Proc. Nat. Acad. Sci. USA
Vol. 72, No. 1, pp. 394–397, January 1975

Cobalt(III), a Probe of Metal Binding Sites of *Escherichia coli* Alkaline Phosphatase

(metalloprotein/cobalt oxidation/spectral properties)

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Contributed by Bert L. Vallee, November 5, 1974

ABSTRACT **To facilitate the study of individual metal binding sites of polymeric metalloproteins, conversion of exchange-labile Co(II) in *E. coli* alkaline phosphatase (EC 3.1.3.1) to exchange-inert Co(III) was examined. Oxidation of Co(II) alkaline phosphatase with hydrogen peroxide results in a single absorption maximum at 530 nm and loss both of the characteristic electron paramagnetic signal and of enzymatic activity. Zinc neither reactivates this enzyme nor displaces the oxidized cobalt atoms. Metal and amino-acid analyses demonstrate that oxidation alters neither cobalt binding nor amino-acid composition of the enzyme. All data are consistent with the conclusion that hydrogen peroxide oxidizes Co(II) in alkaline phosphatase to Co(III). Polymeric metalloenzymes can contain different categories of metal atoms serving in catalysis, structure stabilization, and/or control and exerting their effects independently or interdependently. The *in situ* conversion of exchange-labile Co(II) to exchange-stable Co(III) offers a method to selectively and differentially "freeze" cobalt atoms at their respective binding sites. The accompanying spectral changes and concomitant retardation in ligand exchange reactions may be used to differentiate between specific metal binding sites that serve different roles in polymeric metalloenzymes.**

Alkaline phosphatase [EC 3.1.3.1; orthophosphoric-monoester phosphohydrolase(alkaline optimum)] isolated from *Escherichia coli* contains 4 g-atoms of zinc per molecular weight 89,000. One pair of zinc atoms is involved primarily in catalysis, while the second pair appears to play a predominantly structural role (1). The zinc atoms can be replaced by Co(II), Ni(II), Cu(II), Cd(II), Mn(II), Fe(II), and Hg(II) but, by steady-state kinetics, only the cobalt-substituted enzyme is catalytically active (2). While the zinc enzyme is both a phosphohydrolase and a phosphotransferase, the cobalt enzyme displays only hydrolase activity (3). This enzymatically active, cobalt-substituted enzyme also exhibits a very characteristic visible absorption spectrum (1).

All of the metal ions that have been demonstrated to bind to alkaline phosphatase are exchange-labile (4). However, oxidation transforms Co(II) from the exchange-labile (d^7) to the exchange-inert (d^6) Co(III) state. Preliminary evidence for carboxypeptidase (5) and carbonic anhydrase (6) indicates that the cobalt in Co(II)-substituted enzymes can be oxidized to Co(III), which is exchange-inert.

Chromophoric metal atoms at the active sites of metalloenzymes, e.g., alkaline phosphatase, exhibit spectral properties distinct from those at structural or regulatory sites (7, 8). Yet, it has proven difficult to differentiate the nature and number of ligands that constitute the respective binding sites, e.g., by organic chemical modification in the absence and presence of metals.

In order to explore and differentiate the interrelationships between catalytic and structural metal atoms in alkaline phosphatase, 2 Co(II)† phosphatase was oxidized with hydrogen peroxide to convert the exchange-labile cobalt(II) to exchange-inert cobalt(III), resulting in complete loss of enzymatic activity. Preliminary studies suggest that once the first pair of Co(II) atoms is oxidized, the second pair in 4 Co(II) phosphatase is not similarly susceptible to oxidation. Kinetic measurements, absorption, circular dichroic and electron paramagnetic spectra, as well as metal and amino-acid analyses support the conclusion that the formation of Co(III) alkaline phosphatase accounts for the loss of activity.

