SPECTROSCOPIC AND MAGNETIC STUDIES OF THE COORDINATION ENVIRONMENT AND ELECTRONIC STRUCTURE OF COPPER SITES IN BLUE COPPER OXIDASES

Thesis by

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To my Parents

and

Lynn

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Caltech is justifiably famous for providing the facilities and atmosphere most conducive to research and justifiably infamous for providing little else. Thanks, I guess.

With patience, determination, and luck Harry Gray could occasionally be cornered. When not simultaneously on the phone he could impart a great deal of encouragement, sound advice, useful information and new ideas in a short time. I also appreciate the freedom to work and think independently that Harry has established in the Group.

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ABSTRACT

Low temperature absorption, circular dichroism, and magnetic circular dichroism measurements have been made on the multicopper oxidases Rhus vernicifera laccase, Polyporus versicolor laccase, and human ceruloplasmin. Near infrared bands have been observed that are assigned to ligand field transitions of the type 1 (blue) Cu(II). The low energies of these bands, when considered together with the EPR g values, are consistent only with a tetragonally (D_{2d}) distorted tetrahedral geometry for type 1 Cu(II). Analysis and comparison of data obtained on the blue oxidases and single blue copper proteins indicate that three kinds of blue copper may be distinguished spectroscopically and electrochemically. No evidence of inequivalent type 1 sites was found in native or azide inhibited ceruloplasmin.

Modified forms of the laccases and ceruloplasmin, in which the type 1 copper is specifically reduced or removed, were also investigated. Absorption and circular dichroism bands were observed that are attributed to the types 2 and 3 copper ions. Distorted tetrahedral geometry may be ruled out for these sites as no near infrared bands were present. Observed transitions are consistent with tetragonal six, five or square planar four coordination.

Superhyperfine in the EPR spectra of modified ceruloplasmins established three or four nitrogen atoms as ligands of the type 2 Cu(II). Binding of up to two fluoride ions by ceruloplasmin type 2 copper was also demonstrated.

Magnetic susceptibility measurements on Rhus vernicifera laccase showed the type 3 coppers to be diamagnetic from 5-260 K. If the type 3 site is formulated as an antiferromagnetically coupled Cu(II) pair then $J \geq 500$ cm^{-1}. Data were also obtained on Limulus polyphemus oxyhemocyanin which require a lower limit on J of 550 cm^{-1}. Curie law behavior of the susceptibility of Rhus laccase at low temperatures indicates that types 1 and 2 copper must be separated by several angstroms and are not bridged by a common ligand(s) in the native protein. However, binding of fluoride at the type 2 site in both Rhus and Polyporus laccase perturbs the type 1 circular dichroism spectrum. This may reflect catalytically significant conformational changes designed to facilitate intramolecular electron transfer.

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CHAPTER 1

SPECTROSCOPIC STUDIES OF THE COORDINATION ENVIRONMENT AND ELECTRONIC STRUCTURE OF COPPER SITES IN *RHUS VERNICIFERA* AND *POLYPORUS VERSICOLOR* LACCASE

Extensive spectroscopic studies of the native blue copper proteins, stellacyanin, azurin, and plastocyanin[2] and their Co(II) derivatives[3,4] have resulted in a detailed assignment of the distinctive visible and near infrared spectral features of these proteins. Low temperature absorption, circular dichroism (CD), and magnetic circular dichroism (MCD) results established the coordination geometry of the blue copper ion as tetragonally (D_{2d}) distorted tetrahedral. NMR[5,6] and X-ray photoelectron spectroscopy (XPS)[7] indicated that cysteine sulfur and two imidazole nitrogens were ligands. The intense absorptions at ~13,000, 16,000, and 22,000 cm^{-1} in the visible have been assigned as LMCT charge transfer from nitrogen and sulfur ligands into the $d_{x^2 - y^2}$ orbital of Cu(II). Furthermore, a distorted tetrahedral geometry is consistent with the unusually small $A_{||}$ hyperfine constant characteristic of blue copper.[8] It is probable that the metal site is rather rigid as the electronic stabilization energy associated with a Jahn-Teller distortion strongly favors a square planar geometry around Cu(II). The relatively high reduction potentials of blue copper proteins may be attributed in part to electronic destabilization of the Cu(II) center.[9]

Recently the X-ray crystal structure of plastocyanin has been determined to 2.7 Å resolution[10] and directly confirms many of the properties of the blue copper site determined by spectroscopic methods. The essential features of the crystal

structure are an N_2S_2 ligand donor set in a distorted tetrahedral geometry. The copper ion is coordinated by a cysteine thiol group, a methionine thioether group, and two histidine imidazole nitrogens. In view of the remarkable resemblance of the visible absorption and CD spectra of the Cu(II) proteins and their Co(II) analogs, the presence of a methionine ligand is rather surprising and intriguing as stellacyanin contains no methionine[11] and thus must have at least one different ligand from those observed in plastocyanin.

A 3Å resolution electron density map has recently been determined for *Pseudomonas aeruginosa* azurin also.[12] Although phasing was difficult and the resulting map noisy, the copper ion could be located and the probable ligands identified via use of the amino acid sequence. The coordinating amino acids are cysteine, two histidines, and a methionine. The geometry may be described as tetrahedral. X-ray absorption spectroscopy has also been utilized to investigate the primary coordination environment of *Pseudomonas aeruginosa* azurin.[13] The copper ion ligands were identified as thiolate sulfur, two nitrogens (probably from histidine) and another sulfur, presumed to be thioether. Particularly striking is the very short copper-S (thiolate) distance of 2.10 ± 0.015 Å. The other sulfur-copper distance is 2.26 ± 0.03 Å. These distances are most readily interpretable in terms of a near tetrahedral

geometry. Thus the structures of the blue copper sites in plastocyanin and azurin are remarkably similar.

The laccases are multicopper oxidases that also contain a blue or type 1 copper site.[14,15] The two best characterized enzymes are from the fungus, *Polyporus versicolor*, and the Japanese lacquer tree *Rhus vernicifera*. These oxidases couple the oxidation of a variety of substrates to the four electron reduction of dioxygen to water. The type 1 reduction potential (+415 mV) in *Rhus vernicifera* laccase is slightly more positive than the single blue copper proteins (184-370 mV) but the potential of *Polyporus versicolor* laccase (+767 mV) is significantly more positive. The laccases contain three other copper atoms in addition to the type 1, designated type 2 (1 Cu atom/molecule) and type 3 (2 Cu atoms/molecule). The type 2 copper displays an epr spectrum that is typical of an axial Cu(II) complex.[14,15] No visible absorption or CD bands have previously been associated with this center. EPR measurements established the type 2 copper as an anion binding site.[16] Several experiments indicate that this copper has a key catalytic role, possibly functioning as a mediator of electron transfer into the type 3 coppers.[17] Importantly, superhyperfine has been observed in the type 2 EPR signal, under conditions where the type 1 copper is reduced, that is suggestive of coordination by one or more nitrogen ligands.[17b] Recently, it has been shown that an intermediate in the dioxygen reduction

is bound to the type 2 copper in fungal laccase.[17f] The type 3 coppers are "EPR nondetectable" and are associated with an absorption band in the near UV at approximately 330 nm,[14,15] and together function as a two electron acceptor. Magnetic susceptibility measurements showed the type 3 coppers to be an antiferromagnetically coupled Cu(II) dimer[18] but more recent work indicates that if a binuclear Cu(II) unit is present the coupling must be considerably greater than that previously measured.[19] Because of the two electron transfer capability the type 3 coppers have long been thought to be the dioxygen binding and reaction site. We have recently obtained direct evidence that a catalytically active oxygen intermediate is associated with the type 3 site during turnover conditions.[20] At 3°C and pH = 6.0 a well defined absorption spectrum is obtained displaying a strong band at 340 nm and a weaker band at 475 nm. A positive CD band is also observed at 362 nm. These spectral features are quite similar to the bands observed in the binuclear copper proteins oxyhemocyanin[21] and oxytyrosinase.[22]

We have extended our CD, MCD, and low temperature absorption studies to both the tree and fungal laccases in order to obtain information on the electronic structures of the blue and other copper centers. Bands in the near infrared attributable to d-d absorptions of the blue copper center have been observed and analyzed in terms of the distorted tetrahedral model proposed earlier.[2] Analysis of the type 1 EPR spectra

in both laccases has been carried out and the ligand field contribution to the reduction potential has been estimated. In addition, features in the visible absorption and CD spectra, attributable to the other copper ions in both laccases have been observed. On the basis of these experiments tetrahedral geometry can be ruled out as a possibility for types 2 and 3 copper ions. Finally, we have carefully analyzed and compared all the available spectroscopic data obtained on proteins containing the blue copper center with particular reference to variations in reduction potential and ligand donor set.

Experimental Section

Rhus vernicifera acetone powder was obtained from Saito and Co., Ltd., Tokyo. Laccase was extracted and purified to a ratio $A_{280}/A_{614} \leq 15.4$ by the method of Reinhammar.[23] This material ran as a single band of the correct molecular weight during SDS polyacrilamide gel electrophoresis. We have observed that the A_{280}/A_{614} may be lowered to 11-12 by either extensive dialysis versus pure water or by chromotography on Sephadex G-150. Evidently a low molecular weight UV absorbing material is removed by the above procedures. Laccase from *Polyporus versicolor* was cultured and purified by standard methods[24] from strains provided by Dr. Rölf Brandén and Dr. Lars-Erik Andréasson. Pure fungal laccase provided by Dr. Bo G. Malmström and Dr. Lars-Erik Andréasson was used in some experiments. Fluoride ion was removed from the purified proteins by dialysis against 50 mM acetate buffer, pH = 5.5,

containing 20 mM ascorbate for approximately 10 hours. All fungal laccase samples had A_{280}/A_{610} ratios of 16.5-16.8 and $A_{280}/A_{610} > 2.0$.[24]

Visible region CD and MCD laccase samples were between 5×10^{-5} and 1×10^{-4} M and were run in $\mu = 0.1$ potassium phosphate buffer, pH = 6.0 and 0.1 M sodium phosphate buffer, pH = 6.0, for the tree and fungal laccases, respectively. Instrument baselines were determined with the same buffers. Tree laccase solutions for use in the near infrared region were prepared in D_2O; deuterated 0.1 M sodium acetate buffer, pD = 5.7 was used to prepare fungal laccase samples. Equilibration was carried out by concentrating the protein in an Amicon ultrafiltration cell and then adding approximately a ten-fold excess of D_2O or deuterated buffer. This procedure was repeated 4-5 times before the solution was concentrated to its final value.

Deuterated acetate buffer was prepared by dissolving well dried anhydrous sodium acetate in D_2O and then adjusting the pD to 5.7 at 6°C by addition of DCl. At this concentration of buffer and pD, the total chloride ion concentration is $\sim$ 1 mM, an order of magnitude less than the concentration where inhibition of fungal laccase by this anion occurs.[25] However, to check that this concentration of chloride had no effect on the near infrared measurements spectra of fungal laccase in 0.05 M sodium acetate buffer (pH = 5.5) in H_2O were also obtained. Buffered solutions of fungal laccase were used to

prevent reduction of the type 1 site that occurs at pH > 6.5.[26] Spectra were recorded for samples having concentrations of 1-2 mM in small volume quartz cells with pathlengths of 1 or 2 mm to minimize solvent absorption. Reliable data could be obtained out to ∿ 2.1 μ where D_2O overtones diminished the light level to the limit of detector response. D_2O or deuterated buffer was used to obtain instrument baselines.

Specific reduction of the type 1 copper in fungal laccase was accomplished by titration with NaOH (or NaOD for use in the near infrared) or by extensive dialysis against phosphate-borate buffer (pH = 8.7 @ 4°C). Specific reduction of the type 1 center in tree laccase was achieved via reaction with nitric oxide.[27] Solutions of laccase, ∿ 0.5 mM for visible region experiments and ∿ 2 mM for near infrared work, were placed in cuvettes or EPR tubes that could be directly attached to a vacuum line. Oxygen was purged via 3-4 cycles of gentle evacuation and flushing with argon. Approximately 1 atm of NO was then admitted after passage through a Dry Ice-acetone bath; the protein solution was again evacuated and this procedure repeated at least 3 times to insure complete equilibration. An atmosphere of NO was left over the samples and the cuvette or EPR tube removed from the line. Upon warming to about 45°C for 5 min in a water bath, the blue color was observed to bleach and the laccase solutions became light green. Type 2 depleted laccase was prepared as previously described.[28] The degree of reduction or removal of the copper was checked by EPR. Spectra were

recorded on a Varian E-line Century Series spectrometer at 5 K utilizing an Air Products Heli-Trans system. Frequencies were determined with a PRD Electronics, Inc. frequency meter.

Protein films for low temperature absorption measurements were prepared by evaporating $\sim$ 1 mM solutions of the proteins on plexiglass or quartz disks in a dessicator filled with Drierite at 4°C. Films were not allowed to dry past the stage where they were still slightly elastic. A lightly greased rubber gasket was then placed around the film and a second disk added as a cover. The film was then masked with aluminum foil to prevent light leaks. The assembly was mounted in the sample space of a Cryogenic Technology Model 20 cyrocooler utilizing a mixture of copper filings in silicone grease to insure good thermal contact. Samples were cooled slowly to minimize cracking of the film. Several hours were required for the plexiglass disks to achieve thermal equilibrium with the cold stage of the cryocooler at low temperature (< 70 K).

Absorption spectra were recorded on Cary 17 and 14 spectrometers. Visible CD spectra were taken with Cary 61 and JASCO J-40 instruments. CD spectra in the near infrared were recorded on a laboratory constructed instrument described elsewhere.[29] Standard sensitivity settings were 0.05° and between 5×10^{-4} and 5×10^{-3} ΔA for the visible and near infrared regions, respectively. MCD spectra were obtained using a Varian Associates superconducting magnet at fields of 40 kilogauss. MCD spectra were corrected for natural CD by

computer subtraction of the spectra observed with the field on and off. 10-camphorsulfonic acid was used to calibrate the visible CD. The sign of the CD in the near infrared was determined with a Ni(II) tartrate solution and calibrated with a quarter wave plate. CD spectra are presented in units of $\Delta\varepsilon$ (ℓ mole^{-1} cm^{-1}). MCD spectra are in units of $\Delta\varepsilon$/gauss.

Reagent grade chemicals were used without further purification. D_2O used was 99.8% D (Stohler Isotope Chemicals). Protein concentrations are based on the extinction coefficients at 614 nm (5700 ℓ mole^{-1} cm^{-1}) for tree laccase and 610 nm (4900 ℓ mole^{-1} cm^{-1}) for fungal laccase. A Brinkman Model 101 was used for all pH determinations. pD was calculated from the relation pD = pH meter reading +0.4.

Results

The 20 K absorption spectrum of fungal laccase is shown in Figure 1. The resolution obtained at low temperature is rather disapointing, being only marginally better than that obtained at room temperature. A weak shoulder is resolved at 23,000 cm^{-1}, the shoulder at 13,000 cm^{-1} is more pronounced and a weak absorption in the near infrared, not previously observed, is evident near 10,000 cm^{-1}. Much better results were obtained for tree laccase at 20 K (Figure 2). The intense band at $\sim$ 16,000 cm^{-1} is narrowed considerably at low temperature. A broad, asymmetric peak, probably composed of two overlapping transitions, is clearly resolved to lower energy of the intense "blue" band. Significantly, a less intense band is observed to even lower energies, appearing as a shoulder near 10,000 cm^{-1}.

FIGURE 1

20 K absorption spectrum of fungal laccase on a plexiglass disk.

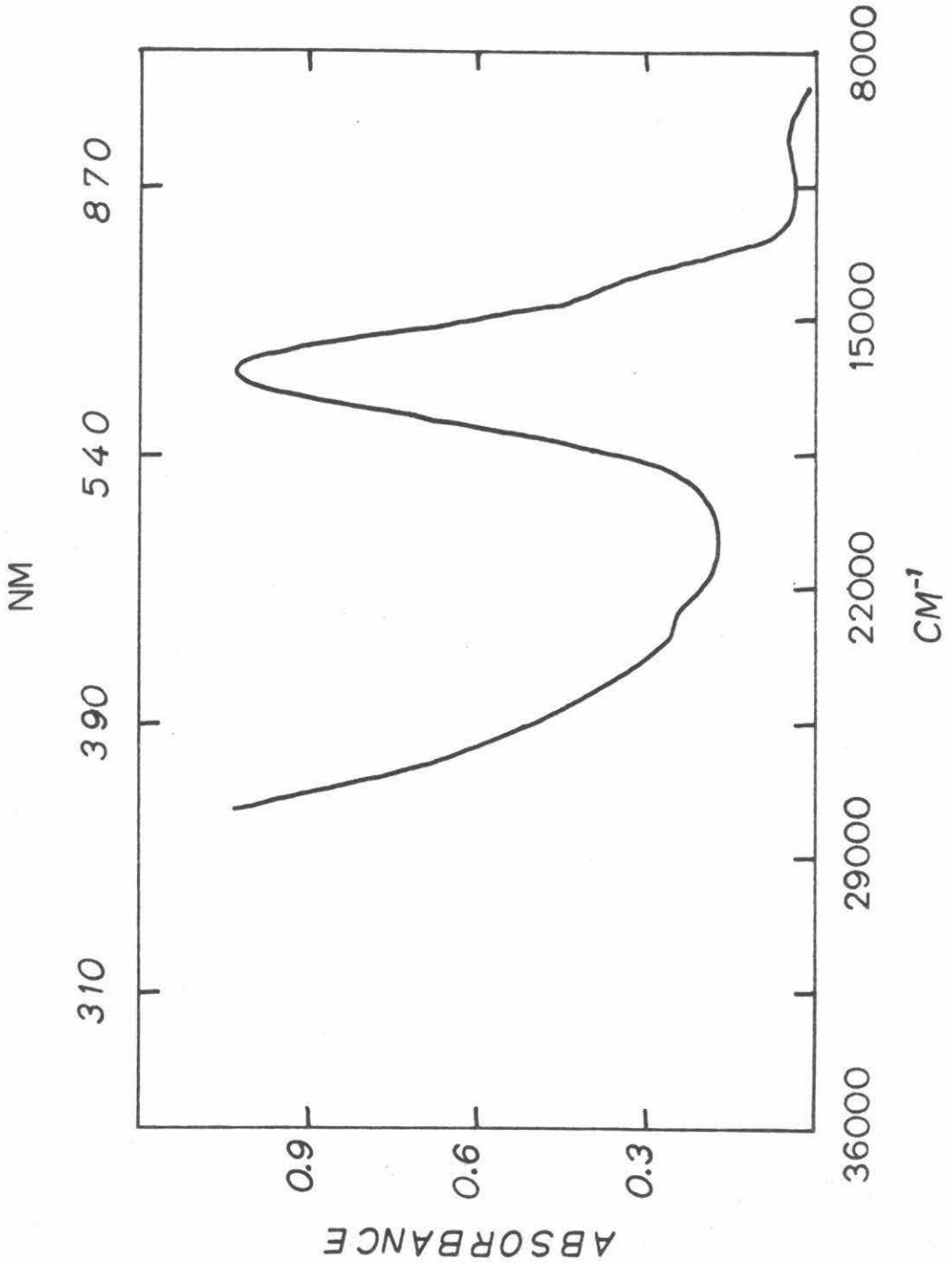
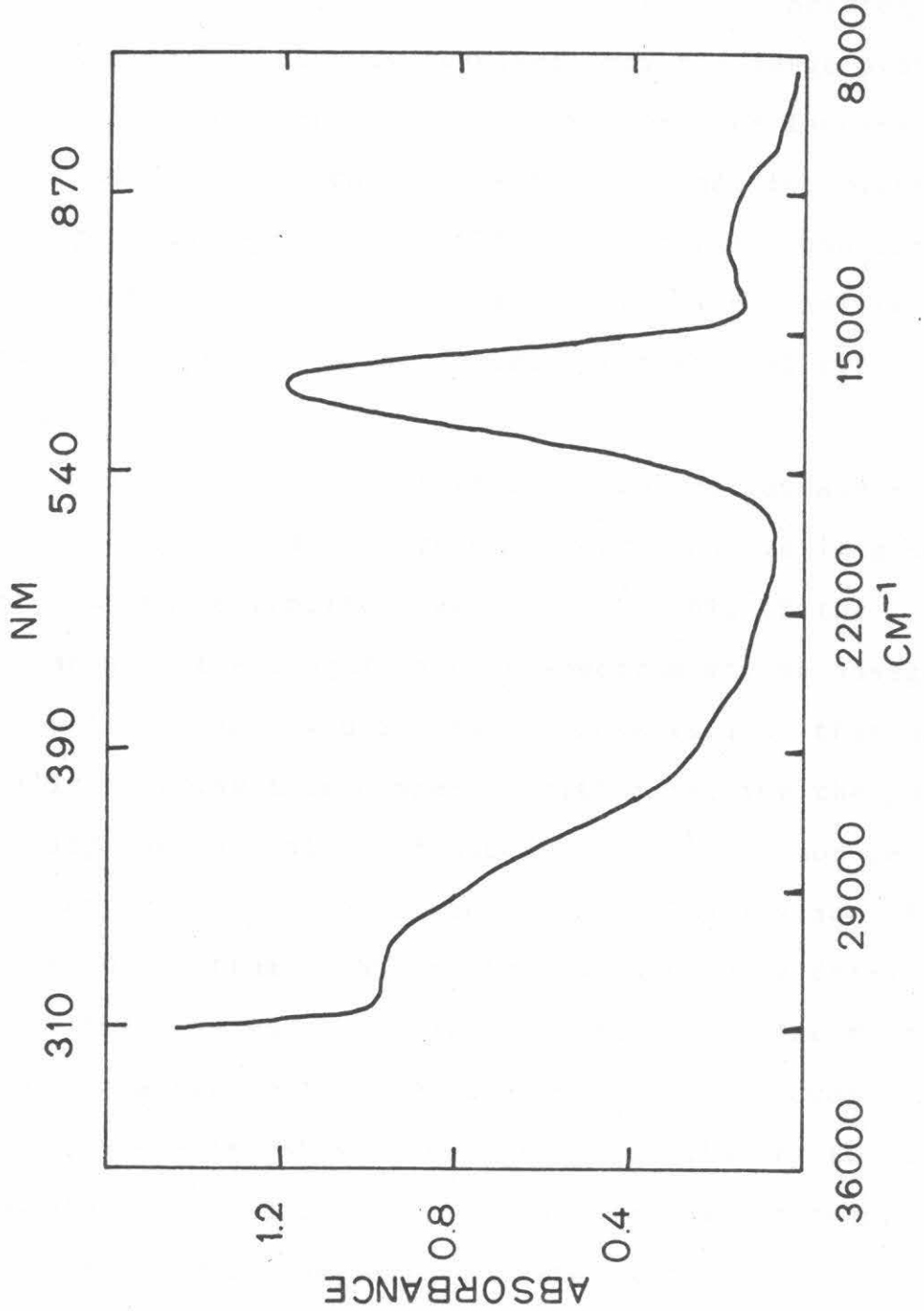

NM
310
390
540
870
ABSORBANCE
0.9
0.6
0.3
36000
29000
22000
15000
8000
CM-1

FIGURE 2

20 K absorption spectrum of tree laccase on a quartz disk.


ABSORBANCE
1.2
0.8
0.4
NM
310
390
540
870
36000
29000
22000
15000
8000
CM-1

A transition at this energy has not previously been detected in tree laccase. At higher energies a weak shoulder is observed near 23,000 cm^{-1} and the near UV band associated with the type 3 coppers is clearly resolved from the large protein absorbance. The low temperature spectrum of tree laccase is similar in many respects to the spectra obtained for plastocyanin, azurin, and stellacyanin.[2,8] The energies and intensities of the weak absorptions in the near infrared are consistent with ligand field transitions of a distorted tetrahedral type 1 copper ion.

The visible CD spectra of tree and fungal laccase are presented in Figures 3 and 4, respectively, and are in good agreement with those reported earlier.[30,31] All of the observed bands in the fungal laccase spectrum may be assigned to the type 1 chromophore owing to the observation that upon specifically reducing this copper by either raising the pH to 8.7 or adding one equivalent of ascorbate to the fluoride inhibited enzyme[32] these bands are absent. Examination of the visible CD spectrum of NO treated laccase (*vide infra*) establishes that the bands in Figure 3 are due to the type 1 site. Such an assignment is reasonable for tree laccase, as its visible CD spectra shows considerable similarity to those of single blue copper protèins. In this context it should be noted that azurin, plastocyanin, and stellacyanin have positive

FIGURE 3

CD spectrum of tree laccase in μ = 0.1 potassium phosphate buffer, pH 6.0.

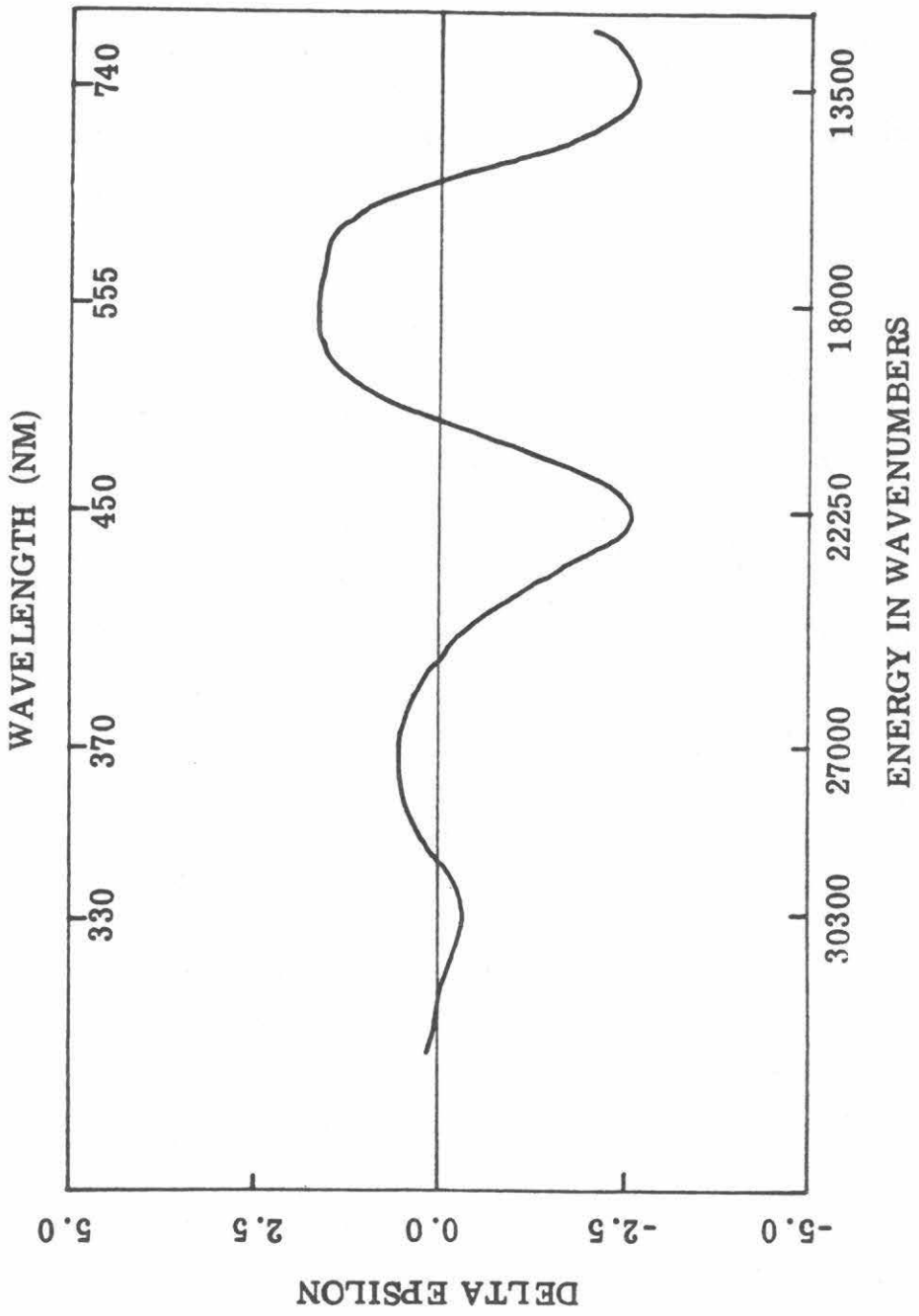
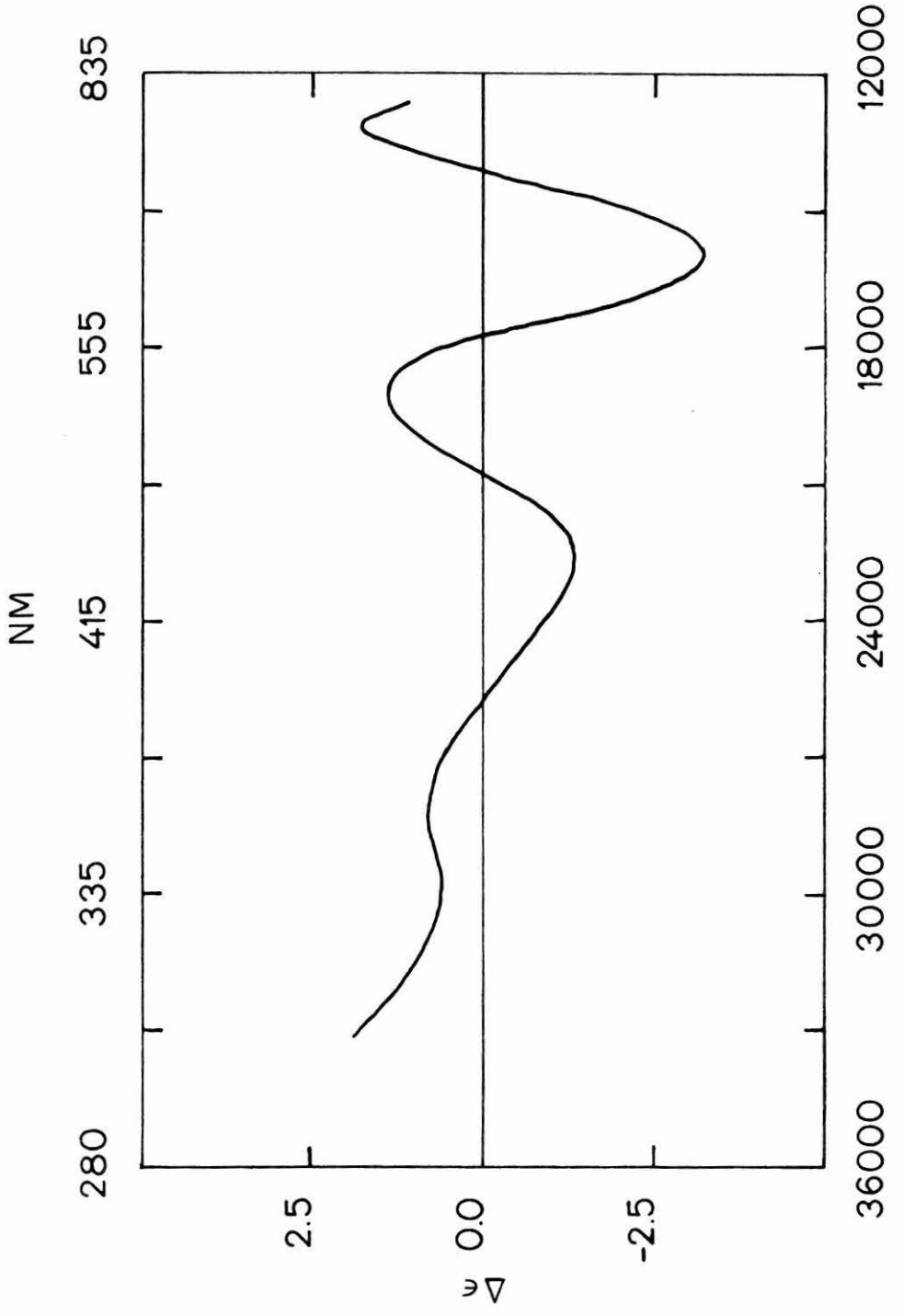

WAVELENGTH (NM)
740
555
450
370
330
ENERGY IN WAVENUMBERS
13500
18000
22250
27000
30300
DELTA EPSILON
5.0
2.5
0.0
-2.5
-5.0

FIGURE 4

CD spectrum of fungal laccase in 0.1 M sodium phosphate buffer, pH 6.0.


