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Utilization of Copper as a Paramagnetic Probe for the Binuclear Metal Center of Phosphotriesterase[1]

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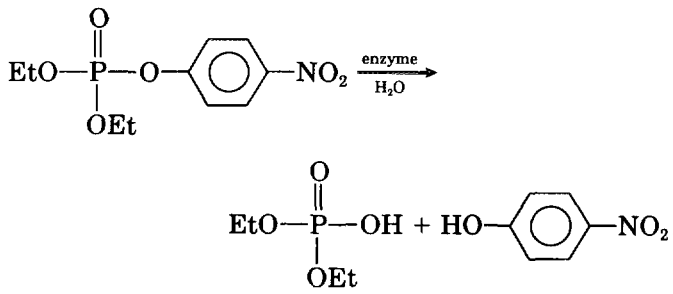
Bacterial phosphotriesterase catalyzes the hydrolysis of organophosphate triesters. To be active, the enzyme requires that two divalent cations are bound. These metal ions are bound in close proximity to one another as a binuclear center. To characterize the structure and function of the binuclear metal binding sites, we have prepared the copper-substituted enzyme. The kinetic data indicate that this enzyme is essentially inactive toward the hydrolysis of phosphotriesters. The EPR signal arising from the copper-substituted enzyme is nearly axial, with $g_{\|} = 2.24$ and $g_{\perp} = 2.05$ and shows at least seven superhyperfine transitions in the $g_{\perp}$ region with $A_{\perp} = 1.45 \times 10^{-3}$ cm^{-1}. These splittings are consistent with the direct ligation of more than one nitrogen to the metal center. The average spin quantitation of copper-substituted enzymes are 0.6 spin/Cu, approximately half of that observed for noninteracting Cu^{2+} ions. The spin intensity increases to ca. 1 spin/Cu when samples are denatured with acid. The binding of metal ions to the designated α and β sites is highly synergistic (i.e., the metal ions bind in pairs). Mixed metal complexes of the type Cu/X and X/Cu were prepared. When X is a diamagnetic ion (Zn^{2+} or Cd^{2+}), the spin quantitation increases, but when X is the paramagnetic Co^{2+} ion, the spin quantitation decreases. This behavior indicates that the low spin intensities observed for copper-substituted phosphotriesterase arise from spin-coupling of the two adjacent Cu^{2+} ions. The addition of dithiothreitol, ascorbate, or dithionite to the copper-substituted phosphotriesterase results in nearly the complete loss of spin intensity. This indicates that the bound coppers can be reduced to the cuprous state.

Key Words: **phosphotriesterase; binuclear metal center; EPR; copper.**

Phosphotriesterase is a bacterial enzyme containing a binuclear metal center (1). The enzyme catalyzes the hydrolysis of organophosphates, such as paraoxon, to *p*-nitrophenol and diethyl phosphate as illustrated below



The enzyme has received considerable attention because of its potential utility for the detoxification of chemical warfare agents and agricultural pesticides. Although there are no known naturally occurring phosphotriesters, the enzyme is remarkably efficient at catalyzing the above reaction. For example, the k_{cat} and k_{cat}/K_m values for the hydrolysis of paraoxon at pH 9 are 2500 s^{-1} and 5 $\times$ 10^7 M^{-1} s^{-1}, respectively. Moreover, it has been demonstrated by solvent viscosity studies that the rate of hydrolysis is limited by diffusion for those substrates with leaving groups having pK_a values of less than 7 (2).

The native enzyme has been found to be associated with 1–2 mol of zinc per mole of protein (3, 4). Removal of the bound metal ions from phosphotriesterase results in the loss of enzymatic activity. The native zinc ions can be substituted with other divalent cations by supplementation of the bacterial growth medium with Co^{2+}, Ni^{2+}, Cd^{2+}, or Mn^{2+}. All of these metal-substituted derivatives are as active as the native zinc-containing enzyme (4). Reconstitution of the apoenzyme with various divalent cations has demonstrated that 2 mol of metal per mole of protein is required for maximum catalytic activity.

Relatively little is known of the actual role of the two metal ions in the mechanism of enzymatic phosphotriester hydrolysis. A stereochemical investigation using

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the enzyme from *Pseudomonas diminuta* has shown that the phosphorus center undergoes an inversion of configuration during the reaction process (5). Therefore, a phosphoenzyme intermediate is not likely to be involved in the reaction mechanism. A critical histidine residue has been implicated at the active site, based upon pH-rate profiles and inactivation studies with diethylpyrocarbonate (6). NMR studies with the ^{113}Cd-substituted enzyme have indicated that the protein ligands to each metal ion are composed of a combination of nitrogen and oxygen atoms (7). These investigations have also shown that binding of the two cadmium ions to the protein is highly cooperative and that both metal sites are solvent accessible. The X- and Q-band EPR spectra of the Mn-substituted enzyme have demonstrated that the two manganese ions are present as an antiferromagnetically exchanged-coupled binuclear metal complex (1). This observation requires that the two metal ions bound to the phosphotriesterase are in close proximity and are most likely bridged by a common ligand. Recently, site-directed mutagenesis investigations (8) have been employed to change each of the seven histidine residues to asparagines.

