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Nitrogen Ligands at the Active Site of Alkaline Phosphatase

(Zn/Cu/*E. coli*/electron spin resonance)

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ABSTRACT **The two Zn(II) ions of native *Escherichia coli* alkaline phosphatase (EC 3.1.3.1) that are necessary for activity have been replaced by ^{63}Cu(II). Titration of apoenzyme with up to 2 eq of Cu(II) gives a homogeneous species with an electron spin resonance typical for Cu(II) in an axially symmetric environment, with A_z = 496 MHz, $g_z = g_{\|}$ = 2.27, and $g_x = g_y$ = 2.05. At least seven nitrogen hyperfine lines, spaced 11 G apart, are clearly resolved on the $M = +{}^3/_2$ Cu(II) hyperfine peak in the parallel region. When more than 2 eq of Cu(II) are added, the electron spin resonance spectrum shows at least two types of Cu(II) binding sites; the additional site, or sites, are characterized by lower g and higher A_z values. When Cu(II) is added to native Zn(II) alkaline phosphatase or to apoenzyme incubated with 2 eq of Zn(II), the electron spin resonance spectrum shows little or no trace of the species with higher g values and nitrogen splitting. These results indicate that the species with higher g represents copper bound at the site normally occupied by the 2 Zn (II) ions necessary for enzyme activity, and that the metal ion at this site has at least 3 equivalent nitrogen ligands, probably histidyl side chains.**

Native *Escherichia coli* alkaline phosphatase (EC 3.1.3.1) is a dimer of two identical subunits (molecular weight 40,000) that contain between 2 and 4 g-atoms of zinc (1–6). A stable apoenzyme can be prepared by treatment of the native enzyme with chelating agents or with Chelex resin (1–3). The enzyme can then be reactivated by the addition of more Zn(II) ions. Several other first transition and II B metal ions have been found to bind to the apoenzyme, including Mn, Co, Ni, Cu, Cd, and Hg. Of these, only Co(II) restores significant activity (4), although Mn and Cd both induce phosphorylation of the specific serine at the active center (3). The two Zn(II) ions of native *E. coli* alkaline phosphatase necessary for activity have been replaced by ^{63}Cu(II). The nuclear superhyperfine structure on the electron spin resonance (ESR) signal that arises from the copper probe has been used for identification of the nature of the ligand donor atoms.

EXPERIMENTAL

Alkaline phosphatase was isolated from *E. coli* CW3747 by the methods described (1). The purified enzyme had a specific activity of 3460 μmol of *p*-nitrophenylphosphate hydrolyzed per hr per mg in 1 M Tris·HCl (pH 8); it ran as a single peak on Sephadex G-100 in 0.01 M Tris·HCl–0.01 M sodium acetate (pH 8). The enzyme was dialyzed against six changes of 100-fold volume excess of 0.01 M Tris·HCl–0.01 M $MgCl_2$ (pH 8) to ensure removal of any contaminating ethylenediaminetetraacetate (EDTA) (2). Apoalkaline phosphatase was prepared by treatment with Chelex 100 (3).

Spectrographically pure $ZnCl_2$ was purchased from Johnson, Matthey, Ltd. London, England; ^{65}Zn in HCl was purchased from New England Nuclear Corp. ^{63}CuO was obtained from Oak Ridge National Laboratories. All other reagents were analytical grade. Zinc analyses were performed by atomic absorption, by use of a Jarrell–Ash spectrometer. ESR spectra were recorded as the first derivative of the absorption on a Varian E-4 spectrometer that operates at 9.1 GHz, and is equipped with a Varian E-257 temperature controller. All spectra were taken at 111°K with 100 kHz modulation frequency, 50 mW power, and 8 G modulation amplitude, unless otherwise noted. Crystalline diphenylpicrylhydrazyl (g = 2.0036, obtained from Varian Associates) was used for calibration of the field.

Preparation of Cu(II) Alkaline Phosphatase. Apoalkaline phosphatase, 30–80 mg/ml in 0.01 M Tris·HCl (pH 7 or 8) was titrated with 0.01 M ^{63}Cu(II) in 1-equivalent (eq) steps until the desired molar ratio of copper to enzyme was reached. Binding of Cu(II) to alkaline phosphatase is weak compared to that of Zn(II), as shown by rapid loss of copper on dialysis and displacement of Cu(II) by Zn(II). All manipulations and measurements of the Cu(II) enzyme were performed in metal-free glassware and buffer solutions (4) to prevent displacement of Cu(II) by Zn(II) or other metals.