NM
280
335
415
555
835
2.5
0.0
-2.5
Δε
36000
30000
24000
18000
12000
CM⁻¹

CD activity at energies greater than 27,000 cm^{-1}. It is surprising that the relatively intense ε(ox-red) = 2800 M^{-1} cm^{-1} absorption at 30,000 cm^{-1} due to the type 3 copper does not have any CD activity associated with it. Considering the remarkable similarity of the visible CD spectra of proteins containing the blue or type 1 copper center,[8,31,33] it is important to note that the spectrum of fungal laccase is decidedly different. In particular, although the features at 28,500 and 22,500 cm^{-1} have the same sign and position as observed for other blue proteins, fungal laccase displays two oppositely signed bands between 21,000 and 14,000 cm^{-1} and a positive maximum at $\sim$13,000 cm^{-1}; all other blue proteins show a broad positive maximum (often asymmetric) between 21,000 and 14,000 cm^{-1} and a negative peak near 13-14,000 cm^{-1}.

Fluoride ion is a potent inhibitor of laccase. The interaction of fluoride with the type 2 copper[16] and its effect on the aneorbic reduction kinetics of tree laccase[17a] have been previously investigated. The binding F^- was shown to perturb the entire visible and near ultraviolet absorption spectrum[16] of tree and fungal laccase. It was also shown that the rate of F^- binding is very slow and independent of F^- concentration. These observations have led to the proposal that F^- traps the enzyme in a conformation which alters the properties of the type 1 and type 3 chromophores.[14] As CD should be more sensitive to small conformational changes affecting the electronic structure of the type 1 copper, we examined the effect of F^-

on the visible CD of tree and fungal laccase (Figures 5 and 6). Only slight changes are observed for tree laccase at less than approximately 21,000 cm^{-1}. However, the intensity at 22,400 cm^{-1} is noticeably decreased and an increase in $\Delta\varepsilon$ is seen above 31,000 cm^{-1}, where the largest and most reproducible change was seen in the absorbance spectrum. The entire spectrum of fungal laccase is slightly altered upon the addition of F^-, although the various bands are apparently affected to differing extents. The largest change occurs above 30,000 cm^{-1}, consistent with changes observed in the absorbance spectrum. Examination of the effect of F^- binding to fungal laccase under conditions where the blue copper is reduced indicates the perturbation of the CD spectrum above 30,000 cm^{-1} is associated with changes in the type 3 chromophore or is due to a new feature associated with the type 2 copper-fluoride interaction as suggested previously.[16] In any case, the data rule out autoreduction of the type 1 copper as an explanation for the effect of F^- on the visible absorption or CD spectrum and indicate that anion binding (and therefore possibly substrate binding) at the type 2 site perturbs the type 1 site.

The visible MCD curves of tree and fungal laccase are presented in Figures 7 and 8, respectively. The spectrum of tree laccase shows a striking resemblance to the MCD spectra of plastocyanin and azurin[8] and ceruloplasmin[33]; a negative maximum is observed at 14,250 cm^{-1} in tree laccase, 14,400 cm^{-1}

FIGURE 5

Difference CD spectrum obtained by subtracting the tree laccase spectrum from that of tree laccase plus stoichiometric fluoride. Incubation time was sufficient for fluoride binding to be complete. Both spectra were taken in $\mu = 0.1$ potassium phosphate buffer pH 6.0.

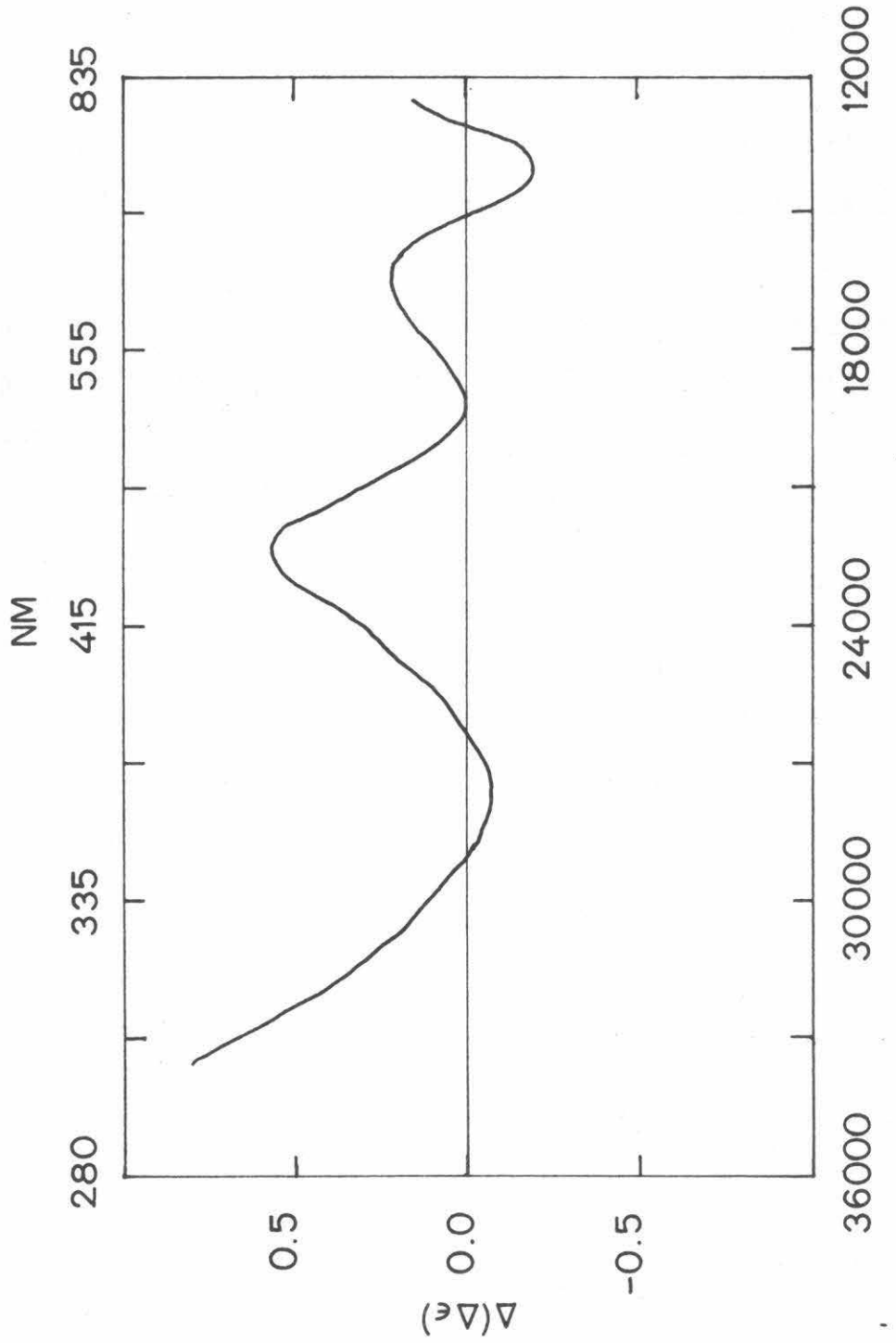

NM
280
335
415
555
835
CM⁻¹
36000
30000
24000
18000
12000
Δ(Δε)
0.5
0.0
-0.5

FIGURE 6

Difference CD spectrum obtained by subtracting the fungal laccase spectrum from that of fungal laccase plus stoichiometric fluoride. Incubation time was sufficient for fluoride binding to be complete. Both spectra were taken in 0.1 M sodium phosphate buffer pH 6.0.

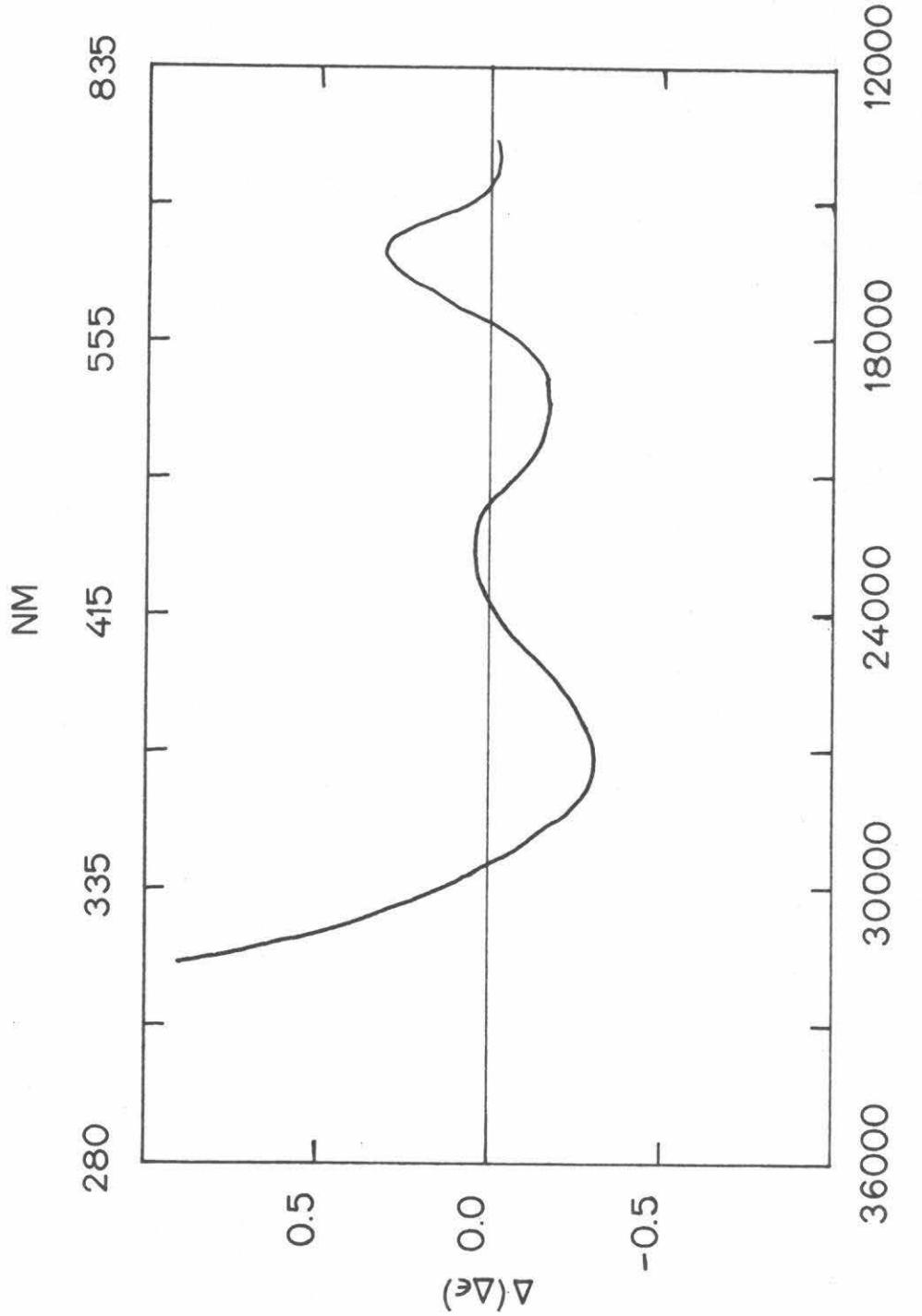

NM
280
335
415
555
835
CM⁻¹
36000
30000
24000
18000
12000
Δ(Δε)
0.5
0.0
-0.5

FIGURE 7

MCD spectrum of tree laccase in $\mu = 0.1$ potassium phosphate buffer pH 6.0. Field was 40 kilogauss.

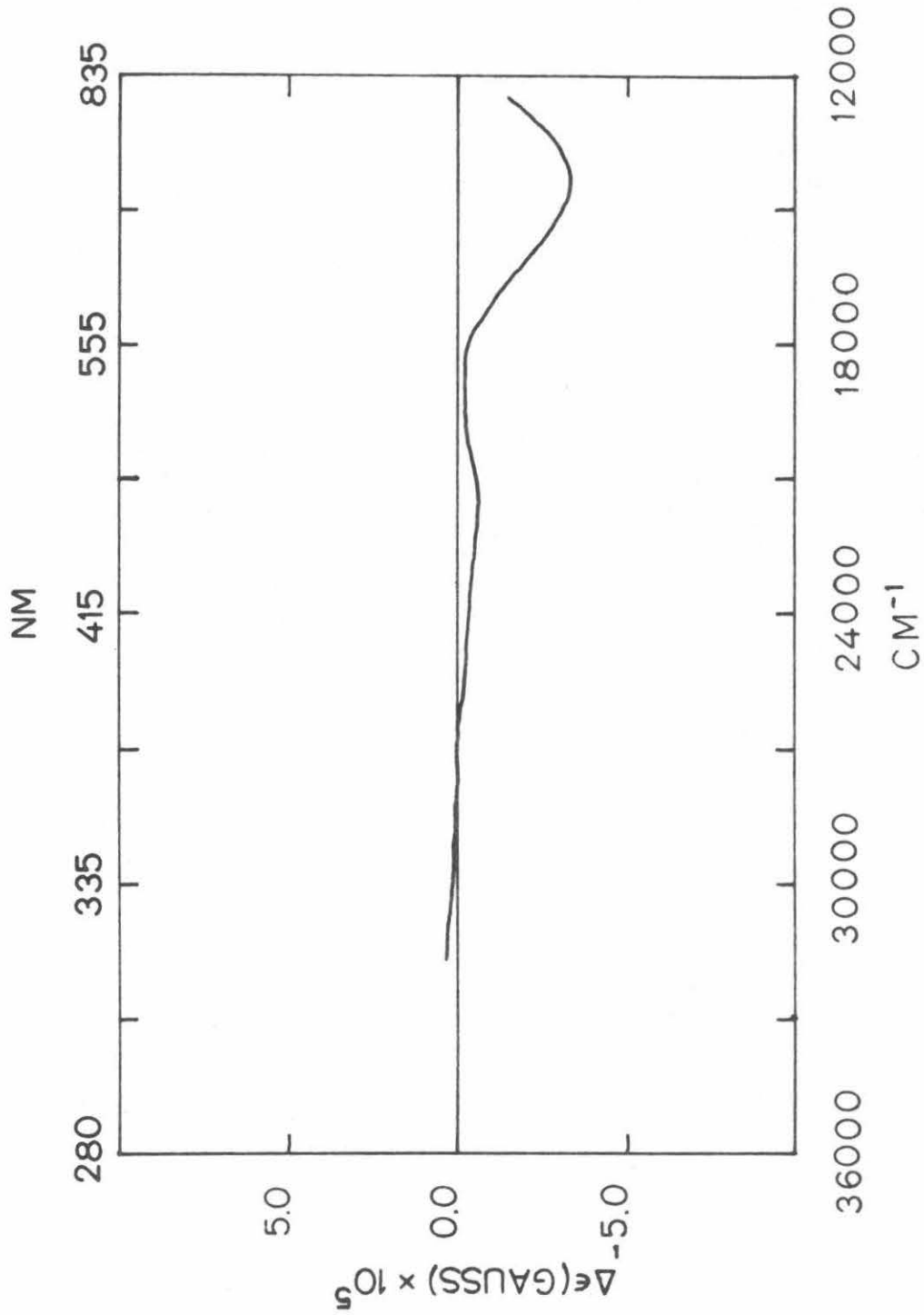
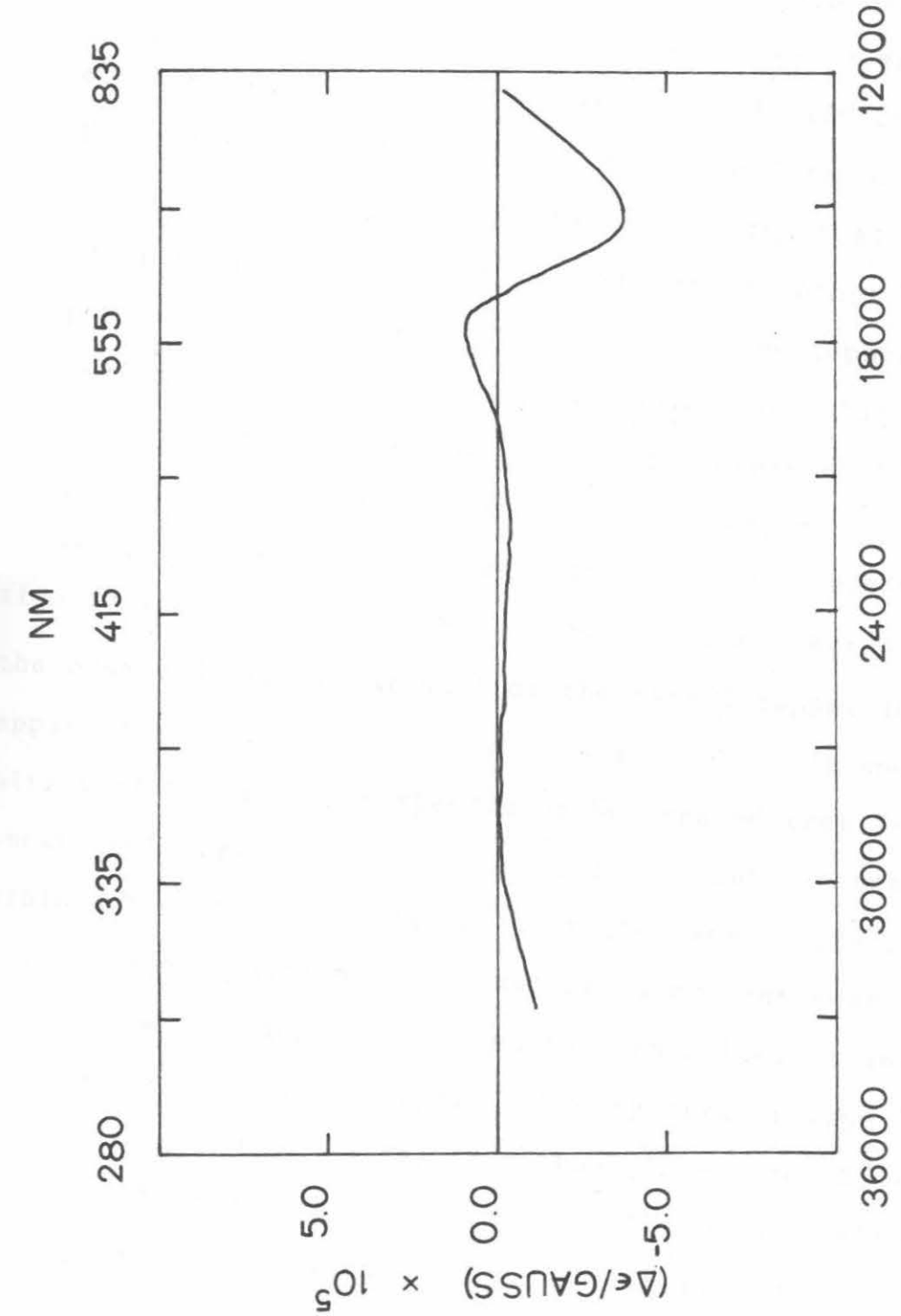

NM
280
335
415
555
835
CM-1
36000
30000
24000
18000
12000
Δε(GAUSS) × 10^5
5.0
0.0
-5.0

FIGURE 8

MCD spectrum of fungal laccase in 0.1 M sodium phosphate buffer pH 6.0. Field was 40 kilogauss.


280
335
415
555
835
NM
36000
30000
24000
18000
12000
CM⁻¹
5.0
0.0
-5.0
(Δε/GAUSS) × 10⁵

in azurin, 14,100 cm^{-1} in plastocyanin, and $\sim$ 14,600 cm^{-1} in ceruloplasmin. In contrast, fungal laccase displays an MCD curve unique among the blue copper proteins examined to date. A negative maximum is observed at 15,150 cm^{-1} and a smaller positive peak at 17,850 cm^{-1}, the curve crossing the baseline at 16,950 cm^{-1}. It should be noted that the MCD spectrum of stellacyanin is also unlike any other blue copper protein. The MCD spectra of all blue copper proteins for which data are available are compared in Table I.

Incubation of tree laccase in the presence of NO at 45°C results in reduction of the type 1 copper.[27] The EPR spectrum (Figure 9) indicates nearly complete reduction as the signals attributable to type 1 copper are not evident. All the observed signals arise from the type 2 copper ion which apparently remains in the cupric state. Visible and near ultraviolet absorption spectra of NO treated tree laccase are shown in Figure 10. The presence of the intense near ultraviolet absorption establishes that the type 3 site is oxidized under these conditions. A shoulder is present near 600 nm (16,700 cm^{-1}) which is assigned to ligand field transitions of the type 2 and type 3 coppers. The spectra of type 2 depleted laccase plus nitric oxide, also shown in Figure 10, indicate the 16,700 cm^{-1} band may be assigned to type 2 copper. In the CD spectrum of NO-treated native laccase a band is clearly seen at 18,500 cm^{-1}, with shoulders near 16,000 cm^{-1} and 20,000 cm^{-1} (Figure 11). These features are also present in NO-treated type 2 depleted laccase spectrum but are less intense, which suggests the bands may be assigned to both types 2 and 3 copper. Additionally, the native protein

Table I. Visible MCD Transitions of Blue Copper Proteins

Blue Protein	$\bar{\nu}$ (cm^{-1})	Sign	$\Delta\varepsilon$/gauss x 10^5
plastocyanin	14,100	(-)	3.5
azurin	14,400	(-)	2.5
tree laccase	14,250	(-)	3.5
ceruloplasmin	14,600	(-)	8.8
stellacyanin	13,500	(-)	3.5
	16,500	(-)	4.0
	22,000	(-)	2.0
fungal laccase	15,150	(-)	4.0
	17,850	(+)	1.0

FIGURE 9

EPR spectrum of tree laccase plus nitric oxide at 75 K and 9.1760 GHz. Microwave power was 20 mW and the modulation amplitude was 1.6 gauss.

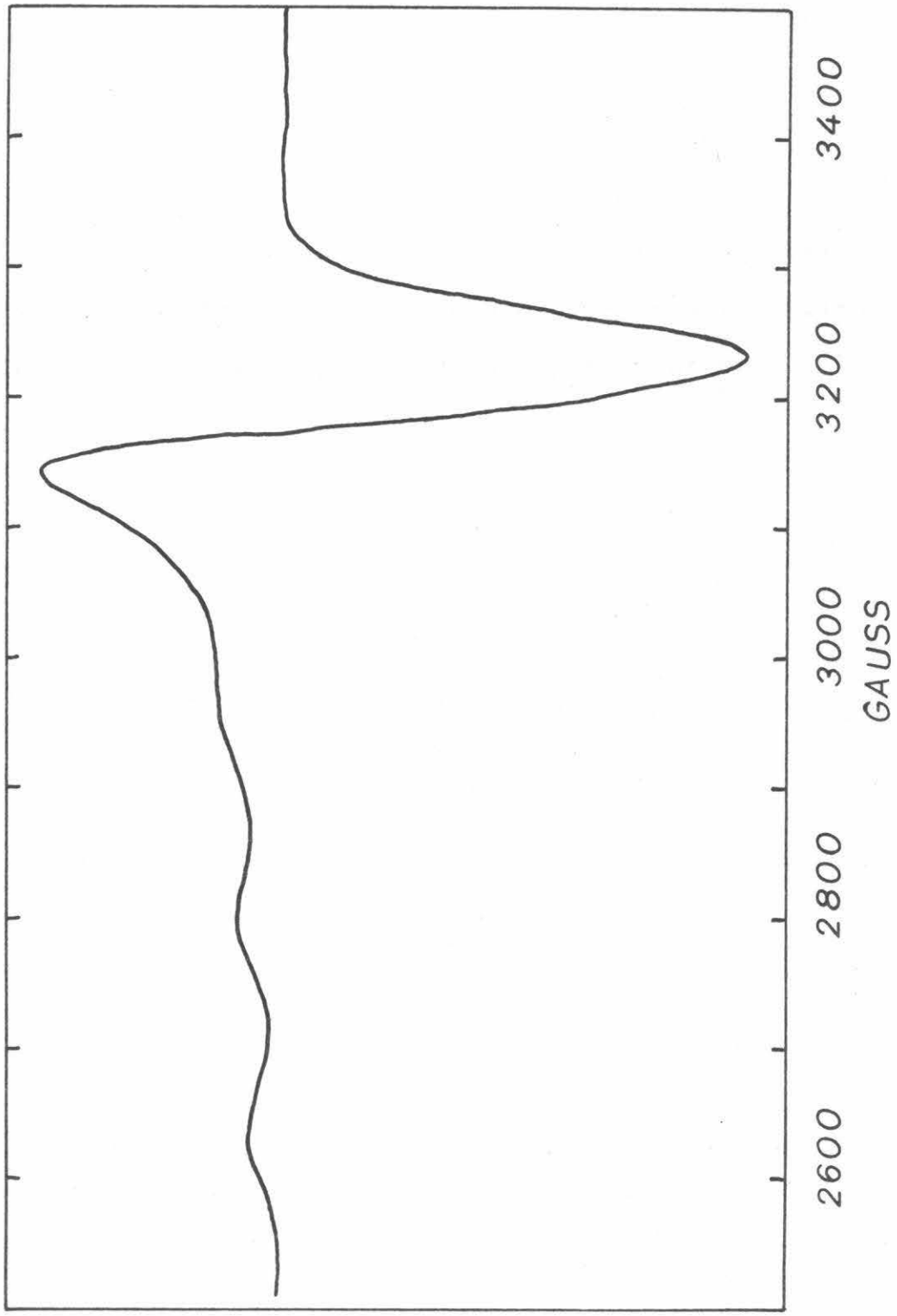

2600
2800
3000
3200
3400
GAUSS

FIGURE 10

Room temperature absorption spectra of native (A) and type 2 depleted (B) tree laccase after reaction with nitric oxide in 0.06 M potassium phosphate buffer pH 7.6.

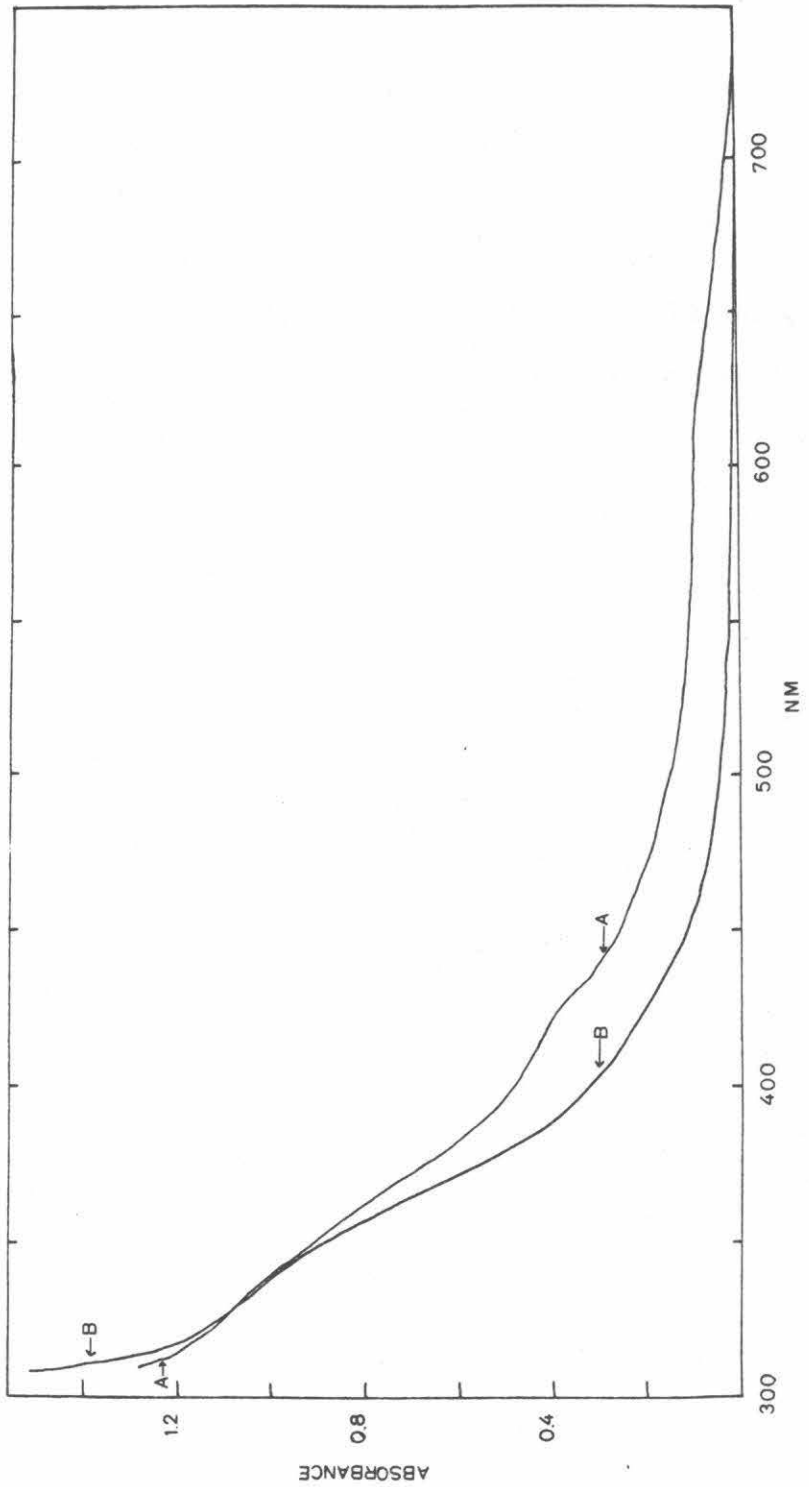
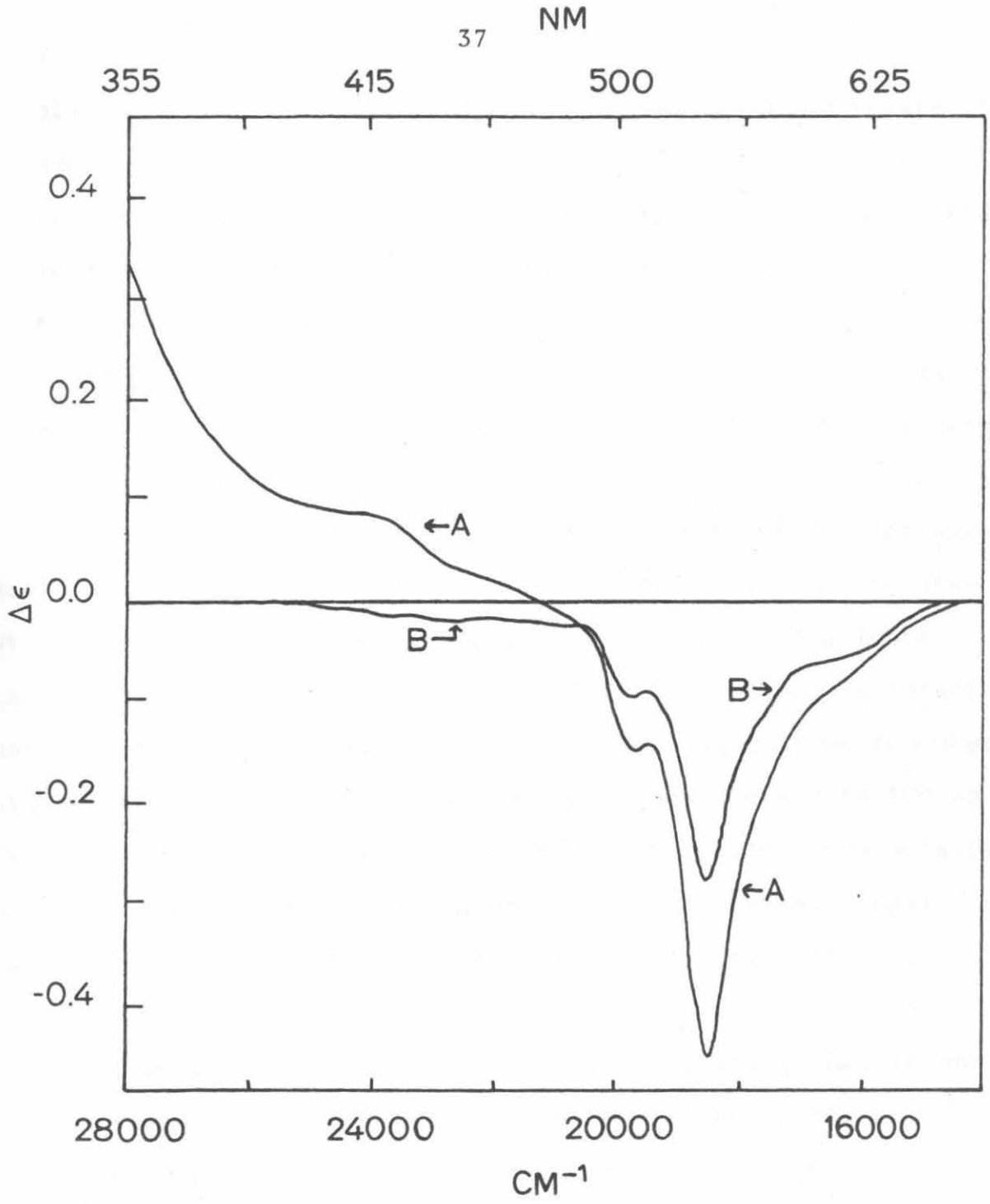

ABSORBANCE
1.2
0.8
0.4
300
400
500
600
700
NM
A
B
A
B

FIGURE 11

CD spectra of native (A) and type 2 depleted (B) tree laccase after reaction with nitric oxide in 0.06 M potassium phosphate buffer pH 7.6.