MATERIALS AND METHODS

Alkaline phosphatase was prepared by DEAE–cellulose chromatography, except that Tris·HCl, pH 7.5, was substituted for imidazole chloride (9). Enzymatic activity was measured with 1 mM *p*-nitrophenyl phosphate substrate, in 20 mM veronal–0.4 M NaCl or in 1 M Tris·HCl, pH 8.0, 25°. The activity of the cobalt enzyme varies from 5 to 10% of that of the zinc enzyme, depending upon the assay buffer (3). The 2 Co(II) enzyme exhibits approximately 35% of the activity of the 4 Co(II) enzyme when assayed in 20 mM veronal–0.4 M NaCl and approximately 25% of that activity when assayed in 10 mM Tris·HCl–1 M NaCl (1). Protein concentrations were determined by the method of Lowry *et al.* (10). A molecular weight of 89,000 served for all calculations involving molarity of alkaline phosphatase (1). Apoalkaline phosphatase was prepared by the procedure of Simpson and Vallee (1), with 8-hydroxyquinoline-5-sulfonic acid. To reconstitute the apoenzyme, metal solutions were prepared from spectrographically pure sulfate salts (Johnson, Matthey Co., Ltd.) dissolved in metal-free distilled water. Zinc and cobalt were measured by atomic absorption spectrometry (11). Buffers,

Abbreviation: EPR, electron paramagnetic resonance.

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† The number of metal atoms added to alkaline phosphatase is identified by an arabic number preceding the metal symbol; e.g., 2 Co alkaline phosphatase and 4 Co alkaline phosphatase refer to the enzyme to which 2 and 4 Co atoms have been added, respectively. Roman numerals indicate the presumed oxidation state; e.g., 2 Co(II) alkaline phosphatase and 2 Co(III) alkaline phosphatase identify the enzyme containing 2 cobalt atoms in the reduced and oxidized states, respectively.

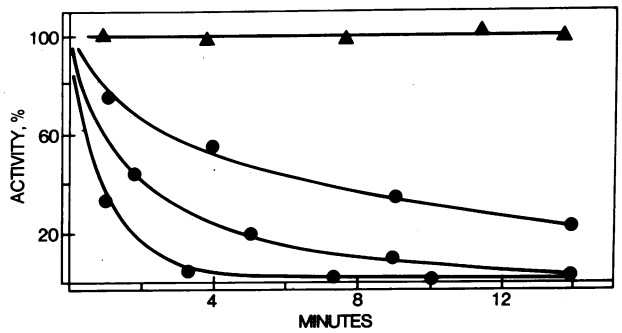

FIG. 1. Hydrogen peroxide inactivation of 2 Co(II) alkaline phosphatase. 2 Co(II) alkaline phosphatase, 2.24×10^{-4} M, in Tris·HCl, 10 mM, pH 8.0, plus (from the bottom in sequence): hydrogen peroxide, 4.45×10^{-2} M, 8×10^{-3} M, and 4.45×10^{-3} M (●). 2 and 4 Zn(II) alkaline phosphatase, conditions as above, plus 4.45×10^{-2} M hydrogen peroxide (▲). Aliquots were removed at times indicated and assayed in 20 mM veronal–0.4 M NaCl as described under *Materials and Methods*. The enzymatic activity before addition of hydrogen peroxide is designated as 100%.

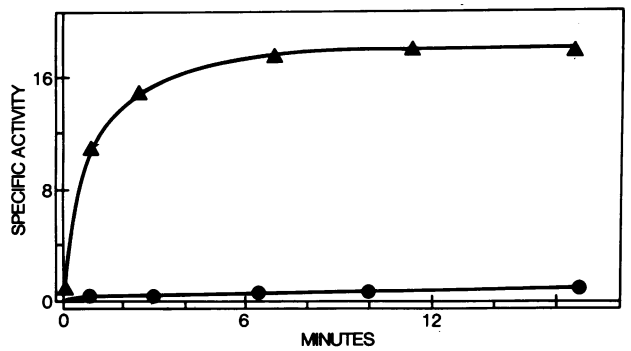

FIG. 2. Effect of Zn addition on activities of 2 Co(II) alkaline phosphatase ± hydrogen peroxide. Zn(II), 1.66×10^{-3} M, was added to 2 Co(II) alkaline phosphatase, 2.24×10^{-4} M, at zero time (▲); same, but enzyme was inactivated with hydrogen peroxide, 1.12×10^{-2} M, prior to addition of Zn (●). Enzyme assays as in legend of Fig. 1.

glassware, and substrate were rendered metal-free as described (12). After metal extraction with dithizone, buffers were stored in the presence of Chelex 100 (10% v/v) to maintain complete freedom from contaminating metals.

Visible absorption spectra were obtained with a Cary Model 14 spectrophotometer equipped with a 0–0.1 absorbance slidewire. Electron paramagnetic measurements were performed at 5°K with a Varian E-9 spectrometer (13).