RESULTS

Apoalkaline phosphatase, 84 mg/ml in 0.01 M Tris·HCl (pH 7), was titrated with 1, 2, and 4 eq of $^{63}CuSO_4$. Allowance was made for the residual 0.05 eq of zinc per mol of enzyme dimer in the apoenzyme preparation. The ESR spectra of the resulting ^{63}Cu(II)-substituted alkaline phosphatase that contains 1, 2, or 4 eq of copper ions per dimer are shown in Fig. 1. Several previous reports on the ESR signals of copper alkaline phosphatase have shown at least two different types of signal arising from copper in two different environments in the protein (5, 6). It is apparent from the spectrum of the enzyme that contains four copper ions that this preparation contains at least two overlapping spectra of almost equal intensity (Fig. 1). When two or less equivalents of Cu(II) are added, the copper appears to occupy only one type of site. The ESR spectrum of this site is characteristic for Cu(II) in an axially symmetric environment of ligands, with $g_z = g_{\|}$ and $g_x = g_y = g_{\perp}$. This spectrum can be fit accurately by computer calculation if we assume an axially symmetric ligand field, and use the g and A values summarized in

Abbreviation: ESR, electron spin resonance.

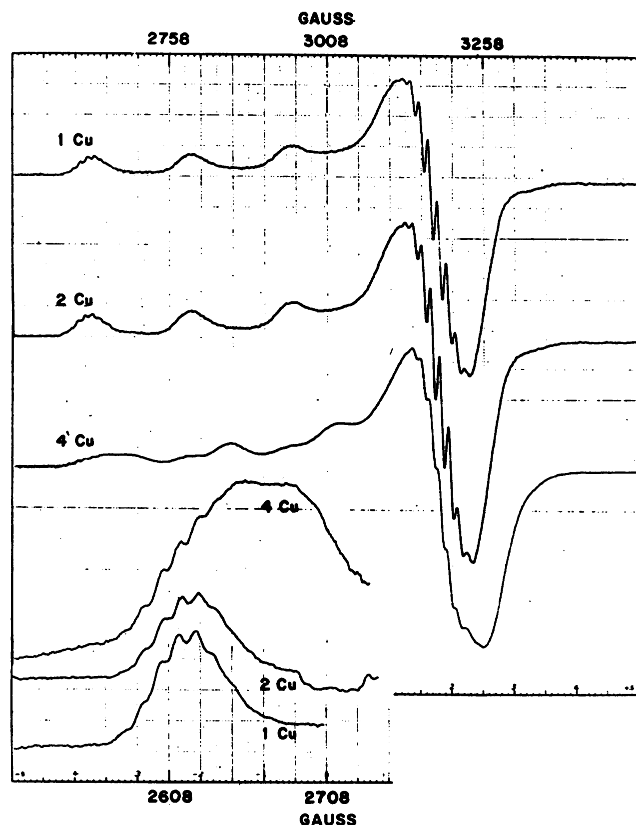

FIG. 1. ESR spectra of alkaline phosphatase during titration with $^{63}Cu(II)$. ESR spectra of apoalkaline phosphatase (0.8 mM) plus 1,2, and 4 eq $^{63}CuSO_4$ are shown. Conditions: 0.01 M Tris·HCl (pH 7). Titrations at pH 8 give identical spectra. Inserts show low-field $g_{\parallel}$ copper hyperfine lines of the same spectra.

Table 1. The second type, or types, of Cu(II) binding site that is apparent in Fig. 1 has an ESR signal with a lower $g_{\parallel}$ value. The binding constant of these secondary sites for Cu(II) is less than that of the first 2 eq of Cu, but is sufficiently close to that of the first type that there is a small amount of type II even in the samples containing 1 and 2 eq of Cu(II). This gives rise to the slight asymmetry of the parallel copper hyperfine lines (*Inserts*, Fig. 1).

