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Spectroscopic Studies of Ceruloplasmin. Electronic Structures of the Copper Sites

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Abstract: Low temperature absorption and room temperature circular dichroism and magnetic circular dichroism spectra have been measured for the blue copper oxidase ceruloplasmin. Azide- and thiocyanate-bound ceruloplasmins have been examined by the two latter techniques. Low energy ligand field transitions have been located in the native (6100, 10 000, and 11 500 cm^{-1}) and azide-bound (8940 and 12 000 cm^{-1}) proteins that are of similar energy to those previously attributed to a flattened tetrahedral type 1 (blue) copper in other blue copper proteins. The MCD spectrum, featuring a single negative band at 14 600 cm^{-1}, is quite similar to those of the other blue copper proteins. The combined spectroscopic evidence suggests that both blue copper sites in ceruloplasmin are structured in the same manner as the CuN_2SS^* (N = His, S = Cys, S* = Met) unit in azurin and plastocyanin. Examination of the ligand field spectra shows conclusively that structural changes in both type 1 coppers occur on anion binding. Analysis of the d-d spectrum of azide-bound ceruloplasmin requires a larger distortion away from a tetrahedral structure than is indicated for native type 1 copper. An ascorbate-modified (type 1 depleted) derivative of ceruloplasmin has been characterized; its absorption (16 500 cm^{-1}), circular dichroism (13 700, 17 000, 19 800, 22 200, 25 300, and 32 000 cm^{-1}), and electron paramagnetic resonance ($g_\perp = 2.06$, $g_\parallel = 2.26$; $A_\parallel = 170$, $A_N = 14$ G) spectra have been measured. The band positions observed in absorption and CD rule out tetrahedral or near-tetrahedral geometries for the type 2 and type 3 coppers. A tetragonal structure for type 2 copper with four nitrogen-donor ligands is suggested by the nine-line superhyperfine splitting pattern seen in the $g_\perp$ region of ascorbate-modified ceruloplasmin.

Ceruloplasmin,[2] the major copper-containing blood plasma protein, is the only mammalian member of a small group of enzymes called the blue copper oxidases.[3-9] Since its original discovery by Holmberg,[10] ceruloplasmin has been intensely investigated, especially after establishment of its clinical importance as the affected protein in the hereditary copper metabolic disorder known as Wilson's disease. Although capable of oxidizing a variety of organic molecules in vitro, its physiological substrate is thought to be ferrous ion.[2] Ceruloplasmin may also function as a copper transport protein,[2] as it will reversibly bind up to ten cupric ions in addition to the intrinsic, catalytically important coppers.[11]

The blue copper oxidases (e.g., ceruloplasmin, ascorbate oxidase, and the laccases) contain at least four copper ions in three spectroscopically distinguishable sites.[3-9] Intensely blue in color, the type 1 (blue) copper has an abnormally small

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copper electron paramagnetic resonance (EPR) hyperfine splitting. The type 2 (nonblue) copper EPR hyperfine splitting is in the range considered normal for simple tetragonal Cu(II) complexes. The type 3 (EPR nondetectable) copper has an intense near-UV (330 nm) absorption band and is thought to be structured as an antiferromagnetically coupled copper pair.[12,13]

The stoichiometry of copper types 1, 2, and 3 is not well established for ceruloplasmin. Initial molecular weight determinations led to an estimate of eight intrinsic coppers;[10,14] recent measurements (130 000–134 000),[15-18] however, are more consistent with six to seven coppers.[18] Forty-four percent of the total copper is paramagnetic,[19,20] a value that suggests three paramagnetic and four nonparamagnetic coppers per enzyme. Quantitative EPR measurements have established the presence of two type 1 and one type 2 copper.[21,22] Therefore, one type 3 pair and an additional EPR nondetectable "type 4" copper[18] are required to fit a stoichiometry of six and two type 3 units are necessary for seven. Huber and Frieden have shown[23] that Chelex treatment leads to loss of one copper without affecting spectral or catalytic properties. With this in mind, Rydén and Björk have suggested[18] that copper stoichiometries above six are due to partial occupation of this "chelexable" site. Further complications include the complex relationship between the electrochemical behavior of the protein and 330-nm (type 3) absorption changes[21,24] and the possible spectroscopic and/or kinetic inequivalence of the two type 1 sites (vide infra).[21,22,25-29]

The polypeptide chain structure of ceruloplasmin has been a matter of some debate. Poulik first reported[30] the presence of several polypeptide chains in denatured ceruloplasmin. Subsequently, several laboratories examined[14,31] the subunit structure of both human and porcine ceruloplasmin. Rydén, however, has shown[18,32] these subunits to be proteolytic fragments not present when proteolytic inhibitors and fresh serum are used. Thus far, no spectroscopic differences have been seen between nicked and intact ceruloplasmin.[21,22] Differences have been seen, however, in their rates of anaerobic reduction.[21] Kingston et al. have qualitatively reproduced the fragment patterns of nicked ceruloplasmins by proteolysis of intact protein.[33] An additional source of heterogeneity is in the carbohydrate part of the protein. It has been shown[34] that chromatography on hydroxylapatite leads to the separation of major and minor forms differing in percent carbohydrate.

Recently, we have investigated the spectroscopic properties of blue copper proteins[35-39] and, in certain cases, their cobalt(II) derivatives[39-40] as well. As discussed in detail elsewhere,[36] these studies in conjunction with X-ray crystal-structure analyses of poplar plastocyanin[41] and *Pseudomonas aeruginosa* azurin[42] have pointed to a distorted tetrahedral CuN_2SS^* (N = His, S = Cys, S* = Met) coordination geometry for the type 1 site. By selectively bleaching the type 1 center, we have been able to study the type 2 and type 3 sites in laccase,[37] ceruloplasmin,[43] and ascorbate oxidase.[44] For ceruloplasmin,[43] the EPR evidence is consistent with the presence of three or four equivalent nitrogen-donor ligands at the tetragonal type 2 Cu(II) site, with two additional coordination positions available for fluoride-ion binding.