To further characterize the structure and function of the phosphotriesterase metal-binding sites, we have now examined the EPR spectra of the copper-substituted enzyme. The native zinc enzyme is spectroscopically silent and thus lacks physical probes that are informative of coordination environment and metal–protein ligands. Copper EPR measurements have aided in establishing the metal ligand donors at the active sites of several zinc metalloenzymes including carboxypeptidase A (9), carbonic anhydrase (10), and alkaline phosphatase (11). Samples of phosphotriesterase were prepared using different molar ratios of Cu^{2+} to protein, at different pH values, with and without inhibitors present. Samples of mixed-metal derivatives of the copper-substituted enzyme were prepared with various molar ratios of Zn^{2+}, Cd^{2+}, and Co^{2+}. Experiments designed to reduce the copper bound to the phosphotriesterase were also performed. These studies have been conducted to provide additional spectroscopic evidence for the existence of potential metal–protein, metal–substrate, and metal–metal interactions.

MATERIALS AND METHODS

Materials. Diethyl-*p*-nitrophenylphosphate (paraoxon) was purchased from Sigma Chemical Co. as a 95% pure oil and purified according to the procedure previously described (4). Copper and other divalent metal ions were purchased as sulfate or chloride salts from Aldrich Chemical Co. Diethyl 4-methoxybenzylphosphonate was purchased from Lancaster Synthesis, and diethyl 4-methoxybenzylthiophosphonate was synthesized as previously described (7). The 99% enriched potassium cyanide ($K^{13}CN$), copper 63 (99 atom %) and cadmium 113 (95 atom %) were obtained from Cambridge Isotope Laboratory. The $^{113}CdCl_2$ and $^{63}Cu(NO_3)_2$ were prepared as previously described (7). All buffers, unless otherwise stated, were purchased from either Sigma or United States Biochemical Corp.

Enzyme preparation. Phosphotriesterase was purified from an *Escherichia coli* strain containing the plasmid pJK01 (8). The bacterial cells were grown in TB medium consisting of 12 g/liter bactotryptone, 24 g/liter yeast extract, 4 ml/liter glycerol, and 100 mM potassium phosphate, pH 7.4, supplemented with 1 mM cobalt chloride. The enzyme was purified as previously described (4) to a specific activity of approximately 1×10^4 units/mg. The protein concentration was determined spectrophotometrically using an $\epsilon_{280} = 2.9 \times 10^4\ M^{-1}\ cm^{-1}$.

Apoenzyme preparation. Apoenzyme was prepared by incubation of the purified phosphotriesterase (1.0 mg/ml) with 2 mM 1,10-phenanthroline for 15 h at 4°C or until the enzymatic activity dropped to less than 1% of its original value. The metal–chelator complex and excess chelator were removed through diafiltration with metal-free 50 mM Hepes buffer, pH 8.3, over an Amicon PM10 membrane until there was less than 0.3 μM 1,10-phenanthroline remaining. The buffers used in this procedure were previously passed through a chromatographic column packed with Chelex-100.

Reconstitution of apoenzyme. Copper-substituted phosphotriesterase samples were prepared by the addition of various amounts of Cu^{2+} to the apophosphotriesterase (~1 mg/ml) at 4°C for a minimum of 24 h. The mixed-metal substituted enzymes were prepared by simultaneously adding both metal ions in the desired ratio.

Sample preparation for EPR spectroscopy. The samples used for EPR spectroscopy were prepared by adding metal ions to the dilute apoprotein and then concentrating the samples by filtration through a semipermeable membrane. However, samples of the Cu-phosphotriesterase for the experiment involving copper spin recovery after protein denaturation were prepared by concentration of the enzyme to the desired level followed by denaturation with 2 M perchloric acid to pH ~1.5. EPR samples used for the pH studies were prepared as follows: The concentrated Cu/Cu enzyme, prepared in 50 mM Hepes buffer, pH 8.3, was passed through a Pharmacia PD-10 Sephadex G-25 column preequilibrated with the desired buffer. The enzyme was recovered and then concentrated by filtration through an Amicon Centricon-10 device. The buffers used were 4-morpholineethanesulfonic acid (pH 6); 1,4-piperazine-diethanesulfonic acid (pH 7); 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes, pH 8); 2-(N-cyclohexylamino)ethanesulfonic acid (Ches, pH 9);[3] and 3-(cyclohexylamino)-1-propanesulfonic acid (pH 10).

The Cu^{2+} ions bound to the phosphotriesterase samples were reduced by adding various molar ratios of freshly prepared dithiothreitol (DTT) to the concentrated enzyme solution. The DTT-treated samples were incubated at room temperature for 15 min prior to freezing by immersion into liquid nitrogen. The Cu^{2+}-phosphotriesterase was also reduced by the addition of 0.25–1.0 equivalents of ascorbate under anaerobic conditions. The enzyme and buffer solutions were degassed with the aid of a Schlenck vacuum line. The ascorbate-treated samples were incubated for approximately 40 min at room temperature before freezing as described above. The reduction of the Cu^{2+} bound to the enzyme was also attempted with 2 molar equivalents of sodium dithionite under both aerobic and anaerobic conditions. Samples were frozen by immersion in liquid nitrogen after 15 min of incubation at room temperature.

Metal analysis. The metal content of the reconstituted proteins was determined by atomic absorption spectroscopy as previously described (4). A Perkin–Elmer Model 2380 atomic absorption spectrophotometer was used with an acetylene/air flame. Perkin–Elmer intensitron lamps were used at the following wavelengths (nm): zinc (213.9), copper (324.7), cobalt (242.5), and cadmium (228.8).

