STUDIES OF THE ENZYME LACCASE

Thesis by

Robert M. Kanne

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ABSTRACT

Rhus Vernicifera laccase was purified to an A_{280}/A_{614} ratio of 15.2. A procedure was then used to selectively remove the Type 2 copper and 70% of it was removed as judged by EPR. The treated enzyme showed decreased absorbance in the 330 nm. region, which is associated with the Type 3 site. The blue color was observed to reversibly bleach on occasion, apparently due to autoreduction of the Type 1 copper. The fluorescence of the Type 2 depleted laccase was increased 60% over that of the native protein. Since fluorescence quenching is often associated with binding of a metal to a protein site, fluorescence was used to monitor the attempted substitution of cobalt and nickel into the Type 2 site. There is some evidence that cobalt can occupy the Type 2 site.

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Introduction

All of the work that will be described in this report was done on the enzyme laccase (p-diphenol : O_2 Oxidoreductase, E. C. 1.10.3.2) from the Japanese lacquer tree, Rhus Vernicifera. Interest in this enzyme stems from its ability to carry out the four electron reduction of oxygen to water. This reaction is extremely important in biology because it is the final step in the catabolism of all aerobic organisms. In mammals, this is accomplished by the enzyme cytochrome oxidase which is more difficult to study due to the fact that it is part of a large multi-subunit, multi-enzyme complex in its native membrane-bound state. The inorganic chemist's interest in laccase is a result of the insights which its study might give on the nature of multi-electron oxidation-reduction catalysis. All four of laccase's copper atoms appear to be intimately involved in the catalytic mechanism, but many questions remain to be answered about how the electrons are passed from the reductant to the three copper sites and then from the copper sites to oxygen.

Rhus laccase has a molecular weight of 110,000 and is a glycoprotein containing 45% carbohydrate.[1] Experimental work on laccase has been summarized in several reviews.[2-6] It contains one Type 1 "blue copper" site (one Cu, narrow hyperfine in the EPR spectrum, visible absorbance at 614 nm.), one Type 2 site (one Cu, "normal" Cu (II) EPR, no visible absorbance), and one Type 3 site (two Cu, no EPR, visible

absorbance at 330 nm.). Several recent experiments on the simple blue copper proteins azurin, stellacyanin, and plastocyanin have done much toward elucidating the structure of the Type 1 site. Spectroscopic studies[7] have shown that the absorption spectrum can be fit using a tetragonally distorted tetrahedral coordination environment where the large absorbance near 610 nm. is due to Cys-S^- to Cu(II) charge transfer. the crystal structure of plastocyanin is consistent with this[8] and the ligands that they found were two histidines, a cysteine, and a methionine. EXAFS[9] of azurin shows two nitrogen atoms at 1.97 A. and one sulfur at 2.10 A.; the fourth ligand has not been located with certainty. EPR work has shown that the Type 2 copper of laccase is almost certainly coordinated to four nitrogens in a square planar arrangement.[10] The Type 3 site consists of two coppers which are strongly antiferromagnetically coupled.[11] By analogy with the molluscan oxygen-carrying protein hemocyanin which also has such a copper pair, the Type 3 site is considered to be the site of oxygen binding and it probably contains no sulfur ligands.[12]

Enzymes that are closely related to Rhus laccase include the fungal laccases. They have the same set of copper sites and the same substrate specificity as Rhus laccase but differ in some detailed properties, such as binding of exogenous ligands. The fungal laccase that is most commonly used is the one found in Polyporus versicolor. The fungal enzyme tyrosinase catalyzes the oxidation of o-diphenols, as opposed to laccase's oxidation of p-diphenols, and has

an additional ability to catalyze the ortho hydroxylation of monophenols. The active site of the tyrosinases from Neurospora crassa and Agaricus bispora appears to be a binuclear copper site similar to a Type 3 site.[13-15] The other members of the blue copper oxidase family, besides laccase, are ascorbate oxidase and ceruloplasmin. They contain Type 1, Type 2 and Type 3 copper centers, but the actual number of each type of copper is still uncertain. Ceruloplasmin, purified from mammalian plasma, contains 5-8 atoms of copper with about twice as many Type 1 sites as Type 2 sites.[16-20] Ascorbate oxidase, usually isolated from zucchini or cucumber, is a dimer containing 8-10 coppers per molecule.[21] Because of laccase's simpler composition, with only one of each of the three types of copper, laccase probably offers the best opportuniy for determining the mechanism of the blue copper oxidases. Ascorbate oxidase is quite specific in its use of ascorbate for the reduction of oxygen but ceruloplasmin's natural reductant is not known with certainty and it is the slowest of the three at reducing oxygen.

The interpretation of the data relating to laccase's mechanism is a complex and difficult task at best. Some experiments involve reoxidation of fully reduced laccase, others involve the steady state reaction of laccase with oxygen and reductant, and still others involve the "anaerobic" reduction of laccase with various reductants. This latter type is always "post steady state" to some extent since there are always small amounts of oxygen present at the start of the experiment. In each of these

care must be taken to decide which "intermediates" are along the catalytic pathway and which are side products or artifacts.

Brändén and Deinum have used reoxidation experiments with $^{17}O_2$ and $^{18}O_2$ to show that: 1) an oxygen intermediate, probably O^-, is produced within milliseconds and that this intermediate has oxidized Type 1 copper and reduced Type 2 copper[22,23]; 2) one of the oxygen atoms from O_2 stays bound to the Type 2 copper indefinitely (or until the enzyme goes through another redox cycle) while the other oxygen atom is released to the solvent as water very rapidly[24,25]; and 3) the O^- radical intermediate's EPR signal is strongly influenced by the pH of the solvent.[26] Fairly similar results are observed for both the tree[26] and fungal [22-25] laccases. A diagram of their proposed model for this reoxidation process is shown below.

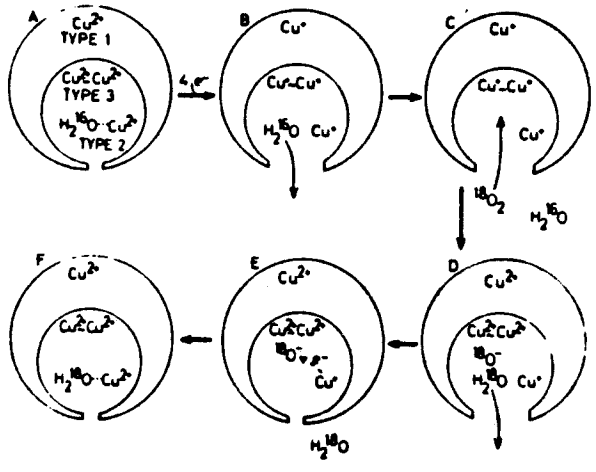


Fig.1. A model for the function of the dioxygen reducing site in laccases when the anaerobically-reduced enzyme reacts with O_2. A and F represent fully oxidized enzyme. B and C represent enzyme anaerobically reduced with 4 e^- equiv. reducing substrate. D and E represent intermediate forms of the enzyme in the reoxidation process.

In contrast to the above intermediate, Baldwin and Gray suggest that the catalytic oxygen intermediates result from the interaction of O_2 with laccase that has Type 1 copper reduced, Type 2 copper oxidized, and Type 3 copper reduced, giving an initial intermediate with a reduced Type 1 copper.[27] Their study of the steady state reaction of laccase with ascorbate and oxygen also detected several species spectroscopically. There was an electronic absorption near 340 nm., a circular dichroism maximum at 360 nm., and an EPR signal similar to the one that the Swedes identified as an O^- radical.[28] Earlier studies of the "anaerobic" reduction of laccase by hydroquinone were interpreted by Gray and coworkers as involving the transfer of electrons through the Type 2 copper to the Type 1 and Type 3 coppers, since the Type 1 and Type 3 chromophores disappear at essentially the same rate.[29] The Swedes interpret similar data in a different way, claiming that the Type 1 site mediates the transfer of at least one of the electrons to the Type 3 site.[30,31]

Other recent papers include a study of the steady state kinetics of laccase which supported a ping-pong ($E + 4e^- \rightarrow E'$, $E' + O_2 \rightarrow E + 2H_2O$) mechanism. They used the rate of oxygen consumption to measure the rates of the reactions.[32] Proposed mechanisms may become even more complex since it has been shown that the Type 3 site can apparently act as a one electron donor/acceptor as well as a two electron donor/acceptor.[33] It has long been thought that formation of superoxide, O_2^-, was unfavorable and that enzymes like laccase would use a two electron

donor like the Type 3 site to bypass this step.[34]

This introduction has hardly scratched the surface of the total amount of work done on laccase. Much more work still needs to be done before a mechanism will be commonly accepted. I am studying the properties of laccase which has had its Type 2 copper removed in an attempt to understand the role of that copper in catalysis and its interaction with the other copper sites. It has been known for a long time that Type 2 copper was necessary for laccase's catalytic activity[35,10] , but its specific role is only gradually becoming clear.

Experimental

Laccase was purified by a method very similar to that of Reinhammer.[1] However, the laccase was not yet pure at the completion of the procedure. The third column, CM Sephadex C-50, was repeated and this improved the A_{280}/A_{614} ratio from 20 down to 18. Extensive dialysis against buffer was tried, as this had improved the ratio in previous preparations of laccase. However, the ratio did not improve. I had observed that the CM Sephadex column spread the laccase into a leading edge of greenish material and a trailing peak of blue material. When the column was eluted at high ionic strength, the blue material always overran the greenish material, mixing them together again. I therefore ran the CM Sephadex column again, but removed the blue material before the ionic strength change step. A very satisfactory ratio of 15.2 was obtained. This laccase was then stored in

liquid nitrogen and aliquots were removed as needed. The ratio has not deteriorated despite months of storage and several freezings and thawings.

Type 2 depleted laccase was prepared in a manner based on that of Graziani, et al.[36] Briefly, this procedure consists of dialysis of a laccase solution anaerobically against a solution of 2×10^{-3} M. dimethylglyoxime and 5×10^{-2} M. ferrocyanide in 0.05 M. pH 5.0 acetate buffer. The laccase is then dialyzed against 1×10^{-3} M. EDTA in the same acetate buffer. The laccase is finally dialyzed against several changes of phosphate buffer. My procedure used a 0.2 mM. laccase solution rather than the 0.5 mM. concentration used in the published procedure and the final dialysis was against pH 7.4 or 6.0 phosphate buffer rather than the pH 7.0 that they used. I also used longer dialysis times (about 40 hours generally compared to their 24 hours) in an attempt to obtain more complete depletion of Type 2 copper than they achieved. Removal of Type 2 laccase was checked on a Varian E-line Century series EPR spectrometer near liquid helium or liquid nitrogen temperature. Some of the laccase samples were concentrated to 0.5 mM. using an Amicon "Minicon" Concentrator before the EPR were run.

Fluorescence measurements were done on a Hitachi-Perkin Elmer MPF-3 spectrofluorimeter at room temperature. Laccase concentrations were in the 1-2 μM. range so that the fluorescence would behave in a linear fashion. I have found that laccase fluorescence becomes very nonlinear when the absorbance at 280 nm. is greater than 0.05. Excitation was

always done at 280 nm., the excitation maximum for laccase, and emission and excitation slit widths were 10 nm. Spectra were run in the direct mode. The fluorescence of the tryptophan and tyrosine residues was then found by scanning the emission spectrum. No emission filters were used.

Substitution of Co(II) and Ni(II) at the Type 2 copper site was attempted using reagent grade $Co(II)(H_2O)_6(ClO_4)_2$ and $Ni(II)(H_2O)_6(NO_3)_2$ dissolved in water. Generally, I used a ratio of metal to laccase of about two to one. Substitution was attempted at concentrations varying from about 10^{-6} to 10^{-4} M. See results section for specific experiments.

Deionized water from a Barnstead Nanopure water purifier was used for all experiments. All buffers had trace metals removed by running them through a column of Chelex 100. UV-visible absorption spectra were done on either a Cary 17 or a Cary 219.

Results and Discussion

Properties of Type 2 Depleted Laccase

Type 2 copper has no significant uv-visible absorption but is paramagnetic so its presence is monitored using EPR. Figure 1 shows the EPR spectrum of native laccase and figure 2 shows the EPR spectrum of laccase that has had about 67% of the Type 2 copper removed. The amount of Type 2 copper removed is determined by comparing the size of the hyperfine absorption near 2720 Gauss in figure 2, which is due solely to Type 2 copper, with that of the hyperfine absorptions

Figure 1

EPR spectrum of 0.3 mM. native laccase.

Microwave frequency, 9.1755 GHz.; modulation amplitude, 10 Gauss; temperature, 80 K; microwave power, 15 mW.; receiver gain was 4×10^3 for (A) and 2.5×10^4 for (B).

