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Chemistry

Coordination environment and fluoride binding of type 2 copper in the blue copper oxidase ceruloplasmin

(nonblue copper/copper proteins/electron paramagnetic resonance/square planar geometry/nitrogenous ligands)

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ABSTRACT **The electron paramagnetic resonance (EPR) spectra of the blue copper oxidase ceruloplasmin [ferroxidase, iron(II):oxygen oxidoreductase, EC 1.16.3.1] and of a derivative having the type 1 (blue) copper centers reversibly bleached are reported. The EPR spectrum of bleached ceruloplasmin has a seven-line superhyperfine structure in the $g_\perp$ region that is attributed to the presence of three nitrogen-donor type 2 copper ligands. The EPR data suggest further that the type 2 copper in ceruloplasmin possesses a tetragonal coordination gometry. In the presence of varying amounts of fluoride, superhyperfine splitting patterns in the $g_\parallel$ region of both ceruloplasmin derivatives indicate that a maximum of two fluorides may be bound to the type 2 copper.**

The blue copper oxidase ceruloplasmin [ferroxidase, iron(II): oxygen oxidoreductase, EC 1.16.3.1] (1, 2) is the major copper-containing mammalian blood plasma protein. As do ascorbate oxidase and laccase, ceruloplasmin catalyzes the four-electron reduction of dioxygen to two molecules of water with concomitant substrate oxidation. Although capable of oxidizing many organic substrates *in vitro*, the physiological substrate of ceruloplasmin is thought to be ferrous ion (2). Ceruloplasmin may also function as a copper transport protein. Indeed, in addition to the intrinsic copper atoms that are involved in the catalytic activity of the enzyme, ceruloplasmin will bind up to 10 cupric ions in reversible fashion (3).

The blue copper oxidases each contain a minimum of four copper atoms in three spectroscopically distinct sites (4–9). The type 1, or blue, copper is characterized by an intense electronic absorption band at *ca* 600 nm and by an abnormally narrow electron paramagnetic resonance (EPR) hyperfine structure. The type 2, or nonblue, copper features a copper EPR hyperfine structure similar to that found in tetragonal copper complexes. Type 3, or EPR-nondetectable, coppers are associated with an intense absorption band at *ca* 330 nm. Ceruloplasmin has six or seven copper binding sites: two type 1, one type 2, and three or four type 3 (10).

Spectroscopic studies on native (refs. 11–14 and unpublished data) and cobalt-substituted (11, 15–17) blue proteins have indicated a near tetrahedral geometry for type 1 copper; for plastocyanin (18) and azurin (19), preliminary x-ray crystallographic results have shown that the copper ligands are two histidines, a cysteine anion, and a methionine. In contrast, much less is known about the exact coordination environment of type 2 copper. Owing to the considerable overlap of EPR, circular dichroism, and optical absorption spectra arising from copper types 1 and 2, selective removal or reduction of the former center greatly aids any detailed study of the latter site. Accordingly, we have examined the EPR properties of type 2 copper through use of a ceruloplasmin derivative lacking type 1 EPR signals (20). Reported herein are the results of these experiments as well as those obtained in an EPR study of the fluoride-binding properties of ceruloplasmin.

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MATERIALS AND METHODS

Reagent-grade chemicals were used without further purification. Nitric oxide was obtained from Union Carbide, and sodium fluoride was from Baker and Adamson.

Ceruloplasmin was purified to electrophoretic homogeneity and a spectral purity ratio (A_{610}/A_{280}) of 0.045 by published methods (10), with minor modifications (unpublished experiments), from Cohn fraction IV kindly provided by Lewis Larsen, Massachusetts Department of Public Health. Enzymatic activity was verified. Nitric oxide-treated ceruloplasmin (NO-ceruloplasmin) was prepared in a quartz EPR tube on a vacuum line using nitric oxide passed through a dry ice/acetone bath to remove impurities. Several gentle pump-purge cycles were performed, first with argon to deoxygenate the protein followed by several more cycles with nitric oxide. In separate experiments, the absorption spectral changes reported by Wever *et al.* (20) were reproduced.