The distinctive feature of the present study is that the ESR signal from the first type of Cu(II) is characterized by a ligand nuclear superhyperfine structure that is highly resolved (Figs. 1 and 2). The low-field edge of the perpendicular signal shows at least seven superhyperfine lines spaced 11 G apart, which can be attributed to splitting by nitrogen nuclei. These nitrogen superhyperfine lines are also well resolved on the $M = +^3/_2$ copper parallel hyperfine line at 2633 G. An expanded recording of this line is shown in the inserts to Fig. 1 and the low-field copper hyperfine line for the enzyme containing 1 Cu(II) per dimer is shown in Fig. 2. Seven superhyperfine lines spaced 11 G apart can be identified on this peak. The slight asymmetry of this signal is caused by the presence of a small amount of the second type of copper that has a lower $g_{\parallel}$ value.

The relationship of the sites occupied by Cu(II) to those occupied by Zn(II) in the native enzyme has been the object of considerable investigation (5, 6). Metal analyses and Cu(II)–Zn(II) exchange studies have shown direct competition between zinc and copper for the high-affinity binding sites on the protein (5). We have also shown by Sephadex-binding studies, using ^{65}Zn, that there is direct competition between ^{65}Zn and copper for the protein binding sites. Extensive studies have now shown that the enzyme containing two Zn(II) ions per dimer is fully active (2). (see *Discussion*). By addition of Cu(II) to the native enzyme and to the Zn(II)-reconstituted apoenzyme, it can be shown that the Cu(II) signal with the higher $g_{\parallel}$ value and the nitrogen superhyperfine structure (Type I) is arising from metal binding sites occupied by Zn(II) in the active enzyme. Apoalkaline phosphatase, 30 mg/ml in 0.01 M Tris·HCl (pH 8), was incubated with 2 eq of Zn(II) per mol of enzyme dimer for 18 hr. 2 eq of $^{63}Cu(II)$ were then added to the reconstituted zinc enzyme (Fig. 3*B*) and to native Zn(II) alkaline phosphatase, which contained 4 eq of Zn per mol of enzyme dimer (Fig. 3*A*). The native enzyme plus 2 eq of Cu(II) gives an ESR signal devoid of nitrogen superhyperfine splitting and having $g_{\parallel} = 2.25$ and $A_{\parallel}$ (Cu) = 574 MHz, compared to $g_{\parallel} = 2.270$ and $A_{\parallel}$ (Cu) = 500 MHz for the primary copper site occupied in the absence of zinc. The reactivated Zn(II) enzyme plus 2 eq of Cu(II) shows a small amount of nitrogen splitting, but is clearly a mixture of several kinds of copper sites. The marked difference between the signal from Type I Cu(II) and the extraneous copper added to the enzyme is best shown in the expanded $g_{\parallel}$ regions of the two spectra (Fig. 3*C*). The signal from copper added to the native Zn(II) enzyme also shows some heterogeneity (Fig. 3*C*).

The small amount of type I signal that is present (perhaps 5–10%) when Cu(II) is added to the reconstituted enzyme suggests that exactly two Zn(II) ions added to the apoenzyme under the experimental conditions do not completely fill all the specific binding sites or that, in the reconstituted enzyme, Cu(II)–Zn(II) exchange may proceed somewhat more rapidly than in the native enzyme that has not been through the Chelex treatment. Both sets of data suggest strongly that the type I signal is that arising from two identical Cu(II) binding sites per dimer that are occupied by Zn(II) both in the native enzyme and the Zn(II) reconstituted apoenzyme. Addition of inorganic phosphate or *p*-nitrophenylphosphate at millimolar concentration to copper alkaline

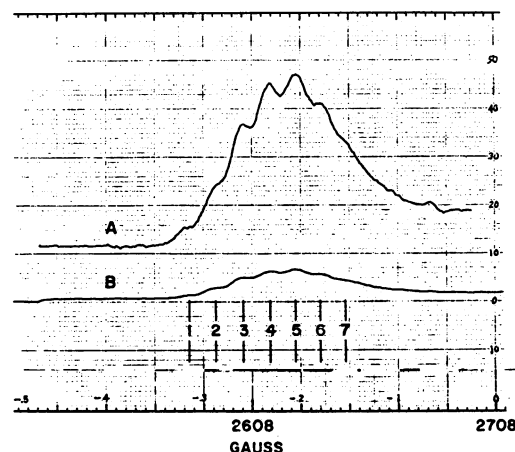

FIG. 2. The low-field $M = +3/2$ peak of copper alkaline phosphatase containing 2 eq $^{63}Cu(II)$. Apoalkaline phosphatase, 0.35 mM plus 0.31 mM $^{63}Cu\ SO_4$–0.01 M Tris·HCl (pH 8).

phosphatase (0.4 mM) containing two Cu(II) ions per dimer causes no detectable alteration in the ESR spectrum.

