# THE STRUCTURE OF THE METAL CENTERS IN CYTOCHROME C OXIDASE

Thesis by

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In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California 91125

1981

(Submitted October 20, 1980)

ACKNOWLEDGEMENTS

I would like to express appreciation to Gary Brudvig, Dave Bocian, Sunney Chan and Randy Morse for the extensive discussions regarding cytochrome c oxidase which gave rise to many of the ideas and experiments described in this thesis. In particular, I would like to acknowledge Gary Brudvig for his excellent overall insight into Biophysics, Dave Bocian for his insight and skepticism and Sunney Chan for his excellent advice and friendship throughout my four year stay at Caltech. Also at this time, I would like to thank all the rest of the Chan Group members not named above for discussions and help throughout this work.

Acknowledgement is also extended to Dr. Hal Beilan for his invaluable advice on the synthesis of the isotopically labeled cysteine. I am indebted to the excellent staff of the shops at Caltech; Eric, Sig and Gabor in the glass shop, Janan Mitchell of the Analytical Lab and the members of the electronics and machine shops.

Finally, I would like to thank the Caltech and Woodbury Soccer Clubs for preserving my sanity. Last but not least, I would like to acknowlege my parents without whom all of this would have been unnecessary.

# ABSTRACT

Mitochondrial cytochrome c oxidase catalyzes the four-electron reduction of oxygen to water. The functional form of the protein contains two copper ions and two heme irons. Two of these metal centers, cytochrome $a_3$ and $Cu_{a_3}$, constitute the oxygen reduction site. The other two metal centers, cytochrome a and $Cu_a$, are involved in electron transfer from cytochrome c to the oxygen reduction site. It is the structure and function of these four metal centers in cytochrome c oxidase which is the subject of this thesis.

The metal centers constituting the oxygen reduction site of cytochrome c oxidase have traditionally been difficult to study because their coupled nature renders them EPR silent. It is shown that nitric oxide can uncouple these metal centers, and under appropriate conditions renders both metal centers observable by EPR. One of the nitric oxide complexes is an NO-bridge complex, demonstrating that the oxygen reduction site is between the two metal centers. Furthermore, this NO-bound complex allows us to calculate a distance between the metal centers of about 5 Å. Finally, with $^{15}N$-his isotopically labeled yeast oxidase it is shown that the fifth endogenous ligand to cytochrome $a_3$ is a histidine.

The copper metal center involved in electron transfer, $Cu_a$, is shown to have a cysteine and a histidine as ligands. The substitution of $^{12}CD_2$-cysteine into yeast oxidase does not perturb the eight line hyperfine pattern first seen on the $Cu_a$ EPR signal at 3 GHz. This demonstrates that the

S=1/2 center interacting with $Cu_a$ is probably cytochrome *a* at a distance of 10-13 Å. These studies represent the first time that ligands to any of the metal centers in cytochrome *c* oxidase have been unequivocally elucidated.

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# CHAPTER I: INTRODUCTION

Cytochrome c oxidase is a membrane-bound protein which catalyzes the last step of cellular respiration, that of transferring electrons from cytochrome c to molecular oxygen in mitochondria[1]. The overall reaction associated with this process is given by

$$4 \text{ Ferrocytochrome } c + O_2 + 4H^+ \longrightarrow 4 \text{ Ferricytochrome } c + 2H_2O.$$

This reaction is now believed to be coupled to the pumping of protons across the mitochondrial membrane by cytochrome c oxidase in the generation of a pH and potential gradient. This potential energy is then somehow coupled to the production of ATP*, the cellular energy source. It is the mechanism of oxygen reduction together with the protein's ability to conserve energy as a pH and potential gradient that have served as the focus for research conducted on cytochrome c oxidase.

Largely because of the multi-subunit and transmembrane (highly hydrophobic) nature of cytochrome c oxidase, it has been difficult to characterize its subunit composition. Recently, Capaldi[2] has carefully characterized the beef heart protein and found that it contains ten subunits, one

*Abbreviations used in this work: ATP, adenosine triphosphate; EPR, electron paramagnetic resonance; ENDOR, electron nuclear double resonance; EXAFS, extended x-ray absorption fine structure; PPD, paraphenylenediamine.

copy each of subunits I-VIII and two copies of subunit IX. The molecular weights of the subunits of the beef heart and yeast proteins are given in Table 1. The discrepancy between the composition of the yeast[3] and beef heart protein subunits probably arises from the lack of a parallel careful study of the subunit structure for the yeast protein. The ten subunits of the beef heart protein sum to a molecular weight of about 130,000. In addition, cytochrome oxidase has also been shown[1] to contain four metal ions per functional unit, two irons in the form of heme a and two copper ions.