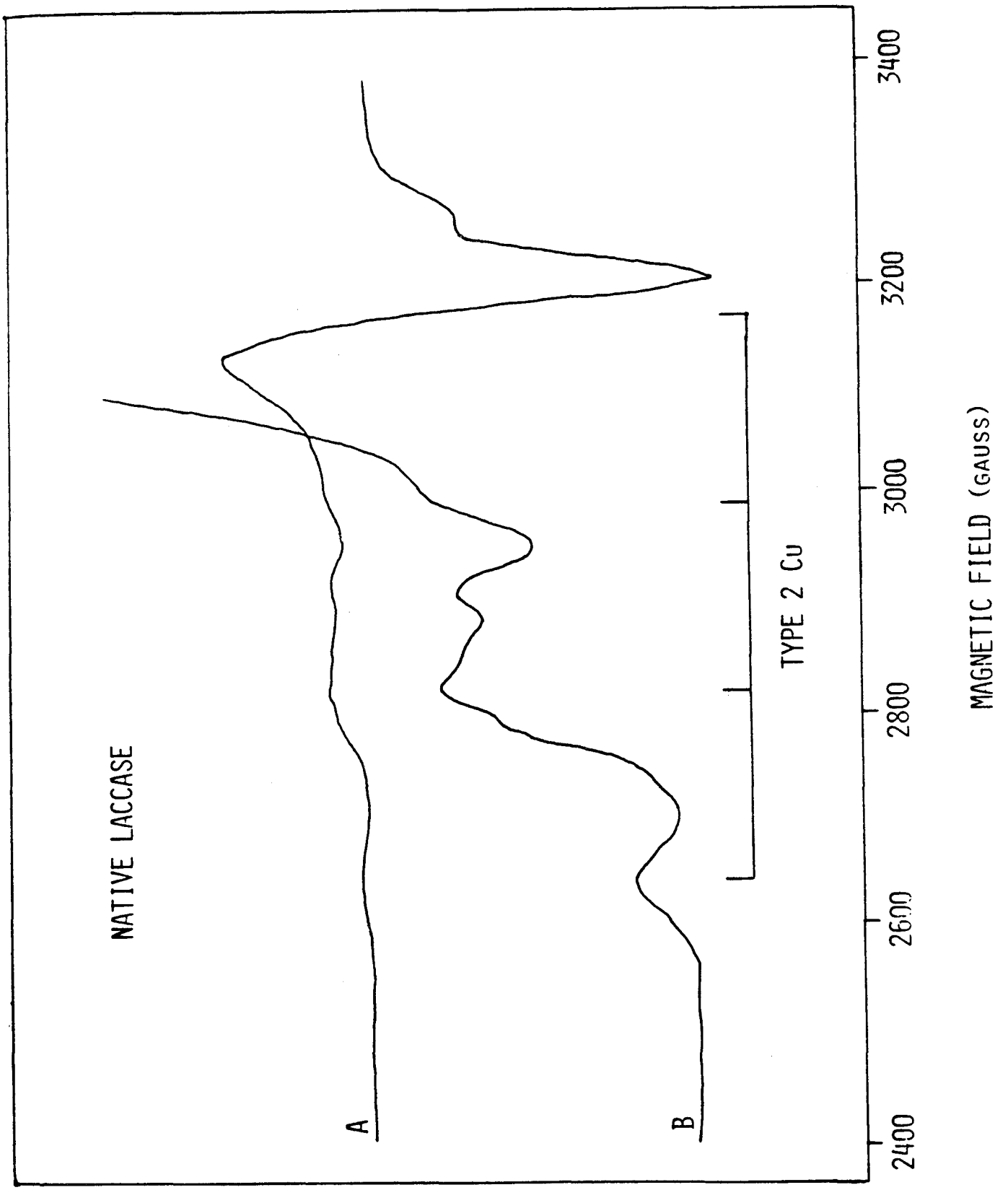
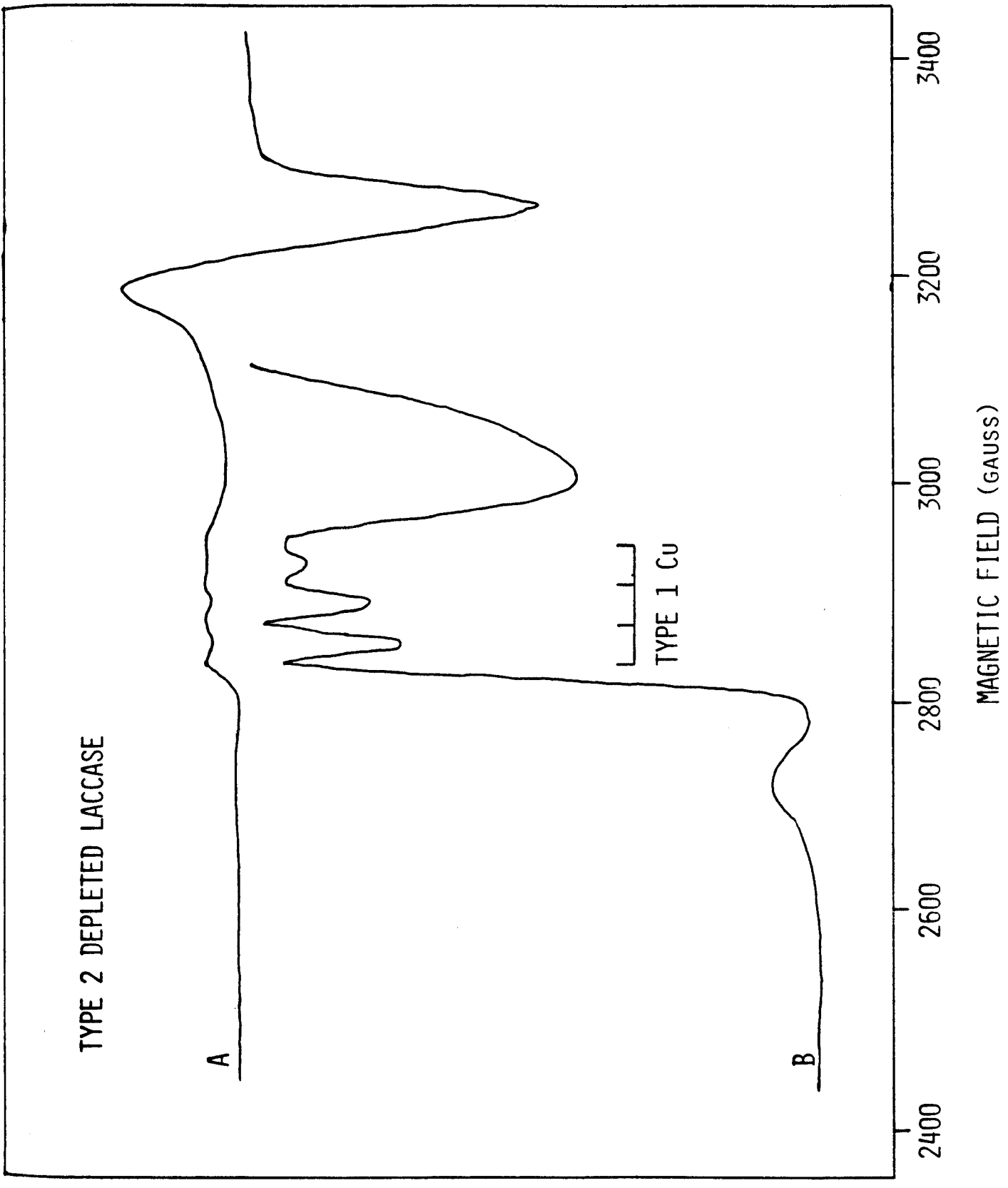

NATIVE LACCASE
A
B
TYPE 2 Cu
2400
2600
2800
3000
3200
3400
MAGNETIC FIELD (GAUSS)

Figure 2

EPR spectrum of 0.5 mM. Type 2 depleted laccase. Microwave frequency, 9.257 GHz.; modulation amplitude, 16 Gauss; temperature, 15 K; microwave power, 1.0 mW.; receiver gain was 6.3×10^3 for (A) and 6.3×10^4 for (B).

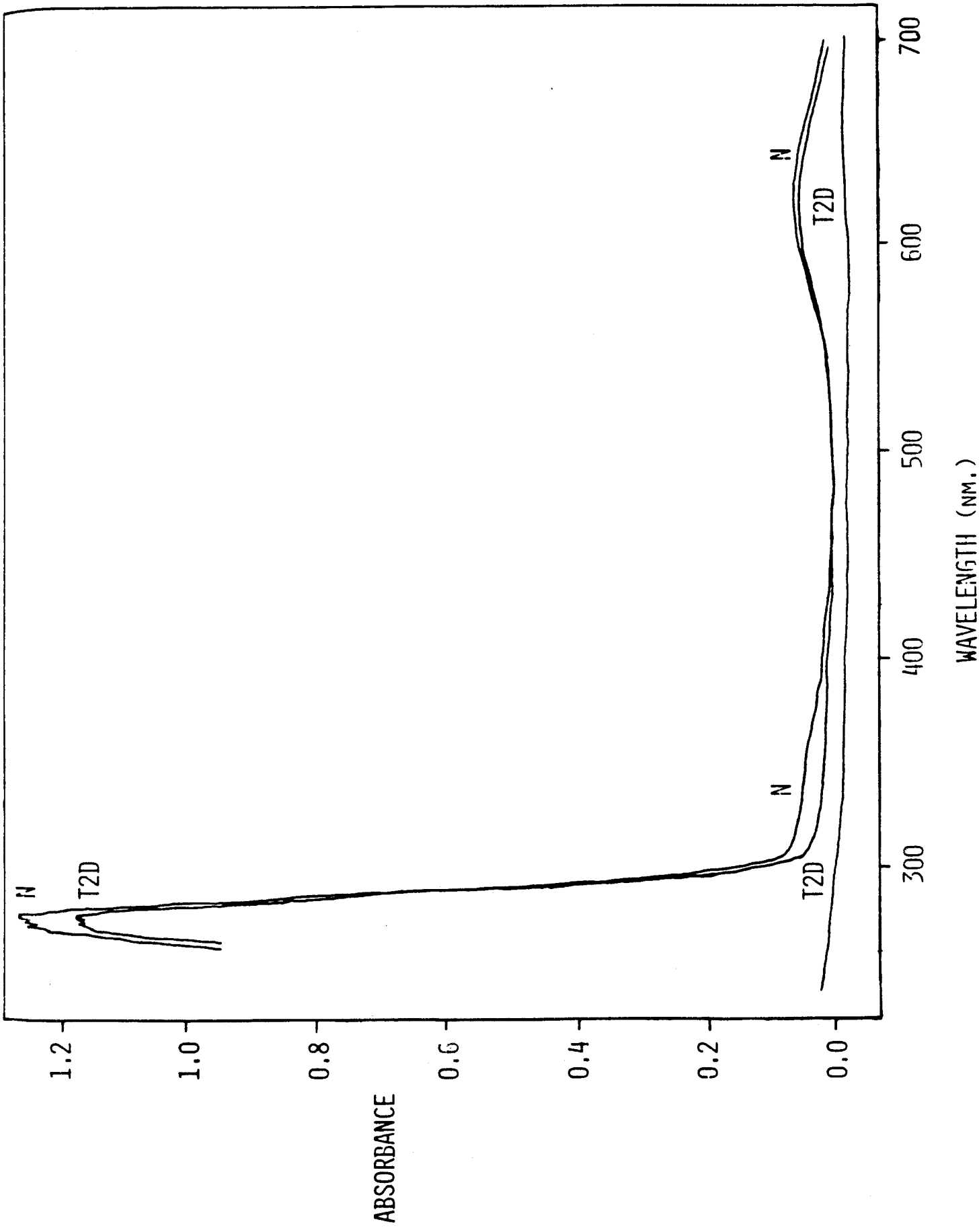

TYPE 2 DEPLETED LACCASE
A
B
TYPE 1 Cu
2400
2600
2800
3000
3200
3400
MAGNETIC FIELD (GAUSS)

near 2910 and 2945 Gauss, which are due predominantly to Type 1 copper. This relationship is then compared with that of the corresponding peaks near 2650, 2870, and 2915 Gauss in figure 1. The positions in terms of Gauss do not correspond exactly because different microwave frequencies were used in the two spectra. The dip in the g_1 region near 3260 Gauss in figure 1 is also indicative of Type 2 copper and this dip is absent in the Type 2 depleted spectrum of figure 2. This amount of Type 2 depletion is equivalent to that generally achieved in the literature reference[36] and by Dave Dooley, who did some work on Type 2 depleted laccase in this group independantly, before I began working on it. Removal of the Type 2 copper makes it very apparent which features in the EPR are due to which coppers and allows one to see the Type 1 copper nuclear hyperfine splittings. The resolution of the four copper (nuclear spin of 3/2) hyperfine absorptions in figure 2 is better than that seen in the original paper or in most of Dooley's work.

Figure 3 shows the uv-visible absorption spectra of native and Type 2 depleted laccase. The Type 1 copper is unaffected by the Type 2 depletion process as is shown by the similarity of their A_{280}/A_{614} ratios, which were identical within experimental error. However, the absorbance at 330 nm., which is a characteristic of the Type 3 coppers, is decreased by almost half in the Type 2 depleted protein. Since some of the absorbance at this wavelength is probably due to background, this observation apparently means that more than half of the Type 3 sites

Figure 3

UV-visible electronic absorption spectra of native (N) and Type 2 depleted (T2D) laccase. Concentrations were approximately 0.2 mM. in a 1 mm. pathlength cell.