Amino-acid analyses were carried out with a Beckman 120 B amino-acid analyzer. Samples were hydrolyzed for 24 hr in evacuated tubes with 6 N HCl at 110°. Tryptophan content was determined by magnetic circular dichroism (14).

RESULTS AND DISCUSSION

Apoalkaline phosphatase was reconstituted with 2 g-atoms of cobalt and then reacted with varying concentrations of hydrogen peroxide to render the cobalt atoms exchange inert. Enzymatic activity is lost completely in less than 5 min when 2 Co(II) alkaline phosphatase, 2.24×10^{-4} M, is incubated with hydrogen peroxide, 4.45×10^{-2} M, in 10 mM Tris·HCl, pH 8.0, 22° (Fig. 1). The patterns of inactivation are identical whether assayed in 20 mM veronal–0.4 M NaCl, pH 8.0, (Fig. 1) or in 1 M Tris·HCl, pH 8.0. Decreasing concentrations of hydrogen peroxide result in progressively slower rates of inactivation.

In contrast, hydrogen peroxide has no effect on the enzymatic activities of 2 Zn or 4 Zn alkaline phosphatase; both remain fully active (Fig. 1). Moreover, incubation of the apoenzyme, 2.24×10^{-4} M, with hydrogen peroxide, 4.45×10^{-2} M, for 1 hr followed by addition of Zn(II), 1×10^{-3} M, results in complete restoration of enzymatic activity equal to that of the native zinc enzyme. Thus, hydrogen peroxide affects neither the functional potential of the apoenzyme to bind zinc nor the activity of the restored zinc enzyme, suggesting that in experiments with 2 Co(II) phosphatase it is cobalt that is being oxidized, not amino-acid residues of the protein moiety.

Amino-acid analyses substantiate this conclusion, since the amino-acid composition of the 2 Co(II) enzyme and of that oxidized with hydrogen peroxide, under the same conditions as those used for inactivation in Fig. 3, are indistinguishable. Tryptophan was determined separately by magnetic circular dichroism (14), and the number of tryptophan residues is also identical in the absence and presence of hydrogen peroxide.

Addition of zinc to the inactive, hydrogen peroxide-treated, cobalt enzyme fails to restore its activity (Fig. 2). In contrast, addition of zinc to the untreated active Co(II) control enzyme increases its activity to that characteristic of the zinc enzyme (Fig. 2).

In the presence of 7.4-fold excess of zinc (zinc to protein, M/M), the 2 Co(II) enzyme binds 3.7 ± 0.3 g-atoms of zinc while the oxidized 2 cobalt enzyme binds only 2.0 ± 0.1 g-atoms of zinc (Table 1). Unlike the control, on addition of zinc the number of g-atoms of cobalt of the peroxide-treated enzyme remains the same. Thus, the exchange-inert Co(III) apparently occupies two of the metal binding sites of the hydrogen peroxide-treated enzyme; while zinc readily binds to the two additional metal binding sites, it does not displace Co(III).

TABLE 1. *Zinc binding capacity of 2 Co(II) alkaline phosphatase ± hydrogen peroxide*

Phosphatase derivative	g-atoms of Zn	g-atoms of Co
2 Co(II) (control)	0.05 ± 0.02	2.1 ± 0.1
2 Co(II) + zinc	3.7 ± 0.3	0.9 ± 0.1*
2 Co(II) + H_2O_2	0.05 ± 0.02	2.1 ± 0.1
2 Co(II) + H_2O_2 + zinc	2.0 ± 0.1	2.1 ± 0.1

Co_2SO_4, 4.7×10^{-4} M, was added to 2.24×10^{-4} M apoalkaline phosphatase in 10 mM Tris·HCl, pH 8.0, to form 2 Co(II) phosphatase in each sample. Hydrogen peroxide, 1.18×10^{-2} M, was added as indicated. Twenty minutes after addition of hydrogen peroxide, zinc was added to achieve a final concentration of 1.66×10^{-3} M. After another 20 min had elapsed, 0.25-ml samples were passed over a 1.7 × 15-cm Sephadex G-25 column equilibrated with metal-free 10 mM Tris·HCl, pH 8.0. All samples were analyzed for protein, zinc, and cobalt (see *Materials and Methods*).

* Binding of cobalt by 4 Zn(II) alkaline phosphatase will be the subject of a further communication (Vallee *et al.*, in preparation).