Measurement of catalytic constants. The enzymatic activity of the

[3] Abbreviations used: Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid; DTT, dithiothreitol.

phosphotriesterase was determined by monitoring the rate of change in absorbance at 400 nm due to the formation of *p*-nitrophenol as paraoxon (1.0 mM) was hydrolyzed at 25°C in 100 mM Ches buffer, pH 9.0. One unit of activity is defined as the hydrolysis of 1.0 μmol of paraoxon/min. The kinetic parameters, V_{max} and K_m, were measured at 25°C in the presence of 100 mM Ches buffer at pH 9.0. The kinetic data were fit to the following equation using the computer programs supplied by Savanna Shell Software:

$$v = V_m A/(K_m + A). \quad [1]$$

In Eq. 1, v is the initial velocity, V_m is the maximal velocity, K_m is the Michaelis constant, and A is the substrate concentration. The competitive inhibition constants were determined from

$$v = V_m A/(K_m(1 + I/K_{is}) + A), \quad [2]$$

where I is the inhibitor concentration, and K_{is} is the slope inhibition constant.

Electron paramagnetic resonance spectroscopy. X-band EPR spectra were obtained on a Bruker ESP 300 spectrometer with an Oxford Instruments ER-910A liquid He cryostat, a HP-535B frequency counter, and a Bruker ER-035M NMR gaussmeter. Modulation frequency was 100 KHz. Samples in the cavity were maintained at either 10 or 100°K. Modulation amplitude was 10 gauss. The nonsaturating microwave power at 10 and 100°K was 0.063 and 2.0 mW, respectively. A solution of 1.0 mM $Cu(ClO_4)_2$ was used as a standard for spin quantitation.

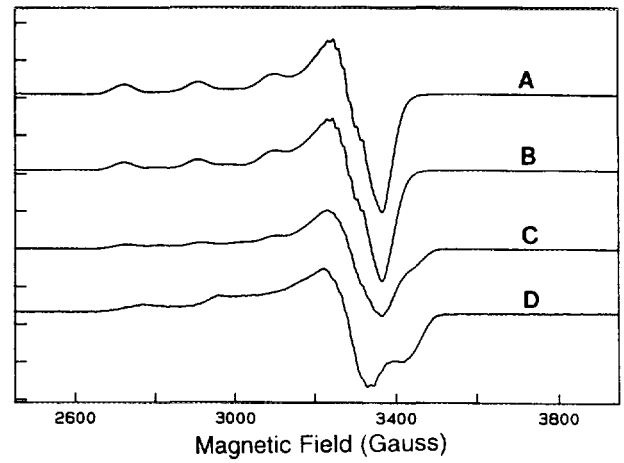


FIG. 1. EPR spectra of the phosphotriesterase reconstituted with various ratios of copper/apoprotein: (A) 0.5 Cu^{2+} enzyme; protein concentration, 0.86 mM (31.0 mg/ml); relative gain, 1. (B) 1 Cu^{2+} enzyme; protein concentration, 0.92 mM (33.2 mg/ml); relative gain, 0.5. (C) 2 Cu^{2+} enzyme; protein concentration, 0.71 mM (25.6 mg/ml); relative gain, 0.25. (D) 4 Cu^{2+} enzyme; protein concentration, 0.23 mM (8.3 mg/ml); relative gain, 1.25. Samples were prepared in 50 mM Hepes, pH 8.3. Spectra were recorded at 9.4 GHz spectrometer frequency, 10 G modulation amplitude, and 100 kHz modulation frequency. (A), (B), and (C) were collected at 10°K with a nonsaturating power of 0.063 mW, while (D) was acquired at 100°K with the same power.

RESULTS

Characterization of Cu-Substituted Phosphotriesterase

Apophosphotriesterase was prepared by incubation of the Co-substituted enzyme with 1,10-phenanthroline. Preparations typically contained less than 0.02 equivalent per protein of Zn, Co, and Fe and had specific activities ≤25 units/mg at pH 9.0. Cu-substituted phosphotriesterase was prepared by incubating apoenzyme with various ratios of Cu^{2+} to protein. The Cu/Cu-substituted enzyme[4] exhibited values for k_{cat} and K_m for the hydrolysis of paraoxon at pH 9.0 of 60 s^{-1} and 0.10 mM, respectively. The metal content of this enzyme preparation was 1.5 Cu^{2+} and 0.03 Zn^{2+} per mole of enzyme. The Cu/Cu-enzyme was competitively inhibited by the substrate analogs, diethyl 4-methoxybenzylphosphonate (K_i = 0.15 mM), diethyl 4-methoxybenzylthiophosphonate (K_i = 0.20 mM), dithiothreitol (K_i = 0.018 mM), and potassium cyanide (K_i = 0.13 mM).

The protein-bound copper seemed fairly weakly bound, dependent on pH, and displaceable by either cadmium or zinc. Only 0.7 Cu^{2+} remained bound to the Cu-substituted enzyme after extensive dialysis (48 h) in 50 mM Hepes, pH 7.7, while no significant loss of copper was observed when the dialysis was performed at pH 8.3. Enzyme reconstituted with 2 Cu^{2+} per protein and then dialyzed for 24 h against a solution containing 0.5 mM $ZnCl_2$ (50 mM Hepes buffer, pH 8.2) had 0.2 Cu^{2+} and 1.9 Zn^{2+} per protein after dialysis, while that dialyzed against 0.5 mM $CdCl_2$, contained 0.1 Cu^{2+} and 1.7 Cd^{2+}/protein after dialysis.

[4] The enzyme consisting of two metal ions/protein will be denoted as M/M protein (for example, the protein containing 2.0 Cu/protein will be denoted as Cu/Cu-substituted phosphotriesterase).