N
T2D
N
T2D
N
T2D
1.2
1.0
0.8
0.6
0.4
0.2
0.0
ABSORBANCE
300
400
500
600
700
WAVELENGTH (NM.)

have either lost their copper or are reduced. This is perhaps the reason why more work has not been done with Type 2 depleted laccase; not only is removal of the Type 2 copper incomplete, but the state of the Type 3 site appears to be changed. Only one paper on Type 2 depleted laccase has been published since the original paper nearly three years ago, and that one was a study of the EPR properties of the Type 1 copper so that the state of the Type 3 copper was not important.[37]

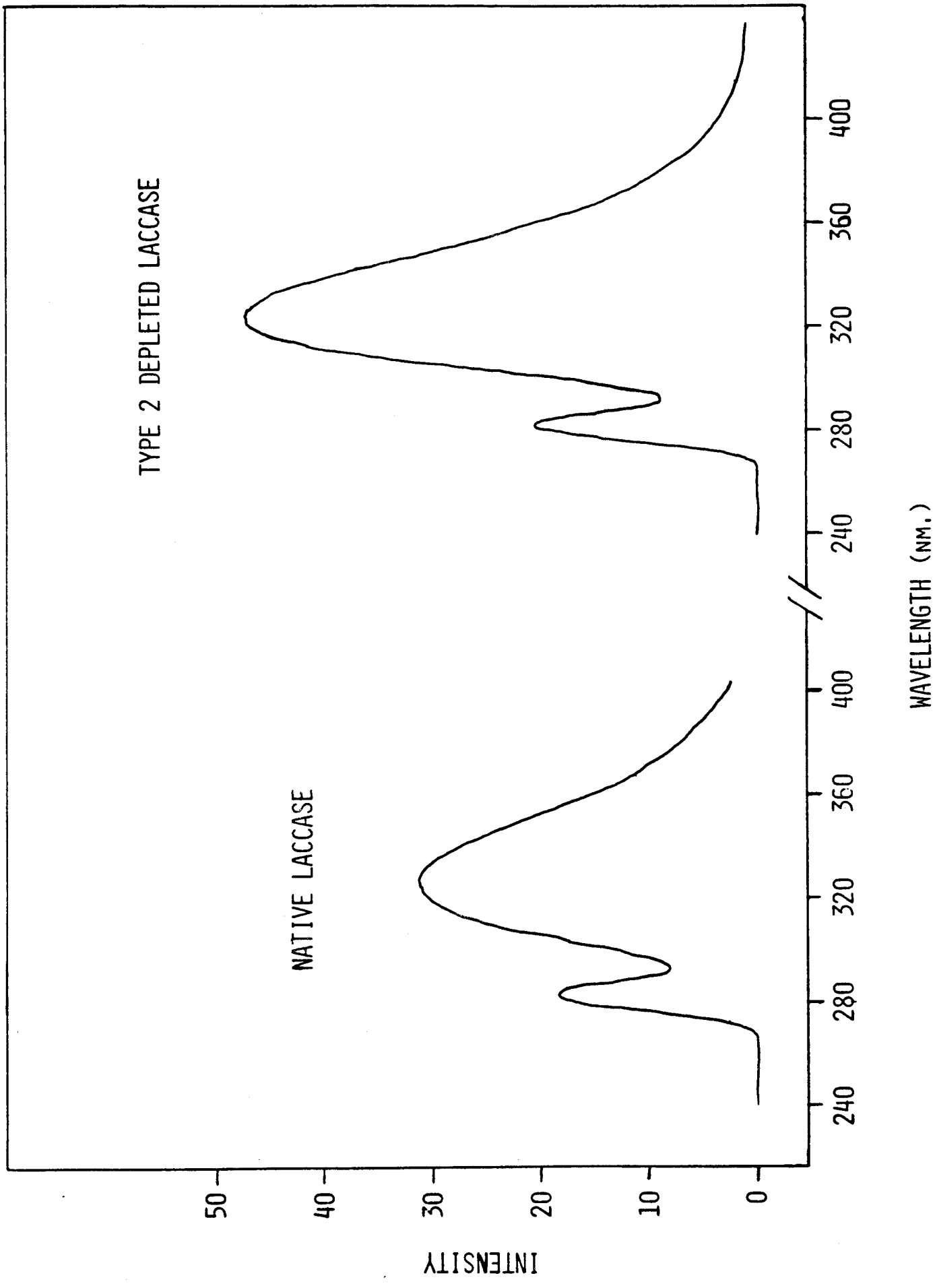
The fluorescence emission spectra of native and Type 2 depleted laccase are shown in figure 4. It can be seen that the fluorescence of this depletion prep is about 50% greater than that of the native enzyme. The increase is actually about 60% because the concentrations of these two solutions were not quite equal. This sort of increase is not uncommon; five to six fold increases are observed when the copper is removed from each of the simple blue copper proteins.[38] The fluorescence of laccase is also increased as the Type 1 copper is reduced.[39] Such fluorescence changes can be caused by conformational movements of the protein as well as quenching due to the presence of the paramagnetic copper ion itself. The fluorescence of tryptophan is very sensitive to the polarity and charge in its vicinity. I will discuss my experiments with metal substitution later on.

Autoreduction of Type 1 Copper in Type 2 Depleted Laccase

I have twice observed Type 2 depleted laccase lose

Figure 4

Fluorescence emission spectra of native and Type 2 depleted laccase at concentrations of 1.98×10^{-6} M. and 1.80×10^{-6} M. respectively in pH 6.0 μ=0.1 phosphate buffer. Excitation wavelength, 280 nm.; sensitivity, 1.0; slits, 10 nm.; direct mode.


TYPE 2 DEPLETED LACCASE
NATIVE LACCASE
INTENSITY
0
10
20
30
40
50
240
280
320
360
400
240
280
320
360
400
WAVELENGTH (NM.)

its blue color while being stored at 4° C. and then have that color return. Native tree laccase has never been reported to autoreduce. However, it has been observed that fungal laccase Type 1 copper does reversibly autoreduce at high pH.[41] It is slightly reduced at pH 6.5 and entirely reduced at pH 9. Color could be restored to the enzyme by lowering the pH or adding one equivalent of oxidant. They have not been able to determine where the reducing electron comes from.

Type 2 depleted laccase in pH 6.0 phosphate buffer in a closed 2 ml. container with about .5 ml. of air space bleached so that only about 20% of its blue color remained after being left alone for three weeks. When the solution was transferred to a cuvette the laccase became blue again within a few seconds. This indicates that the laccase could still be oxidized by oxygen at a reasonably rapid rate. It is extremely unlikely that any exogenous reductant could have caused the reduction since the Type 2 depleted laccase had been extensively dialyzed against chelexed buffer.

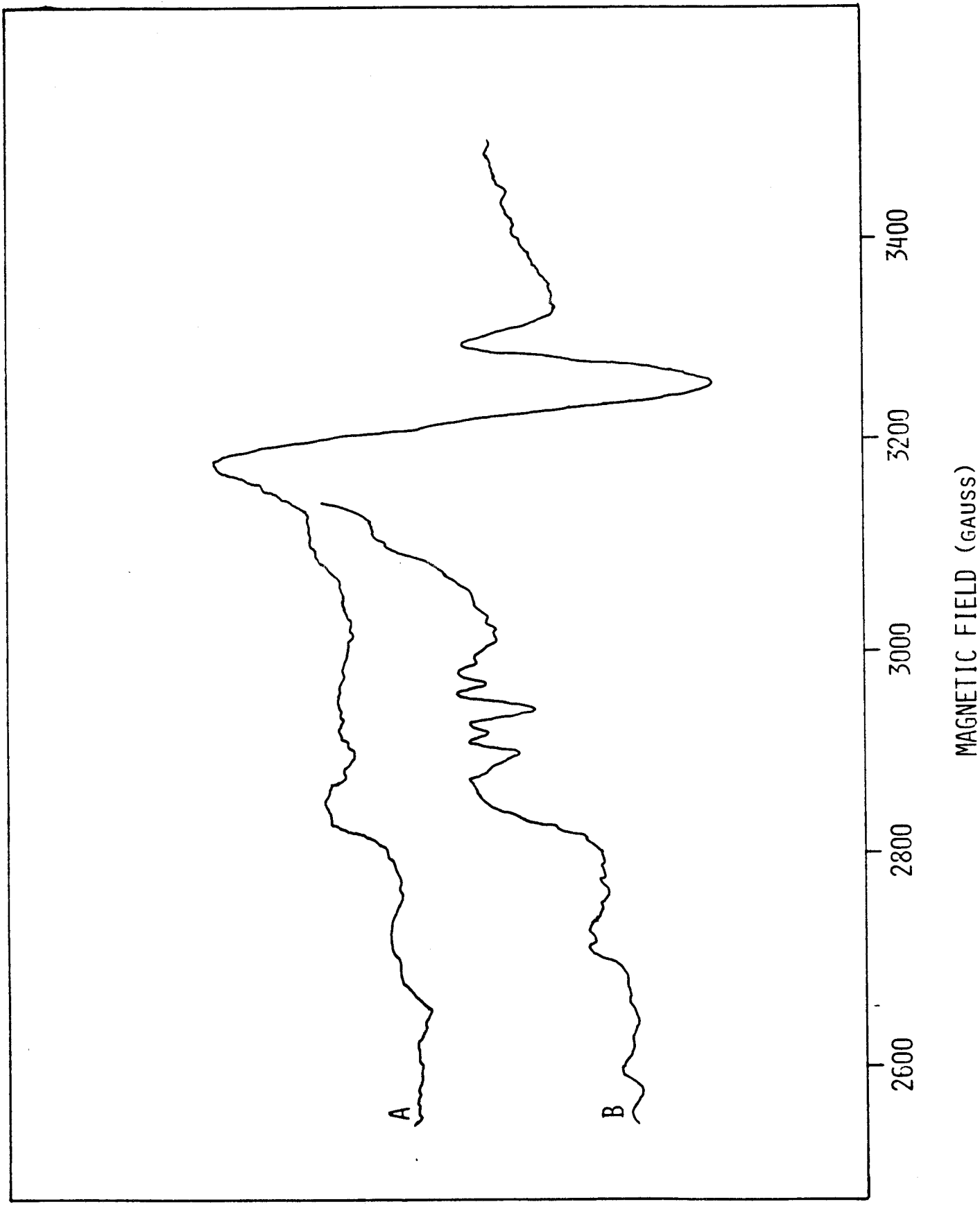
The other time that the protein bleached was in the cold room following the first time that I did the Type 2 depletion procedure. The EPR machine was unavailable for two weeks so I had to wait and I kept the laccase in its dialysis bag dialyzing against pH 7.4 phosphate buffer. After one week I had noticed some slight fading. When I came back after two weeks and was all ready to do the EPR, I was disappointed to find no color in the dialysis bag

and I was fairly sure that the protein was falling apart. I wasn't sure how to revive the protein, if I could at all, so I just moved the 2 l. bottle of buffer with the bag of laccase to the lighted refrigerator on the third floor. When I returned after a week of vacation, I was surprised to find that the laccase was blue again. In addition, it was obvious that some of the protein had simply precipitated out of solution. During this whole time I had kept the bottle covered with a piece of parafilm, although I don't know if it was airtight. I centrifuged the laccase to remove the dead protein and then took an EPR spectrum of it to see if it was depleted of Type 2 copper.This is shown in figure 5. The resolution of this spectrum is poor due to the low concentration and heterogeneity of the sample, but the presence of the features near 2730 and 3350 Gauss clearly show that much "Type 2 like" copper was present. It is hard to say how much of that copper was actually in the Type 2 site and how much was simply extraneous copper from denatured protein, since the EPR spectrum of the latter would probably look almost just like Type 2 copper. The unusual doublet structure of the Type 1 copper hyperfine absorption in the $g_{\parallel}$ region has not been seen in any blue copper protein to my knowledge. It is possible that some of the Type 1 sites were altered by being in the reduced state for so long. Despite the fact that this EPR spectrum was reproducible when repeated, its spectral characteristics were quite dependant on temperature and microwave power.

Figure 5

EPR spectrum of Type 2 depleted laccase after autoreduction, as described in the text.

Microwave frequency, 9.258 GHz.; modulation amplitude, 16 Gauss; temperature, 120 K; microwave power, 200mW.; receiver gain was 5.0×10^3 for (A) and 2.0×10^4 for (B).


A
B
2600
2800
3000
3200
3400
MAGNETIC FIELD (GAUSS)

The Swedes never developed an adequate explanation for the autoreduction of fungal laccase Type 1 copper. Three possibilities that they mention are that the electrons come from oxidation of a carbohydrate of laccase, from denatured protein, or from water. It should be noted that recent experiments in which frozen solutions of native tree laccase were irradiated with 300 to 400 nm. light showed photoreduction of the Type 1 copper.[42] The authors maintain that this demonstrates energy transfer between the Type 3 and Type 1 sites. This is corroborative evidence that laccase contains moieties within the protein structure that can deliver electrons to the Type 1 copper site. If the Type 2 depleted laccase in the small container actually used up all of the oxygen available in the container, then there must be at least several of these electron donors available per protein. The Type 2 depleted protein is apparently not nearly as oxygen sensitive as the native protein. It is possible that the reverse reaction, oxidation of water to oxygen, becomes thermodynamically favored in this protein[41], but this conflicts with the observation that the blue color appeared again when the laccase in the container was exposed to air.

Metal Substitution at the Type 2 Site

Protein fluorescence has proved to be a sensitive indicator of metal binding in the simple blue copper proteins azurin, stellacyanin, and plastocyanin.[40] Both the copper(II) and cobalt(II) substituted proteins are quenched about

five fold with respect to the apoprotein. Because I have measured the fluorescence of Type 2 depleted laccase to be about 60% greater than that of the native enzyme, fluorescence can be used as an indication of metal substitution at the Type 2 site. However, this evidence would need to be supported by EPR (if possible) and atomic absorption spectroscopic evidence consistent with metal substitution.

When both native and Type 2 depleted laccase at 5×10^{-7} M. were mixed with $Co(II)(H_2O)_6(ClO_4)_2$ at 1×10^{-6} M. in pH 6.0 buffer no change in fluorescence was observed over several days. When this experiment was done again at concentrations of 5×10^{-5} M. laccase and 1×10^{-4} M. cobalt, the fluorescence of the Type 2 depleted laccase was only 20% greater than that of the native laccase after 24 hours. This is an indication that some of the cobalt is associated with the laccase, probably near the Type 2 site. When 5×10^{-5} M. laccase solutions were mixed with 1×10^{-4} M. solutions of $Ni(II)(H_2O)_6(NO_3)_2$ at pH 6.0 no change in relative fluorescence between the native and Type 2 depleted enzymes was observed.

These results are somewhat surprising. Co(II) generally prefers either tetrahedral of octahedral coordiantion whereas Ni(II) is commonly found in square planar environments as well as octahedral and tetrahedral ones. The Type 2 site is generally thought of as being four nitrogen atoms in a square planar arrangement so it would seem that nickel substitution would be more likely than cobalt substitution.