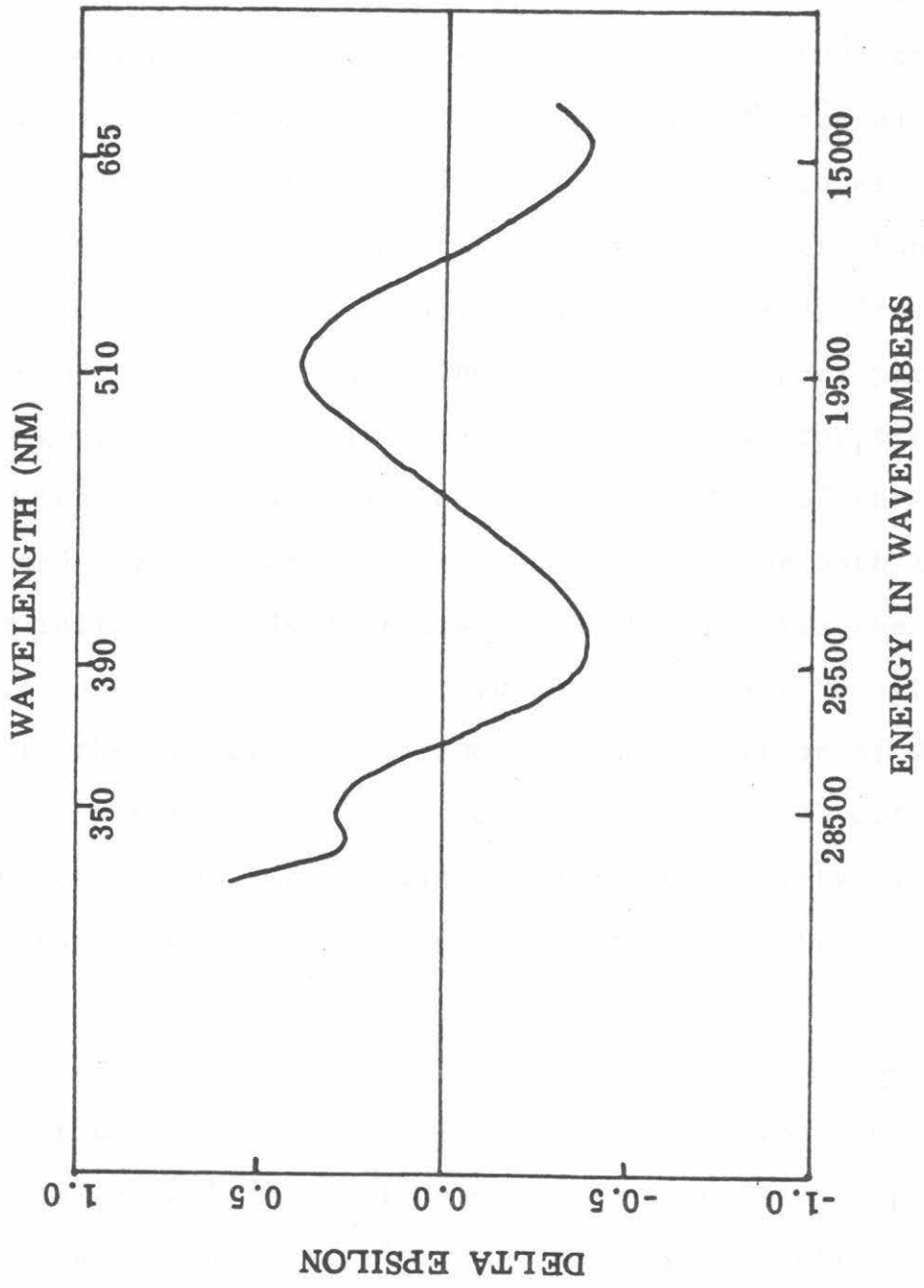

NM
355
415
500
625
0.4
0.2
0.0
-0.2
-0.4
Δε
←A
B→
B→
←A
28000
24000
20000
16000
CM⁻¹

displays strong positive activity above 21,000 cm^{-1} after treatment with NO; no such signals are seen for the type 2 depleted protein, indicating that this activity is a property of the type 2 site. Observed values of $\Delta\varepsilon$ are in the ranges established for d-d transitions of cupric peptide and amino acid complexes.[34,35]

At pH > 6.5 the type 1 copper in fungal laccase is reduced; the type 2 and type 3 coppers remain oxidized.[26] The resulting absorption spectrum is nearly identical to that of tree laccase + NO with a shoulder near 600 nm and then sloping upward at higher energies due to the still intense 330 nm (30,000 cm^{-1}) band in the near UV. However, three peaks are resolved in the CD of a fungal laccase sample that had been titrated with a $NaOD/D_2O$ solution until the blue color disappeared (Figure 12). Negative peaks are observed at 25,000 cm^{-1} and near 15,000 cm^{-1} and a positive peak at 19,500 cm^{-1}. The intensities are of the same magnitude as those seen in tree laccase. Essentially the same CD was observed after dialyzing fungal laccase against phosphate-borate buffer (pH = 8.7) at 4°C, except that some residual intensity was apparent in regions where bands due to type 1 copper occur, indicating that it was not completely reduced. Examination of the EPR spectrum confirmed that some type 1 was still present under these conditions. No MCD was observed for the type 1 reduced protein at fields of 40 kilogauss. Dialyzing the sample against pH 6.0 phosphate buffer restored approximately 70% of the original absorbance at 610 nm.

FIGURE 12

CD spectrum of fungal laccase in deuterated 0.1 M sodium acetate pD = 5.7 after titration with $NaOD/D_2O$.


WAVELENGTH (NM)
350
390
510
665
ENERGY IN WAVENUMBERS
28500
25500
19500
15000
DELTA EPSILON
1.0
0.5
0.0
-0.5
-1.0

Figures 13 and 14 show the near infrared CD spectra of tree and fungal laccase, respectively. Shoulders are observed at about 11,700 and 9500 cm^{-1} on the tail of the negative activity from the lowest energy visible band in the tree laccase spectrum. A peak is clearly resolved at 6000 cm^{-1}. Bands are evident at 11,000 and 7250 cm^{-1} in the fungal laccase CD and positive activity is still evident to the lowest energies obtainable under our experimental conditions. These bands are attributable to d-d transitions, presumably arising from the type 1 copper in analogy with the single blue copper proteins. This assignment may be confirmed in a direct way for tree and fungal laccase by measuring the near infrared CD of the NO treated and high pH forms of these proteins. For both laccases the near infrared bands decrease concomitantly with the visible absorption and CD bands of the blue chromophore. No activity remains if the proteins are reduced by the addition of dithionite. At the concentrations used to record the CD, no MCD activity was observed except for increasing negative activity at energies greater than 10,000 cm^{-1} in the tree laccase spectrum.

Discussion

As noted previously,[2] the low energies of the observed d-d transitions are incompatible with a blue copper center based on tetragonal six or five coordinate and square planar-four coordinate geometries.[8,9,36] Possible geometries derived from trigonal (C_{3v}) distortions of five and four coordinate complexes may be eliminated by consideration of the EPR

FIGURE 13

Near infrared CD spectrum of tree laccase in D_2O

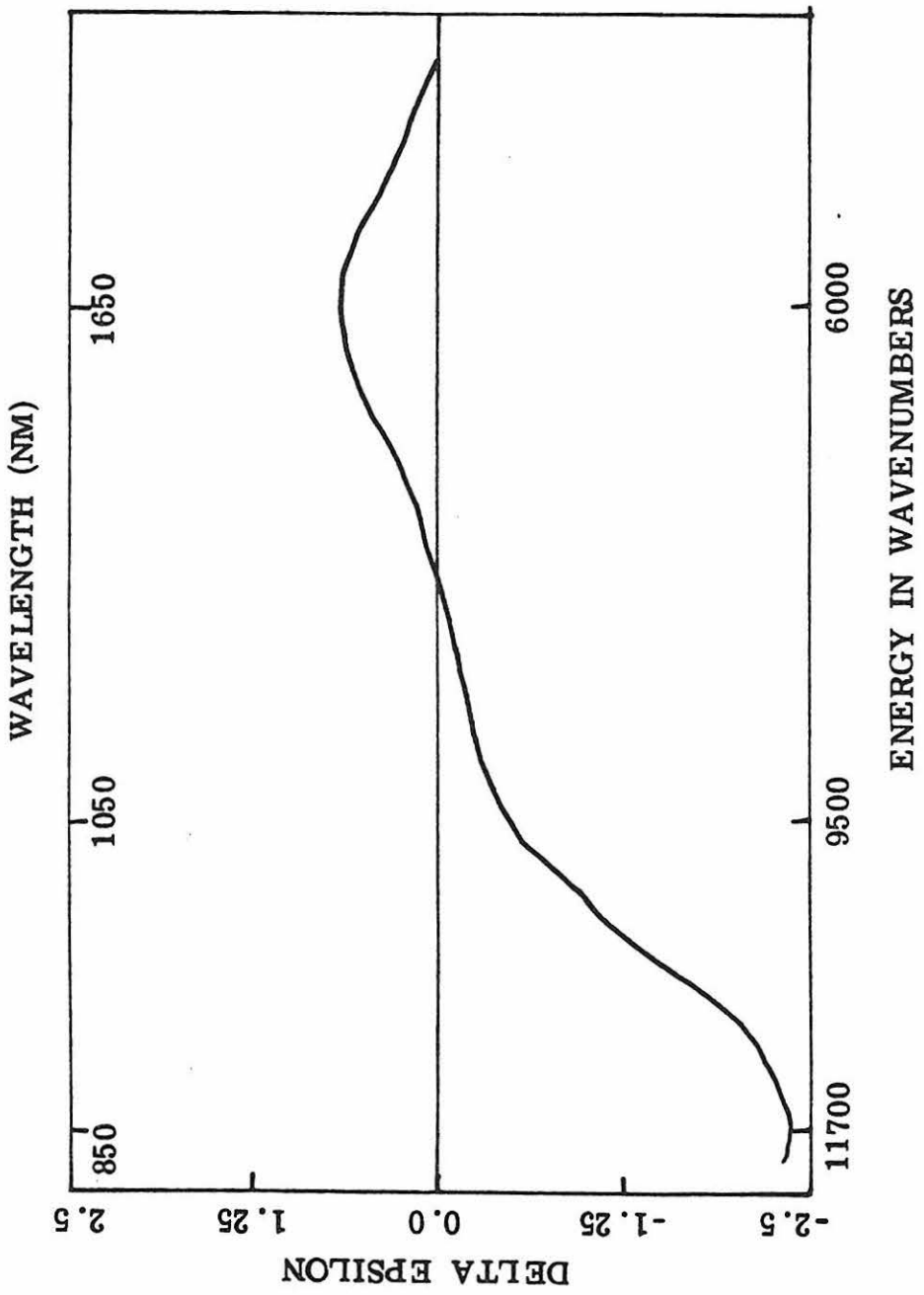
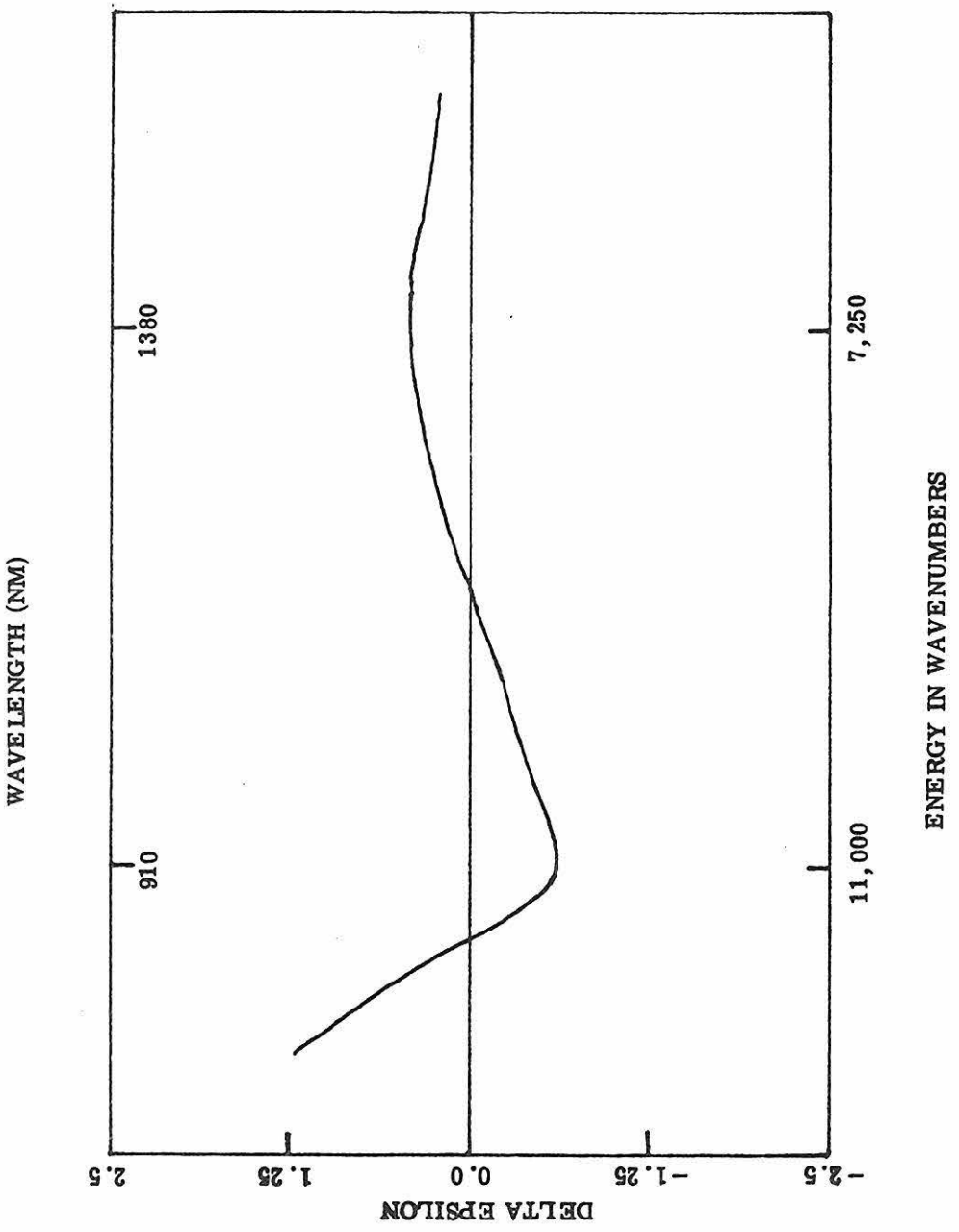

WAVELENGTH (NM)
850
1050
1650
ENERGY IN WAVENUMBERS
11700
9500
6000
DELTA EPSILON
2.5
1.25
0.0
-1.25
-2.5

FIGURE 14

Near infrared CD spectrum of fungal laccase in deuterated 0.1 M sodium acetate pD 5.7.


WAVELENGTH (NM)
1380
910
7,250
11,000
ENERGY IN WAVENUMBERS
DELTA EPSILON
2.5
1.25
0.0
-1.25
-2.5

parameters, particularly the variation of the g values (both magnitude and ordering) and their dependence on the energies of ligand field states.[8] For example, $g_{||} \tilde{-} 2.0 < g_{\perp}$ for axially compressed trigonal bipyramidal Cu(II) complexes, the predicted ordering for a d_{z^2} ground state.[37] A compressed trigonal four coordinate geometry will also have a d_{z^2} ground state for Cu(II) and thus $g_{||} \tilde{-} 2.0$ and $g_{\perp} = 2 - 6k\lambda/\Delta W(^2E_g - {}^2A_1)$.[38] This ordering is observed for copper ZnO[39] and BeO[40] and is opposite to the ordering observed in blue copper proteins. Furthermore, at least one of the values of $|A_{||}|$ or $|A_{\perp}|$[39-41] are much larger than observed for the blue copper sites. More detailed discussions and calculations of g values for axially elongated trigonal five- and four-coordinate geometries (that are also inconsistent with the spectroscopic properties of the blue site) are given in reference 8. However, a tetragonally distorted tetrahedral ligand field satisfactorally accounts for the data. The correct electronic energies (W) of the ligand field states of Cu(II) for a D_{2d} symmetry are

$$W(^2A_1) = W(a_1 = d_{z^2}) = -12(3\cos^2\beta - 1)Ds - 3\delta Dt$$
$$W(^2B_1) = W(b_1 = d_{xy}) = 12(3\cos^2\beta - 1)Ds + \tfrac{1}{2}(35\sin^4\beta - \delta)Dt$$
$$W(^2E) = W(e = d_{xz}, d_{yz}) = -6(3\cos^2\beta - 1)Ds + 2\delta Dt$$
$$W(^2B_2) = W(b_2 = d_{x^2-y^2}) = 12(3\cos^2\beta - 1)Ds - \tfrac{1}{2}(35\sin^4\beta + \delta)Dt$$

where $\delta = 35\cos^4\beta - 30\cos^2\beta + 3$, $Ds = 2e\langle r^2\rangle/21a^3$, $Dt = 2e\langle r^4\rangle/21a^5$, and the ground state for Cu(II) is $^2B_2$. The coordinate system and β are as defined previously.[2] Considerable confidence may be placed in this model as it semi-quantitatively

accounts for the ligand field energies of the tetrachlorocuprates,[8] which are well known to possess geometries varying from D_{2d} distorted tetrahedral to square planar depending on the counter-ion.[42]

Analysis of the single blue copper protein d-d transitions indicated that only values of β near 60° gave reasonable values for $\Delta W(^2A_1 - {}^2B_2)$ in the square planar or tetrahedral limit. Assigning $\Delta W(^2E_2 - {}^2B_2) = 6000\ cm^{-1}$ and $\Delta W(^2B_1 - B_2) = 9500\ cm^{-1}$ for tree laccase, values of $\Delta W(^2A_1 - {}^2B_2)$ may then be calculated for various values of β near 60°. For β = 61°, Ds = 698 cm^{-1}, Dt = 464 cm^{-1}, and $\Delta W(^2A_1 - {}^2B_2)$ is calculated to be 12,150 cm^{-1}. In contrast, values for β of 60° and 64° yield Ds = 897 cm^{-1}, Dt = 483 cm^{-1}, $\Delta W(^2A_1 - {}^2B_2) = 12{,}928\ cm^{-1}$ and Ds = 365 cm^{-1}, Dt = 416 cm^{-1}, $\Delta W(^2A_1 - {}^2B_2) = 9993\ cm^{-1}$, respectively. Falk and Reinhammar reported a negative shoulder at 11,700 cm^{-1} in the CD spectrum of tree laccase[31] and we have observed both absorption and a CD shoulder at this energy; the transition is logically assigned to $\Delta W(^2A_1 - {}^2B_2)$, indicating that the parameters for β = 61° are to be preferred. The 11,700 cm^{-1} absorption band is several times more intense than the 9500 cm^{-1} band, consistent with D_{2d} selection rules; $^2A_1 \rightarrow {}^2B_2$ is electric dipole allowed but $^2B_1 \rightarrow {}^2B_2$ is forbidden. The above calculations serve to illustrate the sensitivity of the d orbital splittings to variations in β near the tetrahedral limit. $\Delta W(^2A_{1g} - {}^2B_{1g})$, the D_{4h} limit, is calculated to be 21,390 cm^{-1}, a quite reasonable value for this transition in square-planar copper complexes with nitrogen donor ligands.[36]

Some ambiguity is present in the analysis of the ligand field transitions in fungal laccase. The EPR spectrum is decidedly rhombic so d_{xz} and d_{yz} cannot be degenerate and the parent ^2E state must be split into two components. Consequently, the 7250 cm^{-1} band may be assigned to one component of $^2B_2 \rightarrow {}^2E$ or to $^2B_2 \rightarrow {}^2B_1$. In the latter case the 11,000 cm^{-1} feature becomes $^2B_2 \rightarrow {}^2A_1$ and both components of the ^2E state are assumed to be lower in energy than 5000 cm^{-1} and thus not observed. However, this assignment is rendered unlikely by the following argument.

The crystal field expressions for the g values in a tetragonally distorted tetrahedral are:

$$g_{||} = 2 - 8k_{||}\lambda^\circ/\Delta W(^2B_1 - {}^2B_2)$$

$$g_{\perp} = 2 - 2k_{\perp}\lambda^\circ/\Delta W(^2E - {}^2B_2)$$

where k and λ°(= -828 cm^{-1}) are orbital reduction and free ion spin-orbit coupling parameters, respectively. Note that the g values, particularly $g_{||}$, are very sensitive to the energies of the $^2B_2 \rightarrow {}^2E$ and $^2B_2 \rightarrow {}^2B_1$ electronic transitions. Values of $k_{||} = 0.38$ and $k_{\perp} = 0.16$ were computed from a fit of the theory to experiment for the EPR of stellacyanin, azurin, and plastocyanin.[8] Very similar values of k adequately fit the observed EPR of tree laccase (*vide infra*). The experimental g values are $g_z = 2.287$, $g_y = 2.077$, $g_x = 2.025$ for stellacyanin,[43] $g_{||} = 2.260$, $g_{\perp} = 2.052$ for azurin,[44] $g_{||} = 2.226$, $g_{\perp} = 2.053$ for plastocyanin,[45] and $g_{||} = 2.298$, $g_{\perp} = 2.047$ for tree laccase[43] and $g_z = 2.190$, $g_x = 2.052$, $g_y = 2.033$ for

fungal laccase.[46] If the values of $k_{||}$ and $k_{\perp}$ for the type 1 copper in fungal laccase are assumed to be approximately the same as for the other blue copper proteins then it is clear that $\Delta W(^2B_1 - {}^2B_2)$ and $\Delta W(^2E - {}^2B_2)$must be correspondingly larger for fungal laccase. Thus $\Delta W(^2B_1 - {}^2B_2)$ would be greater than 10,000 cm^{-1} and $\Delta W(^2E - {}^2B_2)$ greater than 6000 cm^{-1}. Taking $\Delta W(^2B_1 - {}^2B_2) = 11{,}000\ cm^{-1}$ $k_{||}$ is computed to be 0.32. If the higher energy component of the split ^2E is assigned a ΔW of 7250 cm^{-1}, then we can take $k_y = k_x = 0.14$. This places the unobserved lower energy component of ^2E at 4460 cm^{-1} (mean ^2E energy = 5855 cm^{-1}). Positive activity to the low energy side of the 7250 cm^{-1} maximum (Fig. 14) is consistent with another transition of the same sign lying just beyond the observed energy region. Assuming $\beta = 60°$ and taking $\Delta W(^2E - {}^2B_2) = 5855\ cm^{-1}$, $\Delta W(^2B_1 - {}^2B_2) = 11{,}000\ cm^{-1}$, the ligand field parameters are calculated to be Ds = 796 cm^{-1} and Dt = 559 cm^{-1}. The D_{4h} limiting value of $\Delta W(^2A_{1g} - {}^2B_{1g})$ is then 24,690 cm^{-1}. The highest energy d-d transition $^2A_1 \rightarrow {}^2B_2$ is predicted at 13,500 cm^{-1}. This is a region of considerable overlap with intense visible bands associated with the blue chromophore in both the absorption and CD spectra and the expected ligand field transition would be weaker and not resolved.

Calculated ligand field parameters for all the blue copper proteins we have examined are given in Table II. Values of Ds and Dt are those resulting directly from the fit. Calculations were performed using the observed energies of the $^2B_2 \rightarrow {}^2B_1$

Table II. Blue Copper Ligand Field Parameters

Blue Protein	β	Ds (cm^{-1})	Dt (cm^{-1})	D_{4h} Limit (cm^{-1})	LFSE (mV)
plastocyanin	60°	691	465	21,800	+340
azurin	60°	817	518	24,800	+241
tree laccase	61°	698	464	21,400	+269
ceruloplasmin	61°	717	464	21,800	+260
fungal laccase	60°	796	559	24,700	+203
stellacyanin	60°	765	444	22,800	+450

and $^2B_2 \rightarrow {}^2E$ transitions and requiring a reasonable value for the ($^2A_{1g} \rightarrow {}^2B_{1g}$) in the D_{4h} limit. β was allowed to vary. The derived parameters could be evaluated by calculating the energy of the $^2B_2 \rightarrow {}^2A_1$ transition and comparing the calculated and experimental values where possible. Consequently the parameters derived from tree laccase, ceruloplasmin, plastocyanin, and stellacyanin are most satisfactory as all three ligand field transitions were observed in absorption and/or CD spectra. Owing to the previously mentioned ambiguity in the spectra of fungal laccase a wider variety of fits was attempted, primarily by relaxing constraints on the D_{4h} limit. All such attempts gave less satisfactory results than those calculated for $\beta = 60°$ and the values shown in Table II are to be preferred.

It is possible to evaluate the effect of the ligand field on the reduction potential of the type 1 copper in the laccases by evaluating the ligand field stabilization energies (LFSE) relative to the Cu(II) aquo ion.[8,9] The LFSE for tree laccase is -6731 cm^{-1} and a value of -7244 cm^{-1} is calculated for fungal laccase. The value for $Cu(H_2O)_6^{2+}$ is -8900 cm^{-1} so tree laccase is destabilized by 269 mV and fungal laccase 203 mV relative to $Cu(H_2O)_6^{2+}$. As the potential for $Cu(H_2O)_6^{2+}$ is +153 mV, this simple treatment predicts potentials of +422 and +356 mV for *Rhus vernicifera* and *Polyporus versicolor* laccase, respectively.

The predicted and experimental values of the potential for tree laccase are in remarkably good agreement (better than we should expect). Factors other than the ligand field splitting are clearly more important in determining the potential of fungal laccase, as no reasonable values of β, Ds and Dt could be obtained that simultaneously fit the observed ligand field energies and reduction potential. Table II also summarizes values of the LFSE for each blue copper center where such an analysis is possible. Note that LFSE considerations predict markedly positive reduction potentials for all the blue copper centers.

In contrast to fungal laccase, the EPR spectrum of tree laccase is very nearly axial, even at 35 GHz.[43] Values of $g_{||} = 2.265$ and $g_{\perp} = 2.044$ are calculated using the k values derived from analysis of the single blue copper proteins and the observed energy splitting for tree laccase. Increasing $k_{||}$ to 0.43 allows the calculated value of $g_{||}$ to match the experimental value. The orbital reduction factors for all of the blue copper centers examined so far are considerably less than those calculated for square-planar copper peptide complexes, where $0.55 \leq k_{||} \leq 0.64$ and $0.71 \leq k_{\perp} \leq 0.45$,[47] indicating that electron delocalization and covalent bonding may be particularly pronounced in the type 1 copper site.

The unusually low value of $|A_{||}|$ exhibited by the blue copper site is also accounted for by a tetragonally distorted tetrahedral geometry. Previous calculations have shown that

admixture of 4p character into the 3d orbitals, that is allowed in near tetrahedral symmetries but forbidden in most other cases, is responsible for decreasing $|A_{||}|$.[48,49]

Low molecular weight Cu(II) complexes of near tetrahedral geometry are not common, thus little comparative data exists. Copper doped into the C_{3v} distorted tetrahedral sites of ZnO[39] or BeO[40] has been discussed above. Copper occupies tetragonally distorted tetrahedral sites in NH_4F[49] and $Zn[C(NH_2)_3]_2(SO_4)_2$[50] with $|A_{||}| < 4 \times 10^{-4}$ cm^{-1} and $< 5 \times 10^{-3}$ cm^{-1}, respectively and $g_{||} > g_{\perp} \sim 2.1$. This ordering of g values was also observed in the EPR spectrum of the near tetrahedral complex Cu(α,α´-dibormodipyrromethene)$_2$ but $|A_{||}|$ was not resolved.[48] Unfortunately, no optical data could be obtained in these cases. Cs_2CuCl_4 has approximately D_{2d} symmetry with $\beta \sim 62°$. The ligand field bands for this complex occur at approximately 5000, 8000, and 9000 cm^{-1}.[42c] EPR spectra of the pure crystal and of copper doped Cs_2ZnCl_4 show $g_{||} \sim 2.38$, and $g_{\perp} \sim 2.09$ and $|A_{||}| \sim 2.5 \times 10^{-3}$ cm^{-1}.[51] Bis(4-phenylamino-2-phenyliminopent-3-enato-N,N´)Cu(II) also displays a ligand field transition near 6000 cm^{-1}; its EPR spectrum is characterized by $g_{||} \simeq 2.218$, $g_{\perp} = 2.070$ and $|A_{||}| = 10.7 \times 10^{-3}$ cm^{-1}.[52] These observations establish that many of the unique spectral properties of blue copper are a consequence of geometric factors and contradict recent suggestions that ligand donor properties are more important.

Comparison of the experimental reduction potentials and ligand field bands (Table III) for the single blue copper proteins and the type 1 copper containing oxidases is informative.

Table III. Blue Copper Ligand Field Transitions (cm^{-1})

Blue Protein	$^2B_2 \rightarrow$ ^2E	$^2B_1$	$^2A_1$	E° (mV)
plastocyanin	5000	9150	11,200	347
azurin	5800	10,200	--	330
tree laccase	6000	9500	11,700	415
ceruloplasmin	6100	10,000	11,500	535*
stellacyanin	5250†	8570	11,900	184
fungal laccase	7250†	11,000	13,500	787

†Higher energy component of a split ^2E.

*Average of the potentials for the two blue copper ions.

A near tetrahedral ($\beta \sim 60°$) coordination geometry adequately accounts for the measured E° values of plastocyanin, azurin, tree laccase, and ceruloplasmin. However, the observed potentials of stellacyanin and fungal laccase are much lower or higher, respectively, than the values predicted by LFSE arguments. Furthermore the E°'s of these two proteins are strikingly different from other blue copper potentials (Table III and ref. 14). A number of other properties also differentiate stellacyanin and fungal laccase from other blue copper proteins. Apparently the blue site is of lower symmetry in stellacyanin and fungal laccase, as evidenced by a rhombic EPR spectrum which implies a split $^2B_2 \rightarrow {}^2E$ transition. The ligand field in fungal laccase is higher than that of any other blue protein we have investigated. Stellacyanin displays a unique resonance raman spectrum.[53] MCD spectra (Table I) also distinguish stellacyanin and fungal laccase from other blue copper proteins.