DISCUSSION

There has been considerable variation in reports on the number of metal ions required to reactivate alkaline phosphatase; some investigators favor 2 ions per dimer and others favor 4 ions per dimer (1–4, 6, 7). Several physicochemical methods have identified a unique pair of metal-binding sites in the dimer. The visible absorption spectrum of the Co(II) derivative shows a series of *d–d* absorption bands corresponding to two Co(II) ions in a highly unusual environment, probably of relatively low symmetry (8). The apoenzyme binds two manganese ions with loss of the typical manganese ESR signal (9), a finding that also implies sites of low symmetry. Additional manganese ions show the 6-line ESR spectrum typical of the Mn(II) aquo cation (8). More recently, Csopak *et al.* (2) have found that residual EDTA that is bound to the apoenzyme and carried over from the isolation procedure interferes with metal titrations of the apoenzyme. More than two zinc ions are required to restore activity completely if EDTA, as well as enzyme sites, is being titrated. If all EDTA is removed, only two zinc ions are required (2).

Previous studies of the Cu(II) derivative of the enzyme have identified two tight-binding sites for Cu(II), although more weak-binding sites are also present. The same pair of binding sites also bind zinc (5). This is further confirmed here by the finding that two Zn(II) ions primarily replace the first type of copper (Fig. 3). Previously reported ESR signals of the Cu(II) derivatives have been a composite of at least two overlapping copper signals. It is possible that Cu(II)-EDTA complexes have been a source of interference. Copper binding to other sites on the protein is also apparent (Figs. 1 and 3). Previous workers have not observed ligand nuclear superhyperfine structure. Two general difficulties arise when the ESR signals of Cu(II) bound in protein are used to identify ligand nuclear interaction with the Cu(II) ion, particularly the interaction with nitrogen nuclei. (*a*) Since copper so frequently binds nonspecifically to proteins, copper is often present in more than one type of site, giving rise to signals with different *g* values. The resultant displacement of the signals is sufficient to obscure narrow ligand nuclear superhyperfine lines. (*b*) If copper containing the natural abundance of ^{63}Cu and ^{65}Cu is used, ligand superhyperfine structure may be obscured or complicated by the overlap of the spectra that arise from the two isotopes (10). Both these difficulties are present in the case of copper alkaline phosphatase. It is possible to obtain reproducibly an almost homogenous copper species with resolved nitrogen splitting by use of pure ^{63}Cu at concentrations of $\leqq$ 2 eq/mol of apoenzyme, and by rigorously excluding Zn(II) and other metal ions that can exchange with Cu(II).

TABLE 1. *ESR spectral parameters of Cu(II) alkaline phosphatase*

Parameter
$g_{\parallel}$ = 2.270 ± 0.003
$g_{\perp}$ = 2.050 ± 0.003
$A_{\parallel}$ (Cu) = 500 ± 5 MHz
$A_{\perp}$(Cu) = 15 ± 5 MHz
A(N) = 35 ± 1 MHz

These parameters were obtained by fitting a computer-calculated spectrum to the experimental data of Fig. 1.