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APPENDIX I

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Reactions of Nitric Oxide with Tree and Fungal Laccase†

Craig T. Martin, Randall H. Morse, Robert M. Kanne, Harry B. Gray, Bo G. Malmström,‡ and Sunney I. Chan*

ABSTRACT: The reactions of nitric oxide (NO) with the oxidized and reduced forms of fungal and tree laccase, as well as with tree laccase depleted in type 2 copper, are reported. The products of the reactions were determined by NMR and mass spectroscopy, whereas the oxidation states of the enzymes were monitored by EPR and optical spectroscopy. All three copper sites in fungal laccase are reduced by NO. In addition, NO forms a specific complex with the reduced type 2 copper. NO similarly reduces all of the copper sites in tree laccase, but it also oxidizes the reduced sites produced by ascorbate or NO reduction. A catalytic cycle is set up in which N_2O, NO_2^-, and various forms of the enzyme are produced. On freezing of fully reduced tree laccase in the presence of NO, the type 1 copper becomes reoxidized. This reaction does not occur with the enzyme depleted in type 2 copper, suggesting that it involves intramolecular electron transfer from the type 1 copper to NO bound to the type 2 copper. When the half-oxidized tree laccase is formed in the presence of NO, a population of molecules exists which exhibits a type 3 EPR signal. A triplet EPR signal is also seen in the same preparation and is attributed to a population of the enzyme molecules in which NO is bound to the reduced copper of a half-oxidized type 3 copper site.

The laccases are copper-containing oxidases which, like cytochrome *c* oxidase, can reduce dioxygen to two molecules of water (Reinhammar, 1979). The two varieties studied most often are obtained from the oriental lacquer tree *Rhus vernicifera* and from the white-rot fungus *Polyporus versicolor*. They both contain four copper ions per molecule. In the oxidized enzymes two of these ions, types 1 and 2 Cu^{2+}, are detectable by EPR.[1] The type 1 Cu^{2+} ions are characterized by unusually strong optical absorptions around 600 nm, which disappear on reduction, and by EPR spectra with small hyperfine splitting constants ($|A_z| < 0.010$ cm^{-1}). The type 2 Cu^{2+} ions have more normal EPR spectra, and, since they lack strong optical bands, these Cu^{2+} ions can only be monitored by EPR. The two EPR-detectable Cu^{2+} ions are the primary acceptors of electrons from the reducing substrates.

The two copper ions which are not detectable by EPR in the oxidized enzymes, called type 3, constitute an antiferromagnetically coupled binuclear Cu^{2+}–Cu^{2+} unit (Petersson et al., 1978; Dooley et al., 1978) which functions as the dioxygen reducing site. Exchange-coupled binuclear centers are also found in many other proteins capable of reacting with dioxygen. Thus binuclear copper centers are present (Mason, 1976) in another blue oxidase, ceruloplasmin, in the mixed-function oxidase, tyrosinase, and in the oxygen-transporting protein, hemocyanin. In cytochrome *c* oxidase, the dioxygen-reducing site is the copper–heme unit, Cu_B–cytochrome a_3 (Stevens et al., 1979; Malmström, 1979).

Nitric oxide (NO) has been extensively utilized as a spin probe in the study of such dioxygen-reactive centers. For example, direct evidence of the presence of copper pairs has been derived from the investigation of NO complexes with ceruloplasmin (Van Leeuwen et al., 1973; Van Leeuwen & Van Gelder, 1978), hemocyanin (Schoot Uiterkamp & Mason, 1973; Verplaetse et al., 1979), and tyrosinase (Schoot Uiterkamp & Mason, 1973). Furthermore, Brudvig et al. (1980) have found that cytochrome *c* oxidase catalyzes several reactions of NO, and a study of these has yielded significant clues to the mechanisms of dioxygen binding and reduction in this enzyme.

Only limited information is available on the reaction of NO with the laccases. Rotilio et al. (1975) have shown that NO reduces the type 1 Cu^{2+} in tree laccase, and Dooley et al. (1979) have exploited this result to obtain a pure type 2 Cu^{2+} EPR spectrum. In this paper we describe a detailed examination of the interaction of NO with both tree and fungal laccase, as well as with tree laccase depleted in type 2 copper. It has been found that NO can reduce as well as oxidize tree laccase. Some species observed during the reaction cycle give a type 3 Cu^{2+} EPR signal (Reinhammar et al., 1980) and a triplet signal of the type also induced by NO in some other proteins having binuclear O_2-binding sites (Van Leeuwen et al., 1973; Schoot Uiterkamp & Mason, 1973; Van Leeuwen & Van Gelder, 1978; Stevens et al., 1979). We have also found that in the presence of NO, the reduced type 1 copper in tree laccase is reoxidized on freezing. The fungal enzyme is also reduced by NO; however, the reduced form is not oxidized by NO, probably because of the extremely high reduction potentials of the copper sites. We will present evidence suggesting that NO binds to at least one of the reduced copper centers in fungal laccase. These and related findings have enhanced our understanding of the structure and function of the redox active centers in the laccases.

Materials and Methods

The isolation and purification of laccase from the lacquer tree *R. vernicifera* (Reinhammar, 1970; Reinhammar & Oda,

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‡Sherman Fairchild Distinguished Scholar, 1980–1981, California Institute of Technology. Permanent address: Department of Biochemistry and Biophysics, Chalmers Institute of Technology, S-412 96 Göteborg, Sweden.

[1] Abbreviations used: BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; NMR, nuclear magnetic resonance; PPD, *p*-phenylenediamine; $t_{1/2}$, half-time.

1979) and from the fungus *P. versicolor* (Fåhraeus & Reinhammar, 1967) have been described previously. The preparation of tree laccase depleted in type 2 copper has also been described (Reinhammar & Oda, 1979). Bovine serum albumin (Sigma Chemical Co., Fraction V) was dialyzed 8 h against 5 mM EDTA to remove Mn^{2+} contaminant. All samples were prepared in 25 mM potassium phosphate buffer; the pHs were 6.0 and 7.0 for the fungal and tree laccases, respectively, except where noted otherwise.

Nitric oxide (^{14}NO, Matheson Coleman & Bell) was purified by condensing it in a liquid nitrogen trap and then distilling off only the NO before the addition to the samples. ^{15}NO (99.1% isotopic enrichment, Prochem) was found to be essentially free of other nitrogen oxides and was used as received. $Na^{15}NO_2$ (99.1% isotopic enrichment) and $K^{15}NO_3$ (99.1% isotopic enrichment) for use as ^{15}N NMR standards were obtained from Prochem. All other reagents used were of at least reagent grade purity.

In the preparation of samples of enzyme plus NO, oxygen must be strictly excluded to prevent protein denaturation. To this purpose, all samples to which NO was added were first made anaerobic by three cycles of evacuation and flushing with argon on a vacuum line. NO was then added to the samples through an inlet to the vacuum line so as to exclude oxygen completely. Enzyme samples to which NO had been added were judged to have retained their integrity by checking for optical clarity and for restoration of their characteristic blue color upon removal of NO and reoxidation by air. All incubations of the enzymes with NO were carried out at 0–4 °C, apart from the initial addition of NO and the optical experiments, which were done at ~20 °C.

For recording of the time course of the optical changes upon addition of NO to the oxidized laccases, enzyme samples were prepared in quartz optical cuvettes fitted with a ground-glass stopcock. NO was added, as described previously, to a 100-mL bulb above the evacuated sample. For initiation of the reaction, a valve between the bulb and cuvette was opened, the sample was quickly mixed, and the spectrum was immediately run.

For freezing of optical samples, we used quartz 2-mm pathlength cuvettes fitted with glass side arms. Solutions were collected in the side arm, frozen by slow immersion in liquid nitrogen, then thawed, and shaken into the quartz cell for collecting optical data.

For the EPR time course studies with the oxidized laccases, samples were prepared in 5-mm EPR tubes fitted with a ground-glass vacuum joint. NO was added directly to the samples. Within 2 min the samples were removed from the vacuum line and mixed, since in the absence of mechanical mixing, the rate at which NO goes into solution has been found to be extremely slow (Brudvig et al., 1980). The reaction was then quenched by immersing the tube in liquid nitrogen. The samples of reduced enzyme plus NO were prepared as above, except that the enzyme was first degassed once and a solution of 40 mM sodium ascorbate and 4 mM PPD was added from a side arm on the EPR tube. The samples were then made anaerobic as before, and NO was added.

Optical spectra were recorded at room temperature on a Cary 219 spectrometer. EPR spectra were recorded on a Varian E-line Century Series X-Band spectrometer equipped with an Air Products Heli-Trans low-temperature system. For integration or spectral subtractions, spectra obtained under nonsaturating conditions were collected on a Spex Industries SC-32 SCAMP data processor interfaced to the spectrometer. Intensities were obtained by monitoring the low-field portion of the copper EPR signals according to the method of Aasa & Vänngård (1975). EPR spectra were recorded at 40 K unless otherwise noted, since at this temperature the EPR signal at $g = 2$ due to matrix-bound NO is not observed (Stevens et al., 1979).

For NMR and mass spectrometry, samples were prepared in a 5-mL glass bulb fitted with a ground-glass stopcock. A magnetic stir bar was included to continuously mix the sample with the NO gas. For mass spectroscopy, the gas above the sample was fed directly through a ground-glass inlet into a Du Pont 21-492B mass spectrometer. With this procedure, only the gaseous nitrogen compounds NH_3, N_2, NO, and N_2O could be monitored, since other gaseous nitrogen compounds, in particular NO_2, are not stable at room temperature in the presence of water. In these experiments, atmospheric CO_2 is the major contaminant and would have interfered with the observation of the $^{14}N_2O$ parent peak. To alleviate this problem and to allow ^{15}N NMR determination of the soluble products, we used ^{15}NO in our experiments. In each experiment, a blank was also prepared which was identical with the sample except that enzyme was omitted. Quantitation of $^{15}N_2O$ produced was determined relative to the ^{15}NO parent peak.

After mass spectral analysis, the degassed solutions were transferred to 10-mm NMR tubes for complementary analysis of soluble products by ^{15}N NMR. D_2O (15%) was added as an internal lock. The ^{15}N NMR spectrum of the solution was recorded at 25 °C with a Bruker WM500 NMR spectrometer. Precise quantitation of $^{15}NO_2^-$ and $^{15}NO_3^-$ was not possible because the spectra were acquired under partially saturating conditions.

Results

The type 1 and type 3 copper centers of the laccases may be monitored by the optical absorbances near 610 and 330 nm, respectively. EPR spectroscopy allows complementary monitoring of the oxidation state of the type 1 and type 2 copper centers. The reduced forms of all three copper centers are optically and EPR silent. We have used both techniques to follow the changes in the copper centers when the reduced and oxidized laccases are incubated with NO.

Reduced Fungal Laccase plus NO. Reduction of anaerobic fungal laccase by ascorbate with PPD as a mediator is rapid and complete. Addition of NO to the fully reduced enzyme results in no change in absorbance at either 610 or 330 nm, even after 10 h. However, a weak absorption band at 420 nm ($\epsilon = 400\ M^{-1}\ cm^{-1}$) appears rapidly and does not change with time.

The EPR spectra of reduced fungal laccase incubated with 1 atm of NO for up to 10 h at 0 °C show no indication of oxidation of the type 1 or type 2 copper centers. However, a new signal appears near $g = 2$, representing one to two spins per enzyme molecule. This signal, shown in Figure 1A, reaches full intensity within 1 min and does not change with time thereafter. Subsequent degassing of the NO from the solution results in complete loss of this signal. NO added to a blank without enzyme yields no EPR signals at 40 K.

To determine the origin of this new EPR signal, we examined the EPR spectra of the reduced enzyme with isotopically substituted nitric oxide. As seen in Figure 1B, substitution of ^{15}NO ($I = {}^1/_2$) for ^{14}NO ($I = 1$) results in distinct changes in the structure of this signal. For comparison, the spectrum of bovine serum albumin (BSA) incubated with 1 atm of ^{14}NO is shown in Figure 1C. This spectrum is very similar to that exhibited by reduced fungal laccase plus NO, except that it shows no resolvable hyperfine structure. Substitution of ^{15}NO

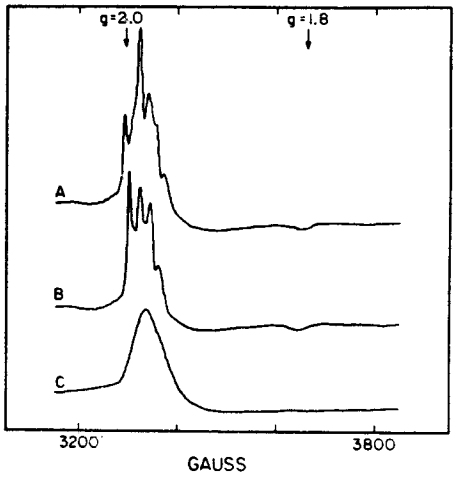

FIGURE 1: EPR spectra of (A) 0.35 mM fungal laccase, pH 6.0, reduced with 4.0 mM ascorbate and 0.4 mM PPD and then incubated 10 min with 1 atm of ^{14}NO, (B) fungal laccase prepared as in (A) but incubated with 1 atm of ^{15}NO, and (C) 0.30 mM BSA incubated 10 min with 1 atm of ^{14}NO. The conditions were as follows: temperature, 40 K; microwave power, 80 mW; modulation amplitude, 5 G; and microwave frequency, 9.22 GHz.