The variation in spectroscopic parameters may be readily rationalized by reference to the X-ray crystal structure data. As stellacyanin contains no methionine at least one ligand must be different from the donor sets established for azurin or plastocyanin. Further, we suggest that tree laccase and ceruloplasmin possess ligand donor sets identical to those described for azurin and plastocyanin as the spectroscopic properties of these proteins are quite similar. The data discussed above indicate that fungal laccase may have a type 1 copper site distinct from any other blue protein; thus ligand variations in its type 1 site, as compared to the known structures, seem

probable. Visible CD data provide additional support for this idea. The visible CD curve of tree laccase (the spectrum of ceruloplasmin is nearly identical[31,33]) resembles the curves of the small blue copper proteins (Figure 3). In contrast the visible CD spectrum of fungal laccase (Figure 4) differs substantially below energies of 21,000 cm^{-1}. Careful comparison of the spectra indicates the difference is primarily that of the two lowest energy transitions in fungal laccase being of opposite sign than observed for all other blue copper sites. It should be noted that stellacyanin, azurin, and plastocyanin display positively signed shoulders at 18,000 - 19,000 cm^{-1} [8]; the corresponding transitions in the laccases are more pronounced and equally as intense as the peak expected near 16,000 cm^{-1}. Consequently a broad (20,500 cm^{-1} to 15,500 cm^{-1}) positive band results in tree laccase. In fungal laccase these two transitions are opposite in sign and well resolved. The unique shape of the fungal CD curve supports the concept of a different blue site structure than observed in plastocyanin and azurin. Since the spectral properties and reduction potentials of stellacyanin and fungal laccase differ as well it appears that at least three types of blue copper sites can be distinguished. Although other factors cannot be absolutely ruled out, most attractive is the possibility that variations in the spectroscopic and thermodynamic properties of the blue sites reflect differences in the copper ligands. These considerations and our present model for the blue copper site are summarized in

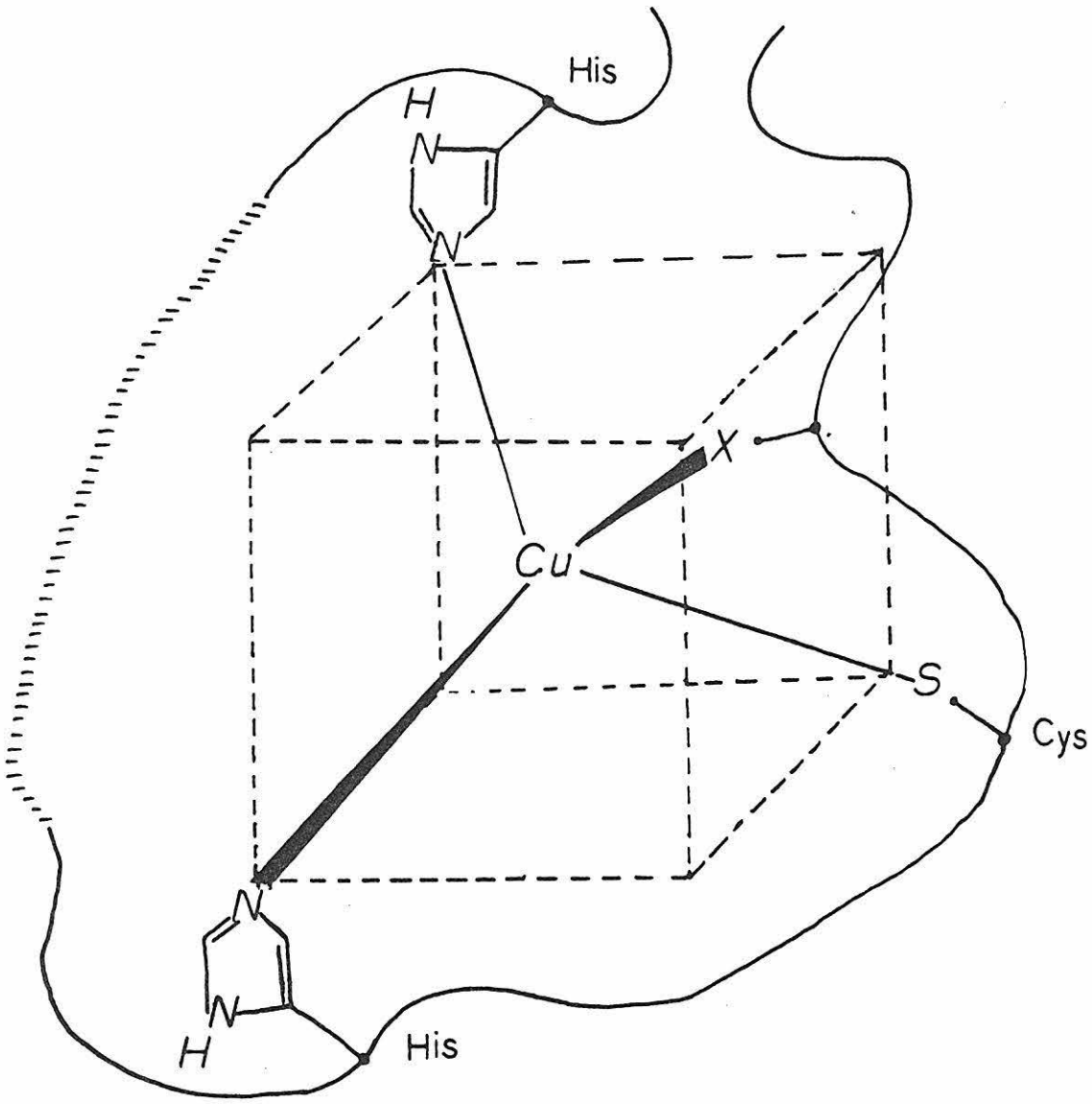
Figure 15.

An approximate MO diagram based on this structure is shown in Figure 16. Assignments of visible absorption bands involving ligands that are known to vary are not considered. Evaluation of the Kuhn anisotropy factor, $\gamma = 4|\Delta\varepsilon|/\varepsilon$, is useful in assigning the various bands as those with magnetic dipole character will display values of γ considerably larger than transitions that are only electric dipole allowed.[54] In a site of approximate D_{2d} symmetry π charge-transfer transitions will be both electric and magnetic dipole allowed whereas σ charge-transfer transitions will be only electric dipole allowed. Values of γ for tree and fungal laccase estimated from room temperature data are shown in Table IV. Reasonable assignments for the bands at about 22,000 cm^{-1} and 14,000 cm^{-1} are $\pi N \rightarrow d_{x^2 - y^2}$ (from imidazole) and $\pi S \rightarrow d_{x^2 - y^2}$ (from thiolate), respectively. The prominent band at $\sim$ 16,000 cm^{-1}, which is responsible for the characteristic blue color, is assigned to $\sigma S \rightarrow d_{x^2 - y^2}$, also from thiolate sulfur. Chemical evidence is consistent with a cysteine ligand for the type 1 copper in tree and fungal laccase.[55]

The types 2 and 3 coppers have been difficult to characterize spectroscopically owing to the intense features of the type 1 copper in both the CD and visible spectra. Specific reduction of the blue chromophore in both tree and fungal laccase allowed the observation of absorption bands and CD activity attributable to the other copper sites. Observed

FIGURE 15

Model of the blue copper site consistent with structural and spectroscopic data.



X = Met-S

plastocyanin
azurin
tree laccase
ceruloplasmin
(ascorbate oxidase)

X varies

stellacyanin
fungal laccase

FIGURE 16

Approximate molecular orbital diagram based on the site structure of Figure 15. Symmetry is assumed to be D_{2d}.

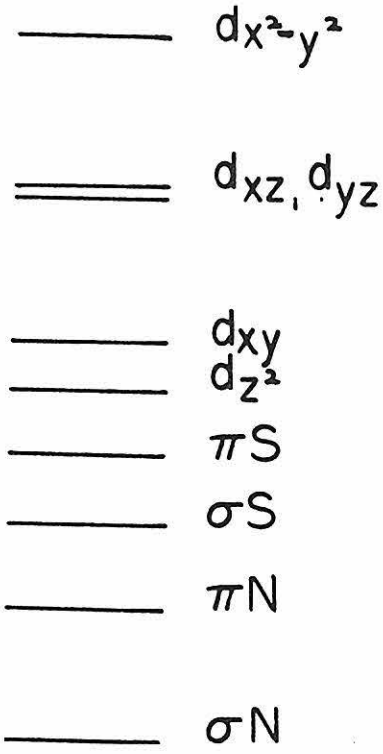

$d_{x^2-y^2}$
d_{xz}, d_{yz}
d_{xy}
d_{z^2}
πS
σS
πN
σN

Table IV. Transition Assignments and Kuhn Anisotropy Factors

Blue protein	ν (cm^{-1})	ε	$\Delta\varepsilon$	γ	Assignment
tree laccase	9500	200	0.25	0.005	$^2B_2 \rightarrow {}^2B_1$
	11,700	600	2.5	0.017	$^2B_2 \rightarrow {}^2A_1$
	13,000	600	2.7	0.018	$\pi S \rightarrow d_{x^2 - y^2}$
	16,125	5700	1.5	0.001	$\sigma S \rightarrow d_{x^2 - y^2}$
	23,000	530		0.020	$\pi N \rightarrow d_{x^2 - y^2}$
fungal laccase	11,000	170	0.6	0.014	$^2B_2 \rightarrow {}^2B_1$
	13,000	830	2.0	0.01	$\pi S \rightarrow d_{x^2 - y^2}$ and $^2B_2 \rightarrow {}^2A_1$
	16,000	4900	3.2	0.0026	$\sigma S \rightarrow d_{x^2 - y^2}$
	19,000	950	1.4	0.006	not assigned
	22,650	800	1.3	0.007	$\pi N \rightarrow d_{x^2 - y^2}$

intensities of the visible absorption and CD spectra indicate the transitions are most likely ligand field in nature, and the energies are consistent with six, five, or square-planar four coordinate Cu(II).[36] Tetrahedral geometry may be ruled out for copper types 2 and 3 in tree and fungal laccase by the lack of ligand field transitions at energies near 10,000 cm^{-1} and lower. As both the types 2 and 3 coppers remain oxidized in high pH-fungal laccase the transitions cannot be assigned to one type specifically. For tree laccase + NO, results with the type 2 depleted protein indicate the 16,700 cm^{-1} absorption is associated primarily with the type 2 site. These observations support the concept that the type 2 and type 3 copper ions possess coordination geometries typical of many small monomeric Cu(II) complexes or bridged binuclear units. Several other metalloproteins are known to contain binuclear or "type 2" copper sites. Oxyhemocyanin[21] and tyrosinase[22] have both absorption and CD bands attributable to d-d transitions of Cu(II) in the region where transitions appear the type 1 reduced laccases. Bovine erythrocyte superoxide dismutase, which contains a $Cu(II)N_4$ chromophore, displays a λ_{max} of 680 nm (14,700 cm^{-1}) in the visible absorption spectrum.[56] Extensive dialysis of ceruloplasmin against ascorbate results in loss of the type 1 chromophore.[33,57] Upon reoxidation the ascorbate modified ceruloplasmin shows an absorption and CD spectra remarkably similar to those of high pH fungal laccase.[33]

Finally, the F^- binding experiments deserve comment. Type 2 copper has been proposed as the substrate binding site and initial electron acceptor[17a]; in addition stopped flow EPR studies suggest the type 2 copper may transfer electrons to the type 3 site, either from substrate directly or from the type 1 copper.[17b] In any case it is apparent that internal electron transfer steps are required in the catalytic mechanism of the laccases. However, magnetic susceptibility measurements have established that the types 1 and 2 copper ions must be separated by several angstroms and are probably not bridged by a common ligand(s).[19] Consequently, perturbations of the type 1 chromophore by anion binding at the type 2 site may have mechanistic significance as such alterations could reflect conformational changes of the enzyme designed to facilitate intramolecular electron transfer. The difference CD spectra (Figures 5 and 6) are consistent with this idea, particularly considering the fact that the bands associated with the type 1 site are not affected equally, suggesting that certain portions of the polypeptide chain are more sensitive to changes at the type 2 site than other type 1 ligand containing sections.

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CHAPTER 2

FURTHER MAGNETIC SUSCEPTIBILITY STUDIES OF LACCASE AND OXYHEMOCYANIN

A number of metalloenzymes that interact with dioxygen are known or postulated to contain a binuclear copper site: hemocyanin (1), tyrosinase (1,2), and the oxidases laccase, ascorbate oxidase, and ceruloplasmin (3). Additionally cytochrome c oxidase has been proposed to contain a coupled heme iron-copper pair as the site of dioxygen binding and reduction (4). In no case has any EPR signal, attributable to a binuclear site, been observed in the native protein (5). Although other structural possibilities are consistent with this observation, it is generally thought that the metal ions involved are strongly antiferromagnetically coupled, resulting in a diamagnetic or even spin ground state (5,6). The temperature dependence of the magnetic susceptibility of such systems is distinctive and may be used to distinguish antiferromagnetic coupling from a truly diamagnetic structure, e.g., a Cu(I) dimer. For two antiferromagnetically coupled S = 1/2 Cu(II) ions, the susceptibility will be a maximum at a temperature that is simply related to the energy difference, J, between the diamagnetic ground state and the paramagnetic excited state (7). Antiferromagnetic coupling between two copper ions has been observed in a wide variety of dimeric Cu(II) complexes (8) and in the four-copper derivative of bovine superoxide dismutase (9).

We have focused our efforts on oxyhemocyanin and laccase. It is generally accepted that the dioxygen binding site in oxyhemocyanin is a binuclear copper(II) peroxo complex, the evidence coming mainly from resonance Raman (10) and other spectroscopic (11,12) experiments. The four copper atoms in laccase are distributed in types 1 (paramagnetic), 2 (paramagnetic), and 3 (binuclear; EPR non-detectable) sites. The type 3 coppers apparently function as a two-electron acceptor and are thought to be the site of interaction with dioxygen (3). Previous magnetic susceptibility measurements showed oxyhemocyanin to be diamagnetic over the temperature range 35-250 K (13,14). Over this same range the susceptibility of Rhus laccase showed, in addition to the Curie law paramagnetism of copper types 1 and 2, non-Curie paramagnetism at temperatures above 80 K that was consistent with an antiferromagnetically coupled Cu(II) pair with $J = 170 \pm 30$ cm^{-1} (13). Owing to the fact that the fit of theory to the observed deviation from Curie law in laccase was not entirely satisfactory (due in part to the low precision of the measurements) and realizing the importance of these observations to understanding the copper-site electronic structure of hemocyanin and laccase, we have remeasured the susceptibilities over a wider temperature range

using susceptometers recently developed at SHE Corporation, San Diego, CA. Although oxyhemocyanin was again observed to be essentially diamagnetic over the entire temperature range, laccase, in contrast to our previous results, displayed only the Curie law behavior of copper types 1 and 2 over the temperature range examined.

MATERIALS AND METHODS

Rhus vernicifera laccase was purified by the method of Reinhammar (15) from the acetone powder (Saito & Co., Ltd., Japan) to a A_{280}/A_{614} value of <15. A sample was then dialyzed extensively against distilled, deionized water and concentrated in an Amicon ultrafiltration apparatus to a final concentration of 2.2 mM, determined from the extinction coefficient (ε_{614} = 5700 M^{-1} cm^{-1}). *Limulus polyphemus* hemolymph was strained through cheesecloth, centrifuged, and then dialyzed against 50 mM Tris-glycine,10 mM EDTA, pH = 8.9, followed by further dialysis against 50 mM Tris, 10 mM EDTA, pH = 8.0. This procedure assured the oxyhemocyanin was disaggregated into a mixture of subunits of molecular weight ~70,000 (16). Copper concentration was determined by atomic absorption and by optical absorption (ε_{340} = 10,000 M^{-1} cm^{-1}) (17) and found to be 4.70 ± 0.05 mM by both methods.

Magnetic susceptibility measurements as a function of temperature were performed using two different prototype instruments constructed at SHE Corporation; these instruments are fully described elsewhere (18). Both versions utilized SQUID detection of the total magnetic moment in a sample. The earlier instrument used a concentric arrangement of the SQUID pick-up coils relative to the sample space and thus was sensitive to the exact sample geometry. (Corrections could be made during conversion of the SQUID output to a volume susceptibility.) The newer instrument uses two counter-wound Helmholtz coil pairs compensated to respond uniformly (within 5%) over a volume 5 mm diameter x 8 mm length, thereby allowing the mass susceptibility to be measured directly. Degassed distilled, deionized water, $HgCo(SCN)_4$, and platinum metal were used to calibrate the susceptometers. The temperature was monitored using a resistance thermometer calibrated <u>in situ</u> against platinum and carbon-glass standards from Lake-Shore cryogenics. At least five measurements were recorded and averaged at each temperature. Temperature regulation was better than 0.4% maximum deviation from the set point over the entire range. The sample holders were measured separately over the same temperature range as the samples and their contribution to the

observed signals subtracted out. No effort was made to subtract the diamagnetism of the water or buffer from the total sample signal.

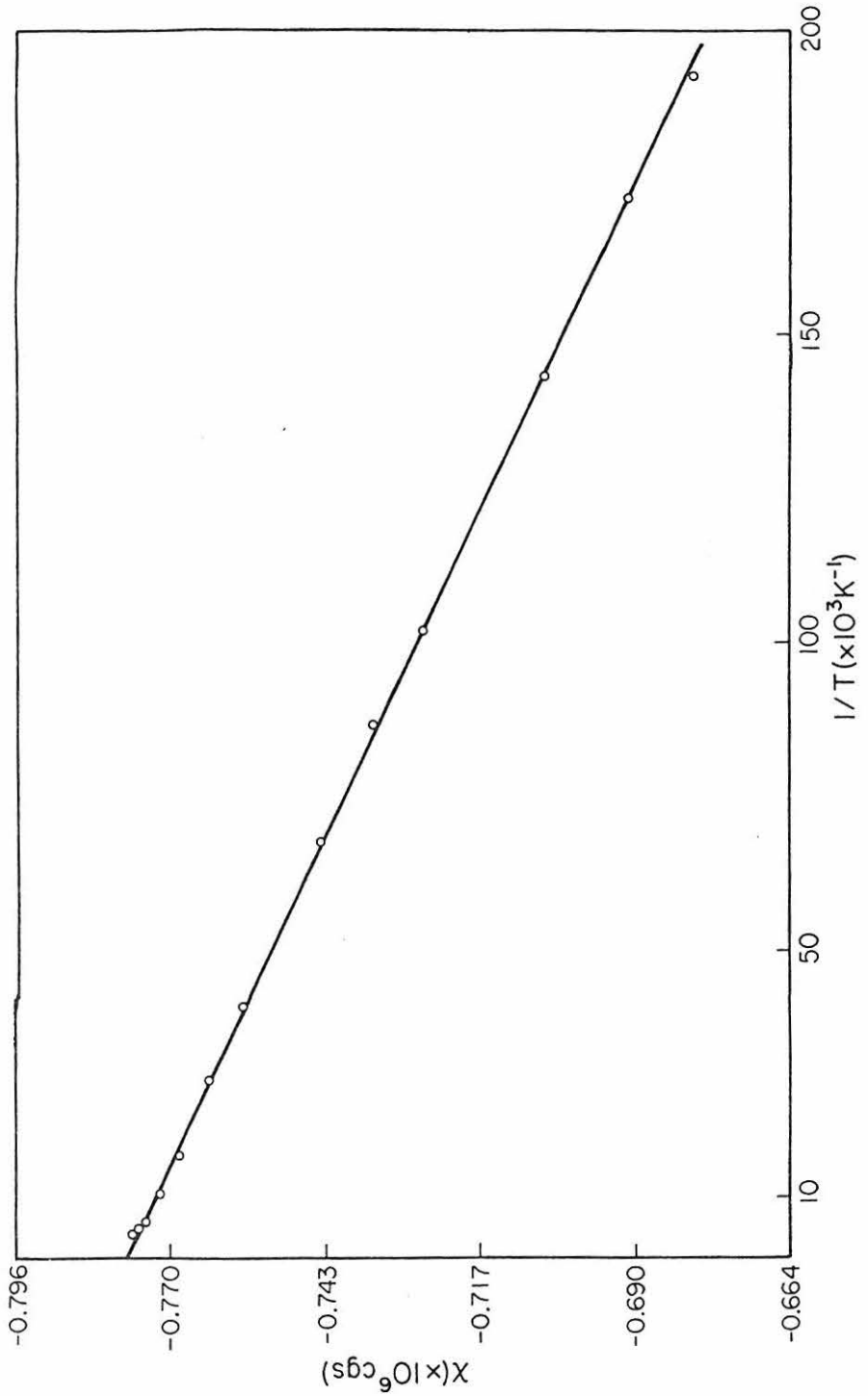
Laccase was degassed in the susceptometer airlock *via* several cycles of complete evacuation followed by flushing with helium. This procedure was avoided for oxyhemocyanin in order to prevent significant deoxygenation. Instead, oxyhemocyanin was exposed briefly to a partial vacuum and then quickly flushed with helium. No changes in the samples were observed to occur during this process. Approximately 0.6 ml of solution was measured with the first prototype, whereas only ~0.05 ml was required on the newer instrument.

RESULTS AND DISCUSSION

The volume susceptibility of *Limulus* oxyhemocyanin as a function of T^{-1} is shown in Figure 1. A weak Curie law ($\chi = CT^{-1}$) paramagnetism, observed as a decrease in the solution diamagnetism, is evident with a least squares fit slope of -0.50×10^{-6} cgs - K. This is less than 25% of the slope calculated assuming 4.7×10^{-3} M Cu^{2+}, $\mu = 1.93$ BM, ($= -2.2 \times 10^{-6}$ cgs - K) and is attributed to the presence of dissolved dioxygen

FIGURE 1

Temperature dependence of the volume susceptibility of Limulus oxyhemocyanin. ○ represents six standard deviations of the SQUID output at each temperature.


-0.796
-0.770
-0.743
-0.717
-0.690
-0.664
χ(x 10⁶ cgs)
10
50
100
150
200
1/T (x10³K⁻¹)

and paramagnetic impurities.* Our present data accord well with previous measurements of Megathura crenulata (13) and Limulus oxyhemocyanin (D. M. Dooley, E. I. Solomon, H. B. Gray, M. Cerdonio, unpublished observations) and taken together establish that the binuclear copper pair in oxyhemocyanin is diamagnetic over the entire temperature range 5-260 K, indicating that the antiferromagnetic coupling must be very strong. Using a Heisenberg-Dirac-Van Vleck hamiltonian $\mathcal{H} = JS_1 \cdot S_2$, to define the exchange energy, the present data require $J \geq 550$ cm^{-1}. This lower limit is somewhat below our estimate for Megathura crenulata hemocyanin ($J \geq 625$ cm^{-1}), owing to the much lower copper concentration in these experiments. Antiferromagnetic coupling is usually mediated via a superexchange pathway involving the atomic or molecular orbitals of bridging groups. Since such large values of

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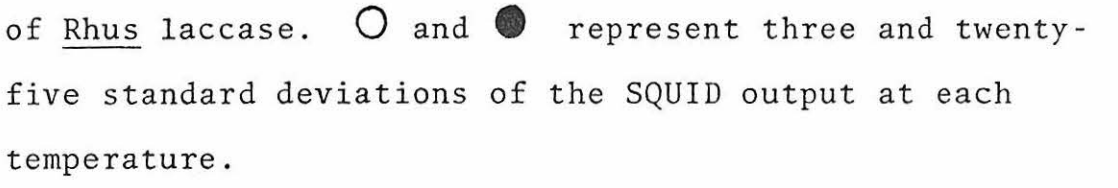
* Assuming the O_2 concentration in solution to be 0.30×10^{-3} M (19) and $\mu = 2.83$ BM, the slope is calculated to be -0.30×10^{-6} cgs - K. Iron content of the oxyhemocyanin solution was determined by atomic absorption to be 2.7×10^{-5} M; if $\mu = 5.9$ BM, then its contribution to the slope is -0.12×10^{-6} cgs - K. Thus, nearly the entire temperature dependence of oxyhemocyanin is accounted for without any contribution from the intrinsic copper.

J have not been observed in any singly-bridged binuclear Cu(II) complexes (20,21,22), a multiply-bridged dioxygen-copper center is a more attractive structural possibility for oxyhemocyanin.

Figure 2 depicts the volume magnetic susceptibility of Rhus laccase versus T^{-1}. In contrast to our earlier results (13), only a simple Curie law paramagnetism is observed from 5 to 260 K. The slope of the least squares fit line is -1.96×10^{-6} cgs - K, which is identical within 5% of the theoretical slope calculated for two paramagnetic Cu(II) ions with μ = 1.93 BM (per Cu) per laccase molecule. The lack of any contribution to the observed temperature dependence from dissolved O_2 or paramagnetic impurities (as seen in the oxyhemocyanin data) is probably due to the more extensive degassing and the dialysis against pure water employed for the laccase sample.

The mass susceptibility of the same sample of Rhus laccase as a function of T^{-1} is plotted in Figure 3. Again only Curie law paramagnetism is observed. The slope of the least squares line is $-0.00787 \frac{cm^3}{g_{Cu}}$ - K,† which accords with the theoretical slope for two paramagnetic coppers ($-0.00733 \frac{cm^3}{g_{Cu}}$ - K). We are confident the present results are correct for two reasons: (1) the measurements (which are identical within experimental

FIGURE 2



Temperature dependence of the volume susceptibility of Rhus laccase. ○ and ● represent three and twenty-five standard deviations of the SQUID output at each temperature.

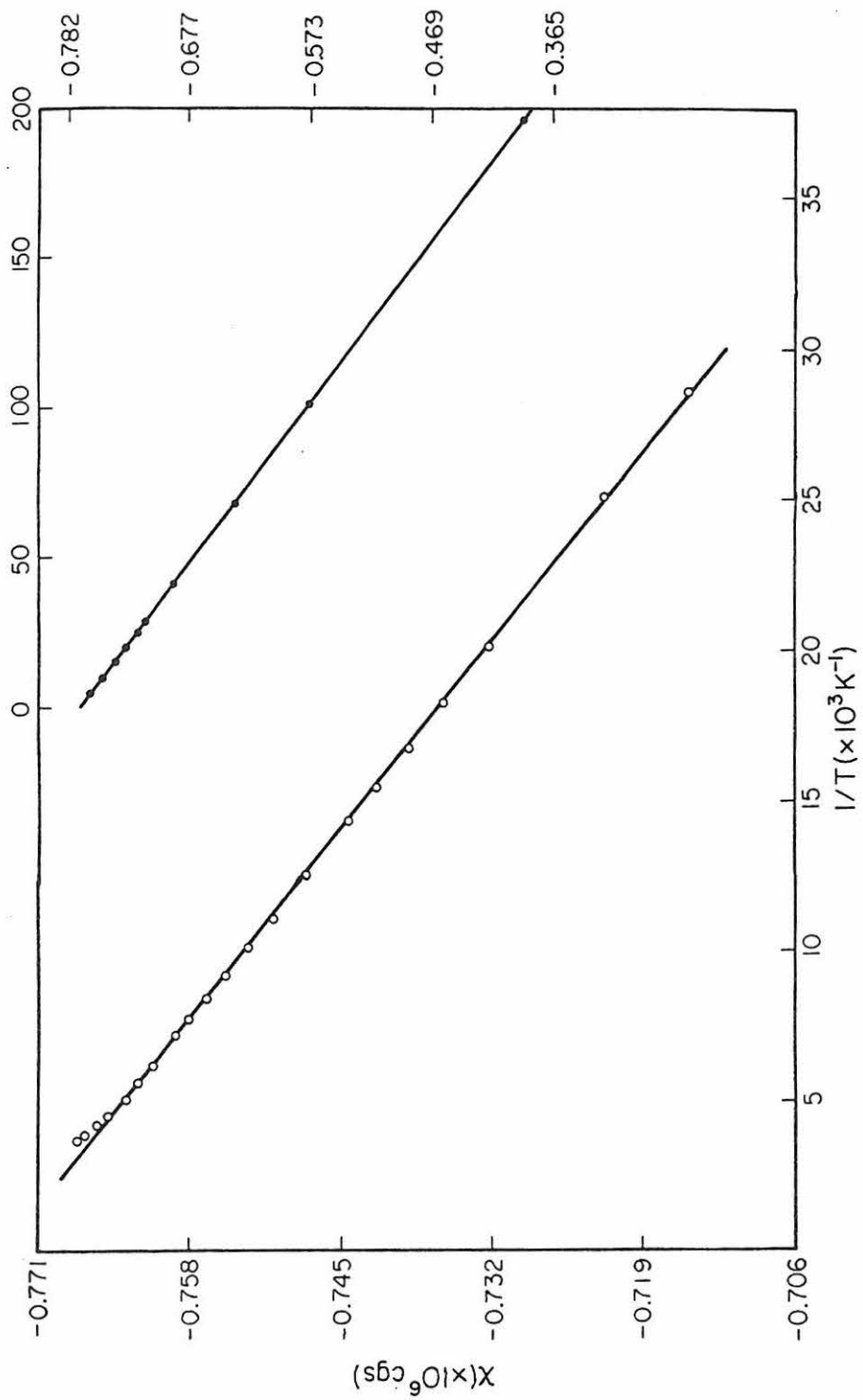
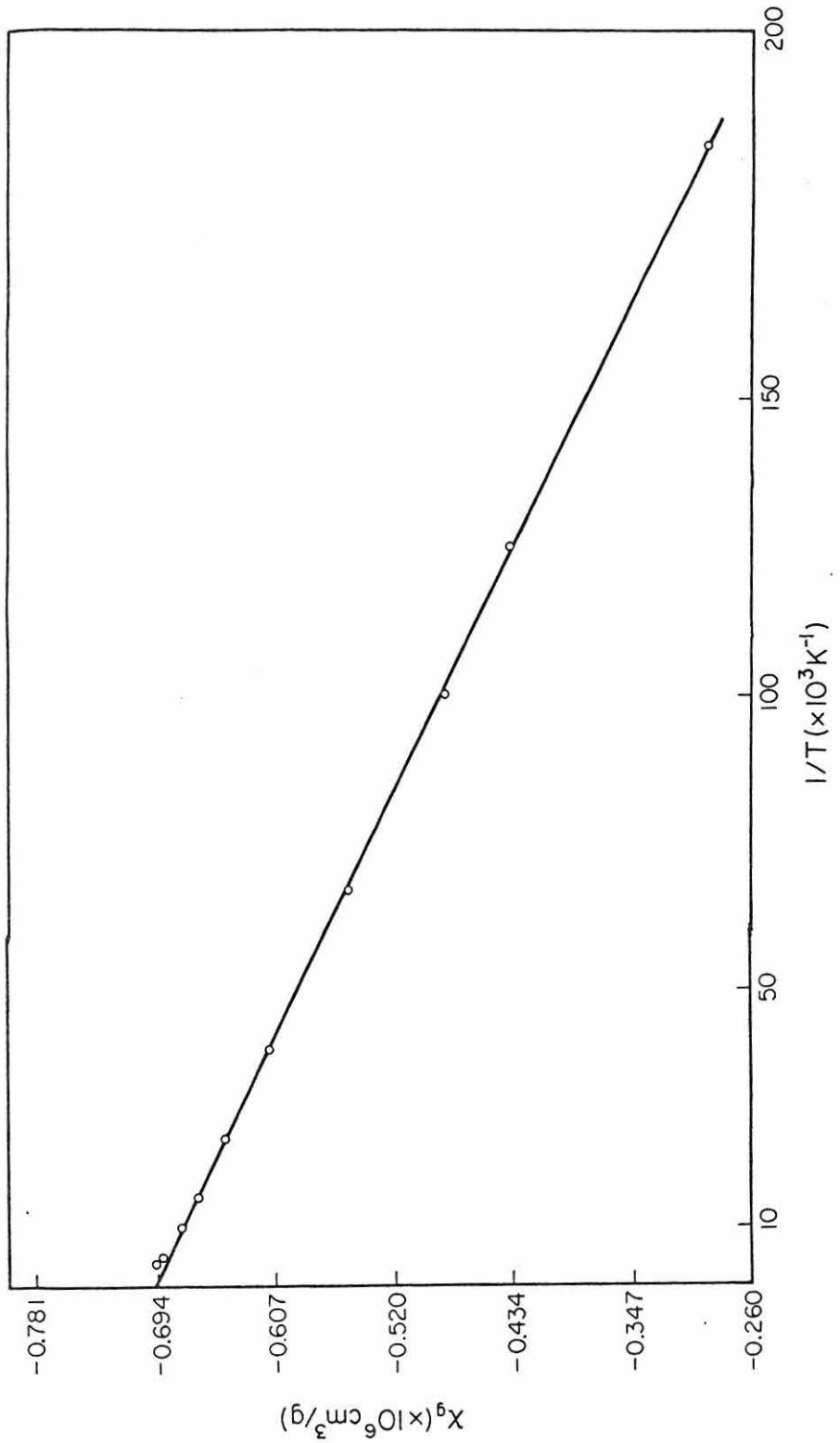

-0.782
-0.677
-0.573
-0.469
-0.365
200
150
100
50
0
35
30
25
20
15
10
5
1/T(×10³K⁻¹)
-0.771
-0.758
-0.745
-0.732
-0.719
-0.706
χ(×10⁶cgs)

FIGURE 3

Temperature dependence of the gram susceptibility (based on total sample weight) of Rhus laccase measured on the new prototype susceptometer. ○ represents approximately fifteen standard deviations of the SQUID output at each temperature.