Cytochrome c oxidase is one of the most complex and yet thoroughly studied membrane proteins. Its assembly into the inner mitochondrial membrane has served as a model for how other multi-subunit transmembrane proteins become inserted into the membrane. It has been found that the three largest yeast cytochrome c oxidase subunits are synthesized in the mitochondria[4], while the remaining subunits are synthesized in the cytoplasm. This result has also been found to be true of the human protein by Hare et al.[5]. In addition, Poyton and McKemmie found evidence that the cytoplasmic subunits are synthesized as a single large precursor polypeptide[6,7] from which the subunits are cleaved in an assembly process that is starting to be unraveled. However, these investigations on the large polypeptide precursor have been called into question[8-10]. Evidence for a precursor to only subunit IV in rat liver[8] and for individual

Table 1: Subunit Composition of Yeast and Beef Heart Cytochrome c Oxidase

| | Molecular Weight | |
|---|---|---|
| Subunit | Beef Heart[a] | Yeast[b] |
| I | 35,400 | 40,000 |
| II | 24,100 | 33,000 |
| III | 21,000 | 22,000 |
| IV | 16,800 | 14,000 |
| V | 12,400 | 12,700 |
| VI | 8,200 | 12,700 |
| VII | 4,400 | 4,600 |
| VIII | 4,400 | |
| IX | 4,400 | |

a) Reference 2

b) Reference 3

precursors to subunits V and VI in yeast[9] provide reason to question the results of Poyton and McKemmie. In addition, Onashi and Schatz[10] were unable to repeat the work of Poyton and McKemmie. In any case, the elucidation of the complex processing and membrane assembly of cytochrome c oxidase is sure to have implications in the growing field of membrane protein assembly.

The macroscopic structure of cytochrome c oxidase has been studied on two-dimensional arrays of the oxidized membrane-bound protein using electron microscopy[11] and variable angle electron microscopy[12]. Electron microscopy revealed very ordered arrays of cytochrome c oxidase units in the membrane. On the other hand, the recent variable angle study revealed a "tooth"-like three dimensional structure for the protein, with dimensions of 80x40x110 Å. The "roots" of the tooth protrude on the matrix side of the inner mitochondrial membrane. The prospect of a high-resolution x-ray crystal structure determination is quite remote, however, due to the large size of the protein and its hydrophobic character. Thus far, there is only one report of obtaining very small microcrystalline arrays[13] and one report claiming the growth of cytochrome c-cytochrome c oxidase complex needle crystals[14], both of which are unsatisfactory for high-resolution x-ray diffraction studies. The lack of high-resolution x-ray structural information for cytochrome c oxidase necessitates the utilization of other techniques to obtain detailed information on the metal

centers, which are believed to be intimately involved in the electron transfer and oxygen reduction processes.

Spectroscopic studies have played an important role in the understanding of the role of the metal centers in the functioning of the protein. These studies began with the original optical characterization of cytochrome a and $a_3$ by Keilin and Hartree[14]. Subsequent investigations have included the use of optical, EPR, x-ray absorption, EXAFS, cryogenic enzymology, ENDOR and several other spectroscopies. From these studies has emerged a nomenclature for the metal centers in cytochrome c oxidase. Two of the metal ions have been referred to as cytochrome $a_3$ and $Cu_{a_3}$, due to their close proximity and combined involvement in the oxygen reduction reaction. The remaining two metal centers have been referred to as cytochrome a and $Cu_a$ due to their exclusive involvement in electron transfer from cytochrome c to the cytochrome $a_3$-$Cu_{a_3}$ oxygen reduction site. These designations are consistent with extensive optical and EPR studies indicating the lack of involvement of either cytochrome a or $Cu_a$ in the binding of any exogenous ligands.

The relative proximity of the four metal centers within the protein matrix is of considerable interest because of the implications on the functioning and mechanism of the protein. However, this has proved to be a difficult problem to address. The proximity of cytochrome a and $Cu_a$ with respect to each other and the cytochrome $a_3$-$Cu_{a_3}$ site has been investigated by Brudvig and Chan[16] using EPR saturation and relaxation

techniques. It was found that cytochrome a and $Cu_a$ are between 10 and 13 Å separated and that both of these metal centers are at least 10 Å (and possible much farther) from the cytochrome $a_3$-$Cu_{a_3}$ site. The assignment of a distance for the separation between cytochrome a and $Cu_a$ remains tentative, however, until the EPR signal from the $Cu_a$ center is better characterized.

A complete understanding of the mechanism of electron transfer and oxygen reduction in cytochrome c oxidase necessitates a knowledge of not only the relative proximity of the metal centers, but also their ligand environments. The endogenous protein ligands will determine a metal center's redox properties, in addition to its ability to be substitutionally labile, and thus influence the metal center's functional role in the protein. For cytochrome c oxidase none of the endogenous ligands to the four metal centers is known. Therefore, the determination of the ligand environment of the metal centers in cytochrome c oxidase should greatly assist in the elucidation of their individual roles in the mechanism of action of this enzyme.

This thesis will concentrate on spectroscopic studies carried out on cytochrome c oxidase. In Chapter II, NO is used as an EPR probe of the structure of the cytochrome $a_3$-$Cu_{a_3}$ oxygen reduction site. Chapter III involves studies on cytochrome c oxidase isolated from auxotrophic yeast with the substitution of the isotopically labeled amino acids, $^{15}N$-histidine and $^{12}CD_2$-cysteine. These investigations have

utilized EPR spectroscopy to study the isotopically substituted proteins in order to elucidate the ligands to and the nature of the $Cu_a$ center.