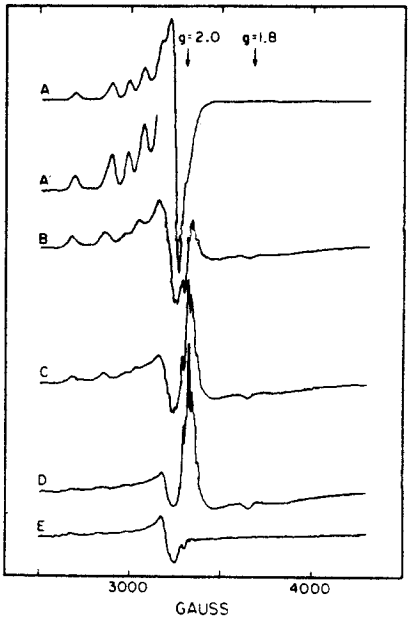

FIGURE 2: EPR spectra of oxidized fungal laccase incubated with 1 atm of NO for various lengths of time. (A and A') 0.35 mM oxidized fungal laccase, pH 6.0; (B) sample A made anaerobic and then mixed with NO for 30 s; (C) sample B mixed and incubated 90 s longer; (D) sample C mixed and incubated 2 h; (E) sample D degassed, with NO replaced by Ar. The conditions were as follows: temperature, 40 K; microwave power, 0.2 mW; modulation amplitude, 10 G; and microwave frequency, 9.22 GHz. Spectrum A was recorded at half the gain of the other spectra.

for ^{14}NO in the BSA sample resulted in only a very slight narrowing of the signal near $g = 2$.

Oxidized Fungal Laccase plus NO. Anaerobic incubation of oxidized fungal laccase with NO at pH 6.0 results in the reduction of the type 1 and type 3 copper centers as followed optically at 610 and 330 nm, respectively. The reduction of both centers follows pseudo-first-order kinetics at room temperature, with $t_{1/2} = 2$ min. A weak absorption band at 420 nm is seen as the enzyme becomes reduced.

The EPR spectra obtained at various times after addition of NO to the anaerobic enzyme solution are shown in Figure 2. The rapid reduction of the type 1 copper observed optically is also seen by EPR spectroscopy. The type 2 copper site is

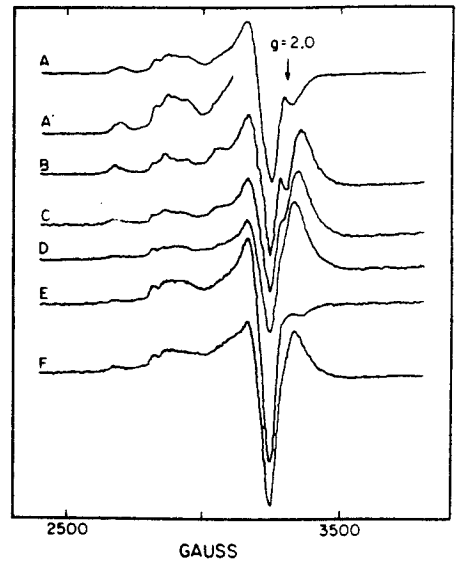

FIGURE 3: EPR spectra of oxidized tree laccase incubated with 1 atm of NO for various lengths of time. (A and A') 0.25 mM oxidized tree laccase, pH 7.0; (B) sample A made anaerobic, mixed with NO, and incubated 5 min; (C) sample B mixed and incubated 30 min; (D) sample C incubated with occasional mixing for 4 h; (E) as in (B), but incubated 20 h without mixing; (F) sample E mixed briefly. The conditions were as follows: temperature, 40 K; microwave power, 0.2 mW; modulation amplitude, 10 G; and microwave frequency, 9.22 GHz. Spectrum A was recorded at half the gain of the other spectra.

also reduced, but more slowly ($t_{1/2} \approx 10$ min). Additionally, the new signal near $g = 2$ seen in the reduced enzyme plus NO also appears slowly with time. After ~1 h of incubation, the type 1 and type 2 copper centers appear almost completely reduced, and the new signal reaches a maximum, as seen in Figure 2D. Subsequent degassing of NO from this sample results in complete loss of the new NO signal with no increase in the copper signals (Figure 2E). Finally, admission of air to the degassed sample results in the complete reoxidation of all four copper centers as measured by both EPR and optical spectroscopy (data not shown).

Essentially the same results were obtained at pH 5 and 7.4. However, at the latter pH, we have sometimes observed an EPR signal attributable to type 3 copper (Reinhammar et al., 1980), after ~1 of incubation with NO. This signal becomes about equal in intensity to the type 2 copper signal but never exceeds 10% of the type 3 copper centers, and its appearance is not reproducible.

Incubation of the enzyme with either 1 or 25 equiv of fluoride, which is known to bind tightly to the type 2 copper, prior to addition of NO, did not affect the reduction of the type 1 copper seen by EPR. The type 2 copper was reduced more slowly in the presence of fluoride.

Oxidized Tree Laccase plus NO. As with oxidized fungal laccase, NO can reduce the oxidized lacquer tree enzyme, as seen by optical spectroscopy. Reduction of the type 1 and type 3 copper centers again occurs simultaneously and follows pseudo-first-order kinetics. However, the reaction is much slower than that of the fungal enzyme, with $t_{1/2} = 70$ min. As with fungal laccase, a weak absorbance appears at 420 nm as the enzyme is reduced; this band is also observed when NO is added to the reduced tree laccase.

Attempts to follow the time course of the reaction by EPR spectroscopy were complicated by the fact that freezing of the sample causes partial bleaching of the residual type 1 blue copper color. The EPR spectra shown in Figure 3A–E confirm that the reduction of the type 1 copper upon freezing is greater than that observed at the same time point in the optical studies.

Subsequent thawing of the EPR samples results in the reappearance of the type 1 blue color. As with the fungal enzyme, EPR studies show a slow reduction of the type 2 copper; for the tree laccase, the time course of the type 2 copper reduction corresponds fairly closely to that of the type 1 copper.

Also seen in Figure 3 is a broad, structureless EPR signal near $g = 2$. This signal resembles that seen in the spectrum of the fungal enzyme with NO, but is featureless. After 20 h of incubation of tree laccase with NO without mixing, the NO signal near $g = 2$ completely disappears, as seen in Figure 3E. EPR spectra at 20 K (data not shown) also display no signal from matrix-bound NO (see Materials and Methods). The type 1 copper signal also increases, after long incubation of tree laccase with NO, to ~50% of the intensity exhibited by the oxidized enzyme (Figure 3E). The type 2 copper remains mostly reduced. Thawing of this sample, followed by brief mixing (~1 min), results in the return of the NO signal to full intensity (Figure 3F). This is accompanied by a small increase in the type 2 copper EPR signal and a slight decrease in the type 1 copper signal.

To determine whether the depletion of NO in solution upon long incubation with tree laccase is due to a turnover of the enzyme similar to that which has been observed for cytochrome *c* oxidase (Brudvig et al., 1980), we looked for evidence of products due to the oxidation and reduction of NO. NO can be oxidized by either the one- or two-electron process

$$NO + H_2O \rightarrow NO_2^- + 2H^+ + e^- \quad (1)$$

$$NO + H_2O \rightarrow NO_2 + 2H^+ + 2e^- \quad (2)$$

NO_2 is unstable, however, in the presence of water and readily disproportionates via the reaction

$$2NO_2 + H_2O \rightarrow NO_2^- + NO_3^- + 2H^+ \quad (3)$$

Accordingly, the production of nitrite and/or nitrate was examined by incubating 2.0 mL of tree laccase with ^{15}NO as described under Materials and Methods. After incubation of the enzyme with NO at 0 °C for 37 h, ^{15}N NMR spectroscopy of the sample solution and the appropriate blank showed substantial production of $^{15}NO_2^-$ relative to the blank. Only a small amount of $^{15}NO_3^-$ was detected (<10% of the $^{15}NO_2^-$ produced). The amount of $^{15}NO_2^-$ produced is too great to be accounted for by a single reduction of the enzyme and must therefore be explained on the basis of enzyme turnover.

The only likely pathway for reoxidation of the enzyme is via the two-electron reduction of NO

$$2NO + 2H^+ + 2e^- \rightarrow N_2O + H_2O \quad (4)$$

To ascertain whether any N_2O had been produced by the enzyme's reaction with NO, we analyzed the atmosphere above the NMR samples by mass spectroscopy. The only significant difference between the mass spectra of the enzyme sample and the blank was a large parent peak at $m/e = 46$. The ratio of intensities of this peak to the ^{15}NO peak at $m/e = 31$ was 0.002 for the blank and 0.085 for the enzyme sample, confirming production of $^{15}N_2O$ in the latter.

Reduced Tree Laccase plus NO. To confirm the reduction of NO to N_2O by the reduced enzyme, we incubated 1.0 mL of tree laccase with ^{15}NO as before, but with the addition of 4 mM ascorbate and 0.4 mM PPD to provide rapid reduction of the enzyme. After 26 h of incubation of the enzyme with NO, mass spectral analysis confirmed the production of $^{15}N_2O$ by the enzyme. The intensity of the peak at $m/e = 46$ relative to the ^{15}NO peak was 0.025 for the enzyme sample vs. 0.009 for the blank. Detailed analysis of the mass spectral data indicates that the rate of production of $^{15}N_2O$ is 2–3 times

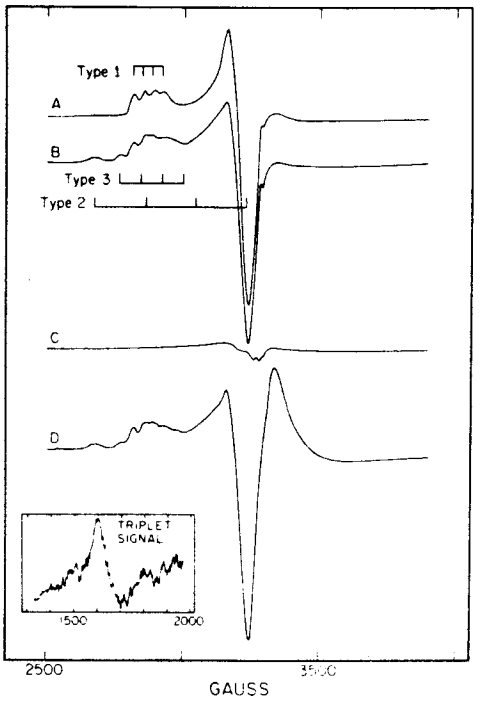

FIGURE 4: EPR spectra of reduced tree laccase incubated with 1 atm of NO for various lengths of time. (A) 0.25 mM tree laccase, pH 7.0, reduced with 4.0 mM ascorbate and 0.4 mM PPD, then mixed with 1 atm of NO, and incubated 1 min; (B) sample A incubated for 30 min without mixing; (C) sample B incubated 21 h without mixing; (D) sample C mixed briefly. The conditions were as follows: temperature, 40 K (A and B) and 20 K (C and D); microwave power, 0.2 mW; modulation amplitude, 10 G; microwave frequency, 9.22 GHz; and gain, 1.0×10^4 (A and B) and 8.0×10^3 (C and D). The inset shows the low-field region of (D), averaged over 10 scans, with conditions as above except temperature, 11 K, microwave power, 20 mW, and gain, 2.5×10^4.

faster in the presence of external reductant than in its absence. The half-time for the reaction in which N_2O is produced, with the assumption that the reduction of the enzyme by ascorbate and PPD is much faster than its reoxidation by NO, is calculated from the mass spectral data to be ~90 min.

Optical spectroscopy of the reduced tree enzyme in the presence of NO shows that the bands at 330 and 610 nm are absent. However, upon freezing of this sample (either in liquid nitrogen or at −20 °C), the type 1 blue color returns to about full intensity. EPR spectroscopy verifies that reoxidation of the type 1 copper center has taken place and is complete (Figure 4A). Note that in this experiment a pure type 1 copper EPR spectrum has been observed from native tree laccase without interference from the other copper centers! Upon thawing, the type 1 copper center is completely reduced again within 1 min, as monitored optically at 610 nm. It was not possible to monitor the type 3 copper absorbance at 330 nm due to the appearance of a large absorption band (ϵ = 20 000 M^{-1} cm^{-1} relative to enzyme concentration) centered at 352 nm. This absorption was also observed in an identical blank without enzyme, if a small amount of O_2 was admitted; therefore, it was not studied further.

To see whether the oxidation of the type 1 copper upon freezing of the reduced enzyme with NO might be due to a change in solution pH upon freezing, we repeated this experiment substituting Hepes buffer (25 mM) for phosphate. Hepes buffer had been reported to minimize pH changes upon freezing of solutions (Williams-Smith et al., 1977). The same EPR spectrum, identical with that of Figure 4A, is observed for two different preparations of the enzyme in either 25 mM Hepes, pH 7.0, or 25 mM phosphate, pH 7.0; an identical EPR

spectrum is seen for a sample at pH 6.0 in 25 mM phosphate buffer. However, when the enzyme solution is frozen as a glass by using 30% ethylene glycol, *no* reoxidation of the type 1 copper takes place; the frozen solution remains colorless.

The reduced enzyme solution plus NO can be frozen and thawed many times, and the same effects observed. However, if a sample of reduced tree laccase plus NO is incubated on ice without mixing, some changes are seen in the EPR spectrum. The NO peak, whether observed at 40 or 20 K, gradually decreases; at the same time, EPR signals of approximately equal intensity gradually appear from the type 2 and 3 coppers (Figure 4B). Eventually, the NO peak completely disappears, as seen in Figure 4C; concomitantly, the type 1 and 2 copper centers are almost totally reduced. At this stage, the solution is colorless (except for some blue color near the meniscus), and it remains colorless when frozen. When a sample which has been incubated for a long time without mixing is then thawed and briefly shaken to dissolve NO in solution again, an EPR spectrum such as that shown in Figure 4D results. The NO peak is restored to full intensity, and the type 1, 2, and 3 coppers exhibit signals of intensity ranging from 30 to 100% of full intensity (70% for type 1, 100% for type 2, and 30–50% for the type 3 copper, approximately). Computer subtraction of the type 1 and type 2 EPR signals shows that the type 3 signal is identical with that observed by Reinhammar et al. (1980) for tree laccase. Furthermore, a weak signal is now observed at $g = 4.0$, as shown in the inset to Figure 4. We have also sometimes observed this signal in the samples after long incubation but *before* remixing. The signal at $g = 4.0$ is most easily observed at low temperature (<20 K) and high power and is not saturated at 80 mW at 20 K (in contrast, at 20 K the copper signals in the oxidized enzyme show the onset of saturation at 0.2-mW power).