-0.781
-0.694
-0.607
-0.520
-0.434
-0.347
-0.260
$\chi_g(\times 10^6 cm^3/g)$
10
50
100
150
200
$1/T(\times 10^3 K^{-1})$

error) were made on the same sample several months apart on entirely different susceptometers; and (2) it is extremely difficult to conceive of effects that would give fortuitous cancellation of a complicated temperature dependence (i.e., $\chi_M = (g^2N\beta^2/3KT)\ (1 + 1/3e^{J/KT})$), due to an antiferromagnetically coupled Cu(II) dimer, in order to obtain precisely the Curie law behavior predicted for the copper types 1 and 2.

As Curie law behavior is observed to ~5 K, any interaction between the copper types 1 and 2 must be quite small, consistent with the idea that these ions are separated by several angstroms (3,23) in the native laccase molecule. Furthermore, it is unlikely that the copper types 1 and 2 redox centers are connected by ligand bridging groups, as such structures would be expected to give rise to measureable superexchange interactions.

Our finding that the type 3 site in oxidized Rhus laccase is magnetically similar to the binuclear copper redox center in oxyhemocyanin may have structural significance. If the type 3 site contains Cu(II), then strong antiferromagnetic coupling ($J \geq 500\ cm^{-1}$) must be present. Further evidence that the type 3 site has some structural similarity to the binuclear copper center in oxyhemocyanin has been obtained recently from spectroscopic examination of laccase-dioxygen intermediates

during turnover conditions. At 3° C and pH = 6.0, a laccase-dioxygen complex is observed with an optical spectrum (λ_{max} = 340 nm, with a weaker peak at 475 nm) that is very similar to that of oxyhemocyanin; what is more, CD maximum is observed for this species at 362 nm, which is a region where signals are present in the spectrum of oxyhemocyanin (D. A. Baldwin, D. M. Dooley, and H. B. Gray, unpublished observations). Further spectroscopic and magnetic studies of this laccase-dioxygen complex are being pursued in our laboratory in an attempt to define the oxidation levels of the coppers and the dioxygen, as well as the relationship, if any, of the type 3 site in this species to the binuclear copper(II)-peroxo unit in oxyhemocyanin.

† This gram susceptibility may be converted to an approximate volume susceptibility by use of the relationship $\chi_g = \frac{\chi}{\rho}$, ρ is the density $\frac{g_{Cu}}{ml}$. A value of -2.2×10^{-6} cgs - K obtains, in good agreement with the measurement on the older susceptometer. The slightly increased paramagnetism could arise from less efficient deoxygenation or from impurities, as the sample was not redialyzed against pure water following several months of storage at -10° C. The A_{280}/A_{614} ratio was ~15.0 after this period, compared to ~13.5 initially.

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INTERMISSION

CHAPTER 3

SPECTROSCOPIC STUDIES OF THE COORDINATION ENVIRONMENT AND ELECTRONIC STRUCTURE OF COPPER SITES IN CERULOPLASMIN

Since the original observation and purification of ceruloplasmin by Holmberg[1,2] this serum protein has been the subject of intense investigation, principally owing to its unusual spectroscopic properties,[3,4] catalytic oxidase activity,[3,4] and its clinical importance as the affected protein in Wilson's disease, a hereditary disorder in copper metabolism.[5] Our interest was stimulated by the similarities between ceruloplasmin and the other blue copper oxidases, laccase and ascorbate oxidase. These enzymes contain a minimum of four copper ions in three spectroscopically distinct sites. Type 1 copper is responsible for the characteristic, intense (ε(~600 nm)$\cong$ 5000 M^{-1} cm^{-1} per copper) blue color of these enzymes. A number of small (MW < 20,000) single blue copper electron transfer proteins are also known whose spectroscopic properties have been investigated in our laboratory.[6,7] The type 2 copper is characterized primarily by its EPR spectrum which is very similar to those usually observed for simple tetragonal Cu(II) complexes. Type 3 copper is associated with a near UV band at 330 nm and occurs in an "EPR nondetectable" state; the stoichiometry of two coppers per enzyme in the case of laccase led to the proposal that the type 3 site is a binuclear, antiferromagnetically coupled pair;[8] recent magnetic susceptibility studies have shown that the coupling

must be very strong if this is the case.[9] Type 3 copper is thought to be the site of reaction with dioxygen.

The stoichiometry of types 1, 2 and 3 copper[10-12] is not as well established for ceruloplasmin as it is for laccase. Total copper stoichiometry has been the subject of numerous investigations and active debate. Uncertainty in the molecular weight of the protein complicated the copper determinations. Measurements of the molecular weight from single crystals[13] and careful sedimentation equilibrium studies[14,15] have finally established the value of approximately 130,000. The generally accepted value for the total weight percent of copper is 0.275 ± 0.009%,[16] which for a molecular weight of 132,000 ± 4100 corresponds to 5.73 ± 0.36 Cu per ceruloplasmin molecule. Rydén and Björk have measured the properties of ceruloplasmin isolated from fresh serum and also found a molecular weight of 134,000 ± 3,000 and a copper content, though somewhat variable, consistent with six g-atoms Cu per mole enzyme.[17] Unfortunately the magnetic data are not entirely consistent with this result. In particular only 44% of the bound copper is paramagnetic,[10,11] a value most easily accommodated by assuming seven coppers per protein molecule. Quantitative EPR studies have established the presence of two type 1 coppers[12,18] and one type 2 copper.[12,18] Therefore a type 3 pair and an

additional "type 4"[17] copper are required to fit a stoichiometry of six and two type 3 sites are required to fit a stoichiometry of seven. Furthermore, electrochemical titrations of ceruloplasmin indicate the 330 nm band, normally associated with the type 3 site, does not behave as a simple two electron acceptor.[12,19] An additional complication arises from the possible inequivalency of the two type 1 sites in native ceruloplasmin. In addition to the intrinsic copper described above, ceruloplasmin can bind up to ten additional cupric ions.[20]

Significant progress has been made in elucidating the structural and electronic properties of type 1 copper. Preliminary X-ray crystal structures have been reported for two single blue copper proteins, azurin[21] and plastocyanin,[22] and intensive spectroscopic studies have provided assignments for the unusual visible bands and located the ligand field transitions of blue Cu(II).[6,7] A D_{2d} distorted tetrahedral arrangement of the ligands most satisfactorally accounts for the data in all type 1 containing proteins we hve examined, including azurin,[6,7] plastocyanin,[6,7] stellacyanin,[6,7] tree and fungal laccase,[23] and ascorbate oxidase.[24] Investigation of copper types 2 and 3 has been hampered by the severe overlap of spectral features with type 1. We have recently obtained CD and absorption spectra for both types 2 and 3 copper in fungal and tree laccase.[23] Spectra

obtained on tree laccase under turnover conditions indicate similarities between type 3 copper and the binuclear copper pair in oxyhemocyanin,[25] as does magnetic susceptibility data on the native proteins.[9]

It has proved possible to study the type 2 copper in ceruloplasmin by EPR techniques via reaction of the native protein with nitric oxide, a procedure that reversibly bleaches the type 1 copper.[26,27] Superhyperfine structure attributable to F^- and nitrogen established equatorial coordination by a minimum of three equivalent nitrogen donors, which are approximately coplanar, and axial coordination by up to two F^- ions.[27] Observed g values in the nitric oxide treated protein are essentially those obtained from simulating the native EPR spectrum and are consistent with a tetragonal Cu(II) complex.[27]

We have initiated a series of spectroscopic experiments designed to further elucidate the nature of the copper sites in ceruloplasmin. Low temperature optical and/or near-infrared CD spectra have been obtained for the native and azide inhibited proteins in order to locate the type 1 ligand field bands. Ascorbate-treated ceruloplasmin,[28] which contains no type 1 copper, has been prepared and studied optically and magnetically in order to further characterize the types 2 and 3 sites.

EXPERIMENTAL

Purification of Ceruloplasmin. Ceruloplasmin was purified from Cohn Fraction IV by a modification of the method of Rydén and Björk.[17] The final preparation displayed a spectral purity ratio A_{610}/A_{280} = 0.045 and was electrophoretically homogeneous under non-denaturing conditions at neutral pH. Enzymatic activity was verified.

Frozen Cohn Fraction IV (ca. 200 g per batch) was thawed and suspended in one liter of 0.015 M sodium phosphate buffer, pH 6.8, containing 0.05 M NaCl and stirred for several hours. Centrifugation and filtration through a 1/2 inch moist pad of Whatman 3 mm filter paper, which had been mulched in a Waring blender, led to a turbid solution which could be applied directly to a 4 x 40 cm DE-52 DEAE cellulose column equilibrated with the above buffer. Elution was carried out with 2ℓ of a 0.05 M to 0.18 M linear gradient of NaCl, buffered as above, and the DEAE cellulose chromatography (3.2 x 32 cm column) was repeated. At this point $A_{610}/A_{280} \simeq 0.026\text{-}0.028$ and some turbidity was still apparent.

Remaining steps were carried out essentially as described previously,[17] except that protein solutions were concentrated for gel filtration by an Amicon stirred cell (PM-30 membrane) and the G-150 column (2.5 x 150 cm) was run in the presence of 1% n-butanol.

Pure fractions from the final column were combined ($A_{610}^{1\ cm}$ ~ 0.3-0.5) and stored at -90° C. Recent work in our laboratory has shown that A-50 DEAE Sephadex affords better separation than DEAE cellulose and that Sephacryl S-200 may be used in place of Sephadex G-150.

Poor fractions from a given column were generally recycled through the same column with the next batch of material. This increased the overall yield substantially and allowed more selectivity in the choice of fractions to subsequently purify. In this fashion, it was routinely possible to isolate over 400 mg per batch of Cohn Fraction IV. Yields as high as 600 mg were achieved on occasion.

Ascorbate Treatment of Ceruloplasmin. The method described by Kasper and Deutsch was used.[28] Dialysis over 2 days with frequent changes of a twenty-fold excess of 1.0 M sodium acetate, pH 5.2, buffer containing 5 mg/ml ascorbic acid was followed by dialysis for an additional day versus the same buffer without ascorbic acid. Finally, the protein was dialyzed for one day against 0.05 M sodium acetate, pH 7.2. At this stage A_{610}/A_{280} was generally less than 0.022. This sample was applied to a DEAE cellulose column (4.4 x 70 cm for a preparation resulting from ca. 300 mg of native ceruloplasmin) equilibrated with 0.05 M sodium acetate, 0.04 M NaCl, pH 7.2. The salt concentration was slowly increased

in a step-wise fashion until the blue color began to spread out on the column (~0.08 M NaCl in the acetate buffer). After the color had spread through half the column, 2ℓ 0f a 0.08-0.14 M linear NaCl gradient in acetate solution was applied to elute the blue color. When $A_{280} < 0.03$ ascorbate modified ceruloplasmin was eluted as a sharp band with 0.3 M NaCl, 0.05 M sodium acetate, pH 7.2. Other methods of elution on DEAE cellulose columns did not accomplish the crucial baseline separation necessary to insure that the ascorbate modified ceruloplasmin was not contaminated with the native protein. Concentration of the very dilute blue fractions yielded apparently pure, native ceruloplasmin ($A_{610}/A_{280} \simeq 0.045$-$0.050$), which could be successfully modified by ascorbic acid.

General Procedures. Absorption spectra were obtained on Cary 17 and Cary 14 spectrometers. JASCO-J40 and Cary 61 instruments were used for the visible and near UV (300-800 nm) CD and MCD measurements. Near-infrared (800 nm-2000 nm) CD were recorded with a laboratory constructed instrument described in detail elsewhere.[29] A Varian E-line Century Series spectrometer, equipped with a 12 inch magnet, was used for all EPR experiments; temperature regulation was provided by an Air Products Heli-Trans system and frequencies were determined with

a PRD Electronics, Inc. frequency meter. A Brinkman Model 101 pH meter with a Metrohm combination glass electrode was used to measure pH.

Protein films for low temperature absorption spectra were prepared on quartz or plexiglass disks as previously described.[23] Samples were mounted on the cold head of a Cryogenic Technology Model 20 cryocooler utilizing a mixture of copper filings in silicone grease to provide thermal contact and were cooled slowly to minimize cracking of the film. Visible CD and MCD spectra were obtained on samples having $A_{610}^{1\ cm} \simeq 1$ in 0.2 M sodium acetate, pH 5.5. Ceruloplasmin solutions for use in the near-infrared were 0.75 mM in deuterated 0.2 M sodium acetate, pD 5.5. The buffers were used to determine instrument baselines.

Reagent grade chemicals were used without further purification. D_2O was 99.8% D (Stohler Isotope Chemicals). DEAE cellulose was purchased from Whatman and Sephadex and Sephacryl gels from Pharmacia. Hydroxyapatite was prepared by a combination of literature methods.[30] Protein concentrations are based on the extinction coefficient at 610 nm (ε = 10,000 $\ell mole^{-1}\ cm^{-1}$).[17] The relation pD = pH meter reading +0.4 was used to calculate pD.

RESULTS

At approximately 20 K the blue (16,000 cm^{-1}) absorption band is significantly narrowed and several new features are resolved (Figure 1). Two prominent shoulders are seen, to higher (~22,000 cm^{-1}) and lower (~13,000 cm^{-1}) energy of the blue band. Three transitions of approximately these energies (and intensities) are characteristic of blue copper sites and have been previously observed in the small blue copper proteins stellacyanin, plastocyanin, and azurin[6,7] as well as in tree and fungal laccase[23] and ascorbate oxidase.[24] Two weak shoulders are evident near 10,000 cm^{-1} and 11,500 cm^{-1}. Bands in this region have not been previously observed in the absorption spectrum of ceruloplasmin. The intensities and energies of these bands are most compatible with an assignment as Cu(II) ligand field transitions. It should be noted that similar transitions have been detected in other blue copper proteins as well.[6,7,23,24]

The CD spectrum of ceruloplasmin in the visible region (Figure 2) accords well with those measured previously.[31,32] Observed values of $\Delta\varepsilon$ for ceruloplasmin are about a factor of two greater than those of blue copper proteins containing only a single type 1 site. The shape of the CD curve is quite similar to that of tree laccase[23,31] and ascorbate oxidase.[24] A completely

FIGURE 1

20 K absorption spectrum of a ceruloplasmin film on a plexiglass disk.

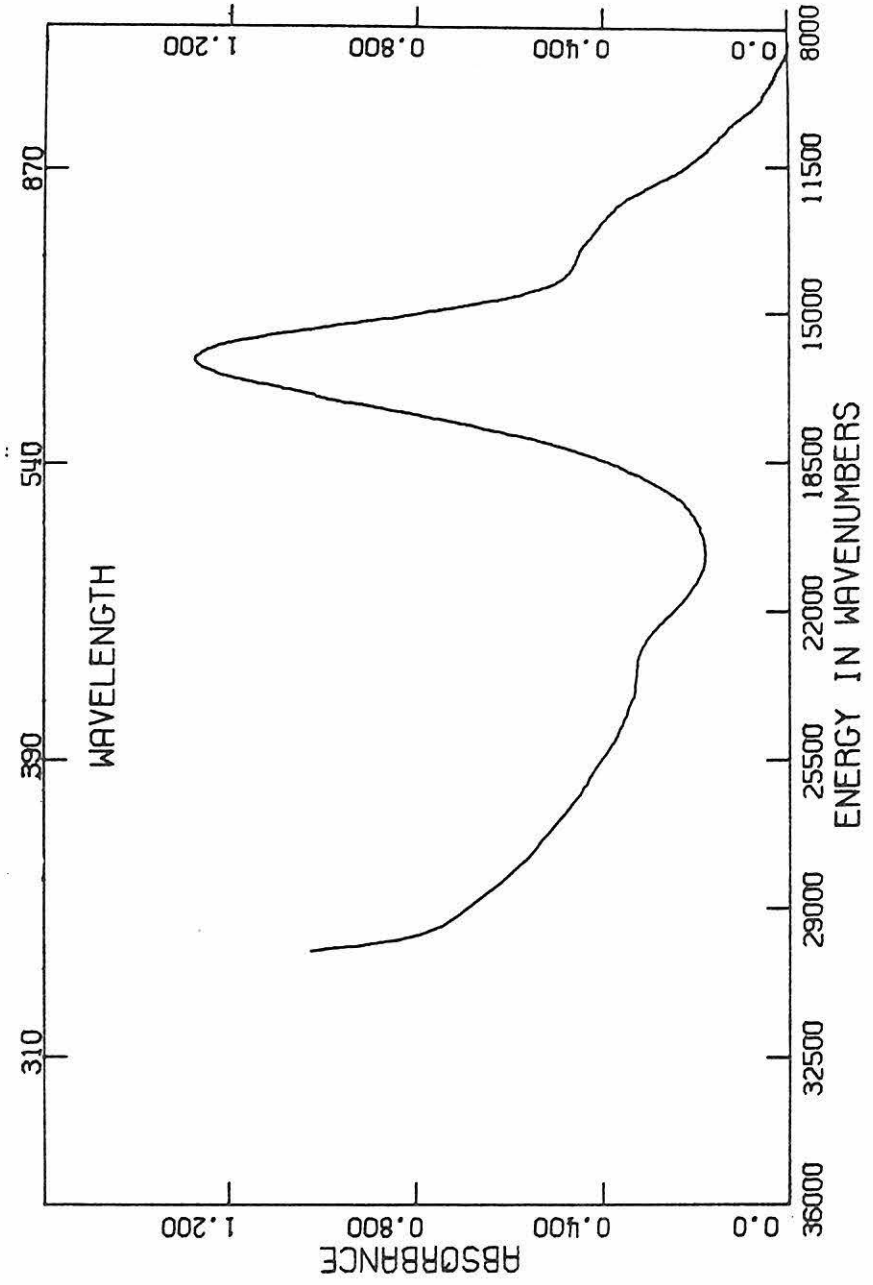
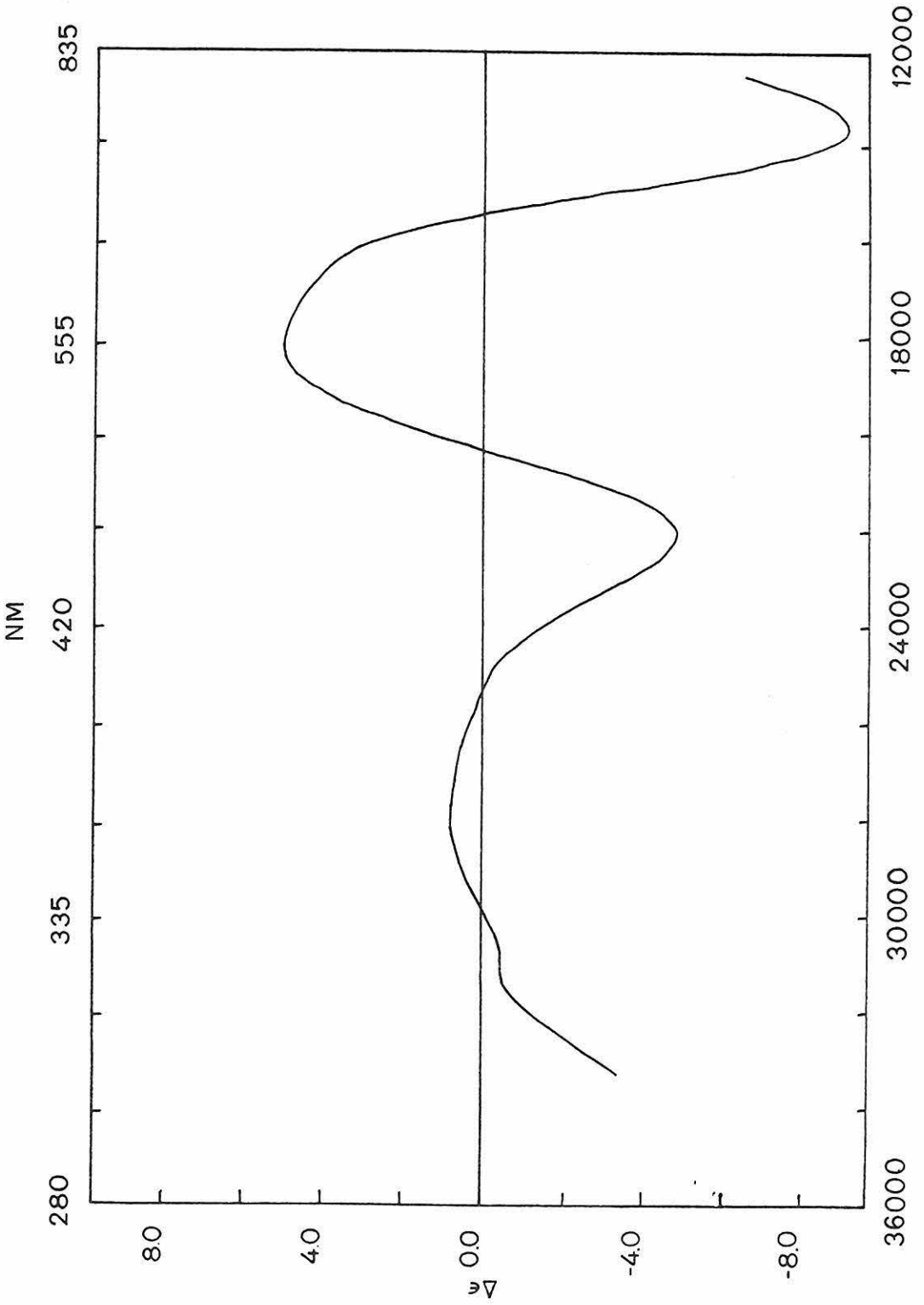

ABSORBANCE
0.0
0.400
0.800
1.200
WAVELENGTH
310
390
540
870
ENERGY IN WAVENUMBERS
36000
32500
29000
25500
22000
18500
15000
11500
8000

FIGURE 2

Visible CD spectrum of ceruloplasmin in 0.2 M sodium acetate pH 5.5. Vertical scale is in units of $\Delta\varepsilon$.


NM
280
335
420
555
835
Δε
8.0
4.0
0.0
-4.0
-8.0
36000
30000
24000
18000
12000

different CD spectrum (vide infra) obtains for the ascorbate-modified ceruloplasmin which lacks the type 1 copper, thus all the bands in Figure 2 may be assigned to transitions in the two type 1 sites. In a similar manner, the intense visible CD bands in both tree and fungal laccase have been unambiguously assigned to the type 1 copper.[23]

The visible MCD spectrum of ceruloplasmin is shown in Figure 3 and is characterized by a strong negative band at 14,600 cm^{-1}. Owing to the similarity of this spectrum to those of plastocyanin and azurin[6,7] the transitions in Figure 3 are reasonably assigned to the type 1 sites. The intensity ($\Delta\epsilon$/gauss) of the 14,600 cm^{-1} band in ceruloplasmin is two - three times greater than the corresponding bands in the single blue copper proteins.[6,7]

We have previously established that the type 1 Cu(II) ligand field transitions occur in the near infrared.[6,7,23] CD data for ceruloplasmin in this region are displayed in Figure 4. A low energy band is clearly resolved at 6100 cm^{-1}. In addition a negative shoulder is apparent at 10,000 cm^{-1}. These transition energies are in excellent agreement with observed energies of other blue copper proteins and taken together with the g values for the type 1 coppers are consistent only with a tetragonally (D_{2d}) distorted tetrahedral coordination geometry for

FIGURE 3

Visible MCD spectrum of ceruloplasmin in 0.2 M sodium acetate pH 5.5. Field was 40 kilogauss. Vertical scale is in units of $\Delta\varepsilon$/gauss.

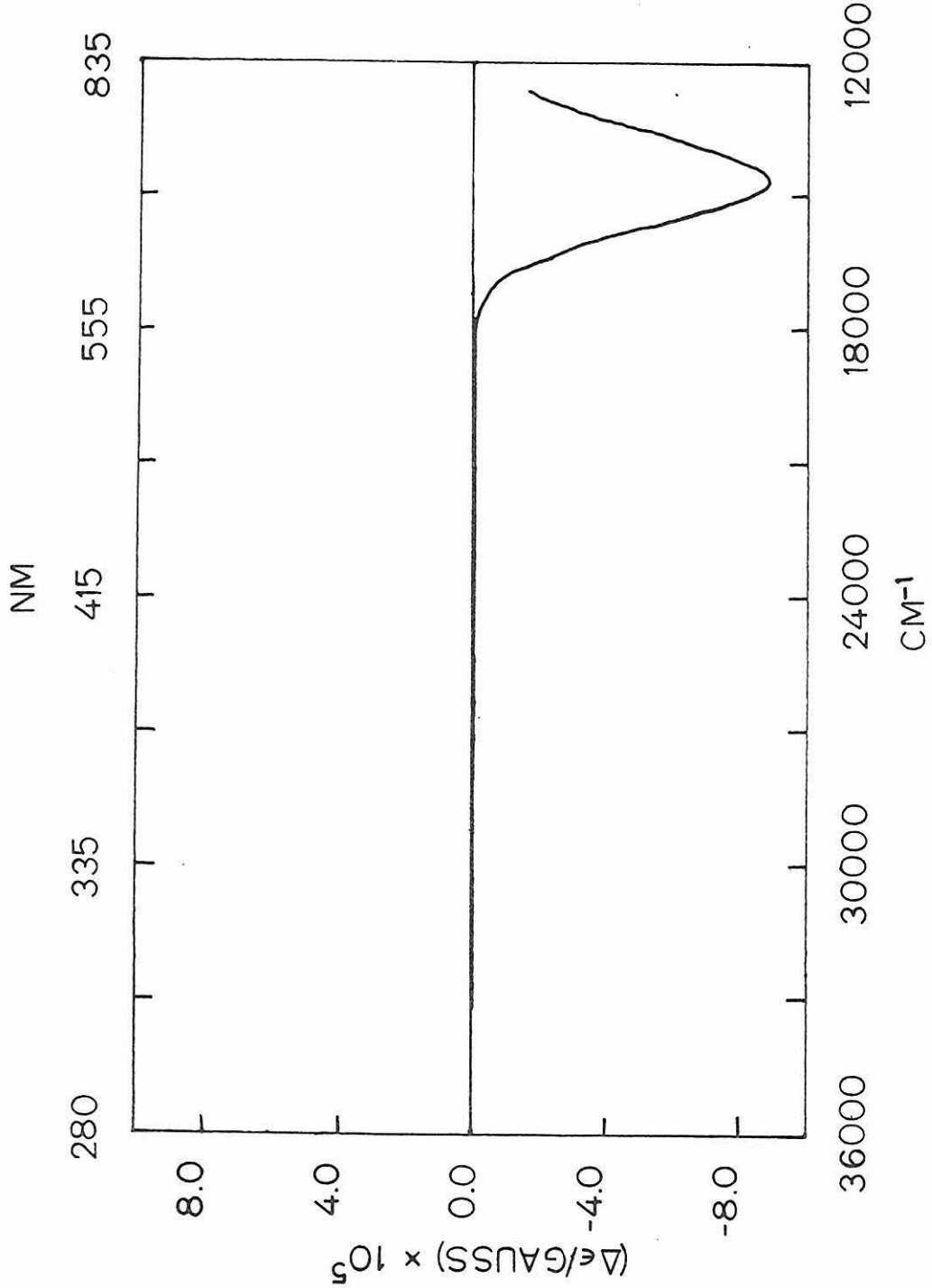
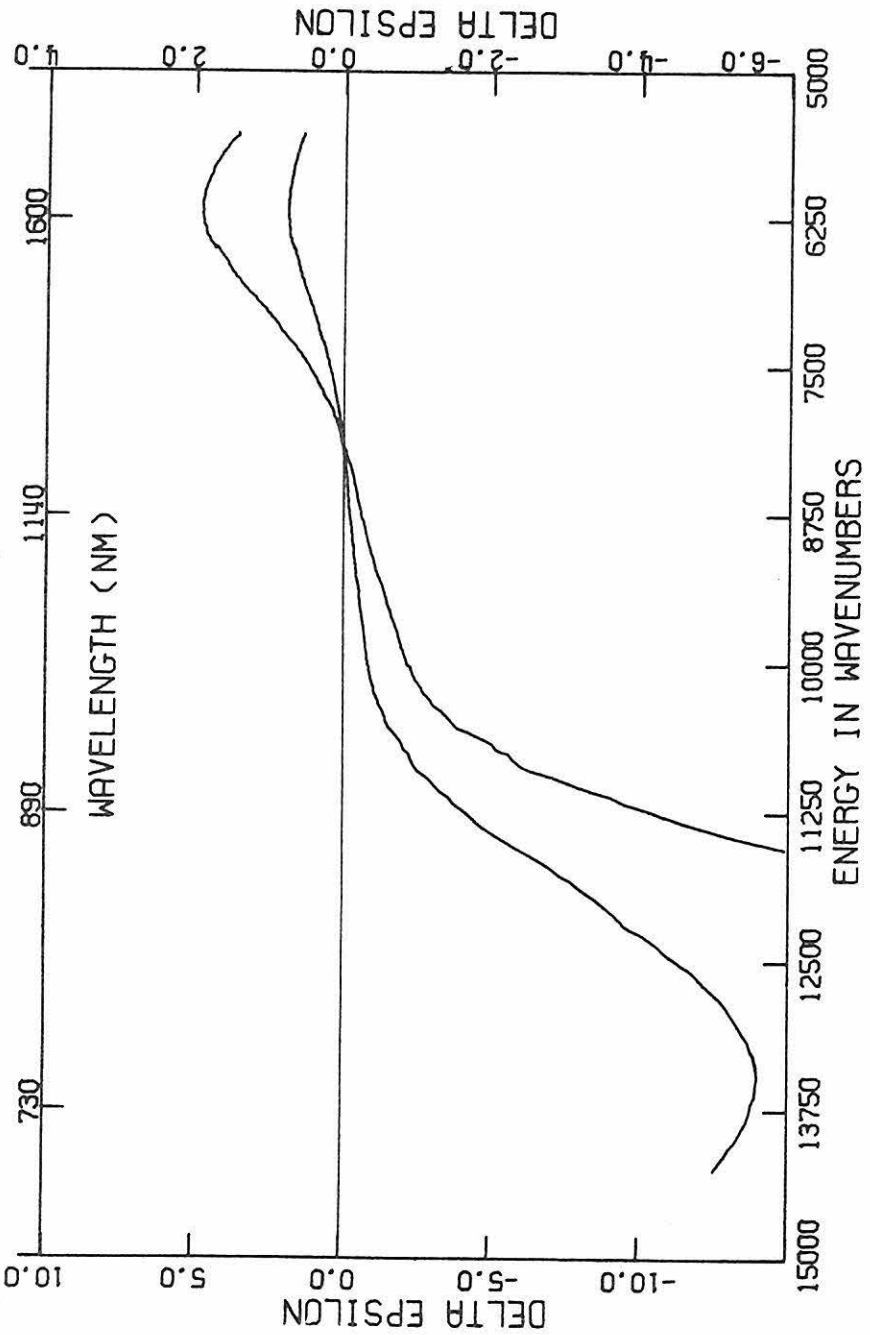

NM
280
335
415
555
835
CM-1
36000
30000
24000
18000
12000
(Δε/GAUSS) x 10^5
8.0
4.0
0.0
-4.0
-8.0

FIGURE 4

Near infrared CD spectrum of ceruloplasmin in deuterated 0.2 M sodium acetate pD 5.5. Vertical scale is in units of $\Delta\varepsilon$.