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# CHAPTER II: STRUCTURE OF THE OXYGEN BINDING SITE

## 1. INTRODUCTION

The oxygen reduction site of cytochrome c oxidase is believed[1] to consist of cytochrome $\underline{a}_3$ and $Cu_{a_3}$. Investigations of these metal centers have been hindered by the fact that neither ion gives rise to an EPR signal in the oxidized protein. It has been shown by magnetic susceptibility measurements[2,3] that these two metal centers are antiferromagnetically coupled ($Fe_{a_3}$(III), S = 5/2, $Cu_{a_3}$(II), S = 1/2) producing a S = 2 ground state. Optical spectroscopic studies of these two metal centers are complicated by the fact that $Cu_{a_3}$ probably does not contribute significantly to the optical spectrum[4] and that it is difficult to deconvolute the heme-dominated optical spectrum to yield the contribution due to cytochrome $\underline{a}_3$. However, since cytochrome $\underline{a}_3$ is known[1] to bind $N_3^-$, $CN^-$, CO, NO and $SH^-$, studies utilizing these ligands in conjunction with optical and EPR spectroscopies have allowed certain features of cytochrome $\underline{a}_3$ to be elucidated.

Cytochrome $\underline{a}_3$ has been found to be high-spin in both the oxidized ($Fe_{a_3}$(III), S = 5/2) and the reduced ($Fe_{a_3}$(II), S = 2) proteins. Cytochrome $\underline{a}_3$ becomes EPR visible as a high-spin heme in the partially reduced enzyme[5], and as a low-spin heme in the $N_3^-$- or $CN^-$-bound partially reduced enzymes. Partial anaerobic reduction results in the reduction of cupric $Cu_{a_3}$ to cuprous (S = 0) in some of the protein molecules allowing the uncoupling of cytochrome $\underline{a}_3$ from $Cu_{a_3}$. A disadvantage of this approach is that only a subpopulation of enzyme molecules

is being observed, usually 5-20%, since most molecules have either zero or two electrons in the cytochrome $\underline{a}_3$-$Cu_{a_3}$ site rendering both ions EPR silent to conventional instrumentation.

On the other hand, very little is known about $Cu_{a_3}$. It was suggested on the basis of X-ray absorption spectroscopy[6] that this copper ion is similar to a type 1 copper, implying that the endogenous ligands are two histidines, a cysteine and a methionine. In addition, this suggestion implies the presence of an intense charge transfer band in the visible spectral region. The interpretation of these x-ray absorption results have, however, been the source of considerable controversy. In this regard, resonance Raman studies[7] indicate that neither copper in cytochrome $\underline{c}$ oxidase is a type 1 copper site. In addition, Brudvig and Chan[8] using $Ag^+$ as a sulfhydryl modifying reagent have demonstrated that $Cu_{a_3}$ does not have a cysteinyl ligand or contribute significantly to the protein's visible spectrum, and thus $Cu_{a_3}$ is not a type 1 copper ion. Further, since $Cu_{a_3}$ has not previously been shown to bind ligands, its structure remains poorly defined.

In an effort to further delineate the structure of the cytochrome $\underline{a}_3$-$Cu_{a_3}$ site, the binding of NO to the reduced and oxidized protein has been investigated[9-13]. NO is a paramagnetic molecule with an unpaired electron in an antibonding $\pi_g$ molecular orbital. This paramagnetism may be taken advantage of in order to break the antiferromagnetic coupling between the cytochrome $\underline{a}_3$-$Cu_{a_3}$ centers and to probe the electronic structures of the metal centers. The binding of

NO to ferrous hemeproteins is well known[11]. In the case of fully reduced cytochrome c oxidase, NO binds to cytochrome $a_3$[9]. The EPR spectrum of this complex is very isotropic, with $g_x$ = 2.09, $g_z$ = 2.006, and $g_y$ = 1.97. The observed hyperfine pattern consists of nine equally spaced lines, and has been interpreted[8] in terms of the superposition of three sets of three lines arising from two non-equivalent nitrogens (I = 1) interacting with the unpaired electron. The larger of the two superhyperfine coupling constants (21.1 G) was assigned[9] to the nitrogen of bound nitric oxide and the smaller coupling constant (6.8 G) was assigned to a nitrogen on the endogenous axial ligand of cytochrome $a_3$. These assignments, while reasonable, need to be confirmed. The presence of an endogenous axial nitrogen ligand on cytochrome $a_3$, as well as the assignment of the coupling constants to two nitrogens, can be verified by investigating the EPR spectrum of $^{15}$NO-ferrocytochrome c oxidase.

The fifth nitrogen ligand on cytochrome $a_3$ could be contributed by any of a number of amino acids, for example histidine, arginine, peptide nitrogen, and lysine. These potential fifth ligands vary greatly in their π-bonding capabilities, with histidine being a strong π-bonding ligand, and the remaining three ligands being predominantly σ-bonding. In this regard, Kon and Kataoka[14] have shown that the g values and nitrogen superhyperfine splittings of the EPR signals of NO-bound hemin are dependent on the π-bonding capability of the axially-bound nitrogen ligand opposite NO. With non-π-bonding ligands, such as amines, the EPR spectra exhibit axial

symmetry, with $g_x = g_y = 2.07$ and $g_z = 2.008$. Also, the observed superhyperfine splitting attributed to the bound $^{14}NO$ nitrogen is typically 16 G, and no superhyperfine splitting is resolved from the nitrogen ligand opposite NO. In contrast, with strong π-bonding ligands, such as pyridine, the EPR spectra exhibit rhombic symmetry, with g values and superhyperfine splittings similar to those of $^{14}NO$-bound ferrocytochrome c oxidase. This result suggests that the endogenous axial ligand of cytochrome $a_3$ is a strong π-bonding ligand, with histidine being the most likely candidate[9].