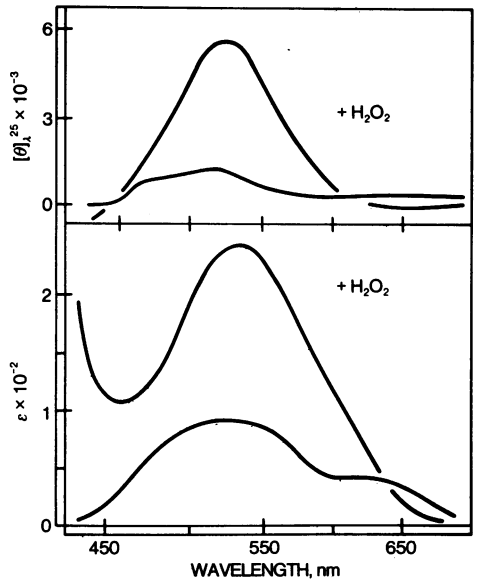

FIG. 3. Absorption and circular dichroic spectra of 2 Co(II) alkaline phosphatase ± hydrogen peroxide. Upper panel: circular dichroism of 2 Co(II) enzyme, 2.24×10^{-4} M, in 10 mM Tris·HCl, pH 8.0 (lower curve); upper curve, same except hydrogen peroxide, 1.12×10^{-2} M, was added. Lower panel: absorption spectrum of enzyme described above (lower curve); upper curve, same except hydrogen peroxide was added. Units for circular dichroism in deg. cm^2 decimole^{-1}.

Fig. 3 illustrates the effect of hydrogen peroxide on the absorption and circular dichroic spectra of 2 Co(II) alkaline phosphatase. The absorption spectrum of the 2 Co(II) enzyme serving as a control is intermediate between those of the apoenzyme reconstituted with either 1.8 or 2.5 g-atoms of cobalt, reported previously (1). In the hydrogen peroxide-treated enzyme (Fig. 3, lower panel), in the range from 400 to 750 nm a single absorption maximum at 530 nm [ϵ = 250 per 2 Co(III)] replaces the bands at 510, 555, and 640 nm of the 2 Co(II) phosphatase control. The circular dichroic spectrum of 2 Co(II) phosphatase exhibits low ellipticity values between 450 and 560 nm (Fig. 3, upper panel). Oxidation with hydrogen peroxide generates a positive extremum centered near 530 nm, $[\theta]_{530}^{23} \cong +5500$, and one negative extremum centered near 620 nm, $[\theta]_{620}^{23} \cong -400$, and another near 400 nm (not shown), indicating significant alterations in optical asymmetry as a result of oxidation, likely reflecting changes in coordination geometry. These large alterations in the absorption and circular dichroic spectra of 2 Co(II) alkaline phosphatase upon addition of hydrogen peroxide are consistent with the formation of Co(III) and constitute significant additional evidence for the oxidation of the cobalt atom.

The loss of the electron paramagnetic resonance (EPR) signal of the cobalt(II) enzyme further documents the oxidation of Co(II) to Co(III) and substantiates the formation of Co(III) alkaline phosphatase‡. The upper curve of Fig. 4

‡ The ground states of octahedral Co(III) complexes are generally diamagnetic. $[Co(H_2O)_3F_3]$ and $(CoF_6)^{3-}$ constitute exceptions, being paramagnetic with four unpaired electrons (17). Most discrete Co(III) complexes known are octahedral, though tetrahedral square-antiprismatic Co(III) complexes have also been observed in a few solid-state situations (18, 19).

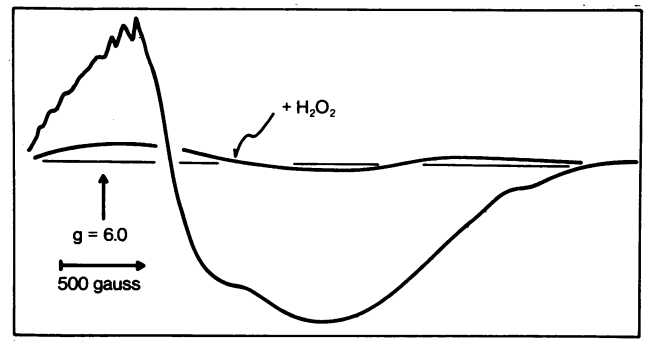

FIG. 4. EPR spectra of 2 Co(II) alkaline phosphatase ± hydrogen peroxide. Conditions are the same as in Fig. 3. Upper curve is before and lower curve after oxidation. Microwave power 10 mW, modulation frequency 100 kHz, microwave frequence 9.39 kHz, 5°K.

shows the EPR spectrum of 2 Co(II) alkaline phosphatase prior to, and the lower one, that subsequent to, oxidation with hydrogen peroxide. The abolition of the EPR signal of the cobalt enzyme upon oxidation is not due to loss of cobalt from the enzyme, as shown by metal analyses which demonstrate that cobalt remains bound to the enzyme after oxidation (Table 1).

