_3 with eight resolved hyperfine lines, characteristic of cobalt with a nuclear spin of $7/2$, seemingly the first cobalt-substituted metalloenzyme in which these features have been observed. The EPR spectrum of the 4-Co 1.6-Mg enzyme is resolved poorly (Figure 7), similar to that observed on addition of solely 4 or 6 g-atoms of cobalt (Figure 3).

Magnesium Titration of Cobalt Phosphatase. To ascertain the amount of magnesium required for both maximal activity and spectral properties, various cobalt enzymes were titrated with magnesium. Magnesium linearly increases the activity of the 2-Co enzyme from 0.6 to 1.4 units to become optimal on addition of approximately 2 g-atoms of magnesium (Figure 4B) which simultaneously maximize the spectral properties. The results with 1- and 4-Co phosphatases are similar. In all instances the sequence of additions of magnesium and cobalt affects neither the final enzymatic activities nor the spectra.

Properties of 4-Zn 2-Co Hybrid Phosphatase. Under the conditions employed (Bosron et al., 1976) zinc binds readily to only four sites, cobalt binds both to these four and the two magnesium sites, a total of six (Anderson et al., 1975). Further, once zinc is bound, cobalt does not readily displace it (Simpson and Vallee, 1968). Apparently, when added to the 4-Zn enzyme, 2 g-atoms of cobalt bind at the magnesium sites, thereby becoming spectral probes of their geometry.

Addition of 2 g-atoms of cobalt to 4-zinc phosphatase results in a broad, featureless absorption spectrum extending from 400 to 600 nm, with a molar absorptivity of less than 30, matched by a similarly weak MCD signal near 510 nm (not shown). However, the well-resolved EPR spectrum of the 4-Zn 2-Co enzyme exhibits hyperfine splitting in g_1 with $A_1 = 0.024$ cm^{-1} and $g_1 = 6.17$, $g_2 = 3.25$, and $g_3 = 2.62$ with an average g value of 4.01, consistent with octahedral cobalt geometry (Figure 8) (Kennedy et al., 1972).

Effect of Phosphate on the Spectra of Cobalt and Metal Hybrid Enzymes. Phosphate is an inhibitor, substrate, and product of alkaline phosphatase and differentially affects the spectra of cobalt phosphatase, depending on the sites which the metal occupies. It does not alter the spectra of the 4-Zn 2-Co enzyme (Figure 8), where cobalt apparently occupies the octahedral-like magnesium sites, and minimally changes the spectra of the 2-Co enzyme (Figure 9), where the major fraction of cobalt atoms are octahedral-like (Figures 1–3); but phosphate significantly alters the absorption (Figure 9), CD, MCD, and EPR spectra (not shown) of the 2-Co 1.6-Mg enzyme, where the majority of cobalt atoms are in a pentacoor-

[4] Under the conditions employed, 2- and 4-Co-alkaline phosphatases bind 1.6 g-atoms of magnesium (see Materials and Methods).

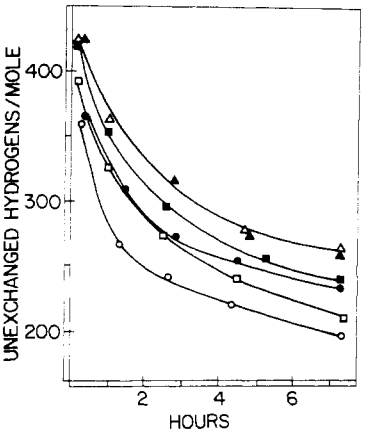

FIGURE 10: Hydrogen-tritium exchange of apo- and cobalt phosphatases ± magnesium. All exchanges were performed in 10 mM Tris-HCl, pH 8.0, 4 °C. Protein concentrations were 10 mg/ml prior to passage over the large column and 1–2 mg/ml during subsequent incubations; apophosphatase (○); 2-Co(II) phosphatase (□); 4-Co(II) phosphatase (△). Closed symbols represent the same enzymes containing magnesium; under these conditions the apoenzyme binds 1.4 g-atoms of magnesium and the 2- and 4-Co(II) enzymes bind 1.6 g-atoms of magnesium.

dinate-like geometry[5] (Figures 5–7). Addition of phosphate to the 2-Co 1.6-Mg enzyme nearly abolishes the absorption band at 640 nm and generates a new maximum at 550 nm (Figure 9). Similarly, it perturbs the spectra of the 4- and 6-Co and the 4-Co 1.6-Mg enzymes in which the pentacoordinate-like sites are also occupied by cobalt. While the intensities of the spectra of these phosphate enzymes vary, overall their characteristics are similar and resemble those of the 2-Co 1.6-Mg enzyme–phosphate complex.