Copper EPR Spectra

EPR spectra of enzyme prepared with 0.5, 1.0, 2.0, and 4.0 Cu/protein are shown in Figs. 1A–1D, respectively. The spectrum of the enzyme prepared with 1.0 Cu/protein (Fig. 1B) exhibits what appears to be a single signal of nearly axial symmetry with $g_{\|} = 2.24$ and $g_{\perp} = 2.05$. The $g_{\|}$ region shows hyperfine splittings ($A_{\|} = 1.91 \times 10^{-2}$ cm^{-1}) from the nuclear spin of 3/2 for $^{65}Cu^{2+}$ and $^{63}Cu^{2+}$. The $g_{\perp}$ region shows at least seven partially resolved superhyperfine lines with $A_{\perp} = 1.45 \times 10^{-3}$ cm^{-1}. No improvement in spectral resolution was obtained when samples were prepared with 99% $^{63}Cu^{2+}$. These superhyperfine transitions appear to arise from nitrogen nuclei (not from copper) and were better resolved at the lower Cu/protein ratios. Expansion of the spectrum that appears in Fig. 1A is presented in Fig. 2. Although the observed spin intensity per copper varied somewhat for the various enzyme preparations, it consistently quantitated to less than unity (0.6 ± 0.2 spins/Cu) for samples containing ≤2.0 Cu/protein. When more than two copper ions were added to the apoenzyme, additional features (Fig. 1D) were evident in the EPR spectra, consis-

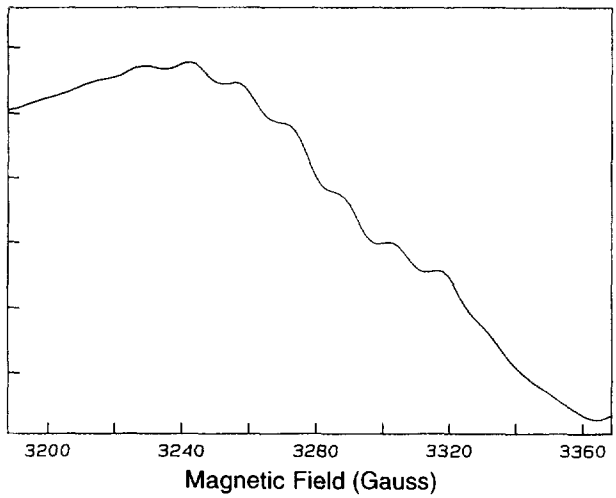


FIG. 2. Expansion of the EPR spectrum presented in Fig. 1A.

tent with adventitious binding of copper ions to the protein. These features were not further characterized.

Increased Spin Intensity after Protein Denaturation

When a sample of the Cu/Cu phosphotriesterase was digested with 2 M perchloric acid, the resulting EPR spectrum was virtually identical to that of Cu^{2+}-perchlorate (at pH 1.8) with $g_{\|} = 2.39$, $g_{\perp} = 2.08$, and $A_{\|} = 1.69 \times 10^{-2}$ cm^{-1} and it quantitated to higher value. The spin intensity of the sample before acid treatment was 0.37 spin/Cu and 0.83 spin/Cu thereafter.

EPR Spectra of Mixed-Metal Substituted Phosphotriesterase

In order to further characterize the copper binding properties of the phosphotriesterase, Cu/Zn, Cu/Cd, and Cu/Co mixed-metal derivatives were prepared and the EPR spectra were obtained (Fig. 3). The specific activities, spin quantitations, and metal composition of these preparations are summarized in Table I. The specific activities of the various Cu/Zn-substituted enzymes increased linearly with the amount of Zn^{2+} bound to the protein. Furthermore, the spin intensities for the 0.5 Cu^{2+}/1.0 Zn^{2+} and 0.5 Cu^{2+}/1.5 Zn^{2+} samples were 1.0 ± 0.2 spin/Cu, significantly higher than those obtained for the 2.0 Cu/protein sample. The overall shape of the EPR spectra for the Cu/Zn mixed-metal enzymes were different from those observed for the Cu/Cu-substituted enzyme. The EPR spectrum (Fig. 3B) of the 1.0 Cu/1.0 Zn enzyme, for example, has $g_{\|} = 2.33$ and $g_{\perp} = 2.09$. The EPR spectra of the Cu/Zn mixed-metal enzymes are also characterized by greater peak intensities in the $g_{\|}$ region and broader $g_{\perp}$ resonances compared to those of the Cu/Cu-substituted enzyme. Furthermore, the hyperfine splittings in the $g_{\|}$ regions of Cu/Zn mixed system ($A_{\|} = 1.20 \times 10^{-2}$ cm^{-1} for 1.0 Cu/1.0 Zn enzyme) are smaller than those observed for the Cu-substituted enzyme ($A_{\|} = 1.91 \times 10^{-2}$ cm^{-1} for 1.0 Cu phosphotriesterase).

Samples of the Cu/Cd-substituted enzyme containing various molar ratios of Cu^{2+} and Cd^{2+} were prepared. There were no significant differences between the EPR spectra for the Cu/Cd and Cu/^{113}Cd-substituted enzymes (data not shown). However, there are significant differences between the spectra of the 1.0 Cu- and 1.0 Cu/1.0 Cd-substituted enzymes. The $g_{\|}$ and $g_{\perp}$ values of the signal from the 1.0 Cu/1.0 Cd enzyme (Fig. 3C) are 2.26 and 2.05, respectively, shifted relative to those of the 1.0 Cu/protein. The $A_{\|}$ value of Cu/Cd-substituted enzyme in the $g_{\|}$ region is 1.76×10^{-2} cm^{-1}, while that of the 1.0 Cu/enzyme is 1.91×10^{-2} cm^{-1}. The peak intensities of the Cu/Cd-substituted enzymes in the $g_{\|}$ region as shown in Fig. 1C were also greater than those measured for the 1.0 Cu/enzyme. However, the signal in the $g_{\perp}$ region is not broader compared to that of Cu/Zn-substituted enzyme. As summarized in Table I, the copper spins observed for the Cu/Cd-substituted enzymes increased as the ratio of Cd^{2+} to Cu^{2+} increased, a phenomenon also observed for Cu/Zn-substituted enzyme.