EPR spectra were run on a Varian E-line Century Series spectrometer equipped with a Varian E-102 microwave bridge and a 12-inch magnet. Frequencies were measured with a PRD Electronics, Inc. (New York, NY) frequency meter. An Air Products Heli-Trans system was used to obtain low temperatures.

All pH measurements were performed at room temperature with a Brinkman pH 101 meter equipped with a Metrohm combination electrode. Experiments with native ceruloplasmin, ceruloplasmin plus F^-, and NO-ceruloplasmin plus F^- were done in 0.2 M sodium acetate buffer (pH 5.5). Experiments on NO-ceruloplasmin were performed in 0.1 M sodium acetate, pH 7.0/0.25 M NaCl. Concentrated protein samples were obtained by ultrafiltration (Amicon) through a PM-30 membrane or by use of a Millipore Immersible Molecule Separator. All EPR spectra were measured on protein of approximately 1 mM concentration. Details of operating conditions are given in the figure captions.

RESULTS

The EPR spectrum of native ceruloplasmin at pH 5.5 (Fig. 1) is essentially identical with those previously reported (21–23). Resolution of the type 1 and type 2 copper hyperfine splitting patterns is indicated. The EPR spectrum of native ceruloplasmin at pH 7.0 is quite similar to that displayed in Fig. 1. Exposure of the native protein at this pH to nitric oxide led to the much simpler spectrum presented in Fig. 2 *upper*. An expan-

Abbreviations: EPR, electron paramagnetic resonance; NO-ceruloplasmin, nitric oxide-treated ceruloplasmin.

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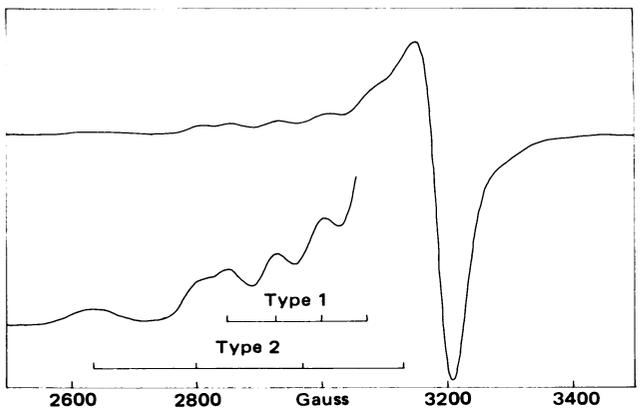

FIG. 1. EPR spectrum of native ceruloplasmin at 5 K and 9.1955 GHz. Microwave power was 5 mW; modulation amplitude was 10 gauss.

sion of the $g_\perp$ (*ca* 3200 gauss) region of this spectrum is shown in Fig. 2 *lower*.

Addition of approximately 15 equivalents of fluoride to ceruloplasmin leads to a splitting of the lowest field EPR transition (Fig. 3). This spectrum, which is better resolved than that reported by Brändén *et al.* (24), was obtained by the sequential addition of small amounts of fluoride over several days. After each addition, and a 24-hr equilibration period (24), an EPR spectrum was run. In order to resolve the entire spectrum, we added nitric oxide to the fluoride-treated protein (Fig. 4). Single addition of approximately 1000 equivalents of fluoride to the native protein led to further splitting of the lowest field transition, as displayed in Fig. 5. As before, addition of nitric oxide led to loss of most of the type 1 EPR signal and resulted in the spectrum presented in Fig. 6.