Effect of Magnesium on the Hydrogen–Tritium Exchange of Apo- and Cobalt phosphatases. Reconstitution with 2 or 4 g-atoms of cobalt per mol of apophosphatase significantly stabilizes the structure of the protein as evidenced by the marked retention of tritium (Figure 10). The rate of tritium exchange of the 2-Co phosphatase differs both from that of the apo- and of the 4-Co phosphatases (Figure 10) (Brown et al., 1974). Magnesium additionally stabilizes the structure both of the apoenzyme and of that containing 2 g-atoms of cobalt, which is then stabilized further by 2 more cobalt atoms to form the 4-Co 1.6-Mg enzyme. Thus, magnesium markedly affects the structural properties of the apoenzyme and of the 2-Co enzyme but has little effect on the structural properties of 4-Co phosphatase.

Discussion

Metals participate in catalysis and structure stabilization of metalloenzymes, but the details of the mechanisms by which they exert their important biological roles remain largely undefined. In particular, the mode of binding, coordination geometry, and other physical-chemical bases of biological function and specificity of zinc enzymes have eluded recognition, in large measure due to the diamagnetism and lack of chromophoric properties of this d^{10} ion. However, the chromophoric, paramagnetic cobalt(II) ion readily substitutes for zinc, yielding enzymatically active enzymes with characteristic absorption, MCD, and EPR spectra which are essential to probe the environment of the metal. In this manner, a number of cobalt(II)-substituted zinc enzymes, including alkaline phosphatase, have served to elucidate the nature of cobalt geometry and, indirectly, that of zinc in zinc metalloenzymes (Vallee and Latt, 1970).

Theoretically, the spectral characteristics of cobalt complexes reflect the number of ligands, their geometry, bonding, and vicinal features; however, even for simple ions, evaluation of details which might lead to specific assignments has proved difficult (Cotton et al., 1963; Carlin, 1965; Kennedy et al., 1972). The assignment of geometry and related features to the multiple metal binding sites of alkaline phosphatase has turned out to be even more complex. The spectra of cobalt alkaline phosphatase have been inferred to reflect two pentacoordinate-like and two distorted octahedral-like sites[5] (Simpson and Vallee, 1968). The present and other recent studies confirm this postulate and further demonstrate a third class of cobalt-binding sites which are occupied by magnesium in the native enzyme (Anderson et al., 1975; Bosron et al., 1975). Thus, there are a total of six metal-binding sites in phosphatase; magnesium binds only to two, four are relatively specific for zinc, but all six can bind cobalt, apparently indiscriminately (Anderson et al., 1975; Bosron et al., 1975, 1976).

Since cobalt will not readily displace zinc (Simpson and Vallee, 1968) but does bind to the magnesium sites, addition of cobalt to 4-Zn phosphatase results in a species whose spectrum contains information regarding the coordination geometry of the cobalt atoms which interact at the magnesium sites (Anderson et al., 1975). As judged from the EPR spectrum of this 4-Zn 2-Co enzyme (Figure 8), cobalt is octahedral-like when bound at the magnesium sites, as substantiated by the difference absorption and MCD spectra of the 6-Co and 4-Co, 1.6-Mg enzymes. While the difference absorption spectrum of these two species is featureless above 600 nm, the molar absorptivities of the 6-Co enzyme between 450 and 575 nm, ϵ_{510} 365 and ϵ_{555} 480, exceed those of the 4-Co 1.6-Mg enzyme, ϵ_{510} 310 and ϵ_{555} 450, consistent with the coordination of additional cobalt atoms to the 6-Co enzyme in an octahedral-like manner. In the MCD spectrum of the 6-Co enzyme, the magnetically induced, negative extremum at 555 nm exhibits the largest ellipticity (Figure 2), while at 605 nm that of the 4-Co 1.6-Mg enzyme is the largest (Figure 6). This difference in the 555-nm region also supports the postulate that compared with the 4-Co 1.6-Mg enzyme the two additional cobalt atoms of the 6-Co enzyme are in an octahedral-like environment (Kaden et al., 1974; Holmquist et al., 1975).