DELTA EPSILON
4.0
2.0
0.0
-2.0
-4.0
-6.0
5000
6250
7500
8750
10000
11250
12500
13750
15000
ENERGY IN WAVENUMBERS
1600
1140
890
730
WAVELENGTH (NM)
10.0
5.0
0.0
-5.0
-10.0
DELTA EPSILON

the blue site. The highest energy d-d transition expected for such a Cu(II) complex is not observed in Figure 4 due to overlap with the lowest energy intense CD band seen in the visible region (Figure 2). There is no indication of different ligand field transitions associated with the two type 1 Cu(II) ions. In fact, the lowest energy CD maximum (6100 cm^{-1}) is slightly more than twice as intense as the lowest energy transitions in other single blue copper proteins,[6,7,23] consistent with the idea that both type 1 sites in ceruloplasmin contribute equally to the 6100 cm^{-1} maximum. CD bands may be confidently assigned to the type 1 coppers as these bands are absent in the ascorbate-treated form of the enzyme (vide infra).

Several anions (e.g. N_3^-, SCN^-) are known to bind the type 2 copper in ceruloplasmin and modify the spectroscopic properties of the type 1 sites.[31-34] Figure 5 shows the visible region CD of ceruloplasmin in the presence of a thousand-fold excess of N_3^-; it accords with spectra measured previously.[31,32] As ligand field transitions are very sensitive to geometric and ligand variations, the near infrared CD spectrum of ceruloplasmin in the presence of the same amount of excess N_3^- was measured and is shown in Figure 6. A positive peak is evident at 8940 cm^{-1}, a negative band at 12,000 cm^{-1}

FIGURE 5

Visible CD spectrum of ceruloplasmin in the presence of a 1000-fold excess of N_3^- in 0.2 M sodium acetate pH 5.5. Vertical scale is in units of $\Delta\varepsilon$.

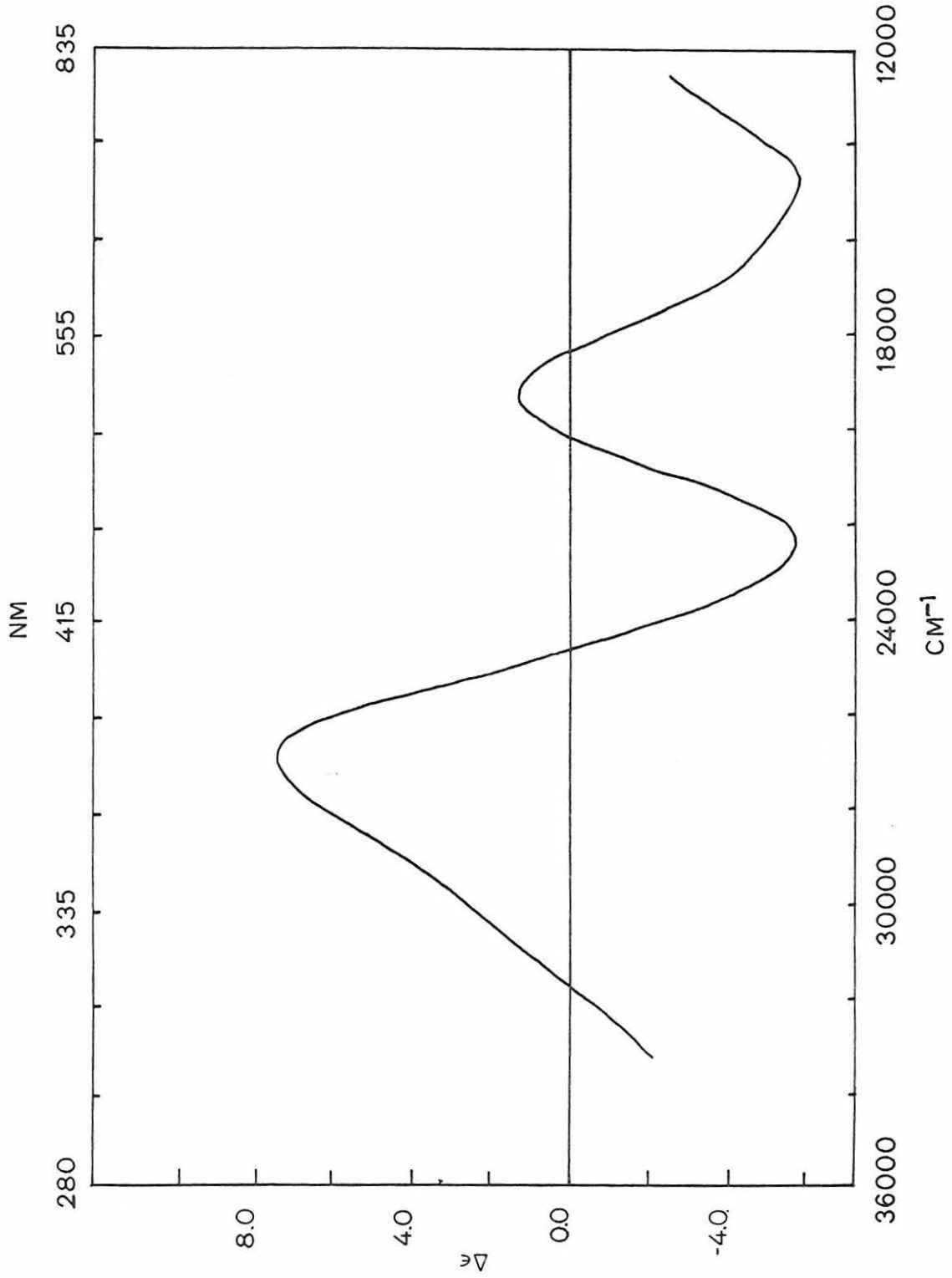
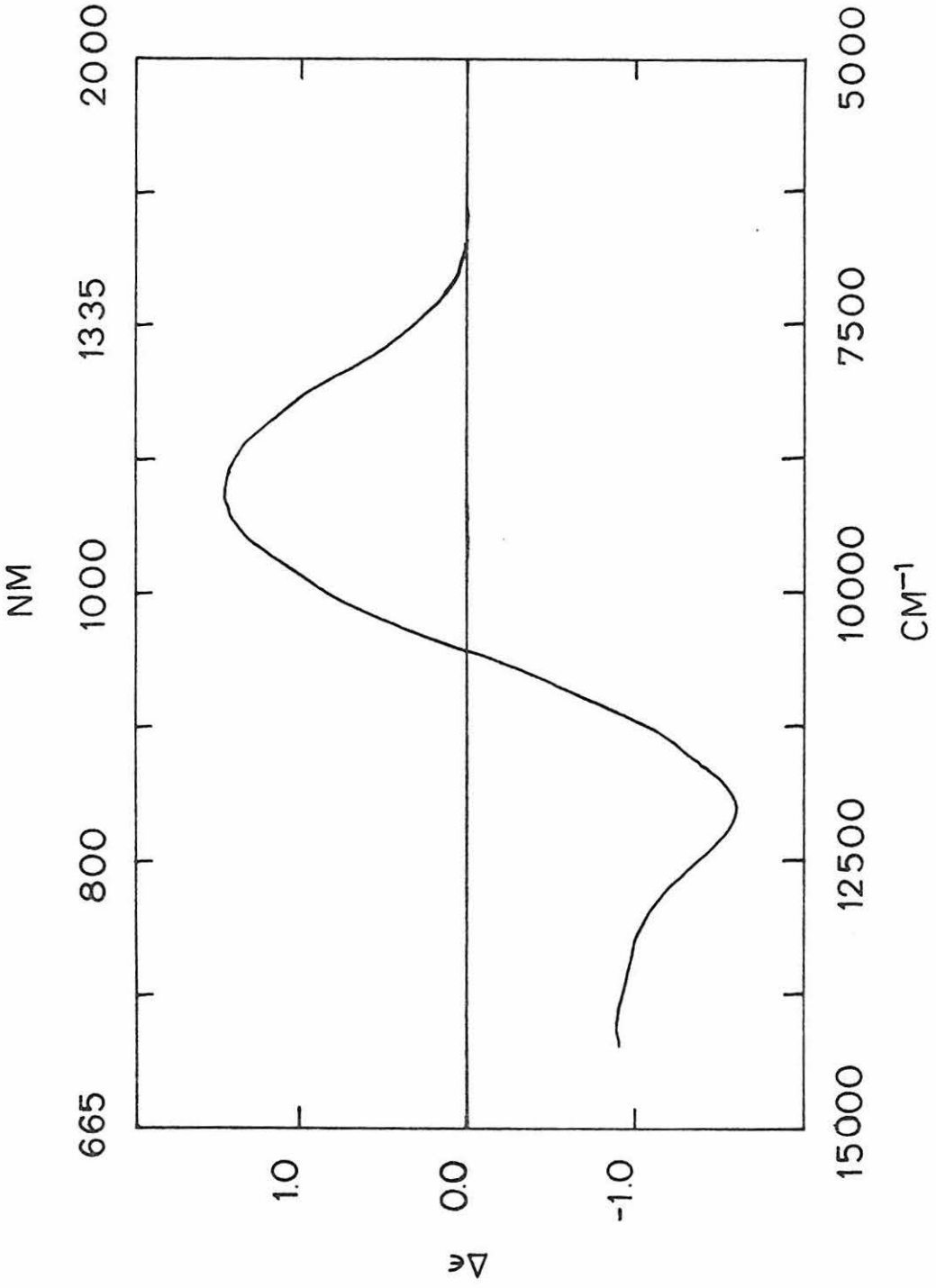

NM
280
335
415
555
835
CM⁻¹
36000
30000
24000
18000
12000
Δε
8.0
4.0
0.0
-4.0

FIGURE 6

Near infrared CD spectrum of ceruloplasmin in the presence of a 1000-fold excess of N_3^- in deuterated 0.2 M sodium acetate pD 5.5. Vertical scale is in units of $\Delta\varepsilon$.


NM
665
800
1000
1335
2000
CM⁻¹
15000
12500
10000
7500
5000
Δε
1.0
0.0
-1.0

with a shoulder near 14,000 cm^{-1}. Intensities are comparable to those observed in native ceruloplasmin.

Dialysis of native ceruloplasmin versus ascorbate results in loss of the type 1 copper,[28] thus permitting spectroscopic examination of types 2 and 3 coppers. Visible absorption and CD spectra of ascorbate-treated ceruloplasmin are shown in Figures 7 and 8, respectively. Significant absorption at 330 nm (30,000 cm^{-1}) is consistent with the presence of the type 3 site in the modified protein. A weaker absorption maximum is also observed near 600 nm (16,000 cm^{-1}). Two observations rule out the possibility that the weak absorption is due to residual type 1 copper: (1) no signals attributable to the type 1 copper are seen in the EPR of the ascorbate-treated protein; (2) no evidence of type 1 was seen in the visible (or near-infrared) CD spectrum of ascorbate-treated ceruloplasmin. Therefore, the 16,000 cm^{-1} band may be assigned to ligand field transitions of types 2 and 3 copper. Several additional bands are evident in the CD spectrum; the data are summarized in Table I. Comparing Figure 8 to Figure 2 it is again clear that no type 1 copper remains in ascorbate-treated ceruloplasmin.

EPR spectra (Figures 9 and 10) provide additional information on the nature of the type 2 copper ion in ascorbate-treated ceruloplasmin. No new signals

FIGURE 7

Room temperature absorption spectrum of ascorbate treated ceruloplasmin in 0.05 M sodium acetate, 0.3 M NaCl, pH 7.2.

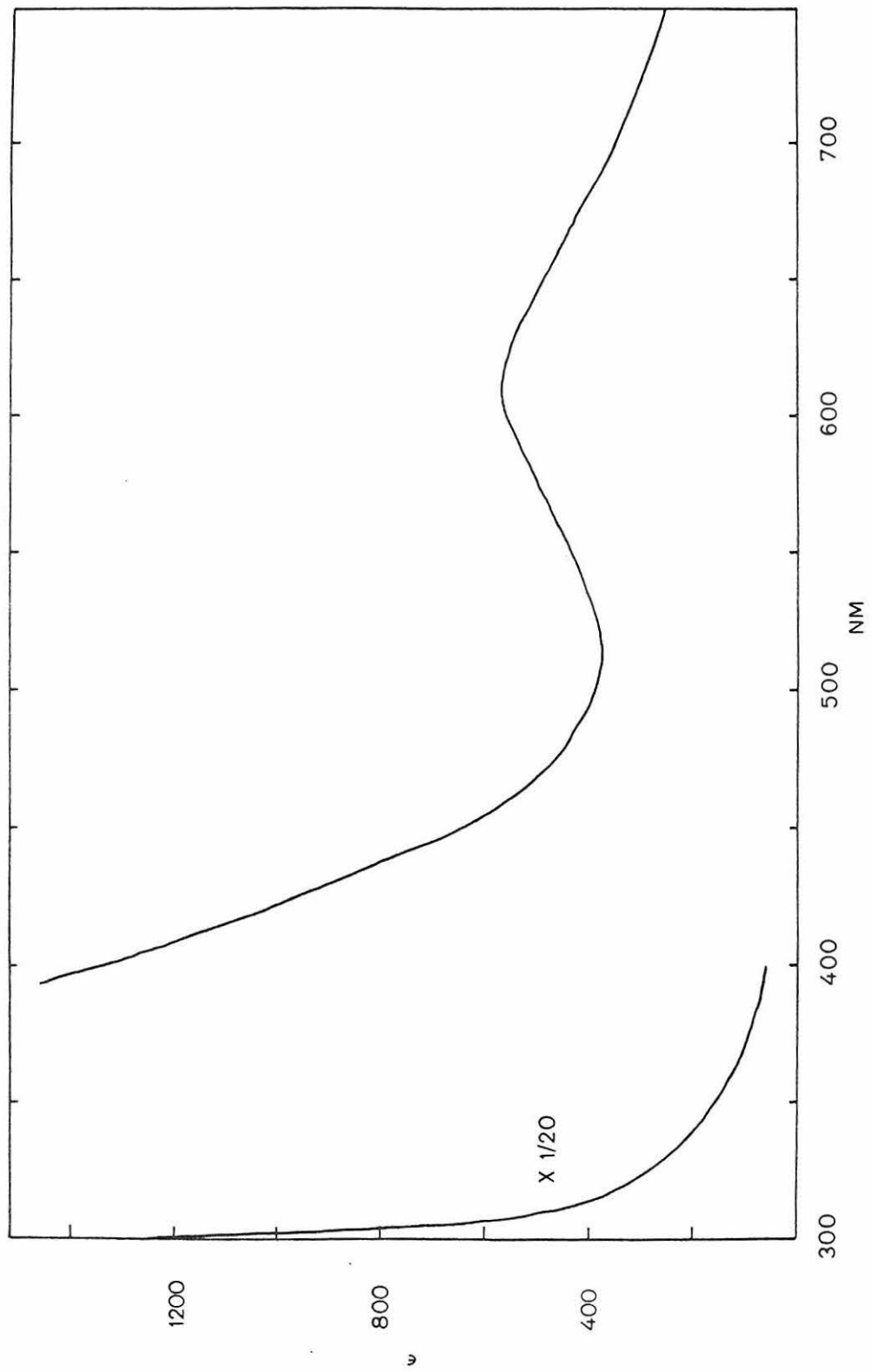

1200
800
400
ε
X 1/20
300
400
500
600
700
NM

FIGURE 8

Visible CD spectrum of ascorbate treated ceruloplasmin in 0.05 M sodium acetate, 0.3 M NaCl pH 7.2. Vertical scale is in units of $\Delta\varepsilon$.

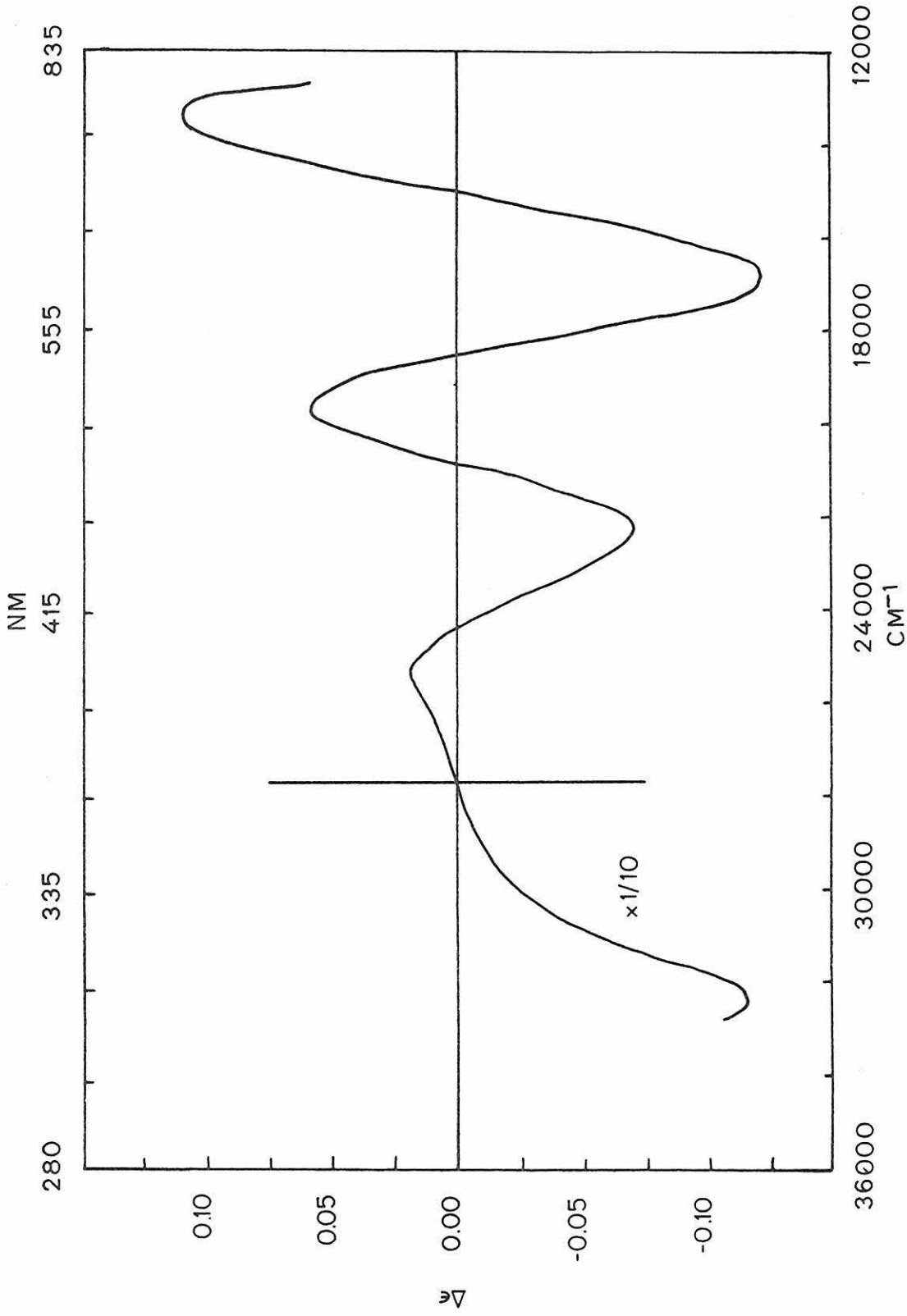

835
555
415
335
280
NM
12000
18000
24000
30000
36000
CM⁻¹
0.10
0.05
0.00
-0.05
-0.10
Δε
x1/10

TABLE I

MODIFIED BLUE OXIDASES VISIBLE CD TRANSITIONS

Ascorbate Treated Ceruloplasmin			High pH Fungal Laccase		
Energy (cm^{-1})	Wavelength (nm)	Sign	Energy (cm^{-1})	Wavelength (nm)	Sign
2500	400	+	28500	350	+
22200	450	–	25500	390	–
19200	520	+	19500	510	+
15600	640	–	15000	665	–
13150	760	+			

FIGURE 9

EPR spectra of ascorbate treated ceruloplasmin at 80 K and 9.1750 GHz. Microwave power was 20 mW and modulation amplitude was 3.2 gauss.

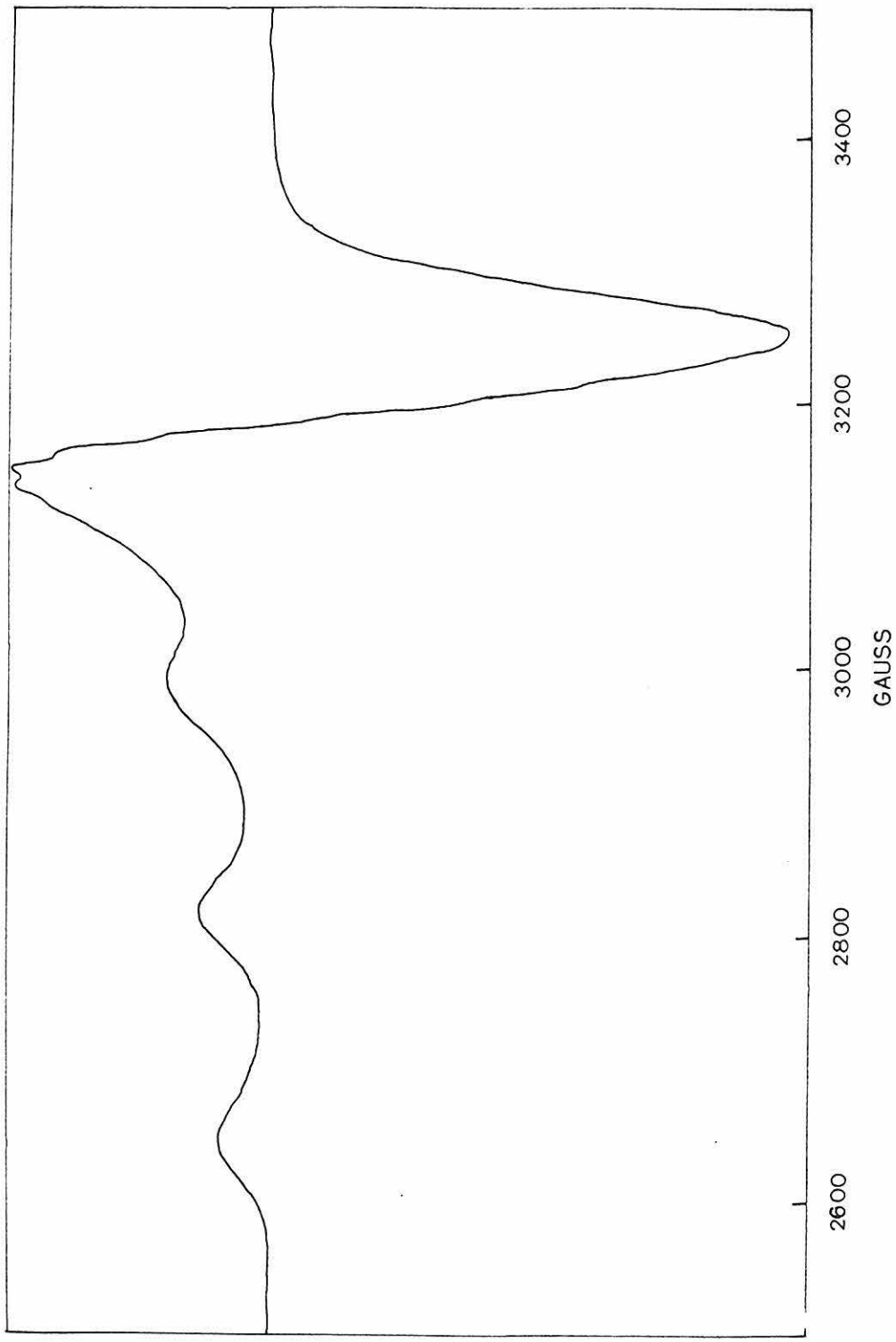
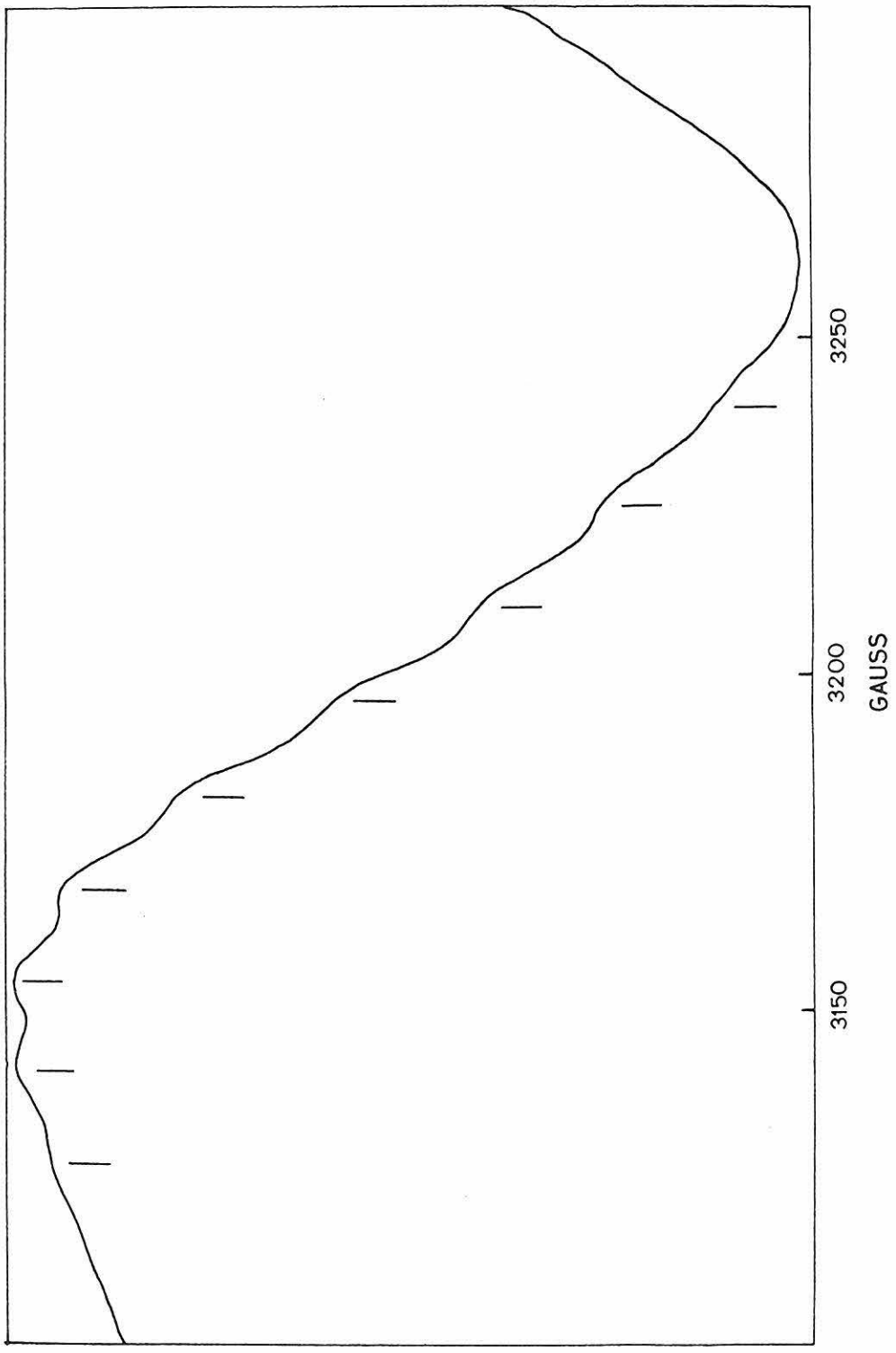

2600
2800
3000
3200
3400
GAUSS

FIGURE 10

The $g_{\perp}$ region of ascorbate-treated ceruloplasmin. Instrument settings were the same as in Figure 9.


3250
3200
3150
GAUSS

attributable to the type 3 site were observed, either near g = 2 or in the half-field g = 4 region, indicating the type 3 coppers remain strongly antiferromagnetically coupled or diamagnetic. Importantly, superhyperfine splitting is observed in the $g_{\perp}$ region (Figure 10). A weak nine line pattern is evident, indicative of four equivalent nitrogen (I = 1) nuclei coordinated to Cu(II). The EPR spectrum of ascorbate-treated ceruloplasmin is similar, but not identical, to that obtained after selective bleaching of type 1 copper in ceruloplasmin by addition of nitric oxide.[27] In contrast to native and nitric oxide-treated ceruloplasmin[27] F^- does not appear to coordinate type 2 copper in the ascorbate-treated protein.

DISCUSSION

The remarkable similarity of absorption, visible CD, and, more importantly, visible MCD spectra of ceruloplasmin to the spectra of plastocyanin, azurin, ascorbate oxidase, and particularly tree laccase indicates the type 1 site structure may be quite similar in these proteins. Ligand field transition energies also accord with this suggestion: bands were observed at 6100, 9500, and 11,500 cm^{-1} in ceruloplasmin; as compared to 6000, 9500, and 11,700 cm^{-1} in tree laccase;[23] 5800, 10,000 cm^{-1} in azurin[7] (the

highest energy band was not resolved), 5000, 8300, and 11,100 cm^{-1} in plastocyanin, and 5800, 10,000 and ~12,000 cm^{-1} for ascorbate oxidase.[24]

Our ligand field model of blue copper based on a tetragonally distorted tetrahedral coordination geometry around Cu(II)[6,7] satisfactorily accommodates the near-infrared data on ceruloplasmin. Energies of the three d-d transitions, assuming D_{2d} symmetry, are functions of the angle between the metal-ligand bond and the z axis, β, and the radial integrals Ds and Dt; assigning the 6100 and 9500 cm^{-1} bands to $^2B_2 \rightarrow {}^2E$ and $^2B_2 \rightarrow {}^2B_1$, respectively, $^2B_2 \rightarrow {}^2A_1$ is calculated at 12,285 cm^{-1} for β = 61°, Ds = 717 cm^{-1}, Dt = 464 cm^{-1}. A weak shoulder was observed near this energy in the 20 K absorption spectrum (Figure 1). In the square planar (D_{4h}) limit $^2B_{1g} \rightarrow {}^2A_{1g}$ is predicted to be 21,800 cm^{-1}. Ligand field parameters are collected in Table II and compared with the ranges observed for these parameters in other type 1 containing proteins. Using the derived values for β, Ds, and Dt together with the expressions for the energies of ligand field states of Cu(II) in D_{2d} symmetry,[21] the ligand field stabilization energy (LFSE) contribution to the reduction potential may be calculated. Ceruloplasmin blue copper is destabilized 260 mV relative to $Cu(H_2O)_6^{2+}$ ($E° = +153$ mV, which predicts a potential $E° = +413$ mV. This value is somewhat lower than those derived

TABLE II

CERULOPLASMIN LIGAND FIELD PARAMETERS†

	β	Ds(cm^{-1})	Dt(cm^{-1})	D_4h limit (cm^{-1})
Native Ceruloplasmin	61°	717	464	21,800
Ceruloplasmin + N_3^-	65°	635	525	20,490
Range for all other blue copper proteins	60-61°	700-820	420-560	21,400-24,800

†Parameters are defined in ref. 7.

experimentally,[10] but considering the approximations involved[7] they are not in unreasonable agreement. Bands in the visible absorption spectrum of ceruloplasmin may be assigned to LMCT transitions analogously to the other blue copper proteins.[6,7,23]

Data for ceruloplasmin in excess azide also accord with a D_{2d} flattened tetrahedral structure for the type 1 site (Table II). Significantly, the site seems distorted slightly more towards a square planar geometry than native ceruloplasmin. LFSE calculations predict a potential of only 161 mV for the azide inhibited enzyme. Apparently binding of azide (and by analogy other anions as well) at the type 2 site induces a protein conformational change resulting in an altered structure for both type 1 coppers. Similar effects were observed in the binding of F^- by tree and fungal laccase.[23,35] F^- has previously been shown to affect the UV-visible absorption spectrym of ceruloplasmin[35] and N_3^-, SCN^-, and OCN^- have drastic effects on the visible absorption and CD spectra.[31-34]

A wide variety of spectroscopic and kinetic measurements have been interpreted in terms of the two type 1 copper ions in ceruloplasmin being inequivalent. Most importantly, the oxidation-reduction potentials have been determined to be 490 and 580 mV,[12] and the EPR parameters, particularly $g_{||}$ and $A_{||}$, are apparently different.[18] Further, resonance raman[36] and CD data[32] of ceruloplasmin in the presence of N_3^-, SCN^-, and OCN^-

have been used as evidence of inequivalent type 1 sites. It has been proposed[36] that binding of these anions at the type 2 site induces a conformational change leading to breaking of the Cu-S bond in one type 1 site, the other site being unaffected. Photoreduction of the blue coppers by laser irradiation has been demonstrated and the calculated quantum yields and rate constants for this process were different for the two blue sites.[37]

Ceruloplasmin type 1 coppers may also differ in their reactivity. Measurements of the proton relaxation time (T_1) versus the extent of copper reduction, measured at 610 nm, were sharply biphasic.[38] Reoxidation of the reduced enzyme by dioxygen was also biphasic when monitored at 610 nm, 330 nm, and 420 nm (where an intermediate in this reaction absorbed).[39] In the presence of N_3^- the differences in rate constants for the two phases observed at 610 and 420 nm were even more pronounced.[39]

However, no evidence of inequivalency is seen in our low temperature absorption spectrum, visible CD and MCD spectra or even in the near-infrared CD spectra of the native or azide inhibited enzyme. These latter observations are particularly significant because d-d transitions should be quite sensitive to variations in ligands and/or geometry. Calculations have shown the energies of ligand field bands to be very sensitive to

exact geometry near the tetrahedral limit,[7] therefore the tetragonal distortion in the blue sites of native and N_3^- inhibited ceruloplasmin must be nearly identical. Additionally the ligand field strengths of the two type 1 sites are identical within the resolution of our experiment, which implies the copper ligands must be very similar.