All spectral and kinetic data presented are entirely consistent with the oxidation of 2 Co(II) to 2 Co(III) alkaline phosphatase by hydrogen peroxide. Further, under the conditions chosen, this agent does not alter the amino-acid composition of the enzyme. The spectral properties, preference for nitrogen donors, and inertness to substitution reactions render Co(III) a valuable probe for the study of enzyme-metal binding sites. Detailed knowledge of the characterization of such sites has assumed special importance in the light of recent developments. It has become increasingly apparent that several different categories of metal atoms may be present in metalloenzymes; these metal atoms may serve in catalysis, structural stabilization, and control, exerting their effects individually and independently or collectively and interdependently (15, 16). In metalloenzymes, metals that affect function directly by participating in catalysis appear to have ligand sites that differ markedly from those of metals that influence function indirectly through modulation of protein structure (7, 8). These different roles have been discerned by inspection of monomeric and polymeric enzymes. The metal atom is known to play a distinct catalytic role, e.g., in the monomeric carboxypeptidase A (20) and carbonic anhydrase (21). A zinc atom also serves in the catalytic mechanism of certain single chain bacterial enzymes, e.g., the neutral protease from *Bacillus subtilis* or thermolysin from *B. thermoproteolyticus*, but, in addition, calcium atoms apparently stabilize their secondary and/or tertiary structures (22). Much less is known about the effects of metals on stabilization of tertiary structure than on their catalytic roles.

The structural roles of metals have been recognized, largely through the extension of such lines of investigation to polymeric metalloenzymes, e.g., *E. coli* alkaline phosphatase (1) or horse liver alcohol dehydrogenase (23). However, differentiation and characterization of metal binding sites that subserve "catalytic," "structural," or "regulatory" roles have encountered difficulties both in experimentation and interpretation of data, owing to a number of factors. These may

include variations in metal content of the native enzyme, oxidation of ligands, and alterations of monomer ⇆ polymer equilibria, conformation, structure, and stability. Such problems are potentiated by uncertainties regarding "on" or "off" rates of one and the same or of different metal species at primarily functional, primarily structural, or primarily regulatory metal binding sites. Hence, procedures that can localize specific metal atoms at any one of these sites will prove important to the understanding of mechanisms of action. Yet, results are often ambiguous and can even imply that such sites may, in fact, be interconvertible, e.g., through local conformational changes or allosteric processes which could take place even in the course of experiments designed to differentiate between such sites.

The *in situ* conversion of exchange-labile Co(II) to exchange-stable Co(III) offers a potential method to selectively and differentially "freeze" cobalt atoms at binding sites, relatively specific for each species, thereby stabilizing a given conformation. This might be feasible owing to the fact that—compared with Co(II)—Co(III) is exchange-inert and shows a distinct preference for nitrogen donors and octahedral coordination geometry, as has been indicated. Hence, the possibility exists that given sufficiently dissimilar ligands with dissimilar constellations at different metal binding sites, the spectral changes and retardation in ligand exchange reactions accompanying oxidation of Co(II) to Co(III) may serve to differentiate among those which serve functional, structural, or regulatory roles in polymeric metalloenzymes. Toward this end, detailed studies with various metal derivatives, including 4 Co(II) alkaline phosphatase, are in progress to ascertain the loci of the oxidized cobalt atoms and will be the subject of a further communication (Vallee *et al.*, in preparation). The distinctive spectral and ligand substitution reactions of Co(III) should prove an important asset in defining and understanding the physical nature and functional potential of the metal binding sites of *E. coli* alkaline phosphatase and, ultimately, of other multichain metalloenzymes.

This work was supported by Grants-in-Aid GM-15003 and GM-02123, and R.A.A. is the recipient of Special Fellowship CA-03037, all from the National Institutes of Health, of the Department of Health, Education and Welfare.

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