This paper presents the results of our spectroscopic investigations of the copper sites in ceruloplasmin. These studies have utilized primarily the nicked form of ceruloplasmin; whenever possible, we have also examined the intact form as well. Low temperature absorption and/or room temperature near-infrared CD spectra have been obtained for the native and azide-inhibited proteins in order to locate the type 1 ligand field bands. The effects of both azide and thiocyanate binding on the visible CD and MCD spectra of ceruloplasmin have been examined. These data on anion-bound ceruloplasmin will be discussed in terms of the structural perturbations on the type 1 sites. Additionally, ascorbate-treated ceruloplasmin,[14] which contains no type 1 copper, has been studied in order to further characterize the type 2 and type 3 sites.

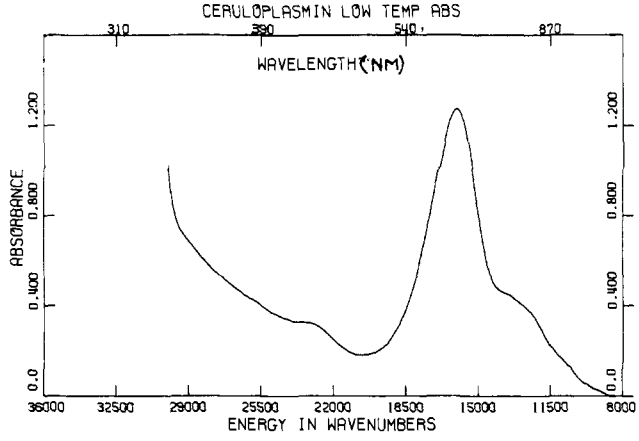

Figure 1. 20 K absorption spectrum of a ceruloplasmin film on a Plexiglas disk.

Experimental Section

Purification of Ceruloplasmin. Ceruloplasmin was purified from Cohn fraction IV[45] and from fresh plasma by modifications of the method of Rydén and Björk[18] to be published elsewhere.[46] To avoid possible heterogeneity due to varying carbohydrate composition,[18,34] hydroxylapatite chromatography is part of the purification. The final enzymatically active preparation displayed a spectral purity ratio $A_{610}/A_{280} \geq 0.045$ and was electrophoretically homogeneous under neutral nondenaturing conditions. The purified Cohn fraction IV protein was found to have six coppers per molecule;[46] the copper stoichiometry (6–7 Cu/mol) of the fresh plasma protein has been determined previously.[18]

Ascorbate Treatment of Ceruloplasmin. Modifications of the method of Kasper and Deutsch[14] to be published elsewhere[46] were used to prepare ascorbate-treated ceruloplasmin. These modifications were designed to achieve complete, base-line separation of the modified protein from intact protein during chromatographic purification. Examination of the copper stoichiometry of ascorbate-treated ceruloplasmin has revealed the presence of three coppers per molecule;[46] as expected,[47] no oxidase activity was found.[46]

General Procedures. Absorption spectra were obtained on Cary 14 and 17 spectrophotometers. JASCO J-40 and Cary 61 instruments were used for the UV-vis (300–800 nm) CD measurements. Near-infrared (700–2000 nm) CD spectra were recorded on a laboratory-constructed instrument.[48] In all cases the sensitivities of the CD instruments between 700 and 800 nm are low, and data taken in this region are reliable only to ±20%. The Cary 61, equipped with a Varian 40-kG magnet, was used for visible MCD measurements. The JASCO J-40 was calibrated as described elsewhere;[49,50] the data obtained were manipulated on a NOVA 840 computer.[50] MCD spectra have been corrected for natural optical activity. A Varian E-12 Century Series EPR spectrometer equipped with temperature regulation (Air Products, Heli-Trans), a Varian E-102 microwave bridge, and a frequency meter (PRD Electronics, Inc.) was used. A Brinkmann Model 101 pH meter with a Metrohm combination glass electrode was employed for pH measurements (all at room temperature). The relationship pD = pH meter reading + 0.4 was used to calculate pD.[51]

Using previously described techniques,[37] a protein film was prepared on a plexiglass disk from concentrated protein in 0.05 M sodium acetate, 0.1 M NaCl, pH 5.5 buffer, and a low temperature absorption spectrum was obtained with a Cryogenic Technology Model 20 cryocooler. Visible CD and MCD and near-infrared CD measurements of native and anion-treated ceruloplasmin utilized protein in 0.2 M sodium acetate (pH or pD 5.5) buffer. Anion additions were done with the solid sodium salts. Appropriate buffers were used to determine the instrumental base lines. Concentrated protein samples were obtained by ultrafiltration (Amicon, PM-30 membrane or Millipore, Immersible Molecular Separator). Native protein concentrations were determined using published extinction coefficients.[18]