Nitric oxide is also known to bind to certain ferric hemoproteins. The ferricytochrome c peroxidase-NO complex has been shown[11] to be EPR silent from 4-296 K, while the ferricytochrome c-NO complex has been shown by magnetic susceptibility measurements to be diamagnetic[15]. In addition, NO can react with both oxidized and reduced copper proteins[16,17]. In particular, the reaction of NO with oxidized ceruloplasmin is reversible after a short incubation time[16] suggesting the formation of a reversible copper-NO complex which is apparently EPR silent.

These studies raise the possibility that NO might interact with at least one of the metal centers in the cytochrome $a_3$-$Cu_{a_3}$ couple in fully oxidized cytochrome c oxidase. If indeed NO does interact with one of the metal centers of the site to form a diamagnetic NO complex, the antiferromagnetic coupling between cytochrome $a_3$-$Cu_{a_3}$ will be broken and the "unaffected" metal center should reveal itself in the EPR spectrum.

We have undertaken EPR studies of the interaction of NO with cytochrome c oxidase as a spin probe of cytochrome $a_3$-$Cu_{a_3}$ site of both the oxidized and reduced states of the protein[10,12,13]. In section 3.1, we report studies of the $^{15}NO$-bound reduced beef heart protein and of the reduced $^{15}N$-histidine substituted yeast protein to elucidate the presence of histidine as the endogenous axial ligand to cytochrome $a_3$. In section 3.2, studies are reported on the interaction of NO with the oxidized protein, in which the antiferromagnetic coupling between cytochrome $a_3$ and $Cu_{a_3}$ is broken. Finally, in section 3.3, we investigate the complex formed upon the addition of NO to the oxidized protein in the presence of azide, wherein new EPR signals are observed for cytochrome c oxidase which shed some light on the structure of the cytochrome $a_3$-$Cu_{a_3}$ site.

## 2. MATERIALS AND METHODS

Beef heart cytochrome c oxidase was isolated by the procedures of Hartzell and Beinert[18] and Yu et al.[19]. All experiments described were carried out with the enzyme isolated by the procedure of Hartzell and Beinert unless otherwise stated. The purified enzyme was stored at 188K until use. The preparations contained 9-11 nmoles heme a/mg protein as measured by the pyridine hemochromagen assay[20]. The purified protein was dissolved in 0.5% Tween 20/50 mM Tris-$HNO_3$, pH 7.4 to a protein concentration of approximately 50 mg/ml with the exception of the Yu et al. preparation which was dissolved in 0.5% Na-cholate/50 mM phosphate-HCl, pH 7.4.

The protein concentrations were then determined by the method of Lowry et al.[21].

The growth of labeled yeast and the subsequent isolation of their mitochondria will be described in detail in Chapter III. Mitochondrial samples studied in section 2 were prepared by placing them in EPR tubes at a protein concentration of from 60-80 mg/ml. These samples were reduced with PPD and ascorbate at concentrations of .2mM and 20mM, respectively, followed by the addition of either $^{14}NO_2^-$ or $^{15}NO_2^-$ to a concentration of 40mM and then freezing, evacuating, and placing the samples under an argon atmosphere. These anaerobic samples were allowed to incubate for 30 min. at 277K followed by freezing at 77K.

The protein-NO samples were prepared by the addition of NO (Matheson) to the anaerobic protein (with or without added ligands) to a pressure of one atmosphere, unless otherwise stated. The samples were made anaerobic by three cycles of evacuation and flushing with argon. These samples were allowed to equilibrate with NO for 10 minutes at 277K before being frozen at 77K. KF or $NaN_3$ were added to the oxidized protein-NO complex from a sidearm on the EPR tube or optical cuvette to yield a final concentration of 100 mM, unless otherwise stated. The oxidized enzyme plus cyanide and NO complex was prepared by the addition of a 1:2 mole ratio mixture of solid KCN and $KH_2PO_4$ to the enzyme while the sample was under vacuum. Then NO was admitted to the anaerobic sample after the desired period of incubation to give a final pressure of one atmosphere

without further evacuation of the enzyme solution. This $KCN/KH_2PO_4$ mixture resulted in a solution with pH 7.4 when added to the enzyme. The amount of KCN added would have given a final concentration of 100 mM. However, the major portion of the cyanide bubbled off as HCN. The actual concentration of dissolved cyanide, estimated from the partial pressure of HCN over the sample and the solubility of HCN in water, was about 2 mM.

The EPR spectra were recorded on a Varian E-line Century Series X-band spectrometer equipped with an Air-Products Heli-Trans low temperature system. Optical measurements were carrier out at room temperature on a Beckman Acta CIII spectrometer.

The intensity of the high-spin cytochrome $\underline{a}_3$ EPR signals were determined relative to an external myoglobin standard and also relative to the low-spin cytochrome $\underline{a}$ EPR signal. The high-spin EPR signals were integrated by the method of Aasa et al.[22]. The low-spin cytochrome $\underline{a}$ EPR signal was integrated by the method of Aasa and Vänngård[23] using the g = 3.0 component to determine the total area. The low-spin cytochrome $\underline{a}$ EPR signal has been shown to correspond to 100% of one heme[22], and on this basis the low-spin heme EPR signal was used as an internal standard. After correcting for the distribution of population among the spin sublevels of the high-spin ferric heme, the cytochrome $\underline{a}_3$ EPR intensities determined using the internal cytochrome $\underline{a}$ standard were found to agree with those determined using the myoglobin standard

to within 10%.