If the reduced tree laccase turns over in the presence of NO, the reductant should eventually be consumed. This was found to be the case; when the reduced enzyme plus NO is incubated at 0 °C and frequently mixed to keep NO in solution, the enzyme solution eventually turns blue and remains so thereafter. EPR spectroscopy shows that, at this point, the sample behaves identically with one made by adding NO to the oxidized tree laccase.

From eq 1, it might be expected that NO_2^- would react with the reduced laccases to produce the NO derivatives. However, no change was seen in EPR spectra of either reduced fungal laccase (pH 6.0) or reduced tree laccase (pH 7.0) after 16 h of incubation with 2 mM NO_2^- at 0 °C.

Three-Copper Tree Laccase plus NO. We also examined the interaction with NO of tree laccase depleted of type 2 copper. As with the oxidized native enzyme, NO reduced the type 1 copper center with $t_{1/2} = \approx 40$–50 min. EPR spectra (not shown) show that the type 1 copper is rapidly reduced on freezing, similarly to the native tree laccase plus NO. However, the type 3 copper center does not appear to be reduced by NO, as seen optically, and no absorption band is seen at 420 nm. After 21 h of incubation with NO without mixing, the type 1 copper is completely reduced, as in the native tree laccase, but in contrast to the results obtained with the native enzyme, there is no observable depletion of NO under these conditions (Figure 5).

The type 1 copper center of three-copper tree laccase can be readily reduced by PPD and ascorbate; however, the type 3 copper center requires ~24 h of incubation for complete reduction (Reinhammar & Oda, 1979). The partially reduced species can therefore be made by short incubation with reductant. Addition of NO to this species again results in no

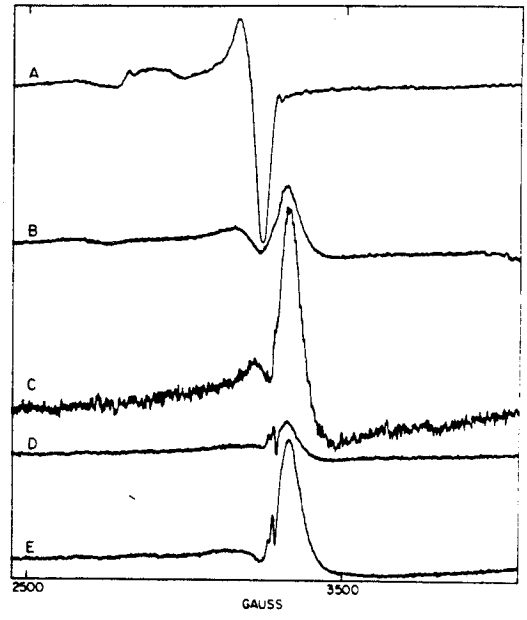

FIGURE 5: EPR spectra of three-copper tree laccase in the presense of NO. (A) 0.25 mM oxidized tree laccase depeleted of type 2 copper (see Materials and Methods) in 25 mM phosphate, pH 7.0; (B) sample A with 1 atm of NO mixed in, incubated 21 h without mixing; (C) as in (A), but reduced 1 min with 0.7 mM ascorbate and 0.07 mM PPD, then NO added, and the sample mixed 2 min; (D and E) as in (A), but reduced 40 h with 0.7 mM ascorbate and 0.07 mM PPD, then NO added, and the sample mixed and incubated 21 h without further mixing. The conditions were as follows: temperature, 40 K (A–D) and 20 K (E); microwave power, 0.2 mW; modulation amplitude, 10 G; and microwave frequency, 9.22 GHz. Spectrum C was recorded with a 4-fold higher gain than the other spectra.

significant reduction of the type 3 copper, as seen optically. Furthermore, freezing of this sample does not result in any significant oxidation of the type 1 copper (Figure 5C), as seen with the fully reduced native enzyme.

The fully reduced three-copper enzyme incubated with NO also shows no significant oxidation of the type 1 copper, either at room temperature as monitored optically or when frozen as observed by EPR spectroscopy (Figure 5D). Again, no absorption band is visible at 420 nm in this sample. It is also interesting to note that this product shows very little enzyme-bound NO EPR signal near $g = 2$. Incubation of the fully reduced species with NO for 21 h without mixing (Figure 5E) again shows no depletion of the EPR signal due to NO in solution.

Discussion

The reaction of NO with oxidized tree laccase has previously been studied by Rotilio et al. (1975), who observed only reduction of the type 1 copper. However, since they did not report the lengths of incubation of the enzyme with NO, it is difficult to compare their results with our own.

The EPR experiments summarized in Figures 3 and 4 demonstrate that oxidized tree laccase can be reduced by NO and, conversely, the reduced enzyme can be oxidized by NO. The most likely route for the oxidation of the reduced enzyme is via the reduction of NO to N_2O (reaction 4). The detection of $^{15}N_2O$ by mass spectroscopy after incubation of reduced tree laccase with ^{15}NO confirms that the oxidation of the reduced enzyme occurs via reaction 4. The reduction of the oxidized tree laccase by NO could occur via the oxidation of NO to NO_2^- (a one-electron oxidation) or to NO_2 (a two-electron oxidation) (reactions 1 and 2). Since NO_2 in solution disproportionates to NO_2^- and NO_3^- (reaction 3), the observation by ^{15}N NMR spectroscopy of only a small amount of

$^{15}NO_3^-$, compared to the amount of $^{15}NO_2^-$ observed, as reaction product of oxidized tree laccase with ^{15}NO implies that the reduction of the enzyme occurs primarily in conjuction with the one electron oxidation of NO to NO_2^-.

Both the oxidation and reduction of tree laccase by NO occur very slowly. The half-time for reduction of the type 1 and type 3 copper centers, as measured by optical spectroscopy, was found to be 70 min; the mass spectroscopy data on the reaction of reduced tree laccase with NO, which indicate ~15 turnovers in 26 h, imply that $t_{1/2}$ for the reaction in which N_2O is produced is ~90 min, on the same order as for the reaction of the oxidized enzyme with NO.

The reaction of oxidized fungal laccase with NO, on the other hand, is much faster. The optical data show that the type 1 and type 3 copper centers are reduced with $t_{1/2} = 2$ min, and the EPR data indicate that the type 2 copper is reduced with $t_{1/2} \approx 10$ min. Furthermore, in contrast to the tree laccase, the reduced fungal enzyme gives no indication of a reaction with NO, even after long incubation. We ascribe this difference in reactivity of the reduced laccases with NO to the difference in their reduction potentials. Fungal laccase, with its extremely high reduction potentials, is a much poorer reductant than tree laccase, so it is not surprising that reduced fungal laccase cannot be oxidized by NO.

Because the reduced fungal laccase did not react with NO, no cyclic reaction analogous to those observed with the tree laccase (in which NO served as oxidant and either NO or ascorbate served as reductant) could take place. Hence, although it seems likely that the reduction of fungal laccase by NO is also accomplished by production of NO_2^-, we were unable to verify this directly.

We also investigated the interactions of NO_2^- with both laccases. Nitrite is known to be reduced to NO by cytochrome *c* oxidase (Brudvig et al., 1980) and nitrate reductase (Wharton & Weintraub, 1980); however, we found no evidence for this reaction with reduced tree or fungal laccase. The lack of reactivity of the laccases with NO_2^- is more likely due to kinetic rather than thermodynamic constraints.

In addition to the *reaction* of NO with tree and fungal laccase, we have also obtained evidence for the specific *binding* of NO, particularly to fungal laccase. The peak observed by EPR spectroscopy at $g = 2$ upon addition of NO to reduced fungal laccase is somewhat similar to that seen in a sample of NO dissolved in buffer solution. The signal differs, however, in that with fungal laccase (1) well-defined hyperfine structure can be seen on the EPR signal at $g = 2$, (2) there is an inflection at $g = 1.8$ associated with the peak, and (3) the signal is observed at temperatures as high as 80 K, whereas no EPR signal is observed from NO dissolved in buffer solution at 40 K. In contrast to the NO signal seen with fungal laccase, the corresponding signal seen with tree laccase is featureless and resembles that seen upon addition of NO to a BSA solution. This latter signal differs, however, from that of NO dissolved in buffer solution, in that it is observable at 40 K. This featureless NO peak, which probably also contributes to the NO signal seen with fungal laccase, is most likely due to a weak association of NO with the protein, perhaps at a hydrophobic region.

The NO signal seen with fungal laccase exhibits structure which changes upon substitution of ^{15}NO for ^{14}NO (Figure 1). The observed structure is thus due to hyperfine interaction between the unpaired electron spin and the NO nitrogen nuclear spin. The signal is remarkably similar to one seen upon addition of NO to soybean lipoxygenase (Galpin et al., 1978), which contains a single iron atom per enzyme molecule, and

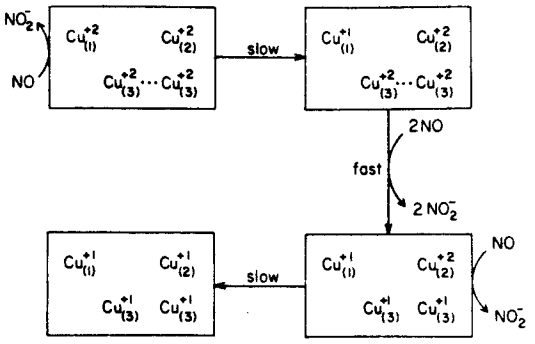

FIGURE 6: Proposed mechanism for the reduction of tree or fungal laccase by NO.

also bears some resemblance in its structural features to an immobilized nitroxide spin-label (Williams et al., 1971). We assign the signal in Figure 1 to NO bound to a reduced copper site. The observed *g* anisotropy is not inconsistent with a bound NO molecule of this structure. The high potential of the fungal laccase copper centers (whichever one the NO is bound to) would prevent a charge transfer from copper to NO, which might otherwise be expected to lead to oxidation of the copper center, as is the case for the tree enzyme.

It is most likely that the NO is bound to the reduced type 2 copper center, since the EPR signal due to this center is observed to decrease concomitantly with the increase in the EPR signal due to the specifically bound NO. The reduction of the type 1 and 3 copper centers by NO occurs considerably more quickly than the appearance of the new NO signal. The type 2 copper center is known to bind exogenous ligands such as F^- and CN^- (Reinhammar, 1979), so it is not surprising that it could bind NO. The 420-nm band we observed when NO was present with either reduced tree or fungal laccase may also be due to an association of NO with the type 2 copper, since the band does not appear in the type 2 copper depleted enzyme plus NO.

The reductions of the type 1 and 3 copper centers by NO occur simultaneously, in both the tree and fungal laccases. In contrast, when fungal laccase is reduced by hydroquinone or ascorbate, the type 1 copper is reduced considerably faster than the type 3 copper, and the rate-limiting step in the latter reduction appears to be an intramolecular electron transfer (Andréasson et al., 1973). Similar results have been obtained for the anaerobic reduction of tree laccase by hydroquinone (Andréasson & Reinhammar, 1979). The slow and simultaneous reduction of the type 1 and 3 copper sites by NO indicates that the first step in the reaction, whether it be reduction of the type 1 or 3 copper center, must be the slow step; the reduction by NO of the second site then follows rapidly. Such a sequence might occur in a number of ways, but the results of adding NO to oxidized, type 2 copper depleted tree laccase suggest a particular mechanism. In the latter experiment, only the type 1 copper center was reduced by NO, with $t_{1/2}$ slightly less than with the normal oxidized enzyme; the type 3 copper center remained oxidized even after long incubation with NO. The simplest mechanism that can account for the observations made with the normal and type 2 copper depleted tree laccase (and the fungal laccase) is shown in Figure 6. The first steps are the slow reduction of the type 1 copper center, probably by an outer-sphere electron transfer, followed by the fast reduction of the type 3 copper site. Apparently, the reduction of the type 3 copper center requires the presence of the type 2 copper center. It may be that, following the reduction of the type 1 copper, two NO molecules in succession are rapidly oxidized to NO_2^- at the type 2 copper,

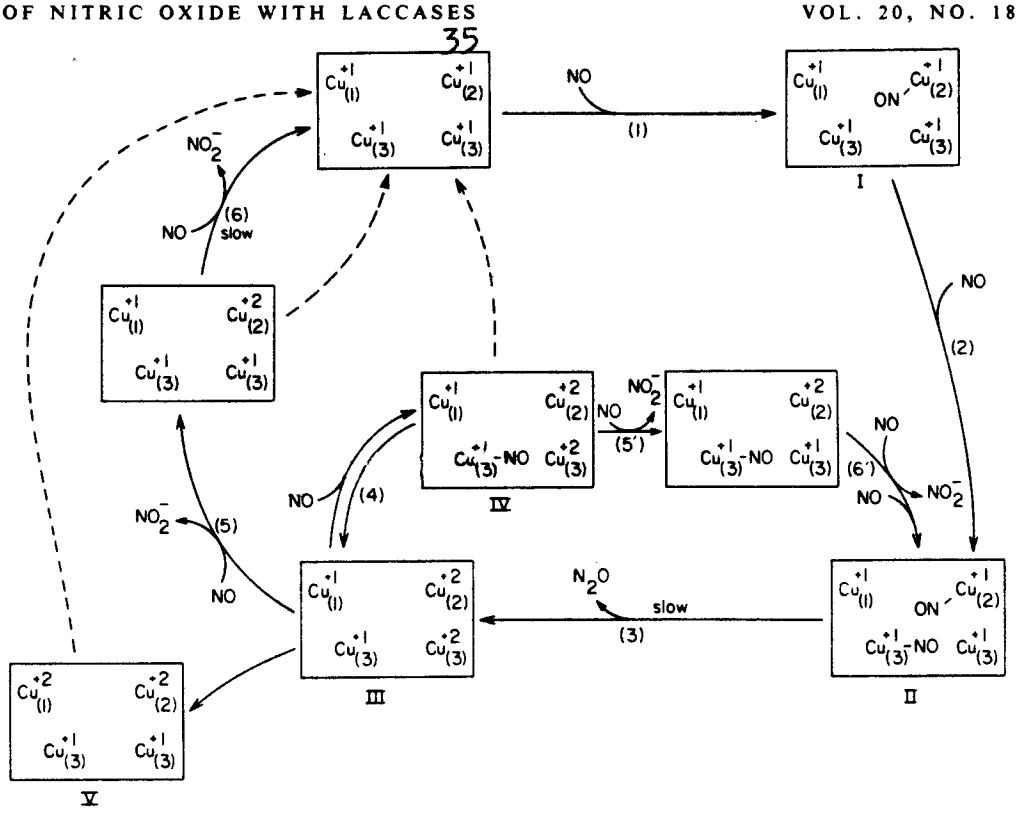

FIGURE 7: The proposed reaction cycle which occurs when tree laccase is incubated with NO. The dotted lines show paths which may be taken in the presence of ascorbate.