Table I. Electronic Spectroscopic Properties of Ceruloplasmin and Anion-Bound Derivatives[a]

protein	absorption		CD			assignment
	cm^{-1}	nm	cm^{-1}	nm	$\Delta\epsilon$	
native[b]			6 100	1640	+2.0	$^2B_2 \rightarrow {}^2E$
	10 000	1000	10 000	1000	−0.9	$^2B_2 \rightarrow {}^2B_1$
	11 500	870				$^2B_2 \rightarrow {}^2A_1$
	13 000	770	13 750	730	−9.0	$\pi S \rightarrow d_{x^2-y^2}$
	16 400	610	16 400	610	+3.4	$\sigma S \rightarrow d_{x^2-y^2}$
			18 350	545	+5.0	$\sigma S^* \rightarrow d_{x^2-y^2}$
	23 000	435	22 000	450	−4.8	$\pi N \rightarrow d_{x^2-y^2}$
			28 000	355	+0.8	*c*
			30 750	325	−0.5	*c*
N_3^- added[d]			8 940	1120	+1.4	$^2B_2 \rightarrow {}^2E$
			12 000	835	−1.6	$^2B_2 \rightarrow {}^2B_1$
	(14 000)[e]	(710)[e]	15 300	655	−6.0	$\pi S \rightarrow d_{x^2-y^2}$[f]
	16 500	605	15 300	655	−6.0	$\sigma S \rightarrow d_{x^2-y^2}$[f]
			19 250	520	+2.6	$\sigma S^* \rightarrow d_{x^2-y^2}$
			22 500	445	−6.7	$\pi N \rightarrow d_{x^2-y^2}$
	26 000	385	27 000	370	+8.0	$N_3^- \rightarrow Cu(II)$
NCS^- added[g]	(13 000)[e]	(770)[e]	13 000	770	−7.8	$\pi S \rightarrow d_{x^2-y^2}$
	16 400	610	16 400	610	−2.1	$\sigma S \rightarrow d_{x^2-y^2}$
			18 750	530	+0.4	$\sigma S^* \rightarrow d_{x^2-y^2}$
	23 500	430	22 000	455	−10.6	$\pi N \rightarrow d_{x^2-y^2}$
	26 650	375	25 600	390	+8.4	$NCS^- \rightarrow Cu(II)$

[a] Values listed correspond to apparent peak and shoulder positions and are not the result of gaussian analyses. With overlapping bands present in some spectra, therefore, the apparent peak and shoulder positions will shift. Except where noted, all band positions are from this work. [b] Figures 1, 2, and 4; absorption spectrum was measured at 20 K. Previous absorption spectral measurements on ceruloplasmin have been made at room temperature (ref 27, 28, and 52-54a). [c] Not assigned. [d] Figures 5A and 7. [e] Shoulders reported in ref 25. [f] The πS and $\sigma S \rightarrow d_{x^2-y^2}$ bands in azide-bound ceruloplasmin are not resolved. [g] Figure 5B.

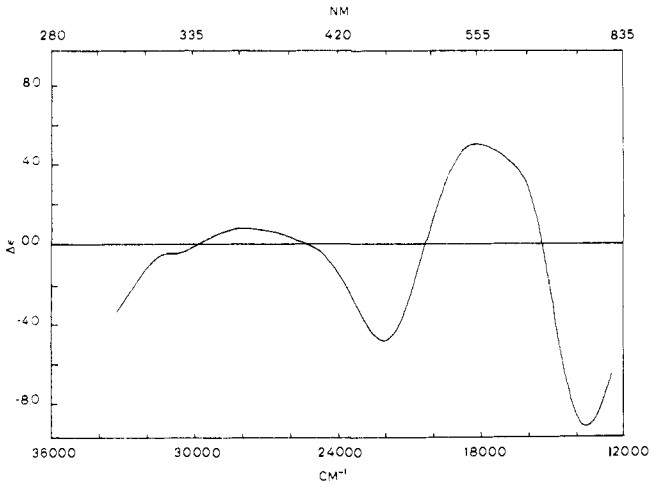

Figure 2. UV-vis CD spectrum of ceruloplasmin in 0.2 M sodium acetate (pH 5.5).

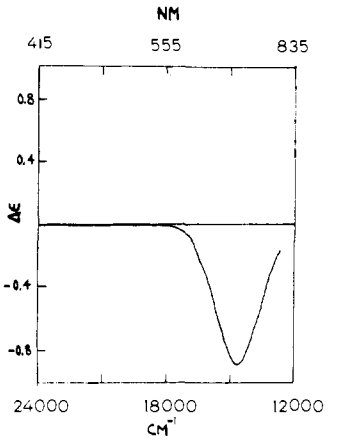

Figure 3. Visible MCD spectrum of ceruloplasmin in 0.2 M sodium acetate (pH 5.5). MCD between 24 000 and 33 000 cm^{-1} is very weak and is not reported.

For ascorbate-treated ceruloplasmin, an extinction coefficient at 280 nm (ϵ 205 000 M^{-1} cm^{-1})[46] was used. Unless otherwise noted, spectral measurements were made on protein isolated from Cohn fraction IV; spectra of protein isolated from fresh plasma were also obtained in some cases, as indicated.

CD and MCD are reported in terms of differential molar extinction coefficients, $\Delta\epsilon$. In the case of MCD, $\Delta\epsilon$ is normalized to a magnetic field of +10 kG.

Reagent grade chemicals were used without further purification. D_2O was 99.8% D (Stohler Isotope Chemicals).

Results

Native Ceruloplasmin. The distinctive blue (16 400 cm^{-1}, 610 nm) absorption band of native ceruloplasmin[27,28,52-54a] is significantly narrowed at 20 K and several new features are resolved (Figure 1). Two prominent shoulders are seen, to higher (~23 000 cm^{-1}, 435 nm) and lower (~13 000 cm^{-1}, 770 nm) energy of the blue band. Three bands of approximately these energies (and intensity per type 1 copper) are characteristic of blue copper sites and have been previously observed in single blue copper proteins[35,36] as well as in tree and fungal laccase[37] and ascorbate oxidase.[38] These bands are attributable[35,36] to $\pi S \rightarrow d_{x^2-y^2}$, $\sigma S \rightarrow d_{x^2-y^2}$, and $\pi N \rightarrow d_{x^2-y^2}$ transitions, in order of increasing energy (Table I). The intense absorption band of type 3 copper[3-8] at 30 000 cm^{-1} (330 nm) is not resolved due to absorption by the plexiglass disk. Two weak shoulders are evident near 10 000 (1000) and 11 500 cm^{-1} (870 nm). Bands in this region have not been previously observed in the absorption spectrum of ceruloplasmin. The intensities and energies of these bands suggest that they be assigned[35,36] to Cu(II) d-d transitions (Table I).