The intensity of the low-spin cyanoferricytochrome $\underline{a}_3$ EPR signal was determined relative to the low-spin cytochrome $\underline{a}$ EPR signal. All three g-values of the cyanocytochrome $\underline{a}_3$ EPR signal are not known since the signal is very anisotropic and the two high-field turning points have not been observed. Therefore, the cyanocytochrome $\underline{a}_3$ EPR signal was integrated by the method of DeVries and Albracht[24], using the g = 3.5 component to determine the total area.

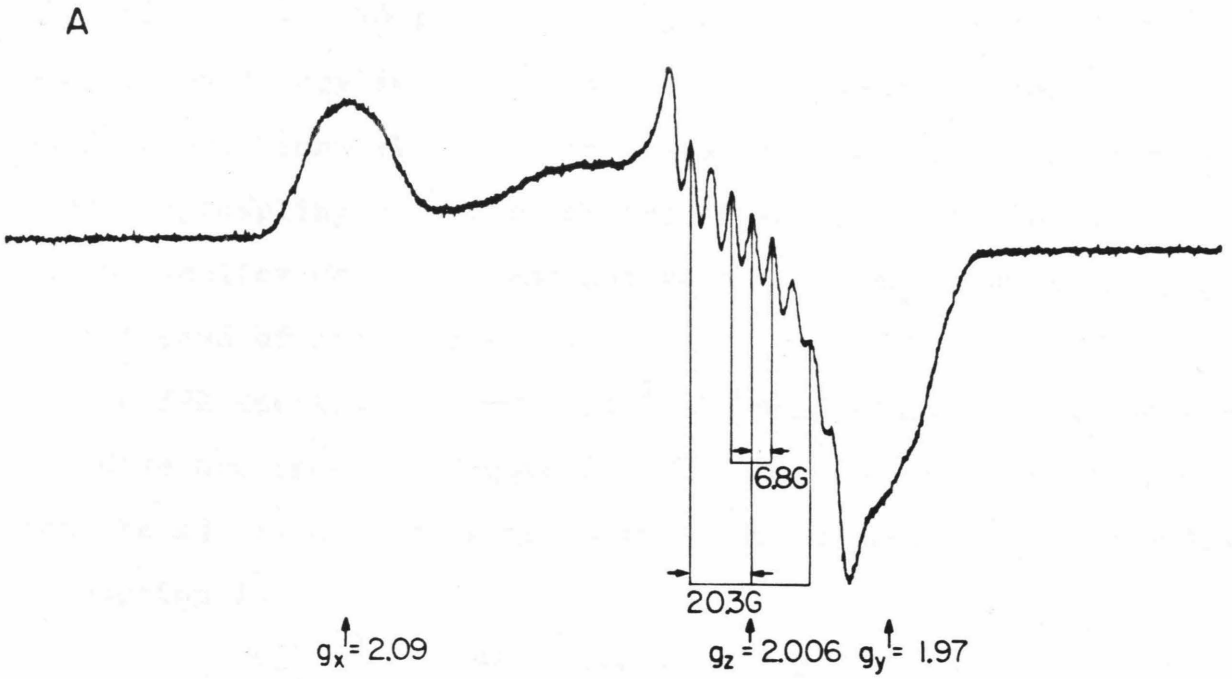
## 3. RESULTS

3.1 Interaction of NO with Reduced Cytochrome c Oxidase. The EPR spectra of $^{14}NO$- and $^{15}NO$-bound beef heart ferrocytochrome $\underline{c}$ oxidase are shown in Figure 1. Both NO-bound complexes exhibit EPR signals with $g_x = 2.09$, $g_z = 2.006$, and $g_y = 1.97$. In contrast, the fully reduced enzyme alone does not exhibit an EPR spectrum. When $^{14}NO$ is bound to reduced cytochrome $\underline{c}$ oxidase, the EPR signal of the complex exhibits a nine-line superhyperfine pattern, which can be interpreted in terms of the superposition of three sets of three lines arising from two non-equivalent nitrogens (I = 1) interacting with the unpaired electron (Fig. 1A). The larger of the two superhyperfine coupling constants is 20.3 G and the smaller 6.8 G. When $^{15}NO$ is used in this experiment, the $^{15}NO$-bound protein exhibits an EPR spectrum with a superhyperfine pattern of two sets of three lines (Fig. 1B). This pattern is consistent with the presence of one $^{14}N$ and one $^{15}N$ nitrogen bound axially to cytochrome $\underline{a}_3$, with a 28.2 G splitting for the $^{15}N$ and a 6.7