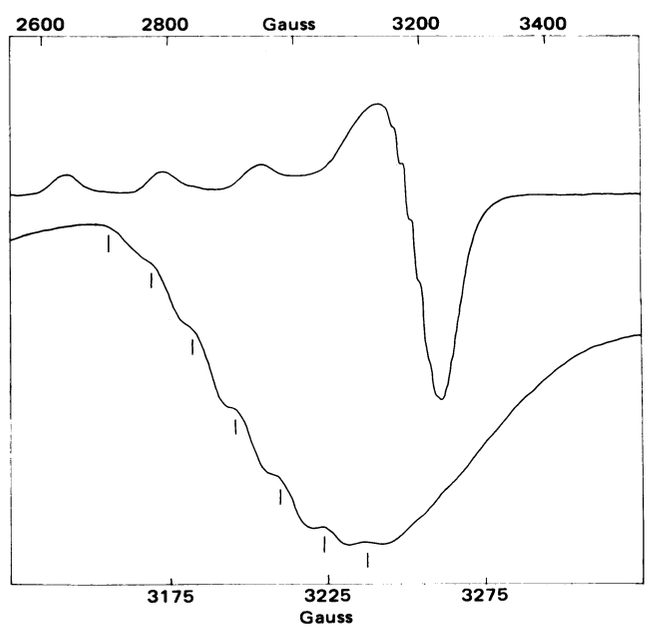

FIG. 2. EPR spectra of nitric oxide-treated ceruloplasmin. (*Upper*) Temperature, 80 K; power, 20 mW; modulation amplitude, 5 gauss; frequency, 9.1760 GHz. (*Lower*) Temperature, 5 K; power, 5 mW; modulation amplitude, 1.6 gauss; frequency, 9.1905 GHz. The scale for the upper curve is at the top; that for the lower curve is at the bottom.

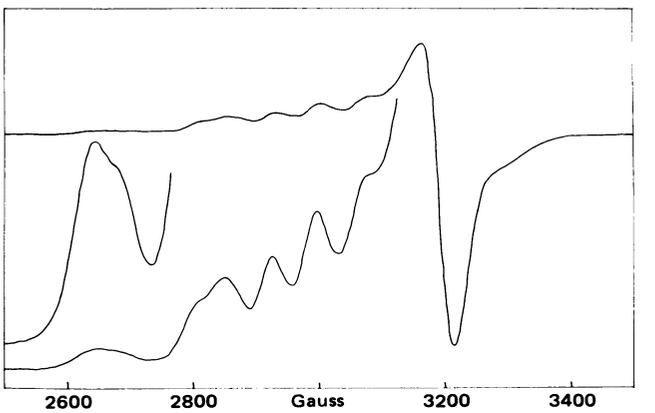

FIG. 3. EPR spectrum of fluoride-treated (15 equivalents) ceruloplasmin at 65 K and 9.0750 GHz. Power was 5 mW; modulation amplitude was 10 gauss.

DISCUSSION

Treatment of ceruloplasmin with nitric oxide results in an axial EPR spectrum (Fig. 2 *upper*) which, due to the lack of type 1 EPR signals, is noticeably simpler than the spectrum of the native protein (Fig. 1). The ordering and magnitudes of the observed parameters, i.e., $g_\parallel > g_\perp > 2.0023$ and $|A_\parallel| \simeq 0.017$ cm^{-1} (160 G), are entirely consistent with a tetragonal coordination geometry for type 2 copper. For such a complex, the unpaired electron is localized principally in a Cu(II) $d_{x^2-y^2}$ orbital, resulting in a $^2B_{1g}$ ground state (assuming approximate D_{4h} symmetry). A weak seven-line superhyperfine pattern in the $g_\perp$ region is evident (Fig. 2 *lower*). No such structure was observed in the $g_\parallel$ (2500–3100 gauss) region, probably because the signal is spread over four $A_\parallel$ lines and thus is too weak to be resolved. The seven-line splitting pattern is most logically attributed to coordination by three nitrogen-donor ($I = 1$) ligands that are magnetically equivalent. Four such ligands may not be definitively ruled out, as it is conceivable that an additional line at each extreme is broadened to the extent that it does not appear. A nine-line pattern was clearly present in the type 2 EPR of ascorbate-treated ceruloplasmin (25) that contains no type 1 copper (unpublished data). Optical data on this form of the enzyme also support the formulation of type 2 copper as a tetragonal coordination complex (unpublished data).