The CD spectrum of ceruloplasmin in the visible region (Figure 2) accords well with those measured previously.[25,27,52,54] Observed values of $\Delta\epsilon$ for ceruloplasmin are about a factor of two greater than those obtained from the visible CD spectrum of tree laccase.[37] The shape of the CD curve is quite similar to that of tree laccase[37,54a] and ascorbate oxidase[38,55]

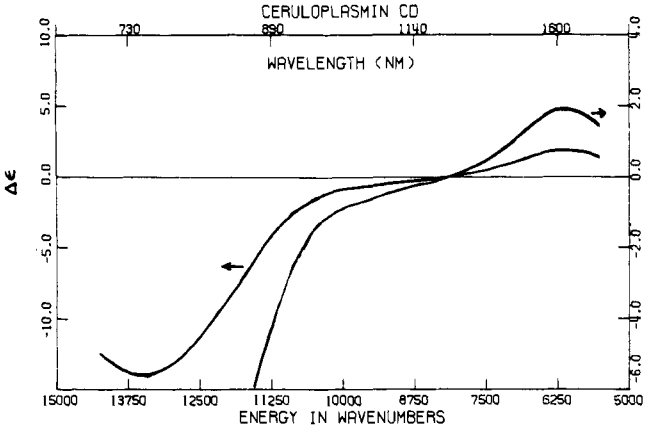

Figure 4. Near-infrared CD spectrum of ceruloplasmin (0.75 mM) in deuterated 0.2 M sodium acetate (pD 5.5).

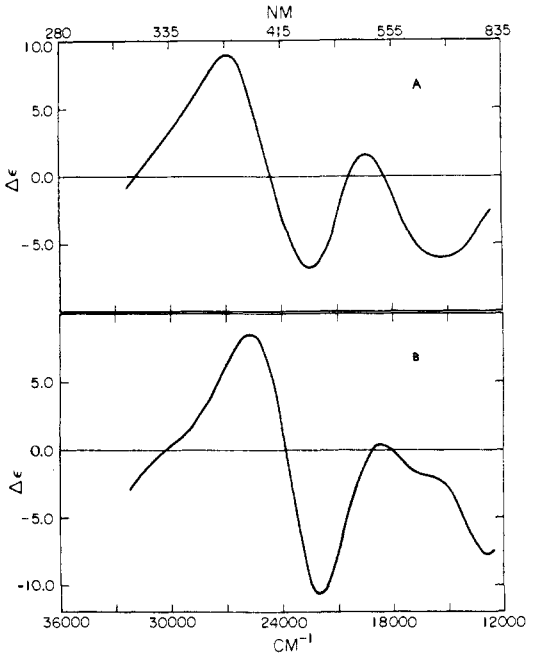

Figure 5. (A) UV-vis CD spectrum of ceruloplasmin (85 μM) in the presence of 85 mM N_3^- in 0.2 M sodium acetate (pH 5.5). (B) UV-vis CD spectrum of ceruloplasmin (78 μM) in the presence of 78 mM SCN^- in 0.2 M sodium acetate (pH 5.5).

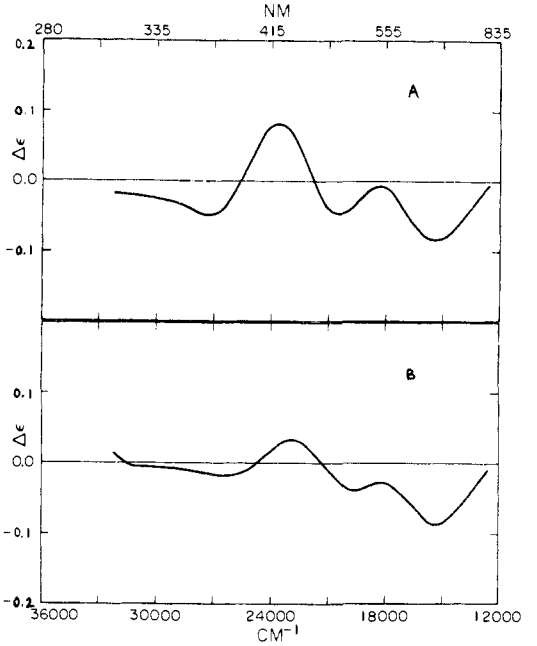

Figure 6. (A) UV-vis MCD spectrum of ceruloplasmin (78 μM) in the presence of 78 mM N_3^- in 0.2 M sodium acetate (pH 5.5). (B) UV-vis MCD spectrum of ceruloplasmin (78 μM) in the presence of 78 mM SCN^- in 0.2 M sodium acetate (pH 5.5).