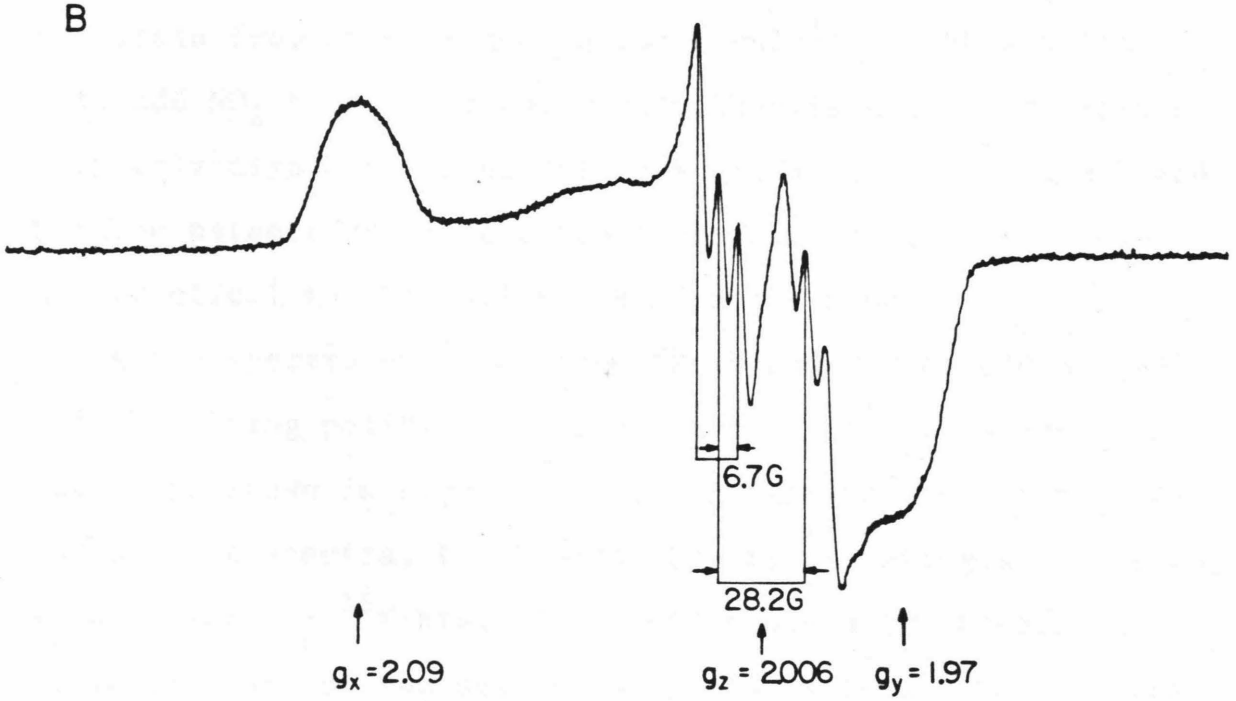
FIGURE 1

EPR spectra of NO-bound reduced beef heart cytochrome c oxidase with (A) $^{14}NO$ and (B) $^{15}NO$, prepared by the addition of either $^{14}NO_2^-$ or $^{15}NO_2^-$ to .16 mM cytochrome c oxidase. Conditions: temperature, 50 K; microwave power, 5 mW; modulation amplitude, 2G; microwave frequency, 9.23 GHz.

BEEF HEART CYTOCHROME c OXIDASE-$^{14}$NO




BEEF HEART CYTOCHROME c OXIDASE-$^{15}$NO



G splitting for the $^{14}N$ ligand. The observed increase of the larger superhyperfine splitting from 20.3 G to 28.2 G upon substitution of $^{15}NO$ for $^{14}NO$ is expected on the basis of the relative magnetogyric ratios of the two nitrogen isotopes. These observations allow one to assign the larger of the superhyperfine coupling constants to the nitrogen of the bound NO and the smaller coupling constant to a nitrogen on an endogenous axial ligand of cytochrome $\underline{a}_3$.

The EPR spectra of $^{14}NO$- and $^{15}NO$-bound yeast ferrocytochrome $\underline{c}$ oxidase are shown in Figure 2. The NO-bound complex results from the addition of nitrite to the reduced protein[25] according to reaction 1.

$$NO_2^- + e^- + 2H^+ \longrightarrow NO + H_2O \qquad (1)$$

It has been found that the addition of $NO_2^-$ to PPD plus ascorbate reduced mitochondria results in the production of the NO-ferrohemoprotein from cytochrome $\underline{c}$ oxidase only[26]. This allows one to add $NO_2^-$ to reduced whole mitochondria and observe essentially only cytochrome $\underline{c}$ oxidase EPR signals. The g values and hyperfine parameters for the yeast protein spectra in Figure 2 are identical to those of the beef heart protein.

The EPR spectra of $^{14}NO$- and $^{15}NO$-bound $^{15}N$-histidine (95% $^{15}N$ in both ring positions) substituted yeast cytochrome $\underline{c}$ oxidase are shown in Figure 3. In contrast to the $^{14}N$-his NO-bound protein spectra, the $^{15}N$-his hyperfine patterns have been altered. For the $^{15}N$-his, $^{15}NO$-bound protein the hyperfine pattern consists of two sets of doublets, with a $^{15}NO$ nitrogen splitting of 27.5 G and a splitting of about 12 G for the $^{15}N$-

FIGURE 2

EPR spectra of NO-bound reduced yeast cytochrome c oxidase with (A) $^{14}NO$ and (B) $^{15}NO$. The samples were prepared by the addition of either $^{14}NO_2^-$ or $^{15}NO_2^-$ to PPD and ascorbate reduced yeast mitochondria. Conditions: temperature, 30K; microwave power, 2 mW; modulation amplitude, 2 G; microwave frequency, 9.21 GHz.