Mondovi *et al.* (26) have measured electron spin-echo decay envelopes of the type 2 copper in ceruloplasmin and have interpreted their results as evidence of histidine nitrogen coordination (from the imidazole moiety). If their interpretation

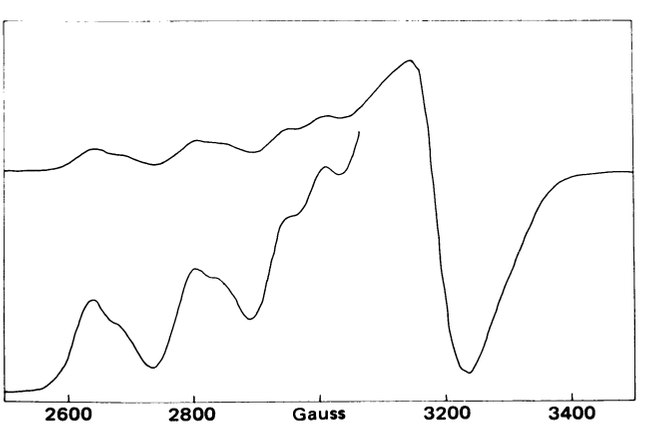

FIG. 4. EPR spectrum of ceruloplasmin, treated with fluoride (15 equivalents) and nitric oxide, at 85 K and 9.1765 GHz. Power was 20 mW; modulation amplitude was 10 gauss.

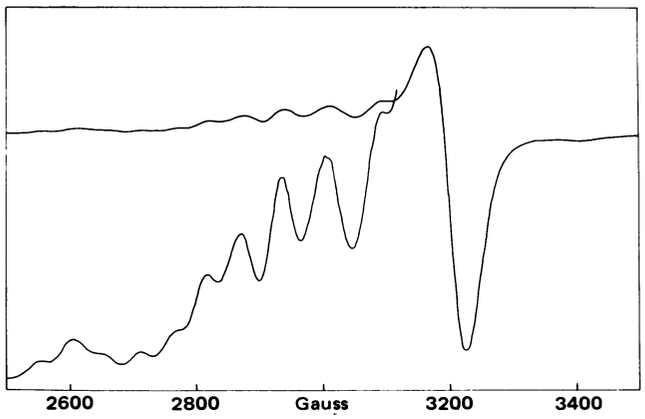

FIG. 5. EPR spectrum of ceruloplasmin in the presence of a 1000-fold excess of F^-. Conditions were the same as those stated in Fig. 1 except that modulation amplitude was 5 gauss.

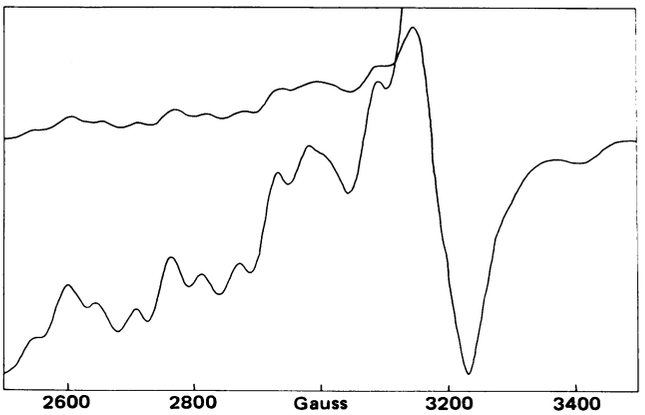

FIG. 6. EPR spectrum of ceruloplasmin, treated with fluoride (1000 equivalents) and nitric oxide, at 80 K and 9.1760 GHz. Power was 20 mW; modulation amplitude was 10 gauss.

is correct, then the nitrogen donors responsible for the superhyperfine structure seen in Fig. 2 *lower* must also be from histidine. Further evidence for histidine coordination of copper with marked similarities to type 2 has come from work on galactose oxidase (27). Superhyperfine structure from two imidazole nitrogens was observed in the native enzyme and additional splittings were resolved in the presence of exogenous imidazole and fluoride.