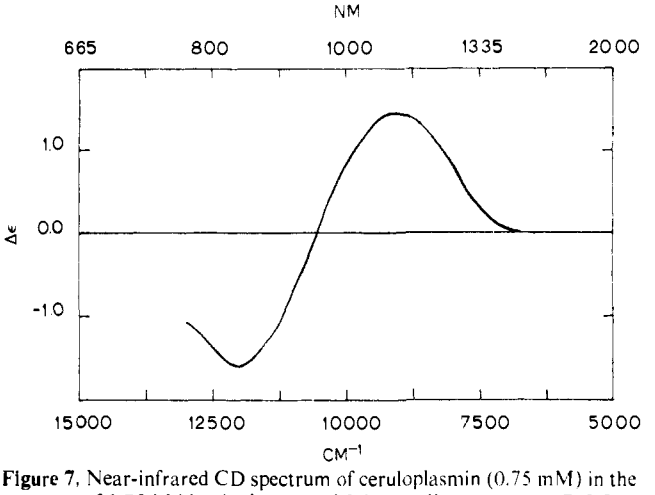

Figure 7. Near-infrared CD spectrum of ceruloplasmin (0.75 mM) in the presence of 0.75 M N_3^- in deuterated 0.2 M sodium acetate (pD 5.5).

with bands at energies (Table 1) corresponding fairly well to the prominent features (>12 000 cm^{-1}) seen in the absorption spectrum (Figure 1). The CD band at about 18 000 cm^{-1} is asymmetric and likely consists of two overlapping transitions. The lower energy component (16 400 cm^{-1}) is due to $\sigma S \rightarrow d_{x^2-y^2}$, whereas the higher energy feature (18 350 cm^{-1}) most likely is $\sigma S^* \rightarrow d_{x^2-y^2}$.[36] The visible MCD spectrum of ceruloplasmin (Figure 3), featuring a strong negative band at 14 600 cm^{-1} (685 nm), is quite similar in shape and energy to those of plastocyanin,[35,36] azurin,[35,36] tree laccase,[37] and ascorbate oxidase.[38] The intensity of this band in ceruloplasmin is about twice that of those in the single blue copper proteins.[35,36] Essentially identical visible CD and MCD spectra have been obtained for ceruloplasmin isolated from fresh plasma.

We have previously established[35-37] that the type 1 Cu(II) d-d transitions occur in the near-infrared region of the spectrum. CD spectral data for ceruloplasmin in this region are displayed in Figure 4. A low energy band is clearly resolved at 6100 cm^{-1} (1640 nm). In addition, a negative shoulder is apparent at 10 000 cm^{-1} (1000 nm). These transition energies are in excellent agreement with those observed for azurin, tree laccase, and ascorbate oxidase.[35-38] The feature at 10 000 cm^{-1} corresponds well with the lowest energy band resolved in the low temperature absorption spectrum (Figure 1). The next highest energy band seen in Figure 1 (11 500 cm^{-1}) is not observed in the CD spectrum (Figure 4) due to overlap with the intense feature that falls at 13 750 cm^{-1} (Figure 2). The lowest energy CD maximum (6100 cm^{-1}) is more than twice as intense as the corresponding bands in other proteins containing a single blue copper center.[35-37]

Anion-Treated Ceruloplasmin. Several anions (e.g., N_3^-, SCN^-, F^-) bind to the type 2 copper in ceruloplasmin; this binding is known to affect the spectroscopic properties of the type 1 sites.[25,53,54a,56,57] Figure 5 shows the visible CD spectra of ceruloplasmin in the presence of 1000-fold excesses of N_3^- and SCN^-. These spectra are similar to those measured previously.[25,54a] The visible MCD spectra of azide- and thiocyanate-bound ceruloplasmin (Figure 6) are more complicated than the spectrum of native ceruloplasmin (Figure 3) with

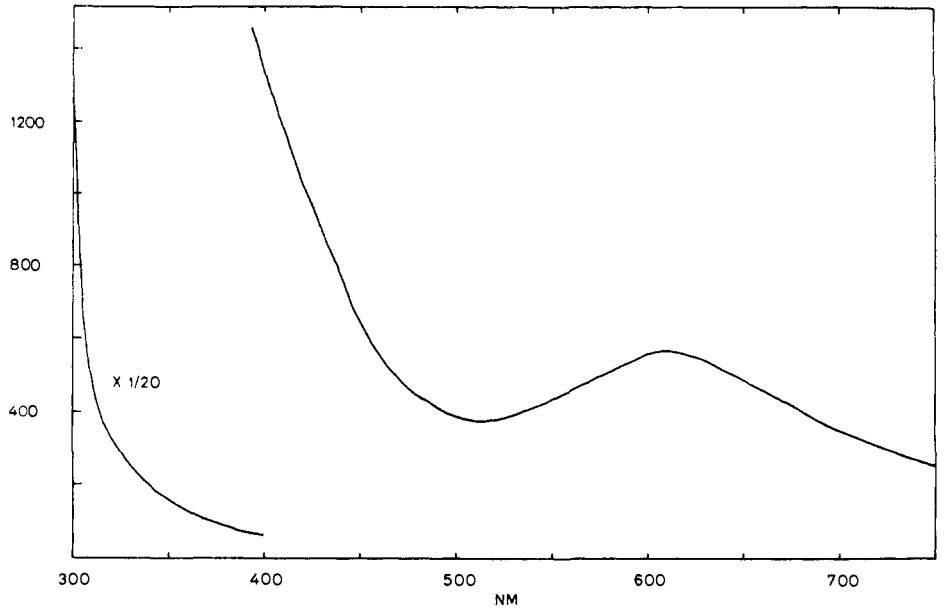

Figure 8. Room temperature absorption spectrum of ascorbate-treated ceruloplasmin (0.7 mM) in 0.05 M sodium acetate, 0.3 M NaCl, pH 7.2.