Samples of the Cu/Co-substituted enzymes were also prepared. The catalytic activity of the Cu/Co-substituted enzymes increased with an increased Co^{2+}/Cu^{2+} ratio of added metals. The overall appearances of the EPR spectra for the Cu/Co hybrids (Fig. 3D) are very similar to the Cu-substituted enzymes. However, the EPR spin intensity of the Cu/Co-substituted enzymes

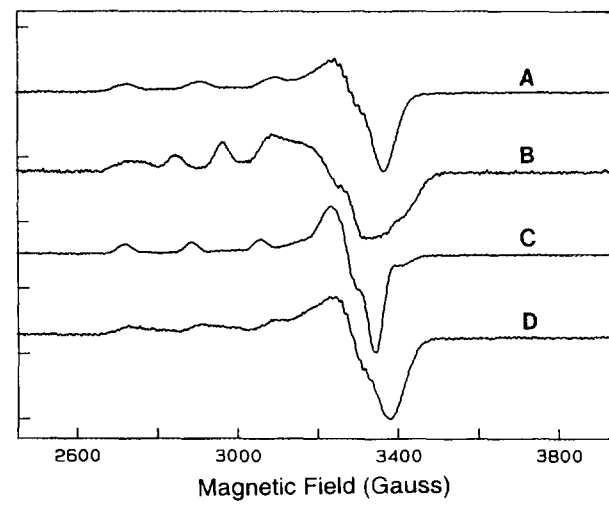


FIG. 3. EPR spectra of mixed-metal substituted phosphotriesterase. (A) 1.0 Cu^{2+} enzyme (0.38 mM protein concn); relative gain, 1. (B) 1.0 Cu/1.0 Zn enzyme (0.11 mM); relative gain, 4. (C) 1.0 Cu/1.0 Cd enzyme (0.22 mM); relative gain, 1. (D) 1.0 Cu/1.0 Co enzyme (0.40 mM); relative gain, 2. Samples were in 50 mM Hepes, pH 8.3. EPR conditions were the same as those described in the legend to Fig. 1. (A), (C), and (D) were collected at 100°K, while (B) was acquired at 10°K.

TABLE I

Summary of the Specific Activities, Spin Quantitations, and Metal Composition of the Mixed-Metal Substituted Derivatives of the Phosphotriesterase

Phosphotriesterase	[Protein][a] (μM)	[Activity][b] (U/mg)	[M][c,d] (μM)	[Cu][d] (μM)	[Spin][e] (μM)	[Spin]/[Cu] (%)
0.5 Cu	150	250	4.4	71	26	37
0.5 Cu/0.5 Zn	146	1030	70	74	45	61
0.5 Cu/1.0 Zn	132	1900	134	55	59	107
0.5 Cu/1.5 Zn	116	2550	151	52	54	104
1.0 Cu	140	250	3.5	129	50	39
1.0 Cu/0.5 Zn	120	900	64	101	62	61
1.0 Cu/1.0 Zn	110	1330	106	94	89	95
1.5 Cu	110	177	3.2	147	65	44
1.5 Cu/0.5 Zn	96	950	52	120	79	66
2.0 Cu	130	150	3.7	192	100	52
1.0 Cu	208	250	—	161	92	57
1.0 Cu/0.5 Cd	242	800	106	189	179	95
1.0 Cu/1.0 Cd	224	800	172	186	197	106
0.5 Cu	330	88	—	147	123	84
0.5 Cu/0.5 Co	390	2630	201	178	117	66
0.5 Cu/1.0 Co	390	4480	341	178	67	38
0.5 Cu/1.5 Co	390	4560	413	167	42	25
1.0 Cu	380	181	—	341	236	69
1.0 Cu/0.5 Co	370	410	187	333	188	56
1.0 Cu/1.0 Co	400	1880	279	325	159	49
1.5 Cu	410	138	—	488	346	71
1.5 Cu/0.5 Co	420	280	170	512	347	68

[a] Determined by OD_{280}.
[b] One unit of activity is defined as the hydrolysis of 1.0 μmol of paraoxon/min.
[c] M represents the other metal ion present in the system.
[d] All metal ion concentrations were determined by atomic absorption spectroscopy.
[e] Determined by EPR integration.

decreased significantly as the ratio of Co^{2+} to Cu^{2+} increased.

Addition of Reducing Agents

The effect of DTT, a strong competitive inhibitor of the enzyme, was explored by titrating the Cu/Cu-substituted phosphotriesterase with this reagent. The spectra shown in Fig. 4 illustrate the effect of adding 0.5–2.0 equivalents of DTT on the EPR spectrum of the Cu/Cu-substituted enzyme; two molar equivalents of DTT resulted in the loss of 95% of the original EPR signal. Similar experiments were performed with the 0.5 Cu/1.0 Zn and 1.0 Cu/1.0 Zn phosphotriesterase preparations. The EPR signal intensities for both samples declined 85–90% after 2 equivalents of DTT was added (data not shown).

The EPR signal intensity also declined when 0.25–1.0 equivalent of ascorbate was added to phosphotriesterase substituted with either 1 or 2 molar equivalents of Cu^{2+} (Fig. 5A and 5B). The spectra indicate that the signal intensities diminish as the ratio of ascorbate/protein increases. The addition of 0.5 equivalent of ascorbate per mole of protein to the 1 Cu^{2+} enzyme reduced the signal by 95%. After the addition of 1.0 equivalent of ascorbate to the 2 Cu^{2+} enzyme only 15% of the original intensity remained. The anaerobic addition of 2 equivalents of dithionite/protein abolished the EPR signal completely (spectra not shown).