Fluoride binding

The anion-binding properties of ceruloplasmin have been extensively studied. Addition of azide, cyanate, and thiocyanate leads to considerable changes in the optical (28), circular dichroism (refs. 29–31 and unpublished data), magnetic circular dichroism (unpublished data), and resonance Raman (32) spectra. Identification of the specific copper site(s) responsible for anion binding on the basis of these spectral changes has proved difficult. Vänngård and coworkers (22, 24) examined the changes in the EPR spectrum upon addition of azide and fluoride and concluded that both ions interact with the type 2 copper. In the presence of azide (22), new EPR transitions were seen, whereas it appeared that the lowest field transition was simply split by interaction with fluoride ($I = 1/2$) (24). Overlap with EPR signals due to type 1 copper allowed observation only of the lowest field type 2 copper transition. Therefore, it is impossible to ascertain whether the spectral changes seen were due to the appearance of new transitions, as was the case for azide binding (22), or whether they were due to a splitting caused by the coordination of fluoride to the otherwise intact type 2 center. Malkin *et al.* (33) have provided evidence that the type 2 copper in laccase possesses two fluoride-binding sites.

In order to clarify the exact nature of the fluoride–type 2 copper interaction in ceruloplasmin, we have examined the EPR spectral changes caused by fluoride addition to both native and NO-ceruloplasmin. Initial exposure to *ca* 15 equivalents of fluoride results in the splitting of the lowest field transition into a doublet of nearly equal intensities (Fig. 3). After addition of nitric oxide (Fig. 4), the splitting of each type 2 copper hyperfine line (see Fig. 1) into a doublet is clearly resolved. With fluoride present in 1000-fold excess, splitting of the lowest field type 2 transition into a triplet with an intensity ratio of approximately 1:2:1 is found (Fig. 5). Again, treatment of this preparation with nitric oxide reveals the splitting of each type 2 copper hyperfine line into a triplet. The preliminary results of Brändén *et al.* (24) pointed to only one binding site for fluoride on type 2 copper, in contrast with the results obtained for laccase (33). The data presented here, however, show that ceruloplasmin type 2 copper also has two fluoride-binding sites. The similarity in the EPR parameters of the type 2 copper before and after nitric oxide addition (Table 1), as well as the fact that the selective type 1 bleaching process is reversible after removal of nitric oxide, indicate that the properties of the type 2 copper are only slightly perturbed in this derivative.

Concluding remarks

The EPR data presented here are most consistent with a square planar, native type 2 copper coordination environment including three (possibly four) nitrogen donors with two axial positions available for fluoride-ion binding. The superhyperfine structure seen in the $g_\parallel$ regions of Fig. 3–6 clearly show that the type 2 copper is readily available for ligation by up to two fluorides. Unfortunately, bleaching by nitric oxide of the type 1 copper in fluoride-treated ceruloplasmin is not complete (Fig. 4 and 6). Thus, while very weak superhyperfine structure in the $g_\perp$ region of Fig. 6 attributable to *at least* two nitrogen-donor

Table 1. Approximate EPR parameters*

			Type 1		Type 2			
Ceruloplasmin†	pH	$g_\perp$	$g_\parallel$	$A_\parallel$, G	$g_\parallel$	$A_\parallel$, G	A_N, G	A_F, G
Native	5.5	2.06	2.21	75	2.27	165		
NO-CP	7.0	2.06			2.28	155	14	
CP + $1F^-$	5.5	2.06	2.21	75	2.26	160		40
NO-CP + $1F^-$	5.5	2.06			2.26	165		40
CP + $2F^-$	5.5	2.06	2.21	70	2.31	160		50
NO-CP + $2F^-$	5.5	2.06			2.30	165		50

* g values are accurate only to ±0.01; $A_\parallel$ values are accurate to ±5 G.
† CP, ceruloplasmin.

ligands was seen, the exact splitting pattern could not be determined due to overlap with the residual type 1 signal. Consequently, we cannot entirely rule out the possibility of fluoride displacement of one of the original nitrogen-donor ligands to form a square planar N_2F_2 coordination unit. More likely, however, the two fluorides coordinate in the axial positions of an overall tetragonal structure.

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