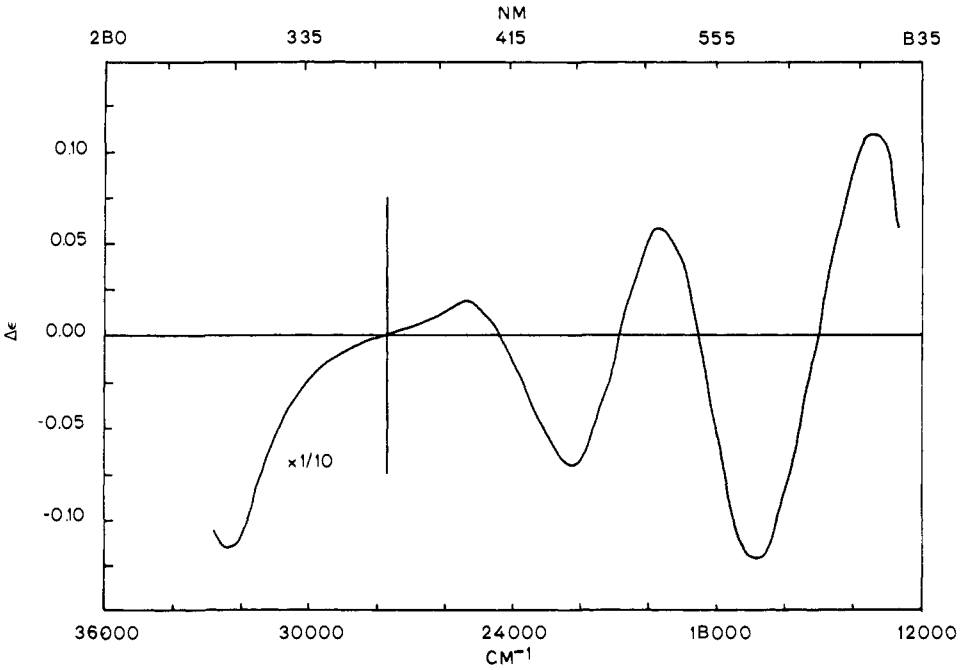

Figure 9. UV-vis CD spectrum of ascorbate-treated ceruloplasmin (0.7 mM) in 0.05 M sodium acetate, 0.3 M NaCl, pH 7.2.

additional higher energy bands present in the spectra of the anion-bound derivatives.

As Cu(II) d-d transition energies are very sensitive to geometric and ligand variations, the near-infrared CD spectrum of ceruloplasmin in the presence of a 1000-fold excess of N_3^- was measured (Figure 7). A positive peak at 8940 cm^{-1} (1120 nm) and a negative band at 12 000 cm^{-1} (835 nm) are observed. Intensities are comparable to those found in the CD spectrum of native ceruloplasmin (Figure 4). The absorption and CD spectral properties of anion-bound ceruloplasmin are summarized in Table I; MCD data are given in Table II.

Ascorbate-Treated Ceruloplasmin. Dialysis of native ceruloplasmin vs. ascorbate results in loss of the type 1 copper,[14,43,46,47] thereby permitting spectroscopic examination of the copper types 2 and 3. Visible absorption and CD spectra of ascorbate-treated ceruloplasmin are shown in Figures 8 and 9, respectively. Significant absorption at about 30 000 cm^{-1} (330 nm) is consistent with the presence of at least one type 3 site[46] in the modified protein. A weaker absorption maximum is also observed near 16 000 cm^{-1} (600 nm). Bands in the CD spectrum are evident at 13 700 (730), 17 000 (590), 19 800 (505), 22 200 (450), 25 300 (395), and 32 000 cm^{-1} (315 nm). No additional CD bands were seen in the near-IR (800–2000 nm) region.

EPR spectra (Figure 10; $g_\perp = 2.06$, $g_\parallel = 2.26$; $A_\parallel = 170$, $A_N = 14$ G) provide additional information on the nature of the type 2 copper ion in ascorbate-treated ceruloplasmin. The spectrum shown in Figure 10A is similar to that reported by Kasper et al.,[47] but the resolution is much improved. No new signals attributable to the type 3 site were observed, either near $g = 2$ or in the half-field ($g = 4$) region, indicating that the type 3 coppers remain strongly antiferromagnetically coupled or diamagnetic. Importantly, a weak nine-line superhyperfine splitting pattern is observed in the $g_\perp$ region (Figure 10B). In