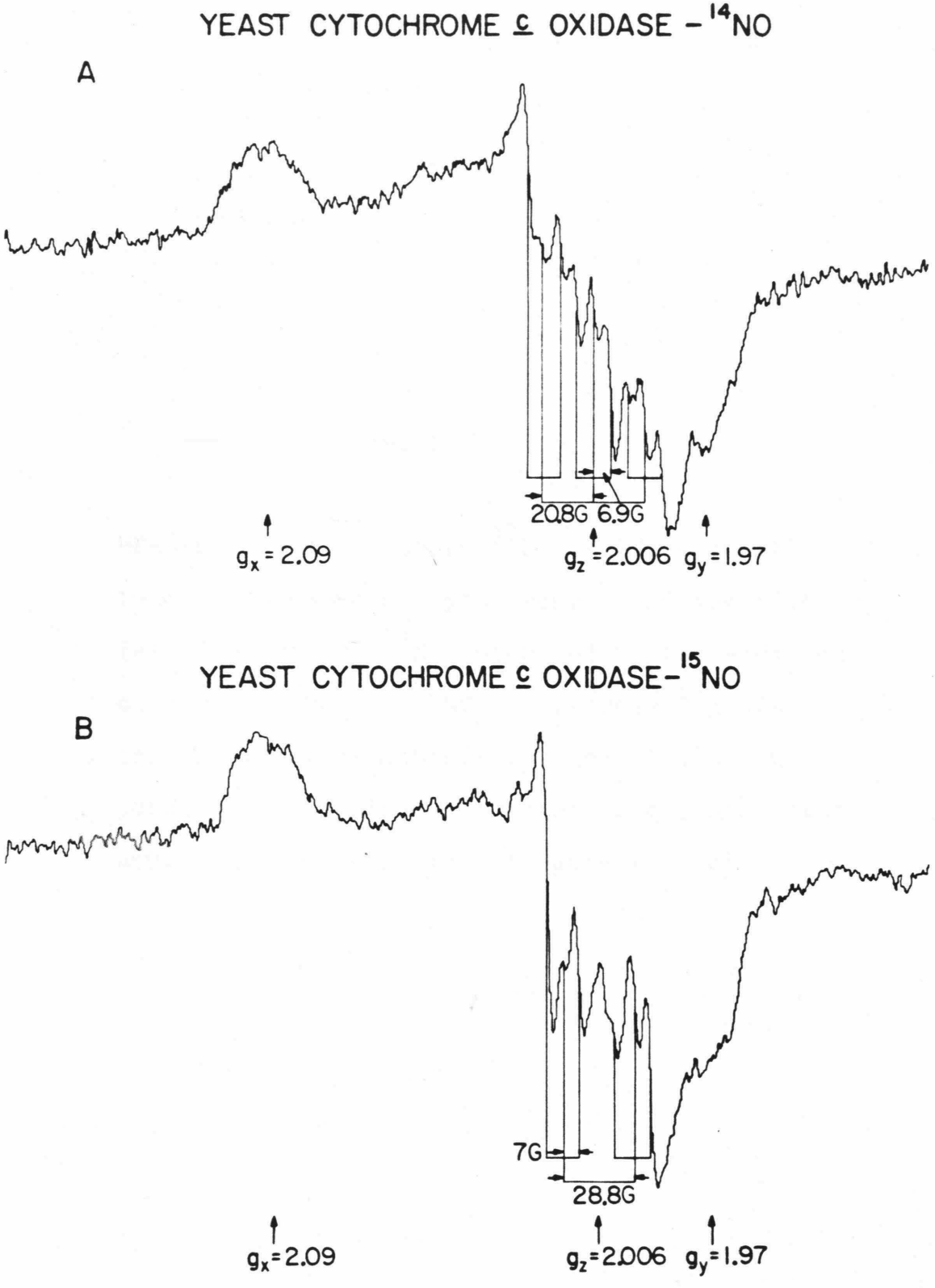
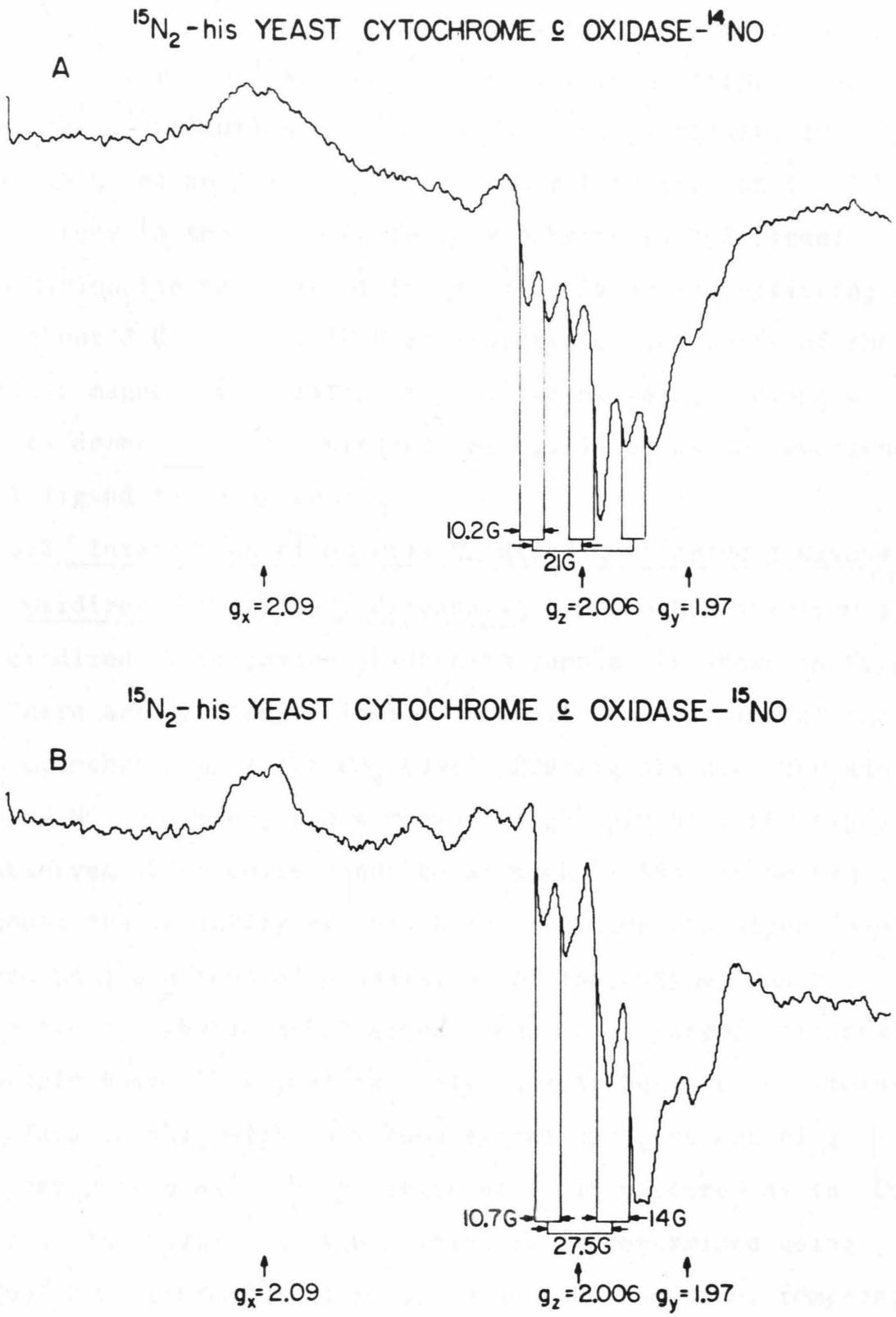