EPR Spectrum of Cyanide-Treated Phosphotriesterase

The effect of $^{13}CN^-$ on the EPR spectrum of the Cu/Cu-substituted enzyme is shown in Fig. 6. The $g_\perp$ value of the signal from Cu-substituted enzyme shifted from 2.05 to 2.03 upon cyanide addition, while the $g_{||}$ value remained unchanged. This suggests that the cyanide anion binds to the copper. Addition of 2 and 4 equivalents of $^{13}CN^-$ to the Cu/Cu-substituted enzyme reduced the EPR signal by 50% and 90%, respectively. No significant difference was observed when $^{12}CN^-$ was substituted for $^{13}CN^-$ (data not shown).

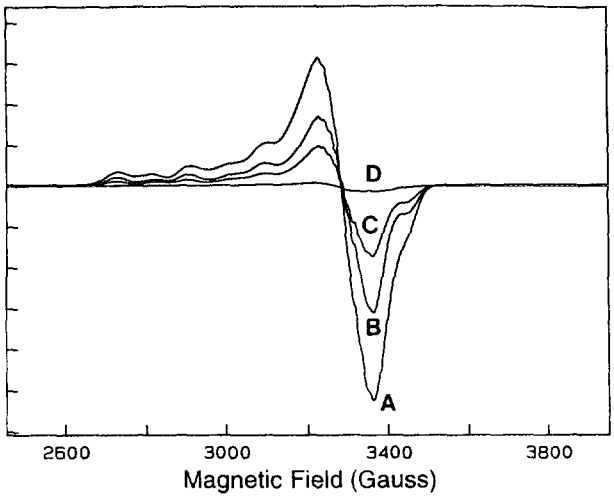


FIG. 4. Effect of dithiothreitol (DTT) on the EPR spectrum of the 2 Cu^{2+} enzyme. DTT was added to the 2 Cu^{2+} enzyme (protein concentration, 0.68 mM, pH 8.3) and the samples were kept frozen in liquid nitrogen after incubating for 15 min at room temperature. The molar ratios of DTT/protein for spectra A, B, C, and D were 0, 0.5, 1.0, and 2.0, respectively. The spectra were collected at 10°K, while other EPR parameters were as described in the legend to Fig. 1.

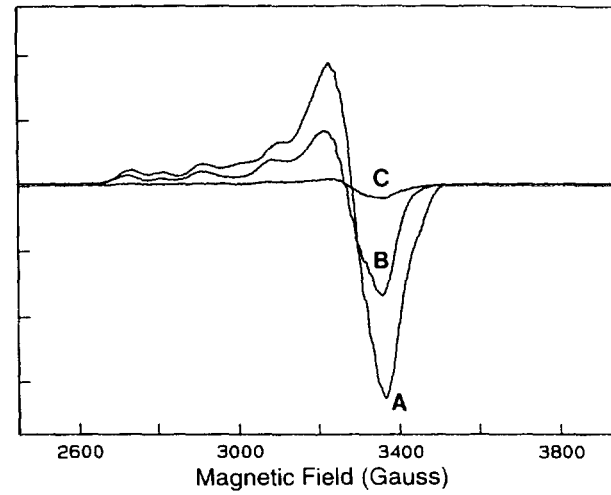


FIG. 6. Effect of cyanide on the EPR spectrum of the Cu/Cu-substituted phosphotriesterase. (A) No cyanide; (B) 2 equivalents of $^{13}CN^-$; (C) 4 equivalents of $^{13}CN^-$. The protein concentration for all samples was 0.65 mM (23.4 mg/ml) in 50 mM Hepes, pH 8.3. All samples were kept frozen in liquid nitrogen after incubating for 15 min at room temperature. All spectra were recorded at 10°K; other conditions are the same as those described in the legend to Figure 1.

Effect of pH on the EPR Spectrum of the Phosphotriesterase

EPR spectra of Cu/Cu phosphotriesterase samples at different pH values are shown in Fig. 7. As the pH is raised, the signals gradually change from axial to rhombic symmetry. The partially resolved nitrogen superhyperfine features are diminished in spectra of samples at pH 6 and pH 10. At pH 5.0 and below, the EPR spectrum of the copper-substituted phosphotriesterase resembles that of copper perchlorate (at pH 1.8), suggesting that the copper has dissociated from the protein.

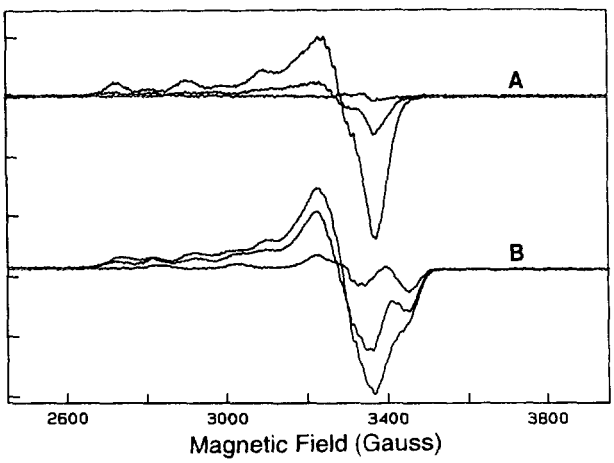


FIG. 5. Effect of anaerobic addition of ascorbate on the EPR spectra of phosphotriesterase with 1 equivalent of Cu^{2+} per protein (A) and 2 equivalents of Cu^{2+} per protein (B). The ratios of ascorbate/protein used were 0, 0.25, and 0.5 for spectrum A and 0, 0.25, and 1.0 for spectrum B. The protein concentration for all samples was 0.33 mM in 50 mM Hepes, pH 8.3. Samples were kept frozen in liquid nitrogen after incubating for approximately 40 min at room temperature. Spectra were recorded at 100°K, and other conditions are as described in the legend to Figure 1.