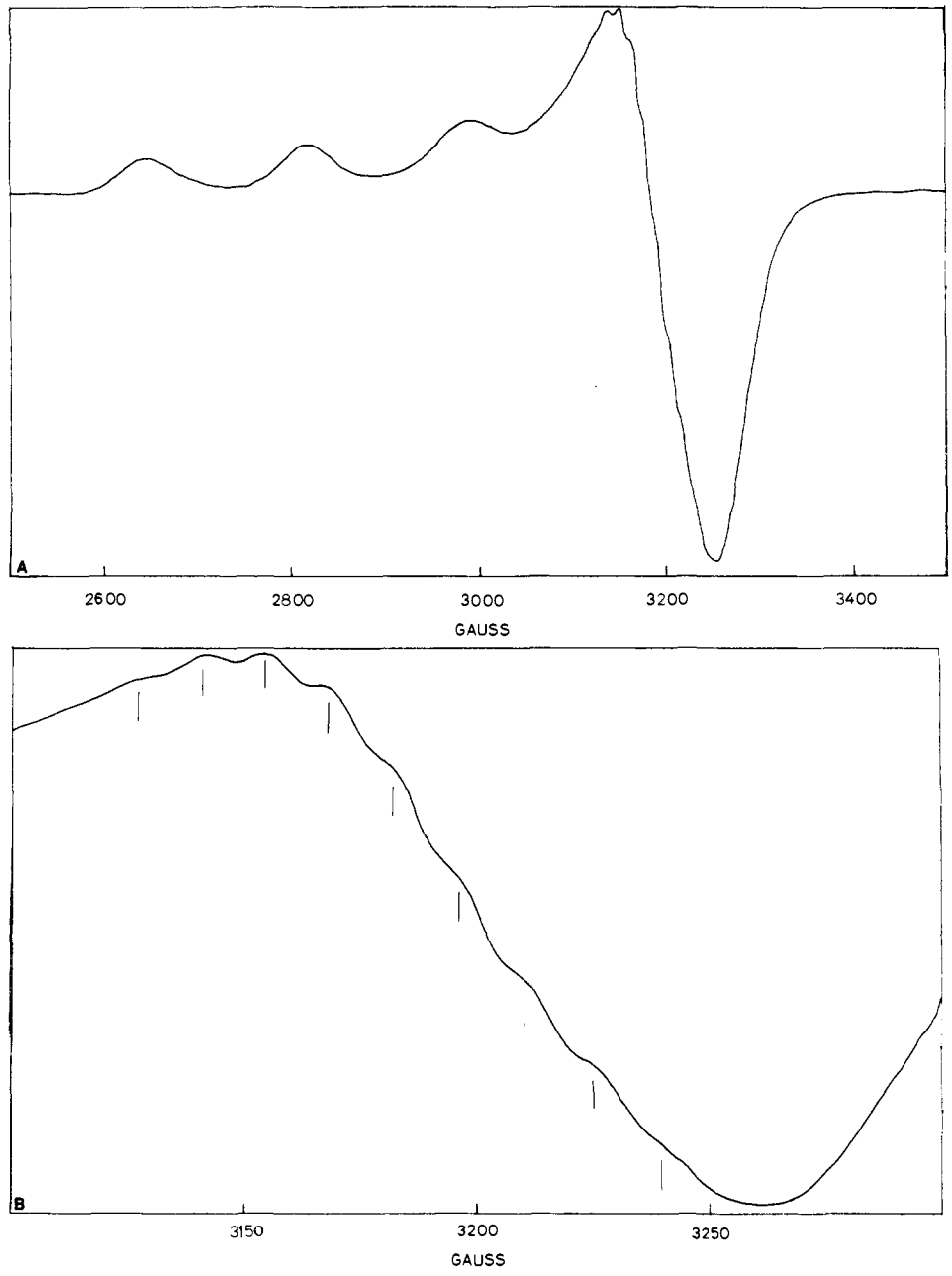

Figure 10. EPR spectra of ascorbate-treated ceruloplasmin (ca. 1 mM; 0.05 M sodium acetate, 0.3 M NaCl, pH 7.2) at 80 K and 9.1750 GHz. Microwave power was 20 mW and modulation amplitude was 3.2 G. (A) Full spectrum. (B) Expanded spectrum in the $g_\perp$ region.

contrast to native and nitric oxide treated ceruloplasmin,[43] F^- does not appear to bind to the type 2 copper in the ascorbate-treated derivative.

Discussion

Type 1 Copper. The type 1 copper d-d bands are at 6100, 10 000 and 11 500 cm^{-1} in ceruloplasmin (Figures 1 and 4). These bands are entirely absent from ascorbate-treated ceruloplasmin, confirming their assignment to type 1 copper. The d-d transition energies are well within the ranges observed for most other blue copper proteins (5000-6100, 8300-10 200, 11 100-12 000 cm^{-1}).[35-38] As demonstrated previously,[35,36] this three-band near-infrared d-d pattern may be analyzed in a satisfactory manner by assuming a flattened tetrahedral (D_{2d}) coordination geometry for Cu(II). In this D_{2d} ligand field the ground state is $^2B_2(d_{x^2-y^2})$ and the energies of the three d-d excited states fall in the order $^2E < {}^2B_1 < {}^2A_1$. Three parameters fix the d-d transition energies, Ds, Dt, and the angle (β) between the metal-ligand bond and the z axis (β = 54.74°, T_d limit; β = 90°, D_{4h} limit). Assigning the 6100- and 10 000-cm^{-1} bands to $^2B_2 \rightarrow {}^2E$ and $^2B_2 \rightarrow {}^2B_1$, respectively, an acceptable fit to the $^2B_2 \rightarrow {}^2A_1$ and D_{4h} limit energies was obtained for β = 61°, Ds = 717 cm^{-1}, and Dt = 464 cm^{-1}. In the calculation the $^2B_2 \rightarrow {}^2E$ and $^2B_2 \rightarrow {}^2B_1$ bands were fit closely, and the $^2B_2 \rightarrow {}^2A_1$ and the D_{4h} limit ($^2B_{1g} \rightarrow {}^2A_{1g}$) were calculated to be 12 285 and 21 800 cm^{-1}, respectively. The observed $^2B_2 \rightarrow {}^2A_1$ energy (Table I) is near the calculated value. The ligand field stabilization energy (LFSE) contribution[36] to the reduction potential of the blue copper in ceruloplasmin is calculated from the derived parameters to be 260 mV relative to $Cu(aq)^{2+}$ (E^0 = 153 mV). Thus, if solvation and other factors were equal (blue copper vs. $Cu(aq)^{2+}$), the potential of ceruloplasmin blue copper would be predicted to be 413 mV. The experimental values (490 and 580 mV)[21] are somewhat higher than 413 mV, indicating that the ligand field destabilization contribution is only part of the explanation of high potential blue copper centers.