Our findings render previous interpretations of certain spectroscopic experiments in terms of inequivalent type 1 sites rather unlikely. The highest energy ligand field transition of blue Cu(II) in native ceruloplasmin was observed near 11,500 cm^{-1}. Consequently, the 450 nm (~22,000 cm^{-1}) band in the CD cannot be a d-d transition as suggested by Herve, et al.[32] This transition is reasonably assigned as LMCT, in analogy with other blue copper proteins,[6,7,23,24] probably from an imidazole moiety. Further, this band is remarkably conserved among all type 1 sites, which suggests that both of these sites in ceruloplasmin contribute to the intensity. As the quantum yields and rate constants for photoreduction of the blue sites were calculated assuming absorption of only one of them at 450 nm,[37] these results should be re-examined. It is also unlikely that N_3^- binding results in breaking one Cu-S bond and leaving the other blue site unaltered.[36] Intensities of the near-infrared CD

bands are only slightly decreased in the N_3^- inhibited enzyme as compared to the native enzyme and it is quite apparent from Figure 5 that the 6100 cm^{-1} transition is completely absent. The most reasonable interpretation of these results is that binding of N_3^- induces a conformational change which causes a distortion in both type 1 sites towards a more square planar geometry.

Due to the severely overlapped nature of the type 1 and type 2 EPR signals, the g values determined from computer simulation assuming two different type 1 coppers are somewhat equivocal.[18] However, the magnitudes are adequately accounted for by a tetragonally distorted tetrahedral geometry and only minor differences in the orbital reduction factors are necessary to quantitatively fit the experimental values.[7,23]

Given the similarity of the spectroscopic properties and thus the electronic structure of the two type 1 coppers, the polypeptide chain must play an important role in mediating the reduction potential and kinetic reactivity of the two sites. Ligand field stabilization energy considerations predict equal potentials. Franck-Condon barriers to electron transfer should also be nearly identical. Consequently reactivity differences between the type 1 coppers may reflect variations in the degree to which the redox centers are "buried" within

the protein and their accessibility to external reagents. Another possibility is that intramolecular electron transfer pathways among the type 1 and other copper sites differ significantly. The proton relaxation data and aerobic reoxidation kinetics are consistent with a requirement for a protein conformational change before all the blue copper can participate in electron transfer processes.[38,39]

Characterization of copper types 2 and 3 has been difficult owing to the spectroscopic dominance of type 1. Unlike the laccases, the near-UV absorption band at 330 nm associated with type 3 copper has been of little assistance in elucidating properties of this site as its redox properties are complex.[12,19] It has also been suggested that additional chromophores, other than type 3 copper, absorb at 330 nm in ceruloplasmin.[12] Only the lowest field hyperfine line of $g_{||}$ is a probe of type 2 copper in native ceruloplasmin,[3,4] making unambiguous determination of its properties difficult. Recently we demonstrated that the type 2 copper could be readily examined via EPR of nitric oxide treated oxidized ceruloplasmin and its F^- binding properties and ligand superhyperfine were analyzed.[27] Ascorbate treated ceruloplasmin also affords an opportunity to study the types 2 and 3 copper in the absence of type 1.

EPR spectra of ascorbate-treated ceruloplasmin (e.g. Figure 9) are approximately axial with $g_{||} > g_{\perp} > 2.0023$ and $|A_{||}| \cong 0.018$ cm^{-1}. These values are consistent with a tetragonal site for Cu(II); the ground state would be $^2B_1$, corresponding to the unpaired electron in a Cu(II) $d_{x^2 - y^2}$ orbital. Visible absorption and CD data (Figures 7 and 8) are consistent with such a structure. Monomeric complexes of Cu(II) with amino acids and peptides display values of $\Delta\varepsilon$[40,41] very similar to those of ascorbate-treated ceruloplasmin in the visible region and are associated with d-d transitions of the Cu(II) center. The multiplicity of bands in Figure 8 suggests that the transitions arise from more than one copper site, as optically active Cu(II) complexes, even with poly(α-amino acids), usually have fewer bands in the visible CD spectrum than observed here.[42] It is interesting that a similar CD spectrum can be obtained for fungal laccase at pH's greater than 8.7.[23] The loss of blue color in this oxidase is substantially reversible.[43] For comparative purposes, these data are also shown in Table I. Near tetrahedral geometry is ruled out by the lack of low energy ligand field bands. Observed energies are consistent with tetragonal six, five, or four coordinate square planar geometries.[44] Monomeric or bridged binuclear Cu(II) complexes most often adopt one of these coordination arrangements.[44,45] More detailed assignments and

correlation with data on monomeric or binuclear Cu(II) complexes are not in order as we cannot distinguish optical or CD transitions of the types 2 and 3 copper with our present data.

Superhyperfine structure from ligand donor atoms is present in the $g_{\perp}$ region of the EPR spectrum (see Figure 10) of ascorbate-treated ceruloplasmin. Close examination reveals a nine line pattern, indicative of four equivalent ligands. The splitting of approximately 14 gauss is in the range usually found for nitrogen donors. A reasonable structure consistent with the data would place the four nitrogen ligands in a pseudo square-planar arrangement about the Cu(II) ion, perhaps with weak axial ligands as well. This pattern of superhyperfine from approximately coplanar donors appearing in the $g_{x,y}$ region of the spectrum has been observed in simple Cu(II) complexes.[46,47] It is also possible that the superhyperfine in the parallel region was too weak to be resolved; in this context it should be noted that nitrogen superhyperfine from imidazole was much more intense in the $g_{\perp}$ region compared to the $g_{||}$ region in galactose oxidase.[48] Such a pattern is expected simply because the superhyperfine intensity is spread over the four copper hyperfine peaks in $g_{||}$ but superimposed upon each other in $g_{\perp}$ where the copper hyperfine is much narrower.

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CHAPTER 4

COORDINATION ENVIRONMENT AND FLUORIDE BINDING OF TYPE 2 COPPER IN THE BLUE COPPER OXIDASE CERULOPLASMIN

Introduction

The blue copper oxidases which include ceruloplasmin, ascorbate oxidase and laccase, are remarkable enzymes owing to their ability to catalyze the four electron reduction of dioxygen to two molecules of water.[1-5] No partially reduced intermediates have been detected during the reaction. These enzymes have a minimum of four copper atoms per protein molecule in three spectroscopically distinct sites.[6] The type 1 or blue copper centers are characterized by an intense (ε ~5000 M^{-1} cm^{-1}) absorption band at ca. 600 nm and by an abnormally narrow copper EPR hyperfine structure. This kind of copper is similar to that found in a number of single blue copper electron transport proteins including azurin, plastocyanin and stellacyanin.[5] The type 2 or non-blue coppers of the blue copper oxidases do not have any well characterized absorption bands, undoubtedly because of overlap with the intense absorption of the type 1 center. The EPR hyperfine structure of the type 2 copper is similar to that found in tetragonal copper complexes. Several non-blue copper oxidases including monoamine, diamine, benzylamine and galactose oxidase display EPR properties similar to the type 2 copper. Finally, the type 3 or EPR non-detectable copper centers of the blue copper oxidases are thought to consist of two copper ions in close proximity.

This two electron accepting unit is associated with an intense near-UV absorption band at ca. 330 nm. No EPR signals have been observed from the type 3 center either because of spin pairing resulting in a diamagnetic ground state or because both centers are permanently cuprous. Dioxygen binding is thought to take place at the type 3 copper center. Some of the properties of hemocyanin, a copper oxygen binding protein, and of tyrosinase, a copper mono-oxygenase, are similar to those of type 3 copper in the blue copper oxidases.

Considerable progress has been made in determining the coordination geometry and ligands of the type 1 center. A wide variety of spectroscopic studies on native and cobalt substituted blue copper proteins have pointed toward a near-tetrahedral geometry with two histidines, a cysteine anion and a deprotonated peptide nitrogen as the four ligands.[7-16] Preliminary x-ray crystallographic results on plastocyanin[17] and azurin[18] support all of these predictions with a methionine sulfur ligand in place of the peptide nitrogen X-ray-absorption-extended-fine-structure spectral examination of azurin[19] and the recent preparation of a model "blue" copper complex[20] provide additional evidence in support of these assignments.

Determination of the geometry and ligands in the type 2 and type 3 copper centers has been much more difficult.

While there do appear to be proteins having only the type 2 or type 3 center present (vide supra), there is considerable variability in spectroscopic parameters from protein to protein. Since the type 2 center lacks the unusual properties of the type 1 copper, a much wider range of geometries and ligand donor sets are possible that will be consistent with the data. Owing to the considerable overlap of EPR, CD and optical absorption spectra arising from the types 1 and 2 coppers, removal or reduction of the type 1 center is required in order to study the properties of the type 2 site when both appear in conjunction. To that end, we have studied the EPR properties of modified forms of the blue copper oxidase, ceruloplasmin. Analysis of these data provides information about both the geometry and protein ligands in the type 2 site. Additional EPR experiments on the anion binding properties of ceruloplasmin are also reported. The application of EPR to the study of metalloenzymes has been reviewed by Vänngård[21] and by Beinert and Palmer.[22]

The blue copper oxidase ceruloplasmin (CP) is the major copper containing mammalian blood plasma protein.[23,24] Like the other blue copper oxidases, it is capable of reducing dioxygen to two molecules of water with concomitant substrate oxidation. Although capable of oxidizing many organic substrates, its physiological substrate is thought to be ferrous ion.[24] CP may also

function as a copper transport protein.[24] Indeed, in addition to the intrinsic copper atoms which are involved in the catalytic activity of the protein, CP will bind up to ten cupric ions in a looser, reversible fashion.[25] CP has six or seven tightly bound copper ions[26]: two type 1 coppers, one type 2 copper and three or four type 3 (two copper pairs or a copper pair and a third (type 4) EPR-silent copper).

This paper presents results obtained from a direct study of CP type 2 copper (via EPR) through use of a derivative lacking type 1 EPR signals. Type 1 "reduced" CP is prepared by preferential, reversible bleaching of the type 1 copper through treatment with gaseous NO[27] (NO-CP). Fluoride binding to the type 2 copper of native CP and NO-CP has also been investigated.

Materials and Methods

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

Ceruloplasmin was purified to electrophoretic homogeneity and a spectral purity ratio (A_{610}/A_{280}) of .045 by published methods,[26] with minor modifications to be published elsewhere,[13] from Cohn Fraction IV kindly provided by Dr. Lewis Larsen, Massachusetts Department of Public Health.

Enzymatic activity was verified. Treatment of CP with NO was carried out on a vacuum line. CP in a quartz EPR tube was exposed to NO passed through a Dry Ice-acetone bath to remove impurities. Several gentle pump-purge cycles were carried out first with argon to deoxygenate the protein followed by several more cycles with NO. In separate experiments the absorption spectral changes reported by Weaver, et al.[27] on exposure of CP to NO were verified.

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. (N.Y., N.Y.) frequency meter. An Air Products Heli-Trans system was used to obtain low temperatures.

All pH measurements were done at room temperature with a Brinkman pH 101 meter equipped with a Metrohm combination electrode. Experiments done with native CP, CP+F^-, and NO-CP+F^- were done in 0.2 M sodium acetate buffer at pH 5.5. Results reported on NO-CP were performed at pH 7.0, 0.1 M sodium acetate with 0.25 M NaCl. Concentrated protein samples were obtained by ultrafiltration (Amicon) through a PM-30 membrane or by use of a Millipore Immersible Molecular 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 CP at pH 5.5 is shown in Figure 1 and is essentially identical to those previously reported.[21,28,29] Resolution of the type 1 and type 2 copper hyperfine splitting patterns is indicated. Addition of approximately 15 equivalents of fluoride leads to a splitting of the lowest field transition as seen in Figure 2. This spectrum is better resolved than that reported by Brändén, et al.[30] and was obtained by the sequential addition of small amounts of fluoride over several days. After each addition, and a 24 hour equilibration period,[30] an EPR spectrum was run. In order to resolve the entire spectrum, nitric oxide was added to the fluoride treated protein (Figure 3). Single addition of approximately 1000 equivalents of fluoride to native CP led to further splitting of the lowest field transition as displayed in Figure 4. As before, addition of nitric oxide led to loss of most of the type 1 EPR signal and resulted in the spectrum presented in Figure 5.

The EPR spectrum of native CP at pH 7.0 is quite similar to that found in Figure 1. Treatment of native CP at this pH with nitric oxide led to the much simpler spectrum presented in Figure 6A. An expansion of the $g_{\perp}$ region of this spectrum is displayed in Figure 6B.

FIGURE 1

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

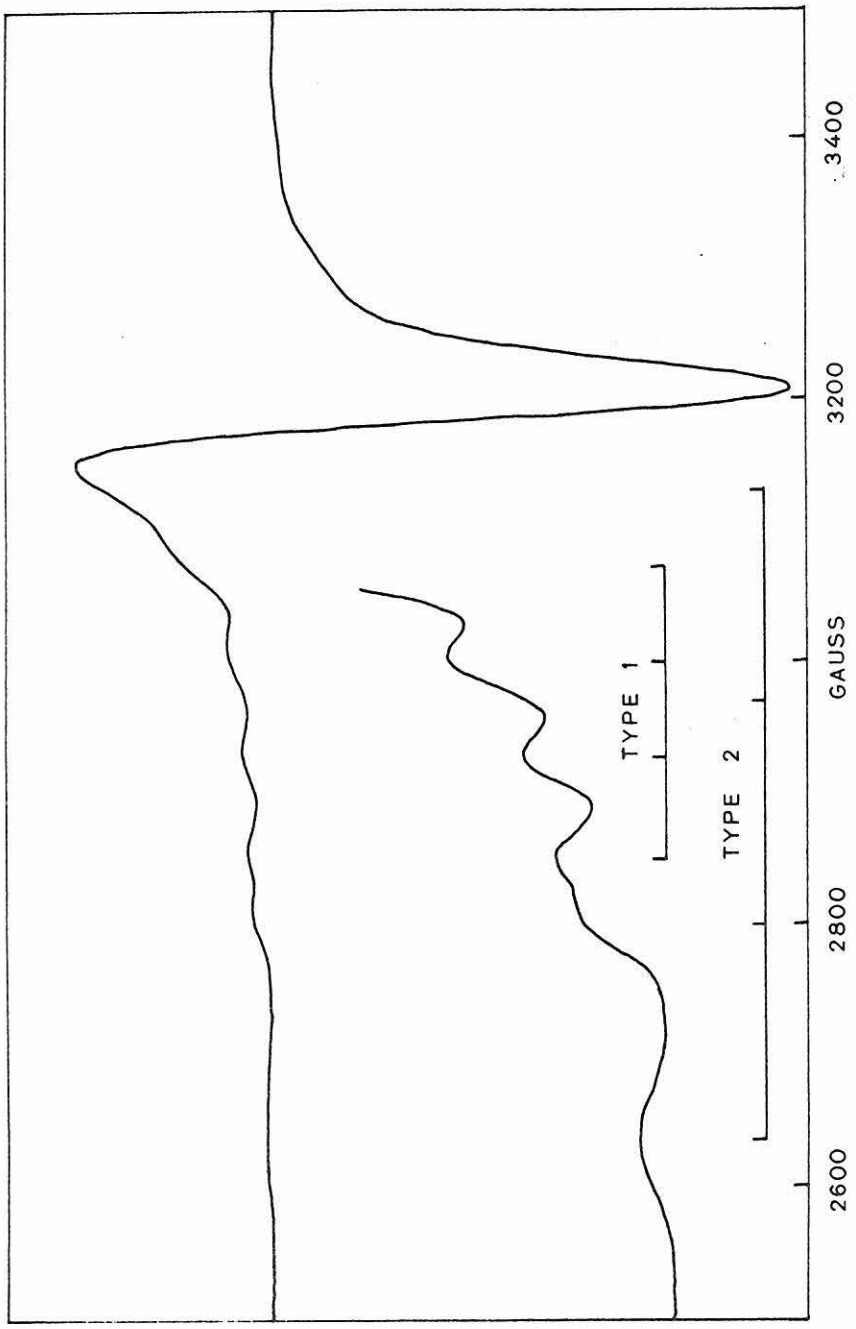

3400
3200
2800
2600
GAUSS
TYPE 1
TYPE 2

FIGURE 2

EPR spectrum of ceruloplasmin plus fifteen equivalents of F^- at 65 K and 9.1750 GHz. Power was 5 mW and the modeulation amplitude 10 gauss.

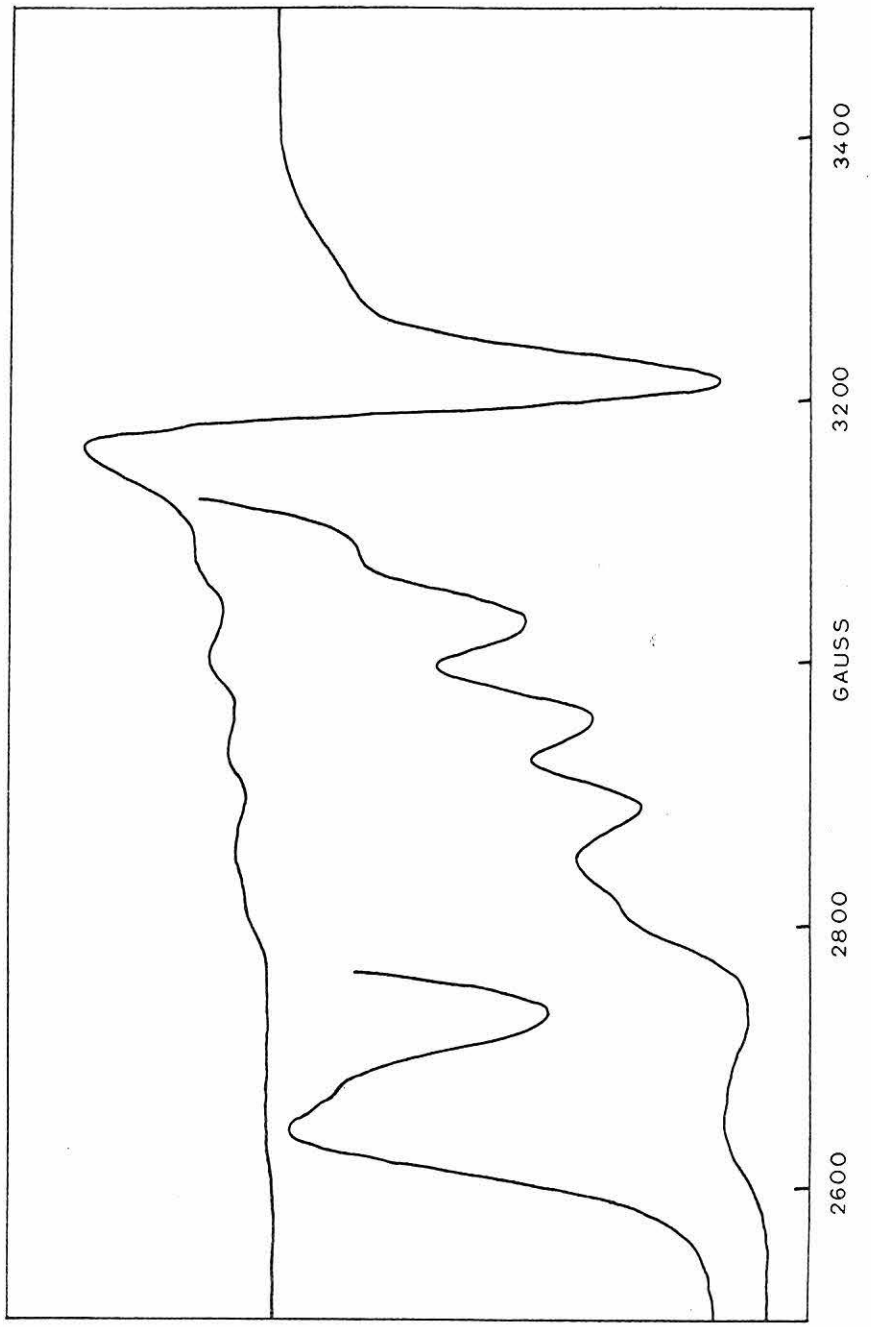

2600
2800
GAUSS
3200
3400

FIGURE 3

Sample of Figure 2 plus NO at 85 K and 9.1765 GHz. Power was 20 mW and the modulation amplitude 10 gauss.

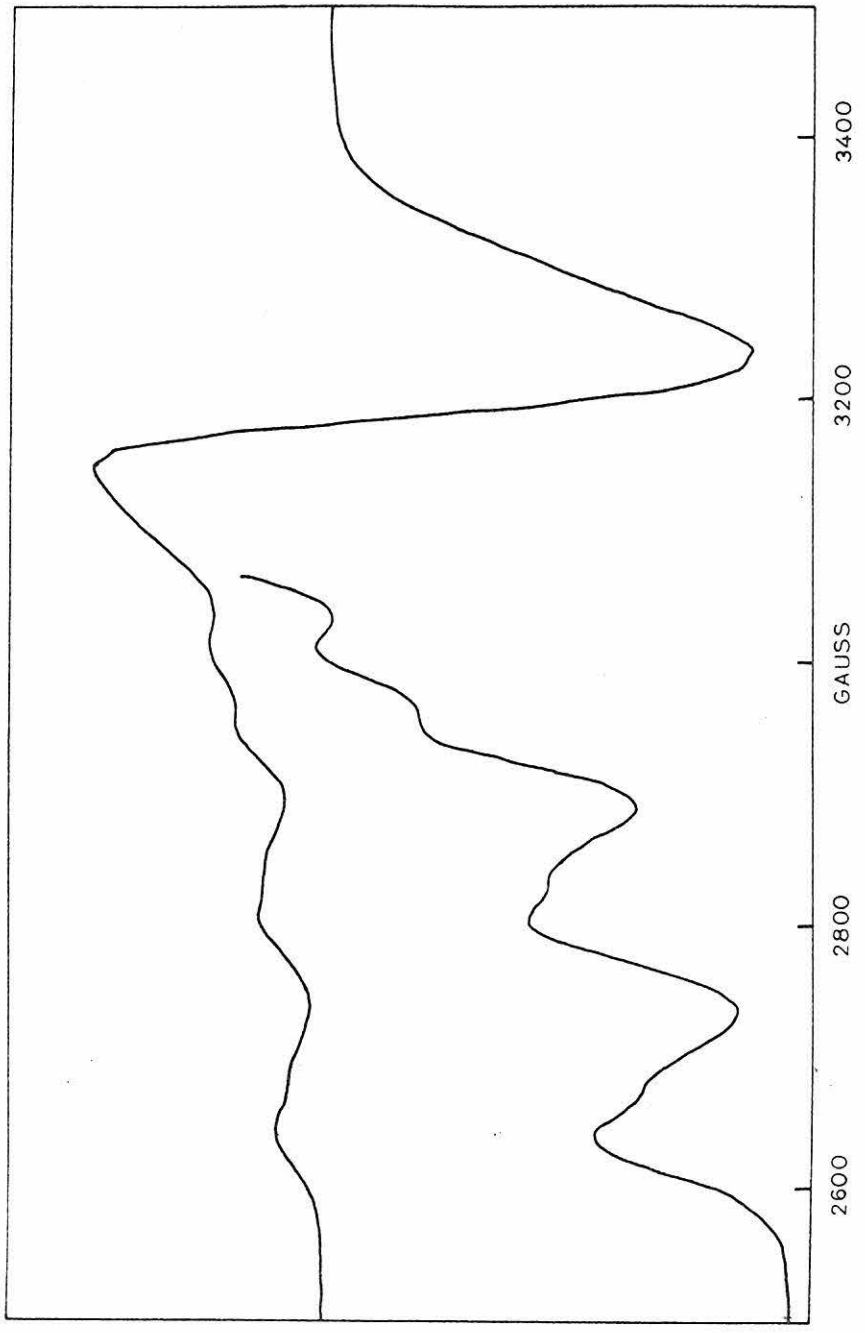

2600
2800
GAUSS
3200
3400

FIGURE 4

EPR spectrum of ceruloplasmin in the presence of a thousand fold excess of F^-. Conditions were the same as Figure 1 except modulation amplitude was 5 gauss.

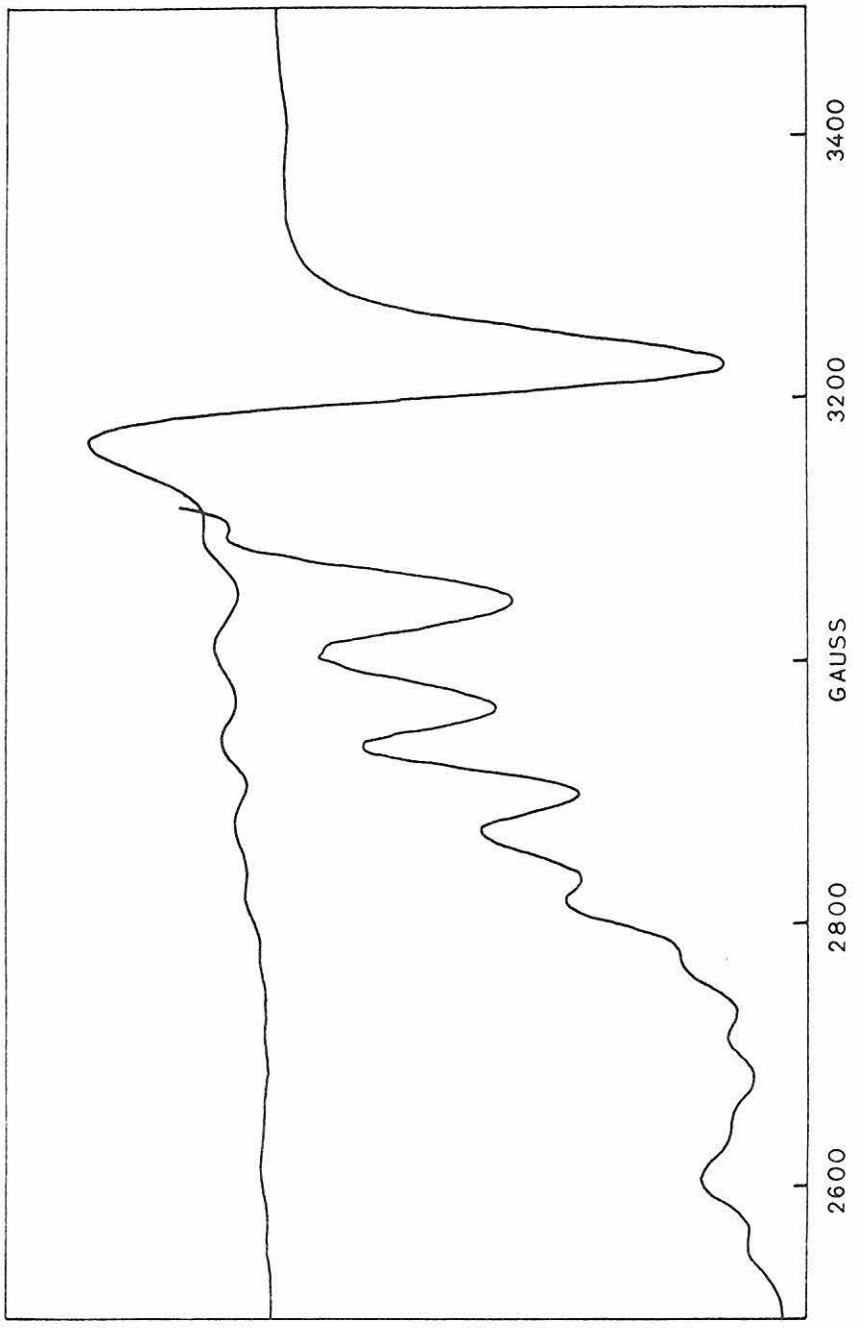

2600
2800
GAUSS
3200
3400

FIGURE 5

Sample of Figure 4 plus NO at 80 K and 9.1760 GHz. Power was 20 mW, modulation amplitude was 10 gauss.