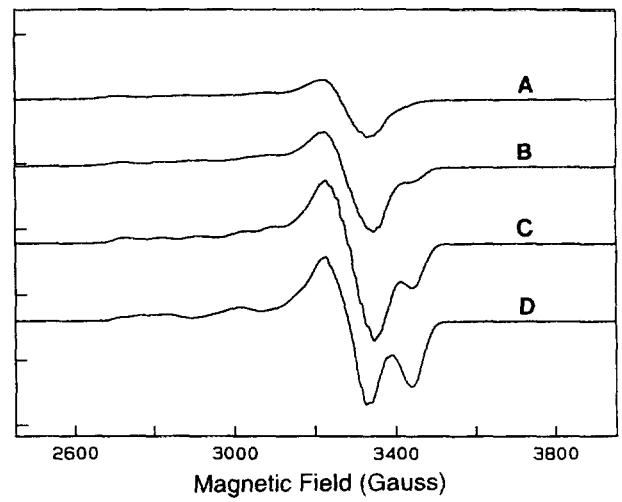
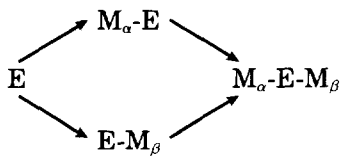


FIG. 7. Effect of pH on the EPR spectrum of copper-substituted phosphotriesterase. The preparation of samples for the pH studies were described in the text. The pH of the samples were (A) 6.0, (B) 7.0, (C) 9.0, and (D) 10.0. The protein and copper concentrations for each sample were (A) 0.59 and 0.77 mM, (B) 0.64 and 0.95 mM, (C) 0.55 and 0.82 mM, (D) 0.58 and 0.96 mM. All spectra were collected at 100°K, and the EPR parameters are as described in the legend to Fig. 1.

DISCUSSION

Bacterial phosphotriesterase requires two divalent metal ions for catalytic activity (7). These metal ions are bound to the protein in close proximity to one another and form a binuclear metal center (1). The individual metal sites have been labeled α and β where the α-site has a higher affinity for zinc than for cadmium and it appears to be the dominant catalytic site. The β-site prefers the binding of cadmium to zinc, but the specific function of this site is less clear. A variety of divalent cations (Zn^{2+}, Ni^{2+}, Cd^{2+}, Mn^{2+}, and Co^{2+}) bind to apoenzyme (4). Their binding is highly synergistic[5] and thus the metal ions add to the active site in pairs rather than sequentially (7).



Therefore, apoenzyme reconstituted with 1 mol equivalent of metal ion is composed primarily of an equal mixture of apoenzyme (E) and a filled binuclear center ($M_\alpha - E - M_\beta$).

Properties of Cu-Phosphotriesterase

The copper-substituted phosphotriesterase can be prepared by incubation of Cu^{2+} with the apoprotein. The specific activity of this derivative, for the catalytic hydrolysis of paraoxon, is approximately 2.4% of the value previously measured for the native zinc-containing enzyme. Analysis of the metal content of the copper-substituted enzyme always indicates a small, but significant, amount of contaminating zinc. The zinc concentration closely parallels the observed enzymatic activity and thus it appears that the copper-substituted enzyme is essentially inactive toward the hydrolysis of phosphotriesters. This conclusion is further supported by the Michaelis constant measured for paraoxon hydrolysis and the competitive inhibition constants for various inhibitors. All of these constants are nearly identical to those previously measured for fully substituted zinc enzyme.

EPR Spectra of Cu-Substituted Enzyme

Since two Cu ions were needed to fully develop the EPR spectrum of Cu-substituted enzyme and the ^{113}Cd NMR spectrum of ^{113}Cd-substituted phosphotriesterase exhibits two distinct peaks (7), it is clear that the enzyme contains two metal sites. EPR spectra of Mn-substituted phosphotriesterase indicate that the two Mn^{2+} ions are near to each other and spin-coupled in a binuclear active site. Thus, the Cu-substituted phosphotriesterase contains two Cu ions bound in a binuclear site. Curiously, the EPR spectra of Cu/Cu-substituted enzyme appears more typical of a single signal, rather than the superposition of two signals, one from each Cu ion. This situation is not unprecedented. The two copper ions in dopamine β-hydroxylase also exhibit what appears to be a single signal (12). The temperature dependence of the signal from Cu/Cu phosphotriesterase follows the Curie Law between 3 and 50°K, indicating a ground state spin system, and no obvious low field features ($\Delta m_s = \pm 2$ transitions) were observed in either the X- or S-band spectra. The EPR spectra of the Cu-substituted enzyme clearly show at least seven superhyperfine transitions in the $g_\perp$ region. These splittings are consistent with the direct ligation of at least three nitrogens to the metal center. This conclusion is consistent with previous studies of the Cd-substituted enzyme. The ^{113}Cd NMR spectra indicated that the metal ions are bound to the protein via a mixture of nitrogen and oxygen ligands (7). The positions of the cadmium chemical shifts also exclude the possibility of ligation by cysteine. The observed values for $g_{||}$ and $A_{||}$ are also consistent with a ligand set of oxygen and nitrogen, according to the correlations compiled by Peisach and Blumberg for model systems and metal centers of proteins of known structure (13).