The ligand field parameters obtained for the type 1 copper

Table II. Magnetic Circular Dichroism Spectral Data for Ceruloplasmin and Anion-Bound Derivatives

protein	cm^{-1}	nm	$\Delta\epsilon$
native[a]	14 600	685	−0.88
N_3^- added[b]	15 300	655	−0.85
	20 500	490	−0.50
	23 700	420	+0.80
	27 300	360	−0.48
NCS^- added[c]	15 400	650	−0.88
	19 600	510	−0.40
	23 000	430	+0.52
	27 000	370	−0.16

[a] Figure 3. [b] Figure 6A. [c] Figure 6B.

in ceruloplasmin are remarkably similar to those extracted from an analysis of the plastocyanin d-d spectrum ($\beta = 60°$; $Ds = 691$, $Dt = 465$, D_{4h} limit = 21 200 cm^{-1}).[36] Thus, it is reasonable to propose that two plastocyanin-like blue coppers, CuN_2SS^* (N = His, S = Cys, S* = Met), are present in ceruloplasmin. Further support for this proposal comes from the very close similarities between ceruloplasmin (Tables I and II) and plastocyanin (or azurin)[36] in the charge-transfer region of the type 1 copper spectra (absorption, CD, MCD). It should also be noted that the spectroscopic evidence points to similar blue copper structures for ceruloplasmin and tree laccase, although there may be significant differences in the ligand composition of the blue site in fungal laccase.[37]

Addition of azide and thiocyanate to ceruloplasmin results in considerable changes in its electronic spectroscopic properties (Figures 5–7). Particularly striking is the fact that absorption spectral measurements have revealed a 50% decrease in the extinction coefficient of the blue band in these anion-bound derivatives.[25,53,54a] These observations plus changes seen in the blue copper resonance Raman spectra of anion-bound ceruloplasmin have led[26] to the proposal that only one of the type 1 sites is affected by anion binding. However, it is clear that the 6100-cm^{-1} CD band (Figure 7) is not present after addition of azide, which is inconsistent with the proposed disruption of only one of the two type 1 centers. Thus, our spectroscopic data require a somewhat altered structure for both type 1 sites. An anion-induced conformational change that perturbs the blue site geometry is a likely source of these variations, although ligand substitution at the type 1 copper cannot be ruled out. The positions of the d-d bands in azide-bound ceruloplasmin suggest that both blue sites are distorted more toward a square planar geometry than in the native protein. Analysis of the spectrum yields $\beta = 65°$ ($Ds = 635$, $Dt = 525$, D_{4h} limit = 20 490 cm^{-1}) and a predicted (LFSE-corrected) 161 mV potential for the blue coppers.

Ceruloplasmin type 1 coppers are known to differ in their reactivity. Measurements of the proton relaxation time (T_1) vs. the extent of copper reduction, measured at 610 nm, are sharply biphasic.[28] Photoreduction of the blue coppers by laser irradiation has been demonstrated and the calculated quantum yields and rate constants for this process are different for the two blue sites.[27] Reoxidation of the reduced enzyme by oxygen is also biphasic when monitored at 610, 330, and 420 nm (where an intermediate in this reaction absorbs).[29] In the presence of N_3^-, the differences in rate constants for the two phases observed at 610 and 420 nm are even more pronounced.[29] Given the close electronic structural similarity of the two type 1 coppers required by our spectroscopic examination of ceruloplasmin, it is reasonable to suggest that the aforementioned reactivity differences reflect variations in the degree to which the redox centers are buried in the protein and/or the placement of each type 1 site relative to the other coppers. The former presumably would directly affect the reactivity of type 1 centers with external reagents, whereas the latter would influence the rate of intraenzyme electron transfer.

Types 2 and 3. The EPR spectrum of ascorbate-treated ceruloplasmin (Figure 10A) is approximately axial with $g_\parallel > g_\perp > 2.0023$ and $|A_\parallel| \simeq 0.018$ cm^{-1}. These values are consistent with a tetragonal site for Cu(II);[36,37] the ground state is $^2B_1$, corresponding to the unpaired electron in a Cu(II) $d_{x^2-y^2}$ orbital. Visible absorption and CD spectral data (Figures 8 and 9) are consistent with such a structure. Monomeric (tetragonal) complexes of Cu(II) with amino acids and peptides display[58] values of $\Delta\epsilon$ in the visible region that are very similar to those of ascorbate-treated ceruloplasmin. The multiplicity of bands in the latter derivative (Figure 9) suggests that the transitions arise from more than one type of copper site, as optically active Cu(II) complexes, even with poly(α-amino acids), generally exhibit[59,60] fewer visible CD bands than we have observed. Near-tetrahedral geometry for type 2 and type 3 copper is ruled out by the lack of low energy d-d bands, but tetragonal six-, five-, or four-coordinate square planar structures[61] remain as possibilities.

The EPR spectrum of ascorbate-treated ceruloplasmin is similar, but not identical, with that obtained after selective bleaching of type 1 copper by addition of nitric oxide to the protein.[43] A superhyperfine structure attributable to ligand donor atoms is present in the $g_\perp$ region of the EPR spectrum (Figure 10B) of the ascorbate-treated protein. Close examination reveals a nine-line pattern, indicative of the presence of four equivalent ligands. The splitting of approximately 14 G is in the range usually found for nitrogen donors.[62] A reasonable structure consistent with the data would place the four nitrogen ligands in a square-planar arrangement about the Cu(II) ion, perhaps with axial ligands as well. This pattern of superhyperfine structure from approximately coplanar donors appearing in the $g_{x,y}$ region of the spectrum has been observed in simple Cu(II) complexes.[63,64] It is also possible that the superhyperfine structure in the parallel region was too weak to be resolved; in this context it should be noted that nitrogen superhyperfine structure from imidazole is much more intense in the $g_\perp$ region compared to the $g_\parallel$ region in the EPR spectrum of galactose oxidase.[65] Such a pattern is expected simply because the superhyperfine intensity is spread over the four copper hyperfine peaks in $g_\parallel$ but is overlapped in $g_\perp$ where the copper hyperfine is much narrower.

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