The effects of magnesium on the coordination geometry of cobalt atoms bound to the zinc sites are evident from comparisons of the spectra and activities of the enzymes which either contain solely cobalt or cobalt plus magnesium. When magnesium is absent, the absorption, MCD, and EPR spectra of the 1- and 2-Co enzymes reflect a marked predominance of octahedral-like geometry, though they suggest that a fraction of atoms are in a pentacoordinate-like geometry (Figures 1–3), but in its presence, the spectra are consistent with the predominance of this geometry (Figures 5–7). These spectral changes are accompanied by a threefold increase in activity (Figure 4). In the 4-Co enzyme, magnesium increases the activities (Figure 4), absorptivities (Figure 5), and magnetic molar ellipticities of the respective spectra (Figure 6), but there is no suggestion of alterations in coordination geometry. The addition of two more cobalt ions to 2-Co 1.6-Mg phosphatase

[5] "Pentacoordinate-like sites" is used to denote those sites which bind cobalt and generate spectra with absorption, and MCD extrema near 640 nm. "Pentacoordinate-like" is not intended to constitute a specific assignment or identification of these sites but is to serve solely as an operational definition to describe the sites which might actually be 4 or 5 coordinate-like. Similarly, "octahedral-like" is used to denote sites which bind cobalt and yield spectral properties similar to those observed for octahedral model compounds (Kennedy et al., 1972; Kaden et al., 1974).

decreases the amplitude of the EPR signal (Figure 7), possibly due to dipolar broadening, which may reflect magnetic interactions of cobalt—not changes in geometry. Such broadening becomes evident when cobalt occupies more than two zinc sites and could suggest that these are close to one another, perhaps resembling the metal sites of superoxide dismutase (Richardson et al., 1975) or of concanavalin A (Becker et al., 1975).

Phosphate, a substrate, product and/or inhibitor, affects the largely octahedral-like spectra of 2-Co phosphatase to a minor extent (Figure 9), but it significantly alters the pentacoordinate-like cobalt ions of 2-Co 1.6-Mg (Figure 9), 4-Co (Simpson and Vallee, 1968), and 4-Co 1.6-Mg phosphatases (not shown). Apparently phosphate perturbs the spectra of the pentacoordinate-like cobalt ions but not those in an octahedral-like geometry. The failure of phosphate to alter the spectra of cobalt bound to the magnesium sites and its failure to bind to the enzyme which contains only magnesium reinforce this conclusion (Bosron et al., 1976).

Spectral titrations, metal-binding and metal-activity titrations qualitatively differentiate three pairs of metal-binding sites, among which the metal atoms distribute in the course of titrations. The approaches employed thus far cannot determine accurately the degree to which this distribution is ordered, random, the result of metal migration and equilibration or of conformational changes of the protein or all of these. Further, save for the involvement of histidine (Tait and Vallee, 1966; Taylor and Coleman, 1972), the identities of the metal binding sites are unknown, and, clearly, more precise knowledge of the identity of the ligands of the various metal species would greatly facilitate their localization. Unfortunately, there are no suitable models which bear on the origins of such remarkable interrelationships between different metal atoms and each of their roles in enzymatic catalysis, structure stabilization, and regulation of alkaline phosphatase. It might have been hoped that the stabilities of the various metal complexes with the different types of binding sites would be distinctive for each category of metal resulting in distinctive site occupancies. If this had proved to be the case, the remarkable effects of magnesium both on activity, spectra, and coordination geometry could have been explained directly.

The present and previous data demonstrate (Anderson et al., 1975), however, that both cobalt and magnesium—but not zinc—bind readily to the magnesium sites, but the known complex ion chemistry would not lead to the expectation of this finding.

In this regard, it should be kept in mind, of course, that the spectral evidence for magnesium-induced changes in coordination geometry might be *apparent* only since spectra from which such changes could be inferred are confined to those species in which some metal binding sites are vacant. They do not seem to occur in derivatives where all six sites are occupied and, hence, the possibility cannot be dismissed that addition of magnesium to the 2-Co phosphatase could induce such spectral changes either through an ordered redistribution of metal ions between the occupied and unoccupied sites or changes in overall rather than strictly local conformation.

Some of the experimental observations are not inconsistent with such a postulate. As gauged by hydrogen–tritium exchange, magnesium stabilizes the structure of 2-Co phosphatase, but hardly affects that of the 4-Co (Figure 10) or 4-Zn enzymes (Anderson et al., 1975). Further, it prevents both the time-dependent loss of activity in 2-Zn 1.6-Mg phosphatase and the accompanying increased rate of H $\rightleftarrows$ T exchange of 2-Zn phosphatase. Metal migration and an ordered redistribution could account for these observations, both regarding the catalytic and structural properties of the 2-Zn (or 2-Co) species. However, the activities of the 4-Zn (or 4-Co) enzyme which are enhanced by magnesium would then have to be postulated to be mechanistically unrelated.