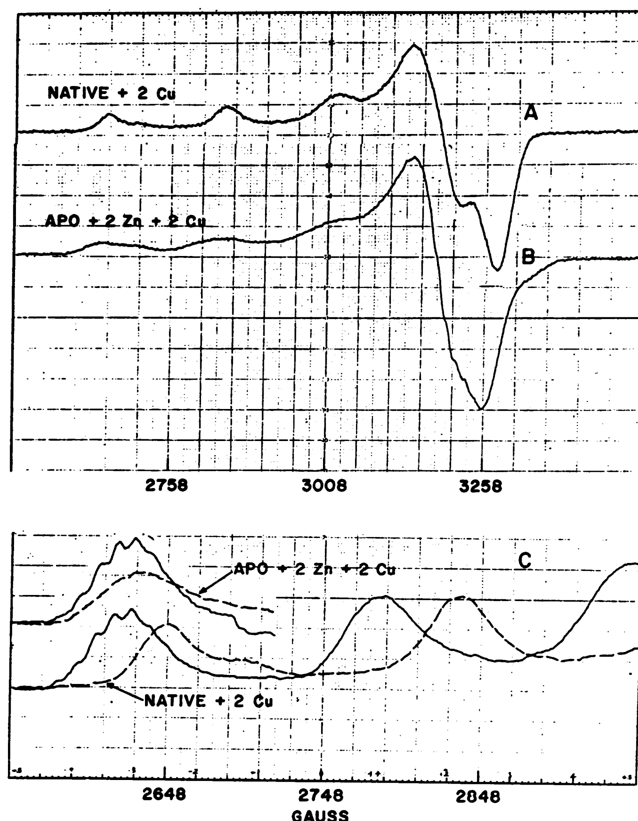

FIG. 3. ESR spectra of alkaline phosphatase after addition of ^{63}Cu(II) to enzyme containing Zn(II). (*A*) Native alkaline phosphatase, 0.33 mM plus 0.62 mM $^{63}CuSO_4$. (*B*) Apoalkaline phosphatase, 0.33 mM, incubated with 0.62 mM $ZnCl_2$ at 4° for 18 hr, plus 0.62 mM $^{63}Cu SO_4$. (*C*) *Solid lines*, low-field $g_{\parallel}$ hyperfine lines for 0.33 mM apoalkaline phosphatase plus 0.62 mM $^{63}CuSO_4$. *Dashed lines*, $g_{\parallel}$ lines from upper spectra as marked. Conditions: 0.01 M Tris·HCl (pH 8).

The spectra shown here were all obtained on the enzyme from the CW3747 strain of *E. coli*. The amino-acid composition of the enzyme from this strain is identical to that of the enzyme from *E. coli* strain C-90. Extensive N-terminal and C-terminal amino-acid sequences of the enzyme from the two strains are also identical (Schlesinger, M.L., personal communication). A study of the enzyme from the C-90 strain, by use of ^{63}Cu and ^{65}Cu in natural abundance, also showed nitrogen superhyperfine structure. Purity of the preparation affects the resolution due to copper-binding impurities or denatured molecules. Purity, as judged by highest specific activity, is consistently better for our preparations from the CW3747 strain.

The nitrogen atoms appear to produce an axially symmetric environment for the copper ion (Table 1). A square-planar arrangement of ligands would appear likely for Cu(II), although distant vertical ligands might be present. Whether a fourth ligand is contributed by the protein or by solvent water is unclear at present. Although changes in the ESR signal induced by phosphate have been reported, we observe no effect of phosphate or substrate on the ESR signal of the homogeneous species in Fig. 1 that occupies the active zinc sites in alkaline phosphatase. This may simply mean that the

copper enzyme is a poor phosphate binder, as was indicated by earlier data from our laboratory on the binding of $H^{32}PO_4^-$ (3). Alternatively, a phosphate oxygen might substitute for a water oxygen with little change in the signal. The present Cu(II) ESR data do not shed light on whether phosphate contributes a group to the inner coordination sphere of the active metal ions.

The Cu(II) ESR does give information on the ligands at the active site. The spectrum shows at least seven nitrogen superhyperfine lines on the single copper hyperfine line at 2633 G (Figs. 1 and 2). The asymmetry of the peak due to the slight amount of the other species of copper and the broadening of the multiplet lines at higher field makes it difficult to determine the center of the multiplet. Hence, it is impossible to say whether there are seven or nine lines in the multiplet. The seven nitrogen lines indicate at least 3 equivalent nitrogen atoms as ligands to the metal ion. Four cannot be ruled out. Nitrogens of histidyl residues appear to be likely candidates as the coordinating ligands. Some evidence for this exists in that histidyl residues are more readily destroyed by photooxidation in the apoenzyme than in the zinc enzyme (11). Tait and Vallee (11) showed that two or three histidyl residues per dimer out of the 16 present were protected from photooxidation by the presence of Zn(II). If the 3 equivalent nitrogen ligands appearing in the ESR signal are assigned to histidyl side chains, then six histidyl residues per dimer are involved in metal binding. The assumption that the ligands to Cu(II) occupying the active site are the same as when Zn(II) occupies this site appears to be a reasonable one; however, more definitive proof of this must await high-resolution x-ray diffraction studies currently underway (12). Even if the same ligands are involved, all features of the coordination geometry may not be the same in the various metallophosphatases. Copper may induce some shift in the geometrical arrangement of the ligands, for example, as is apparently the case in carbonic anhydrase (10).

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