and two electrons are quickly transferred to the type 3 site. Alternatively, the two electrons can be transferred to the type 3 site from the type 1 and type 2 coppers immediately after one NO molecule is oxidized to NO_2^- at the type 2 site. Our data do not permit us to distinguish between these two pathways. In any event, is is likely that after the initial reduction of the type 1 site, the next two electrons enter via the type 2 copper, since there is no precedent for the reactivity of the type 1 site varying with the redox states of the type 2 or 3 sites. On the other hand, stopped-flow experiments on the anaerobic reduction of tree laccase indicate that the reactivity of the type 2 copper site varies with the redox state of the type 1 copper (Andréasson & Reinhammar, 1979). We find that the reactivity of the type 2 copper is dependent on the redox states of both the type 1 and 3 coppers. The final reduction of the type 2 center was found to be quite slow, in agreement with earlier kinetic experiments showing that anaerobic reduction of the type 2 copper becomes much slower once the type 1 and 3 copper centers have been reduced in either tree laccase (Andréasson & Reinhammar, 1979) or fungal laccase (Brändén & Reinhammar, 1975).

The foregoing scheme deals only with the reduction of the oxidized tree (or fungal) laccase by NO. We have shown, however, that NO can also oxidize the reduced tree laccase. We now develop a scheme for the complete cyclic reaction in which tree laccase is both oxidized and reduced by NO. Such a scheme must embrace the following observations: (1) when ascorbate is used as reductant with PPD as mediator, $t_{1/2}$ for the reaction in which N_2O is produced is ~90 min; (2) in the absence of ascorbate or PPD, so that the enzyme is reduced by NO, the production of N_2O is 2–3 times slower; (3) the blue color remaining in the oxidized tree laccase plus NO after a long incubation accounts for 10–15% of the original optical absorbance at 610 nm in the oxidized enyzme (when the enzyme is kept at 4 °C); (4) upon long incubation with NO, the reduced enzyme exhibits small type 2 and type 3 copper EPR signals of about equal intensity; (5) only the type 1 copper of the type 2 copper depleted tree laccase is reducible by NO; and (6) reduced type 2 copper depleted tree laccase gives no indication of being oxidized by NO, even after long incubation.

A simple reaction scheme which accounts for the above observations is depicted in Figure 7. This scheme emphasizes the requirement for the type 2 copper in both the oxidation and reduction of laccase by NO, as dictated by the results obtained by using the type 2 copper depleted tree laccase. Beginning with the fully reduced enzyme, we show in steps 1–3 the binding of NO to reduced type 2 (species I) and 3 (species II) copper sites and the reaction to produce N_2O, respectively. At least one of these steps, most likely step 3, must be slow. The reaction of step 3 almost certainly involves two bound molecules of NO, as shown. First, we know that the type 2 copper is required for this step from the results obtained with the type 2 copper depleted enzyme. Second, there is no precedent for an exogenous ligand binding to a type 1 copper in any enzyme, and it is unlikely that the second molecule of NO could come from solution, since this would result in a two-electron reduction taking place at a single copper atom.

Species III, shown in Figure 7 following step 3, in which the type 2 copper and one type 3 copper are oxidized, is most likely reponsible for the type 3 EPR signals that we have observed (Figure 4). A molecule of NO binding to the type 3 site in this half-oxidized enzyme would result in the appearance of species IV, which could be expected to exhibit, in addition to a type 2 EPR signal, a triplet EPR signal due to a weak coupling between the NO molecule bound to the reduced type 3 copper and the remaining oxidized type 3 copper atom. This triplet species would account for the "half-field" EPR signal observed at $g = 4.0$. Compared to the type 3 signal exhibited by reduced tree laccase in the presence of NO, which is almost as large as the type 2 signal, the triplet signal is quite small (Figure 4), suggesting that species IV accounts for only a small fraction of the enzyme molecules. From this result, we infer that the equilibrium III

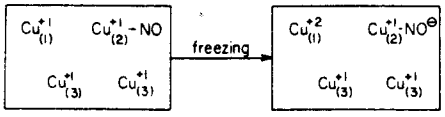

FIGURE 8: Proposed mechanism for the oxidation of the type 1 copper of tree laccase upon freezing of the reduced enzyme in the presence of NO.

⇌ IV is toward the left. Presumably this equilibration is rapid.

Both forms of the half-oxidized enzyme, namely, species III and IV, are expected to be reduced in two successive one-electron oxidations of NO (steps 5, 5′, 6, and 6′) to give the fully reduced enzyme and complete the reaction cycle. Steps 5 and 5′, which result in the enzyme being three-fourths reduced, should be fast, to maintain consistency with our earlier results on the reduction by NO of the oxidized enzyme (Figure 6). The relatively high reactivity of the redox state of the enzyme in which the type 3 site is only half-reduced is probably related to the specific function of that site, which is to react with O_2, when both coppers are reduced, to yield peroxide as the first intermediate in oxygen reduction (Malmström, 1981). The reduction of the type 2 copper shown in steps 6 and 6′ again is presumably slow.

Finally, to account for the 10–15% of the blue copper observed even after long incubation of the oxidized tree enzyme with NO, we introduce species V. Since both the type 1 and 2 coppers are oxidized in this species, the type 2 copper center is expected to be relatively inert toward NO; hence, species V is not shown to be active in the redox cycle.

The observations made upon freezing reduced tree laccase in the presence of NO can also be rationalized by this scheme. If ascorbate is present, the half-oxidized enzyme will be rapidly reduced, so that species I and/or II in the reaction cycle predominates in the steady state. Upon freezing of the solution, we propose that the species I and II of the enzyme undergo a conformational change which increases the relative reduction potential of the type 2 copper center and facilitates electron transfer between the type 1 and 2 copper sites.

Transfer of an electron from the reduced type 1 copper site to the NO which is bound to the type 2 copper, as shown in Figure 8, would result in the restoration of the type 1 copper EPR signal to full intensity. Note that this observation implies that step 1 of Figure 7 is fast. When the solution is thawed again, the enzyme slowly returns to its original conformation, so that after ~90 s the solution again becomes colorless. The involvement of the type 2 copper center is implicated by studies on the reduced type 2 copper depleted enzyme, which remains colorless upon freezing. Here, of course, there can be no $Cu_{(2)}^{+}$–NO unit to which an electron can be transferred. Furthermore, when a solution of reduced native tree laccase plus NO is frozen as a glass, the type 1 copper remains reduced. This could be due to our hypothesized conformational change being induced by a reordering of the liquid water solvent structure around the enzyme upon freezing, whereas the solvent structure in the frozen glass is not expected to induce this same conformational change.

The schemes of Figures 6–8 contain certain structural implications. First, the type 2 and 3 copper centers must be close, to allow the reaction of two bound NO's to produce N_2O as in Figure 7, step 3. These two copper centers must act in concert in the oxidation of tree laccase by NO. Second, NO appears able to bind at the type 2 copper site, as in Figure 8, and at the type 3 site, as indicated by the appearance of the triplet EPR signal in the experiments with reduced tree laccase plus NO, but we have obtained no evidence that NO binds at the type 1 copper.

The interactions of tree and fungal laccase with nitric oxide show some similarities but also some substantial differences from those which have been observed with other copper proteins, including cytochrome *c* oxidase. Hemocyanin (Schoot Uiterkamp, 1972; Schoot Uiterkamp & Mason, 1973) and tyrosinase (Himmelwright et al., 1980), both of which contain only a type 3 copper site, exhibit triplet EPR signals upon treatment with NO, as does ceruloplasmin (Van Leeuwen & Van Gelder, 1978; Van Leeuwen et al., 1973), which contains type 1, 2, and 3 copper centers. However, the triplet signal exhibited by tree laccase (Figure 4) differs from the triplet signals exhibited by these other enzymes, in that it is substantially narrower (<300 G) and does not exhibit the seven-line hyperfine splitting expected for an interacting pair of Cu(II) ions. Regarding the reactions of NO with copper-containing proteins, Van der Deen & Hoving (1977) have shown that the active site of hemocyanin can be singly oxidized by reaction with either nitrite or nitric oxide in the presence of ascorbate, and Verplaetse et al. (1979) have shown that the oxidation of reduced hemocyanin from *Helix pomatia* is accompanied by production of N_2O. The type 1 copper of ceruloplasmin can also be reduced by NO (Van Leeuwen & Van Gelder, 1978), but reaction products have not been characterized. Clearly, further work will be required before useful comparisons can be made regarding the reactions of the copper-containing proteins with NO.

The effect upon freezing of reduced tree laccase plus NO in our study is unique among the blue copper proteins. Morpurgo et al. (1981) recently reported a change in the *g* values and hyperfine splittings in the EPR spectrum of tree laccase upon freezing, which may be related to the effect of freezing on reduced tree laccase plus NO reported here. Reduced fungal laccase plus NO does not behave like the tree laccase upon freezing, but it may be that the much higher reduction potentials of the copper centers in the fungal laccase preclude an electron transfer similar to that of Figure 8.

Finally, cytochrome *c* oxidase is remarkably similar to tree laccase in its reactions with NO (Brudvig et al., 1980). In the presence of reductant, both cytochrome *c* oxidase and tree laccase are oxidized by NO to the half-oxidized state. In cytochrome *c* oxidase, the oxidized enzyme is also slowly reduced by NO and slowly reoxidized to form a cycle similar to that of Figure 7 deduced for tree laccase. However, all four metal centers of tree laccase (and fungal laccase), as we have seen, are completely reduced by NO, whereas only Cu_B and cytochrome a_3 of cytochrome *c* oxidase are reduced by NO. Furthermore, in the cyclic reaction of oxidized tree laccase with NO, all three copper centers are involved (Figures 6 and 7), whereas only the oxygen-binding site is involved in the analogous cycle of oxidized cytochrome *c* oxidase with NO. The similarity of cytochrome *c* oxidase to the laccases in its reactions with NO is undoubtedly related to its similar function of catalyzing the four-electron reduction of oxygen to water. The differences, on the other hand, may reflect the unique role of cytochrome *c* oxidase as an energy-conserving protein with proton-pumping capabilities (Chan et al., 1979) to create a transmembrane proton gradient during its catalytic cycle (Wikström & Krab, 1979).

Acknowledgments

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APPENDIX II

Preparation and Reactions of Aryldiazo Complexes of Cobalt(I). Formation of a Spin-Triplet Cobalt-Dioxygen Cation.

Gabriele Albertin,[1a] Emilio Bordignon,*[1a] Robert M. Kanne,[1b] and Harry B. Gray*[1b]

Contribution from the Istituto di Chimica Generale ed Inorganica dell'Universitá, 30123 Venezia, Italy and Contribution No. 6705 from the Arthur Amos Noyes Laboratory, California Institute of Technology, Pasadena, California 91125

Abstract. The reactions of a cobalt(I) aryldiazo complex, $[Co(4\text{-}CH_3C_6H_4N_2)L_4](BPh_4)_2$ ($L = PhP(OEt)_2$), with CO, NO, NO^+, CNPh, and O_2 have been investigated. The reaction with O_2 produces a pink complex, $Co(O_2)L_4^+$ ($\bar{\nu}(O_2)$ = 1224 cm^{-1}), which is a spin-triplet (μ_B = 3.4).

Preparation and Reactions of Aryldiazo Complexes of Cobalt(I). Formation of a Spin-Triplet Cobalt-Dioxygen Cation.