The low spin intensity of the EPR signal from the Cu/Cu enzyme is almost certainly the result of magnetic interactions between the two paramagnetic Cu^{2+} ions within the binuclear metal center. All of the expected spin intensity can be recovered if the protein is denatured with acid and thus the protein matrix is clearly required for the juxtaposition of the two metals in a way that facilitates this interaction. The diminution of the observable spins from two paramagnetic centers in close proximity could be due either to the dipolar interaction described by Leigh (14) or to an incomplete spin pairing between the two $S = 1/2$ centers.[6] A dipolar interaction of the type described by Leigh has been observed be-

[5] The ^{113}Cd NMR spectrum of 1.0 ^{113}Cd-substituted enzyme exhibits two distinct peaks with similar signal intensities (7). If the added Cd bound predominantly to either the α- or β-site, only one NMR peak would have been observed. Moreover, the EPR spectrum of 0.5 Mn and 1.0 Mn phosphotriesterases afforded fully coupled signals (Chae *et al.*, unpublished observations), indicating formation of a binuclear center. Had the Mn ions bound in a stepwise fashion, the EPR spectrum would have primarily exhibited signals of uncoupled mononuclear complexes.

[6] The working model for the binuclear metal center of the phosphotriesterase has two divalent cations bridged by a common ligand. The metals are coordinated to the protein primarily by the imidazole side chains of histidine. This model resembles to a certain degree that for type 3 copper centers which are EPR silent. Type 3 copper centers are each ligated by three histidine ligands, and are strongly antiferromagnetically coupled ($S = 0$ ground state) and are totally EPR silent. The two Cu ions are thought to be bridged by an oxygen ligand as a means of effecting the strong coupling. The coupling between Cu ions in phosphotriesterase appears to be weaker, as the site exhibits an EPR signal of reduced intensity. The distances between the Cu ions might be greater in phosphotriesterase, and/or the bridging group might be larger (histidine or aspartate).

tween the two Mn^{2+} ($S = 5/2$) bound to glutamine synthetase (15), and antiferromagnetic coupling has been observed in a binuclear Cu site of 4 Cu^{2+}-substituted superoxide dismutase (16).

Since the binding of metals to the binuclear center of phosphotriesterase is highly synergistic, we have been unable to observe the signal of Cu^{2+} bound to one site with the other site unoccupied. We have been able to make mixed metal complexes where the fraction of the Cu/Cu protein is diminished and the relative amount of Cu/X (and/or X/Cu) is increased. In cases where the other metal ion is diamagnetic (Zn^{2+} or Cd^{2+}) the spin quantitation invariably increased to nearly 1 spin/Cu. This clearly indicates that the loss of signal is magnetic in nature. It is interesting to note that the spectrum observed for the Zn/Cu protein is significantly different than the spectrum of the Cu/Cd protein. The Zn/Cu protein is nearly as active as the Zn/Zn protein (i.e., substituting Cu in the β-site has little effect on activity), while the Cu/Cd protein is significantly less active than the Cd/Cd protein (i.e., substituting Cu in the α-site strongly affects activity). This may reflect the preference of Zn for the α-site (the site predominantly responsible for activity) and Cd for the β-site.

When the paramagnetic Co^{2+} is paired with Cu^{2+}, spin quantitation is further diminished relative to what is observed for the Cu/Cu protein. This effect may be due to magnetic coupling caused by a greater local electronic spin of the Co^{2+} ($S = 3/2$). The spin quantitation is also diminished in the presence of cyanide. These results suggest that the binding of cyanide to the binuclear copper center enhances the coupling interaction between the two metals and the EPR signal is further reduced. Experiments are in progress to explain in more detail the precise mechanism for the interaction of two paramagnetic ions in this protein.

Effect of Reducing Agents

The addition of either dithionite, ascorbate, or dithiothreitol[7] to the Cu/Cu phosphotriesterase results in nearly complete loss of the EPR signal. These results suggest that the copper bound to the phosphotriesterase can be reduced to the cuprous state. At present it is unknown whether the Cu(I) phosphotriesterase can react with molecular oxygen at a kinetically significant rate or whether the Cu/Cu phosphotriesterase can be transmutated from a simple hydrolase to an oxidoreductase.

[7] The observed effect of DTT on the Cu/Cu-substituted enzyme may be due to the reduction of Cu^{2+} to Cu^{1+} or to an increase in the coupling between the two coppers caused by complexation of DTT. When 2 equivalents of DTT were added to the Cu/Zn hybrid enzyme, the signal intensity decreased by 90%. Since there can be no magnetic interactions between Cu^{2+} and Zn^{2+}, this suggests that DTT is reducing the copper.

Summary

Two copper ions bind apophosphotriesterase, forming a binuclear site. The copper ions bind in pairs, not sequentially, and the resulting Cu-substituted enzyme is inactive toward the hydrolysis of paraoxon. The Cu ions can be reduced by DTT, ascorbate, or dithionite. The EPR signal from the binuclear Cu site was affected by cyanide and pH. The EPR spectrum of the Cu-substituted enzyme exhibits a single, nearly axial signal, with low spin intensity almost certainly due to the spin-coupling of the two Cu ions. The spin intensity increases when the protein is denatured and when one Cu is replaced with a diamagnetic metal ion (Zn^{2+} or Cd^{2+}). The spin intensity decreases when one Cu is replaced with another paramagnetic ion, Co^{2+}. The mechanism of spin-coupling responsible for this behavior is under investigation.

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