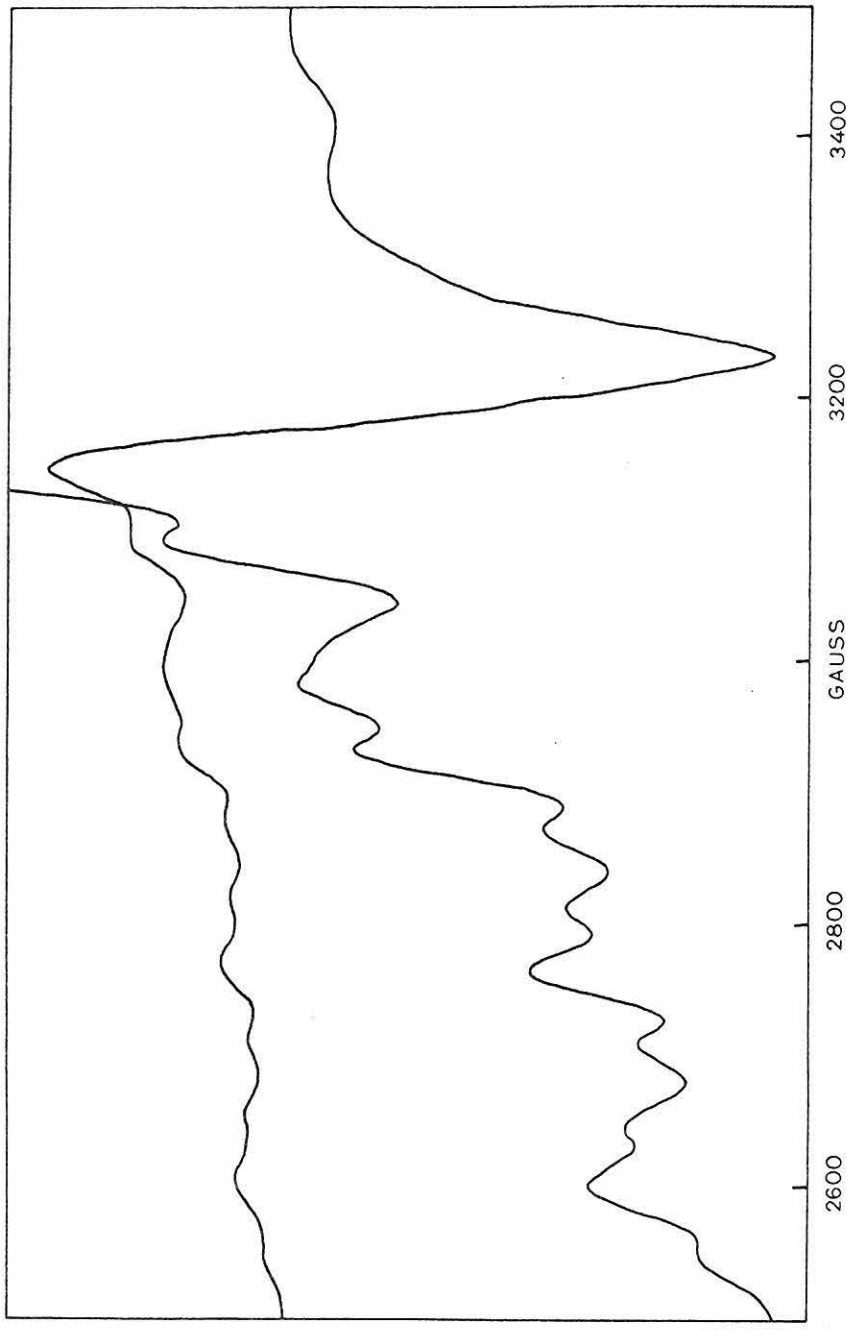
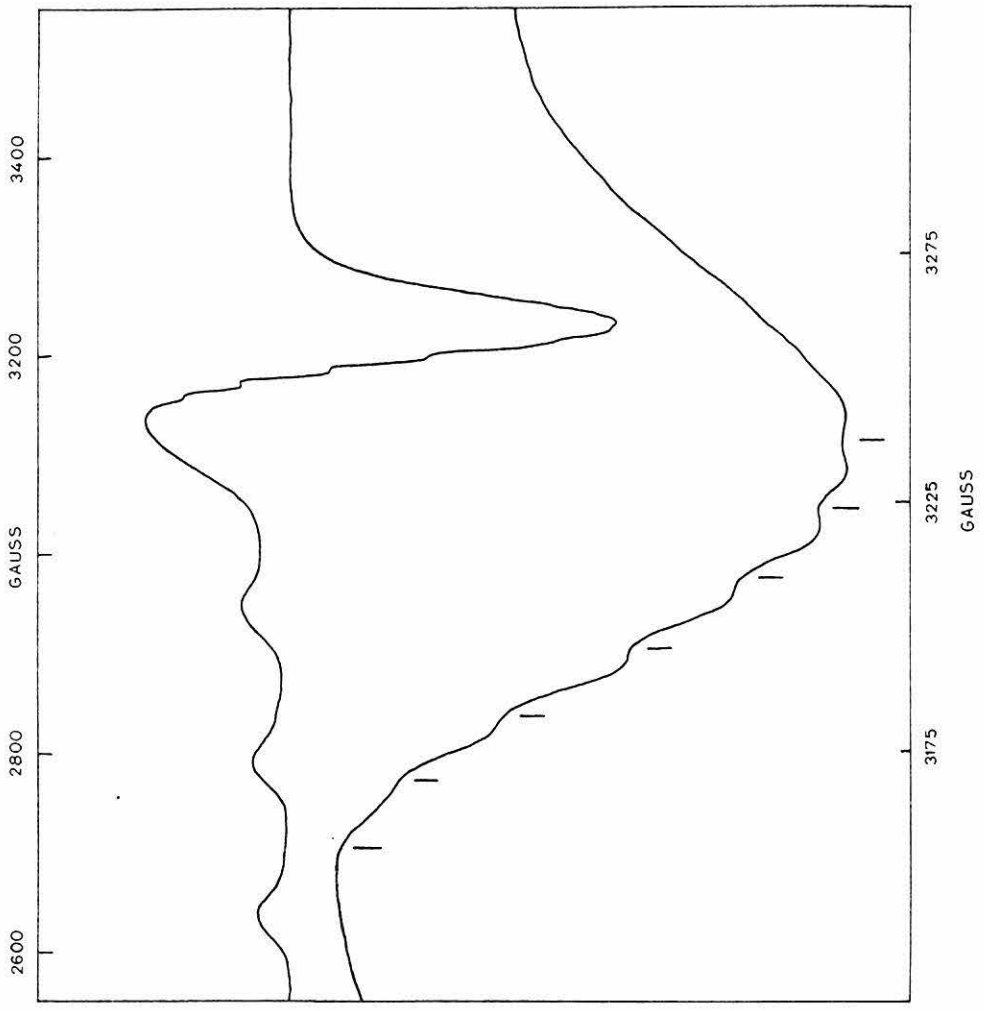

2600
2800
GAUSS
3200
3400

FIGURE 6

EPR spectra of NO-treated ceruloplasmin. Conditions for A (top) were: temperature 80 K, power 20 mW, modulation amplitude 5 gauss; B (bottom): temperature ~5 K, power 5 mW, modulation amplitude 1.6 gauss. Frequencies were 9.1760 GHz and 9.1905 GHz for A and B respectively.


3400
3200
2800
2600
GAUSS
3275
3225
3175
GAUSS

Discussion

Fluoride Binding

The anion binding properties of ceruloplasmin have been extensively studied. Addition of azide, cyanate and thiocyanate to CP leads to considerable changes in the optical,[31] circular dichroism[32-34] and resonance raman[35] spectra. Identification of the specific copper site(s) responsible for anion binding on the basis of these spectral changes has proved difficult. Vänngård and co-workers[28,30] examined the changes in the EPR spectrum of CP upon addition of azide and fluoride and concluded that both ions interacted with the type 2 copper of CP. In the presence of azide,[28] new EPR transitions were seen, whereas it appeared that the lowest field transition was simply split by interaction with fluoride ($I = \frac{1}{2}$).[30] 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 be sure whether the spectral changes seen were due to the appearance of new transitions, as was the case for azide binding,[28] or whether they were due to a splitting caused by the coordination of fluoride to the otherwise intact type 2 center. Malkin, et al.[36] have provided evidence for two fluoride binding sites on the type 2 copper in laccase.

In order to clarify the exact nature of the fluoride-type 2 copper interaction in ceruloplasmin, we have re-

examined the EPR spectral changes seen on fluoride addition to both native and NO-CP. The data in Figures 1 - 5 clearly show that, like laccase,[36] CP can bind up to two fluoride ions. Initial exposure of CP to ca. 15 equivalents of fluoride (Figure 2) results in the splitting of the lowest field transition into a doublet of nearly equal intensities. Following addition of nitric oxide (Figure 3), the splitting of each type 2 copper hyperfine line (see Figure 1) into a doublet is clearly resolved. With 1000-fold excess fluoride present (Figure 4), splitting of the lowest field type 2 transition into a triplet with an intensity ratio of approximately 1:2:1 is found. 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.[30] pointed to only one binding site for fluoride on type 2 copper, in contrast with laccase.[36] As can be seen from the data presented here, however, 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 I) as well as the fact that the selective type 1 bleaching process is reversible after removal of nitric oxide likely indicate that the properties of the type 2 copper are only slightly perturbed in this derivative.

TABLE I

APPROXIMATE EPR PARAMETERS[†]

		TYPE 1			TYPE 2			
	pH	$g_{\perp}$	$g_{\|\|}$	$A_{\|\|}$(G)	$g_{\|\|}$	$A_{\|\|}$(G)	A_N(G)	A_F(G)
native CP	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-CO+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.

$A_{||}$ values are accurate to ±5G.

In apparent contrast to the situation with native CP (Figure 6), the bleaching of the type 1 copper of fluoride treated CP is not complete (Figure 3 and 5). Superhyperfine splitting such as seen in the $g_{\perp}$ (ca. 3200 gauss) region due to ligands of the type 2 copper (vide infra, see Figure 6B) is obscured by the remaining type 1 signal. While very weak superhyperfine in the $g_{\perp}$ region of Figures 3 and 5 was seen (data not shown), the exact splitting pattern could not be determined.

Coordination Environment of Type 2 Copper

EPR spectra of NO-CP are axial and noticeably simpler than spectra of native CP due to the lack of type 1 EPR signals. The ordering and magnitudes of the observed parameters, i.e. $g_{||} > g_{\perp} > 2.0023$ and $|A_{||}| \simeq 0.017\ cm^{-1}$ (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 (Figure 6B). No such structure was observed in the $g_{||}$ region, probably because it is spread over four $A_{||}$ lines and thus too weak to be resolved. The seven line splitting pattern is most logically attributed to coordination by three nitrogenous (I=1) ligands which 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 modified ceruloplasmin (which contains no type 1 copper).[13,37] Optical data on this form of the enzyme also support the formulation of type 2 copper as a tetragonal coordination complex.[13]

Mondovi et al.[38] 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 is correct, then the nitrogen donors responsible for the super-hyperfine seen in Figure 6B 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.[39] 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.

Ceruloplasmin type 2 copper must possess labile ligands, in addition to the nitrogen donors, as subsequent to fluoride addition superhyperfine is resolved on each $A_{||}$ line. This result is most readily explained by coordination of a maximum of two magnetically equivalent fluoride ions. Taken together the data are most consistent

with an approximately coplanar arrangement of three (possibly four) nitrogen donors with the labile ligands coordinated perpendicular to the Cu-N plane. Complexes of Cu(II) with simple peptides and amino acids most often adopt this type of structure.[40] Water has been identified as the exchangeable ligand of type 2 copper in fungal laccase[41]; it is an attractive possibility here as well.

We are confident the results obtained on NO-CP are also applicable to the native protein as bleaching of the type 1 site is completely reversible and the EPR parameters for native CP and NO-CP are identical within our experimental error.

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APPENDIX A

Calibration of First SHE Susceptometer

Water from a Millipore purification system was throughly degassed, equilibrated with helium, and used as the primary calibrant for measurements of the volume susceptibility. Data were obtained on three separate samples over the range 5-260 K. A representative experiment is shown in Figure 1. The susceptibility of the high purity quartz holder (obtained from Wilmad) used was measured separately (see Figure 2).

Coupling of magnetic flux to the SQUID is dependent upon the sample geometry owing to the single turn pickup coils used. In particular the cross-sectional area and length of the sample are important. Both of these effects may be corrected for by choosing a standard set of dimensions and then converting the measured signal from any sample to a magnitude based on the standard. Specifically the correction for variation in cross-sectional area is just the ratio of the standard diameter to the sample diameter, quantity squared. Figure 3 is necessary to account for length variations; a value proportional to the mutual inductance between the sample and pickup coil is read off the vertical scale for a given sample length; the correction factor is simply the ratio of the value for the standard to that of the sample.

FIGURE 1

Magnetic susceptibility of pure degassed, distilled, deionized water in a quartz holder. Vertical scale is direct SQUID output in volts.

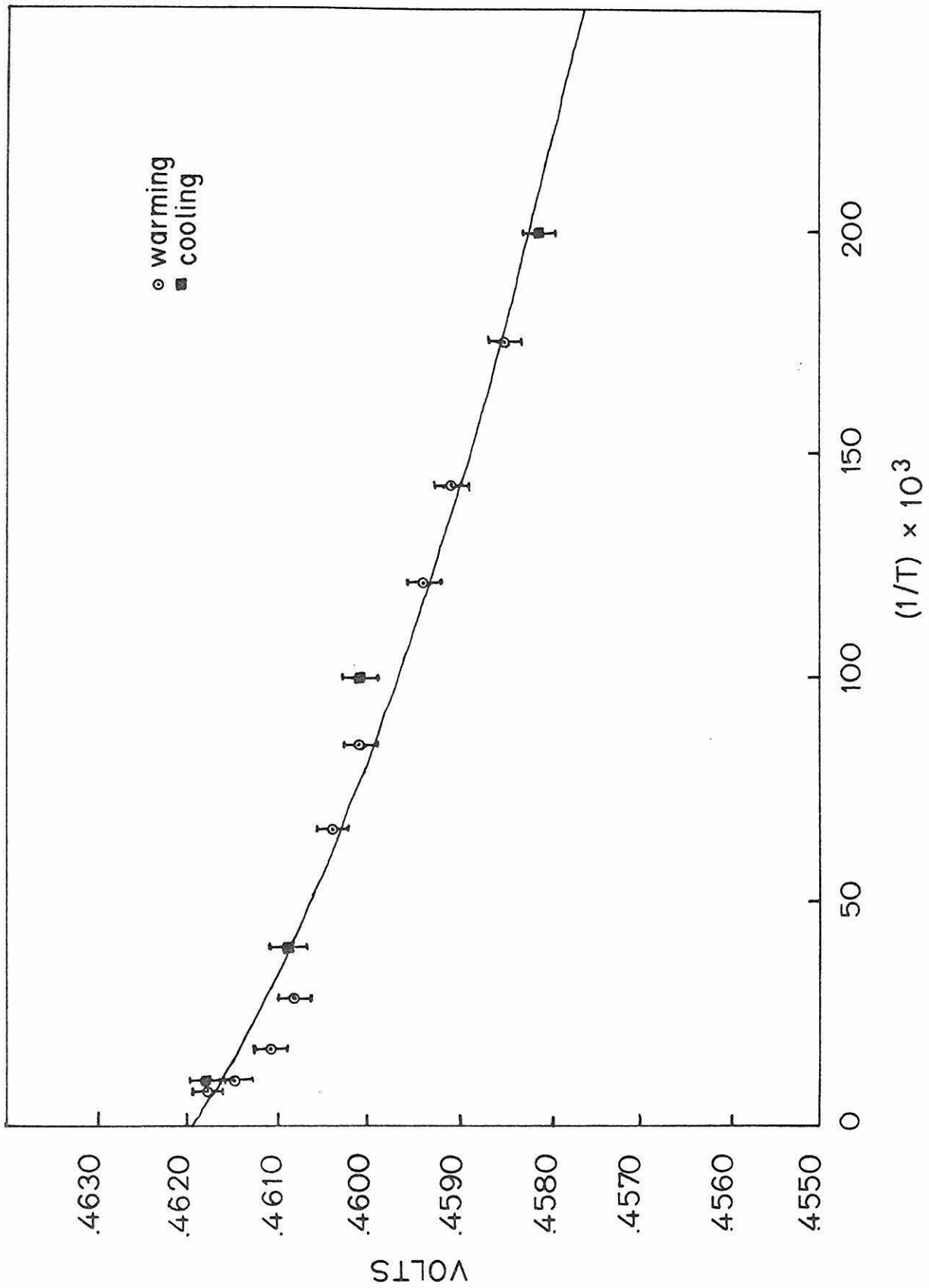

warming
cooling
VOLTS
.4630
.4620
.4610
.4600
.4590
.4580
.4570
.4560
.4550
0
50
100
150
200
(1/T) × 10³

FIGURE 2

Magnetic susceptibility of the quartz holder used in calibrations with pure water. Vertical scale is direct SQUID output in volts.

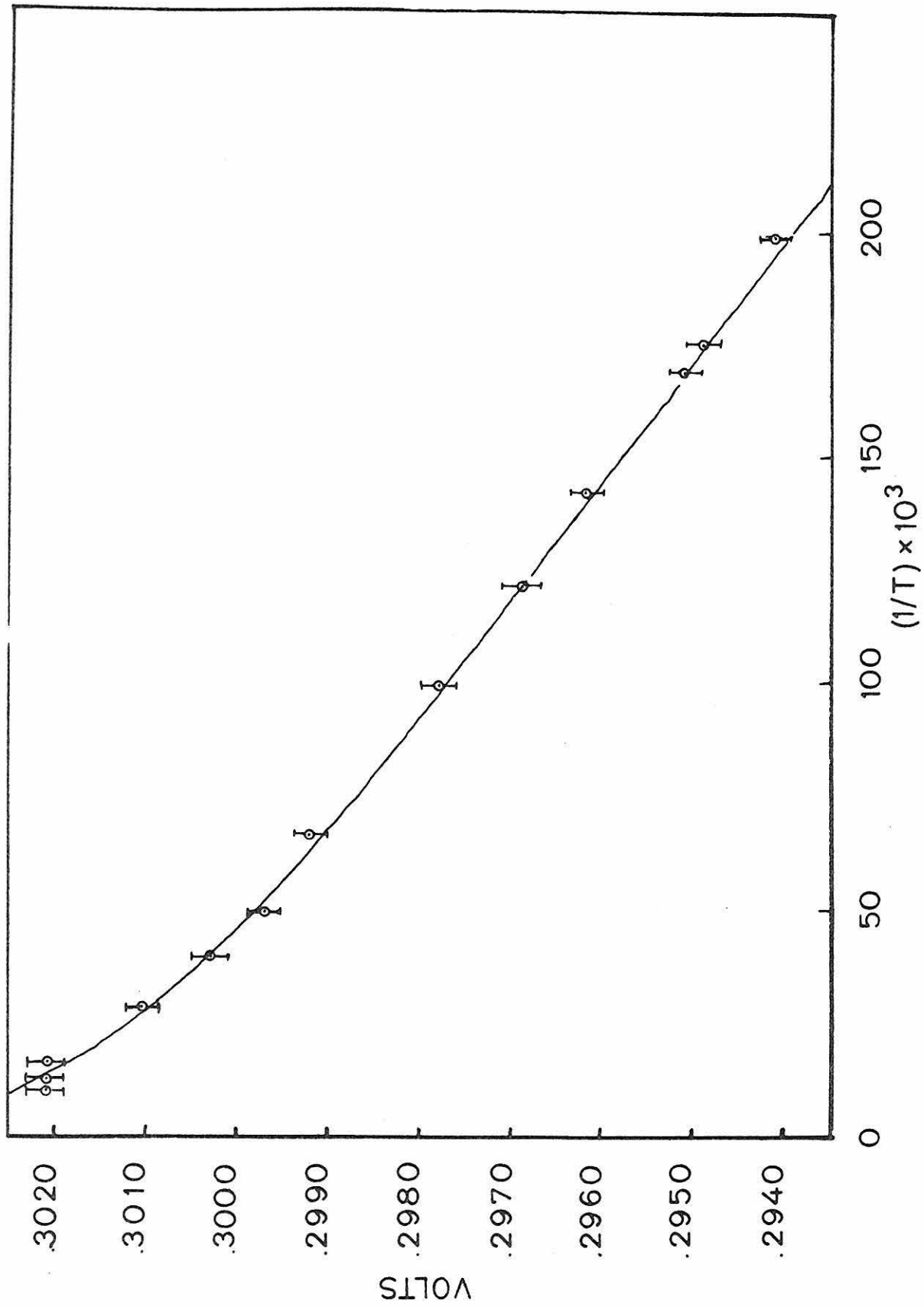
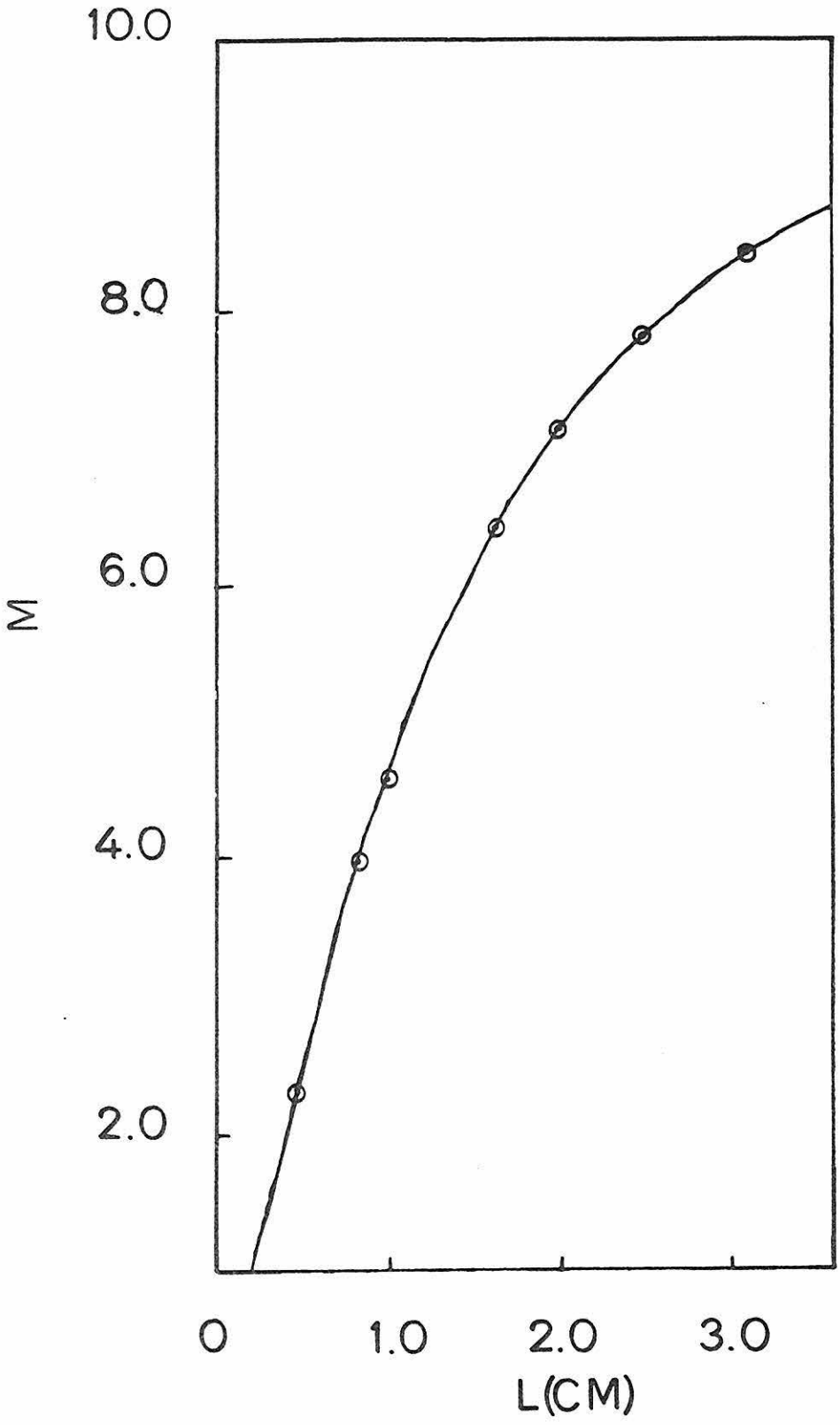

VOLTS
.3020
.3010
.3000
.2990
.2980
.2970
.2960
.2950
.2940
0
50
100
150
200
(1/T) x 10^3

FIGURE 3

Mutual inductance between a 2 cm diameter SQUID pickup coil and a solenoid of length ℓ.


10.0
8.0
6.0
4.0
2.0
M
0
1.0
2.0
3.0
L(CM)

From the three water samples we choose one of them to standardize upon: the sample 0.33 cm long and diameter = 0.452 cm (measured frozen). Voltages are those extrapolated for the intercept ($T^{-1} = 0$) in a χ vs. T^{-1} plot and are corrected for sample holder contributions.

$$\text{I: } 1.404 \text{ volts} \times \underbrace{\left(\frac{.452}{.558}\right)^2}_{\substack{\text{correction for} \\ \text{diffrent cross-} \\ \text{sectional areas}}} \times \underbrace{\left(\frac{.175}{.797}\right)}_{\substack{\text{correction for} \\ \text{different lengths}}} = 0.203 \text{ volts}$$

Sample I was 2.619 cm long and had a diameter = 0.558 cm.

$$\text{II: } 1.4225 \text{ volts} \times \left(\frac{.452}{.558}\right)^2 \times \left(\frac{.175}{.838}\right) = 0.195 \text{ volts}$$

Sample II was 3.10 cm long with diameter = 0.558 cm.

III: 0.207 volts (the standard)

Sample III was 0.33 cm long with diamter = 0.452 cm.

The average value is 0.202±0.006 volts. Considering the variety of effects that must be compensated for, the agreement is reasonable (the values differ at most by only 6% of the average).

Clearly, variations in sample geometry will be temperature dependent. No attempt was made to correct the data for thermal effects as they are expected to depend critically on the exact composition of the sample (e.g. pure water vs. protein solution), the concentration of any solutes, and possibly the identity of the protein under investigation. Empirically, from measurements similar to those in Figure 3, the SQUID signal would be $V_\ell(1+.597\Delta\ell/\ell)$ for a sample with ℓ = 2.54 cm and $V_\ell(1+.187\Delta\ell/\ell)$ where ℓ = 0.9 cm. V_ℓ is the measured voltage for a sample of length ℓ and $\Delta\ell$ is the change in ℓ induced by some effect. Thus for small $\Delta\ell$ the signal will not vary appreciably. Ray Sarwinski of SHE Corporation may be contacted for further information.

Data for laccase and oxyhemocyanin may then be calibrated as shown using the value for the susceptibility of water:

$$\chi_{H_2O} \cong -0.72 \times 10^{-6} \text{ cgs (at 273 K for liquid } H_2O, \underline{\text{CRC}}\ 51^{\underline{\text{st}}} \text{ ed.)}$$

This value is calculated from the relation $\chi_M = M\chi/\rho$ where $\chi_M \equiv$ molar susceptibility; $M \equiv$ molecular weight and $\rho \equiv$ density in grams per cc. The laccase sample was 2.54 cm long and 0.558 cm in diameter so the signal would be (relative to the standard)

$$V = 0.202 \text{ volts} \times \left(\frac{.558}{.452}\right)^2 \times \left(\frac{.785}{.175}\right) = 1.381 \text{ volts}$$

$$\text{and} \quad \chi = \frac{-0.72 \times 10^{-6} \text{ cgs}}{1.381 \text{ volts}} = -0.52 \times 10^{-6} \frac{\text{cgs}}{\text{volt}}$$

Oxyhemocyanin was 2.41 cm x 0.558 cm so

$$V = 0.202 \text{ volts} \times \left(\frac{.558}{.452}\right)^2 \times \left(\frac{.770}{.175}\right) = 1.356 \text{ volts}$$

$$\text{and} \quad \chi = \frac{-0.72 \times 10^{-6} \text{ cgs}}{1.356 \text{ volts}} = -0.53 \times 10^{-6} \frac{\text{cgs}}{\text{volt}}$$

Calibration of Second SHE Susceptometer

The primary improvements of this version compared to the earlier instrument are ease of operation and uniform response over the sample volume. Essentially this instrument (with some minor improvements) will be available commercially.

$HgCo(SCN)_4$, prepared as described by B. N. Figgis and R. S. Nyholm, J. Chem. Soc. A, 190 (1958), was used as the calibrant. This substance follows a Curie-Weiss law over the range 5.8 - 293 K with $\theta = +2K$ (see H. -St. Rade, J. Phys. Chem., 77, 424 (1973)). The gram susceptibility is χ_g (paramagnetic) = 16.45×10^{-6} cm^3/g at 293 K. χ_g (diamagnetic) = -0.1667×10^{-6} cm^3/g so χ_g (total) = 16.283×10^{-6} cm^3/g (293 K). A transfer function (assumed to be temperature independent) may

now be calculated. Its form is

$$F \equiv \frac{(\chi_g{}^{293K}(\text{total}))(m)(H)}{V^{293}(\text{calibrant})}$$

where m = mass in grams, H = field in gauss and V^{293} is the voltage reading of the SQUID output at 293 K corrected for sample holder contributions. For the actual calibration sample m = 0.0899 g and H = 2000 G. Therefore:

$$F = \frac{(1.6283 \times 10^{-5})(.0899)(2 \times 10^{3})}{(30.298)} = 9.663 \times 10^{-5} \frac{cm^3\text{-}G}{volt}$$

Any voltage at a given field and sample mass may be converted to a gram susceptibility via the following formula

$$\chi_g(\text{total}) = \frac{(F)(V)}{(m)(H)} \quad \text{with units of } \frac{cm^3}{g} \text{ as required.}$$

A separate transfer function was calculated from data on platinum metal at 293 K. Its value is in close agreement with that based on $HgCo(SCN)_4$. See Joe Ellinghaus of SHE Corporation for further details.

Susceptibility measurements were made on a laccase sample with the characteristics below:

concentration = 2.2×10^{-3} M

density = 1.054 g/ml

total weight = 0.0557 g

Using the transfer function calculated above the slope of the χ vs T^{-1} plot for laccase is (per gram Cu)

$$\frac{(-2.411 \text{ volt} \cdot \text{K})(9.663 \times 10^{-5} \text{ cm}^3\text{-gaus volt}^{-1})}{(2000 \text{ gauss})(1.48 \times 10^{-5} \text{ g}_{Cu})} = -0.00787 \frac{\text{cm}^3}{\text{g}} \cdot \text{K}$$

Cu content was calculated from the experimental concentration, density, and weight of the sample.

APPENDIX B

OXYHEMOCYANIN VOLUME SUSCEPTIBILITY

T(K)	V(-holder)	$T^{-1}(x10^3)$
259.2	1.4633	3.858
198.5	1.4609	5.038
160.6	1.4586	6.230
99.3	1.4538	10.07
60.0	1.4474	16.67
34.5	1.4376	29.00
24.25	1.4267	41.24
14.74	1.4017	67.84
11.55	1.3847	86.58
9.84	1.3691	101.6
8.05	1.3492	124.2
6.98	1.3293	143.3
5.80	1.3028	172.4
5.11	1.2794	195.7

LACCASE VOLUME SUSCEPTIBILITY

T(K)	V_{sample}	V_{holder}	$V_{sample-holder}$	$T^{-1}(x10^3)$
269.5	1.6994	0.2256	1.4738	3.711
259.2	1.6981	0.2257	1.4724	3.858
239.5	1.6964	0.2258	1.4706	4.175
219.5	1.6950	0.2263	1.4687	4.556
198.5	1.6923	0.2268	1.4655	5.038
179.5	1.6901	0.2265	1.4636	5.571
160.6	1.6874	0.2262	1.4612	6.227
129.2	1.6812	0.2262	1.4550	7.740
118.9	1.6783	0.2262	1.4521	8.410
109.1	1.6753	0.2261	1.4492	9.166
99.3	1.6713	0.2260	1.4453	10.07
80.0	1.6618	0.2262	1.4356	12.50
70.0	1.6548	0.2263	1.4285	14.29
65.0	1.6504	0.2264	1.4240	15.38
60.0	1.6450	0.2264	1.4186	16.67
55.0	1.6392	0.2263	1.4129	18.18
49.7	1.6316	0.2262	1.4054	20.12
39.8	1.6126	0.2260	1.3866	25.12
34.5	1.5984	0.2259	1.3725	29.00
24.25	1.5512	0.2251	1.3261	41.24
14.74	1.4494	0.2211	1.2283	67.84
9.84	1.3210	0.2202	1.1008	101.6
5.11	0.9658	0.2150	0.7508	195.7

LACCASE GRAM SUSCEPTIBILITY

T(K)	V_{sample}	V_{holder}	$V_{sample-holder}$	$T^{-1}(x10^3)$
5.11	1.0097	0.6720	0.3377	195.7
8.00	1.1896	0.68525	0.50435	125.0
10.00	1.2466	0.6897	0.5569	100.0
15.0	1.3330	0.6959	0.6371	66.7
25.0	1.4044	0.70105	0.70335	40.0
40.2	1.4461	0.7040	0.7421	24.9
65.0	1.4695	0.7058	0.7637	15.4
100.0	1.4856	0.70675†	0.7789	10.0
200.0	1.5034	0.70775†	0.7957	5.00
250.0	1.5090	0.7080†	0.8010	4.00

As χ vs. T^{-1} was reasonably linear for the holder below 100 K values of V_{holder} were graphically interpolated for temperatures where a direct measurement was not made.

†These values obtained from extrapolation of low temperature line.

LACCASE GRAM SUSCEPTIBILITY: SAMPLE HOLDER

T(K)	V	$T^{-1}(x10^3)$	
4.50	0.6671	222.0	
5.00	0.6710	200.0	
10.0	0.6897	100.0	
15.0	0.6959	67.7	
25.0	0.7010	40.0	
35.0	0.7033	28.6	
40.0	0.7040	25.0	
50.0	0.7048	20.0	
65.0	0.7058	15.4	
100.0	0.7041	10.0	
130.0	0.7034	7.69	
203.0	0.7052	4.93	outgassing problems, probably from N_2 frozen in dewar.
240.0	0.7062	4.17	
300.0	0.7084	3.33	