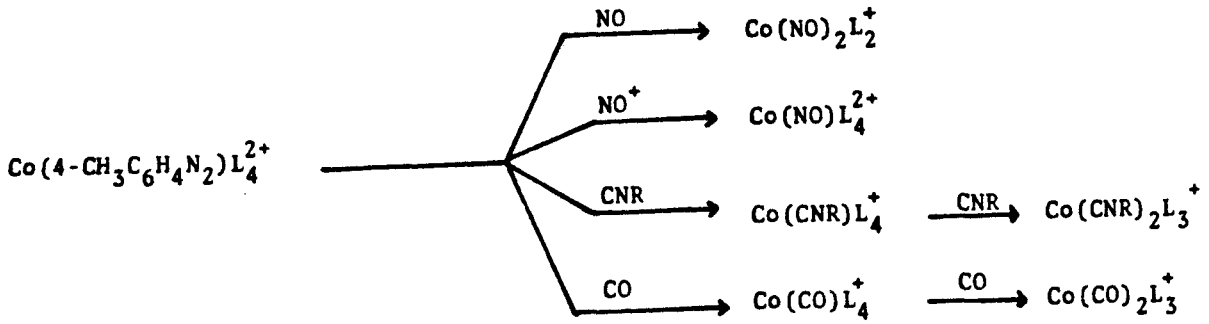
Gabriele Albertin,[1a] Emilio Bordignon,*[1a] Robert M. Kanne,[1b] and Harry B. Gray*[1b]

Contribution from the Istituto di Chimica Generale ed Inorganica dell'Universitá, 30123 Venezia, Italy and Contribution No. 6705 from the Arthur Amos Noyes Laboratory, California Institute of Technology

Previous investigations[2,3] of the coordination chemistry of aryldiazo cations have not included cobalt[4] as a central metal. In view of this absence we wish to report that in the course of our work on cobalt compounds[5] we have found that the monocarbonyl complex 1 (L = $PhP(OEt)_2$)[5a] reacts at room temperature in dichloromethane or acetone solution with an excess of diazonium salt to give a monoaryldiazo cobalt(I) derivative (2), according to eq. 1:[6,7]

$$\underset{\underline{1}}{Co(CO)L_4^+} + 4\text{-}CH_3C_6H_4N_2 \rightarrow \underset{\underline{2}}{Co(4\text{-}CH_3C_6H_4N_2)L_4^{2+}} + CO$$

The back reaction ($\underline{2} \xrightarrow{CO} \underline{1}$) also is facile, but $Co(CO)_2L_3^+$ is formed if excess CO is present. In general we have found that the aryldiazo cation in 2 is a good leaving group. Some reactions in which this is the case are outlined below (R = Ph or 4-substituted Ph):



Loss of N_2 accompanies heating or irradiating 2 in the solid state or in solution. Evolution of N_2 also occurs by treating 2 with HCl or $HClO_4$ (formation of aryldiimines was not observed by IR or NMR spectroscopy).[3a] Green solutions of 2 in CH_2Cl_2 react at room temperature with air or O_2 (1 atm) (absorption of 2 at 595 nm (ε = 164) is replaced by a band at 555 nm ($\varepsilon = 1.7 \times 10^3$)). Measurements of gas uptake and gas-chromatographic analysis both in the pink solution and in the corresponding vapor phase indicate that the dioxygen adduct is $Co(O_2)L_4^+$ (eq. 2):

$$\underset{\underline{2}}{Co(ArN_2)L_4^{2+}} \xrightarrow[CH_2Cl_2]{+O_2} \underset{\underline{3}}{Co(O_2)L_4^+} \xrightarrow{-O_2 \text{ (or } O_2^-)} \underset{\underline{4}}{[CoL_4^{+/2+}]}$$

Strong evidence that 3 is an authentic dioxygen adduct is our observation that it reacts with CO to give 1 in essentially quantitative yield. Attempts to crystallize a salt of 3, however, produced only a pink material that is an

oil at room temperature.[8] The EPR spectrum of 3 at 15 K shows a broad signal near g = 4.00 (Figure 1a), and susceptibility measurements on CH_2Cl_2 solutions of 3 at 307 K gave 3.4 μ_B (3.2 μ_B for the pink oil), in accord with expectation for a spin-triplet complex. Removal of O_2 from 3 yielded a mixture of Co(I) and Co(II) species (4; the EPR spectrum (Figure 1B) is that of a Co(II) species containing four equivalent P-donor ligands).

The infrared spectrum of 3 in CH_2Cl_2 solution exhibits an intense band at 1224 cm^{-1} that is attributable to $\bar{\nu}(O_2)$. This band also is observed in the IR spectrum of the pink oil, whereas it is absent in the spectrum of 1 (Figure 2). Moreover, its assignment to $\bar{\nu}(O_2)$ is strongly supported by the appearance of an analogous peak at 1160 cm^{-1} in the spectrum of a sample (3) prepared with $^{18}O_2$.

The spectroscopic and magnetic results strongly suggest end-on coordination for O_2 in 3.[9] Side-on $Co(O_2)^+$ complexes are spin-singlet peroxo species, with much lower energy (and much less intense $\bar{\nu}(O_2)$ absorptions.[10] It is not unreasonable to speculate that as cobalt(I)-dioxygen chemistry[11] is developed further that end-on binding will be preferred by systems containing bulky π-acceptor ligands. But we cannot rule out the possibility that $Co(O_2)L_4^+$ will turn out simply to be an isolated curiosity.

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4. Aryldiazo ligation to cobalt(0) has been proposed previously based only on an IR spectrum of an unstable material (Carrol, W. E.; Lalor, F. J. J. Organometal. Chem. 1973, 54, C37).

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6. Although the reaction is quantitative, the yield of pure solid compound is low (40 to 50%), because part of the product decomposes during crystallization. The BPh_4^- salt of 2 is a green crystalline material that is stable in the solid state and in polar organic solvents. Anal: ($C_{95}H_{107}N_2Co_1P_4O_8B_2$). Calcd: C, H, N: 70.90; 6.70, 1.74; Found: 71.04, 6.80, 1.74. 2 (BPh_4) is diamagnetic (solid state, Faraday method; solution, Evans method) and a 1:2 electrolyte (116.6 Ω^{-1} M^{-1} cm^2 in 10^{-3} M nitromethane solution at 25°C). The infrared spectrum of 2 (BPh_4) (Nujol mull, KBr pellet) shows two bands (1778(s) and 1764(s) cm^{-1}) that indicate the presence of a singly-bent aryldiazo group.[3] In CH_2Cl_2 solution the bands fall at 1780(s) and 1764(s) cm^{-1}, and a new absorption attributable to a linear isomer appears at 1950 cm^{-1}. The intensity of the 1950-cm^{-1} band increases for 10-20 min at the same time the absorptions at 1780 and 1764 cm^{-1} decrease. The intensity ratio of the two sets of bands depends on the nature of the solvent and on the concen-

tration. It is possible to obtain solid samples of 2 in good yield (90%) from a dichloromethane solution containing both the proposed isomers. The related IR spectra (KBr) indicate that the principal species in the sample is the bent isomer. The ^1H NMR spectrum of 2 in CD_2Cl_2 solution at room temperature exhibits two singlets (7.70 and 7.50 τ) in the methyl proton region. The intensity ratio of these signals changes with time in the same way as observed for the "$\bar{\nu}(N_2)$" bands. Furthermore, a triplet at 8.87 τ appears in the phosphite methyl region, which at -40°C is split into two triplets (8.67 and 8.92 τ). Finally, the phosphite signals do not change with time.

7. By means of a slightly modified procedure, $[CoL_4(N_2C_6H_4R)](BPh_4)_2$ (L = $PhP(OEt)_2$ and R = H, 3-CH_3, 4-CH_3O, 4-Cl, 4-F; L = $P(OEt)_3$ and R = 4-CH_3, 4-F) complexes have been prepared.
8. For example, a pink material separated when a pink CH_2Cl_2/n-pentane solution containing BPh_4^- was cooled to -40°C (at room temperature this material is an oil). Both the pink solution and the oil are stable for several days if they are kept at -30°C.
9. The assignment of oxidation levels in the end-on (possibly linear) $Co(O_2)^+$ ground state may not be meaningful, because extensive $d\pi$-π^*O_2 mixing is expected. Such mixing is likely to be important in determining the

intensity of the 555-nm absorption of 3, which may be attributed to $d\pi \rightarrow \pi^* O_2$ excitation (Lever, A. B. P.; Gray, H. B. Acc. Chem. Res. 1978, 11, 348).

10. In this context we suggest that the enhanced π-acceptor nature of L relative to a phosphine disfavors the extensive electron transfer from cobalt that accompanies side-on dioxygen binding. The diamagnetic $[Co(2\text{-phos})_2(O_2)]BF_4$ ($\bar{\nu}(O_2)$ at 909 cm^{-1} in $CHCl_3$ solution) is an example of side-on (peroxo-type) coordination (Terry III, N. W.; Amma, E. L.; Vaska, L. J. Am. Chem. Soc. 1972, 94, 653).

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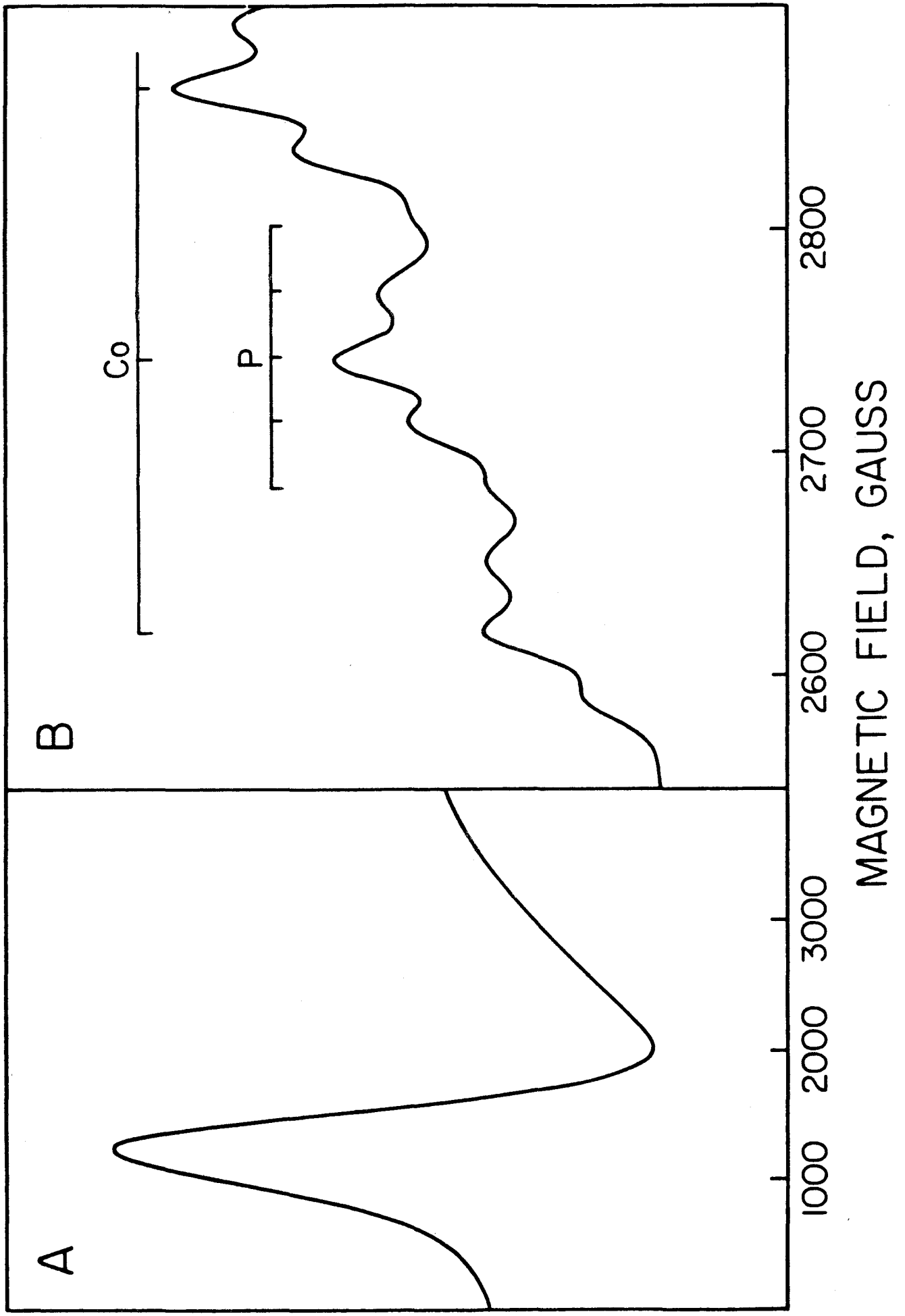
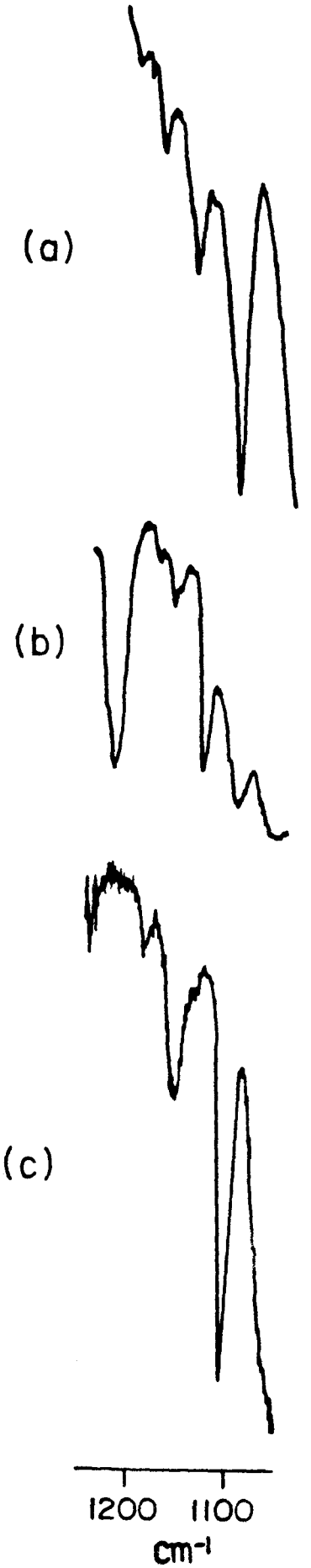
Figure Captions

Figure 1. A. EPR spectrum of 3 in CH_2Cl_2 at 15 K (m.f. 9.243 GH_z). B. Low-field part of the EPR spectrum of 4 in CH_2Cl_2 at 13 K (m.f. 9.226 GHz; power 10 mW; mod. amplitude 16 gauss).

Figure 2. Infrared spectra in the 1250-1050 cm^{-1} region of 2 (a), 3 (b), and 1 (c) in CH_2Cl_2 solutions at room temperature.


A
B
Co
P
1000
2000
3000
2600
2700
2800
MAGNETIC FIELD, GAUSS


(a)
(b)
(c)
1200
1100
cm-1