A possible role of metal migration can be examined by oxidation of Co(II) to Co(III) (Anderson and Vallee, 1975). Co(II) is exchange labile relative to the exchange inert Co(III) and, hence, the potential for migration of the latter is greatly reduced. Further, Co(III) distinctly prefers nitrogen donors and octahedral coordination geometry. Hence, given sufficiently dissimilar ligands at different metal-binding sites, the spectral changes and retardation in ligand exchange rates which accompany the conversion of Co(II) to Co(III) might aid in identifying and differentiating the classes of metal-binding sites of polymeric metalloenzymes.

The present and previous data (Anderson et al., 1975; Bosron et al., 1975, 1976) document a major role of magnesium in regulating alkaline phosphatase. It remains to assess and control the mutual effects of metals and their relationship to monomer $\rightleftarrows$ polymer equilibria, subunit interactions, structure stabilization, local or overall protein conformation and other sources of experimental uncertainties that may vary in the course of experiments designed to define their existence.

The present studies reorient the nature and scope of the critical questions to be asked regarding the diverse roles of metals in catalysis, structure stabilization, and regulation of *E. coli* alkaline phosphatase and other polymeric metalloenzymes and of the experiments that can be designed to answer them.

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Role of Bound Calcium Ions in Thermostable, Proteolytic Enzymes. Separation of Intrinsic and Calcium Ion Contributions to the Kinetic Thermal Stability†

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ABSTRACT: The total kinetic thermal stability of a protein molecule, expressed as the total free energy of activation in thermal denaturation reactions, can be separated into an intrinsic contribution of the polypeptide chain and a contribution due to the binding of calcium ions. The theory for this procedure is applied to thermal denaturation data, obtained at the pH of optimum stability, for the serine proteases, thermomycolase and subtilisin types Carlsberg and BPN′, and for the zinc metalloendopeptidases, thermolysin and neutral protease A. The results, obtained from Arrhenius plots at high and low free calcium ion concentrations, reveal a considerable variation in the calcium ion contribution to the total kinetic thermal stability of the various enzymes. In the serine protease group, at 70 °C, the stability is largest for thermomycolase, mainly due to a relatively high intrinsic contribution. For the metalloendopeptidases the total kinetic thermal stability is largest for thermolysin, the difference between thermolysin and neutral protease A being dominated by bound calcium ion contributions. The intrinsic kinetic thermal stability of the polypeptide chain of thermolysin is considerably smaller than that of any of the serine proteases and is probably of the same order of magnitude as that of neutral protease A. Thus, the well known total kinetic thermal stability of thermolysin is due mainly to a *single* calcium ion (Voordouw, G., and Roche, R. S. (1975), *Biochemistry 14,* 4667) that binds with high affinity even at very high temperatures ($K \simeq 6 \times 10^7$ M^{-1} at 80 °C).

The reason for the successful survival of thermophilic microorganisms at the high temperatures preferred or needed by these organisms is intriguing. One may suggest that an increased stability of many intra- and extracellular components is a likely explanation. In the case of the proteins, such an enhanced stability, compared to similar protein molecules isolated from a mesophilic source, has indeed frequently been demonstrated. The data presented are generally limited to the kinetics of the denaturation process: the enhanced stability is thus kinetic and not necessarily thermodynamic in origin. The observation of slow-denaturation kinetics for enzymes isolated from thermophilic microorganisms compared to those from their mesophilic counterparts has led to the description of the former as "thermostable" enzymes. The distinction between "thermostable" and nonthermostable is, however, largely subjective.

Nevertheless, the marked difference in kinetic thermal stability that is observed even between extensively sequence homologous and thus probably conformationally homologous enzymes asks for an explanation. One current strategy aimed at the solution of this problem is to elucidate, in as much detail as is presently possible, the three-dimensional structures of homologous enzymes isolated from a thermophilic and a mesophilic source. By comparing the structures one hopes to obtain pertinent information relevant to the kinetic thermal stability of the "thermostable" enzyme. This approach has led, for instance, to the elucidation of the sequence (Titani et al., 1972) and three-dimensional structure of thermolysin, the thermostable zinc metalloendopeptidase produced by *B. thermoproteolyticus* (Matthews et al., 1972a,b; Colman et al., 1972; Matthews and Weaver, 1974; Matthews et al., 1974). Sequence studies of neutral protease A, the zinc metalloendopeptidase produced by *B. subtilis*, have advanced considerably (Pangburn et al., 1973, 1975, 1976; Pangburn, 1973) and, with 54% of the sequence completed, indicate a high de-

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