YEAST CYTOCHROME c OXIDASE - $^{14}$NO
A
20.8G
6.9G
$g_x$ = 2.09
$g_z$ = 2.006
$g_y$ = 1.97
YEAST CYTOCHROME c OXIDASE- $^{15}$NO
B
7G
28.8G
$g_x$ = 2.09
$g_z$ = 2.006
$g_y$ = 1.97

FIGURE 3

EPR spectra of NO-bound $^{15}$N-histidine substituted reduced yeast cytochrome c oxidase with (A) $^{14}NO$ and (B) $^{15}NO$, prepared by the addition of either $^{14}NO_2^-$ or $^{15}NO_2^-$ to reduced $^{15}$N-his labeled yeast mitochondria. Conditions: temperature, 30K; microwave power, 2 mW; modulation amplitude, 4G; microwave frequency, 9.21 GHz.


$^{15}N_2$-his YEAST CYTOCHROME c OXIDASE-$^{14}$NO
A
10.2G
21G
$g_x$=2.09
$g_z$=2.006
$g_y$=1.97
$^{15}N_2$-his YEAST CYTOCHROME c OXIDASE-$^{15}$NO
B
10.7G
14G
27.5G
$g_x$=2.09
$g_z$=2.006
$g_y$=1.97

his nitrogen. The $^{15}$N-his, $^{14}$NO-bound protein hyperfine pattern consists of three sets of doublets, with splittings of 21 G and 10.2 G for the $^{14}$NO and $^{15}$N-his nitrogens, respectively. Thus, the substitution of $^{15}$N-his for $^{14}$N-his results in the involvement of an I = 1/2 $^{15}$N nucleus rather than an I = 1 $^{14}$N nucleus in the NO-bound ferrocytochrome $\underline{a}_3$ EPR signal. In addition, the increase of the smaller hyperfine splitting from about 7 G to about 10 G is expected on the basis of the relative magnetogyric ratios of the two nitrogen isotopes. These results demonstrate the presence of histidine as the endogenous axial ligand to cytochrome $\underline{a}_3$.

3.2 Interaction of NO with Oxidized Cytochrome c Oxidase.

(1) Oxidized Cytochrome c Oxidase-NO. The EPR spectrum of the oxidized cytochrome c oxidase-NO complex is shown in Figure 4. There are no changes in the intensity or position of the low-spin cytochrome a or the $Cu_a$ center EPR signals upon the addition of NO. However, a new rhombic high-spin heme EPR signal is observed which corresponds to as much as 58% of one heme, although the intensity of this high-spin heme EPR signal does depend on the method of preparation of the enzyme (Table 1). Since the cytochrome a EPR signal remains unchanged, the new high-spin heme EPR signal can only be attributable to cytochrome $\underline{a}_3$. This rhombic high-spin heme signal has g values of $g_x$ = 6.16 and $g_y$ = 5.82. The position of $g_z$ is obscured by the $Cu_a$ signal. The high-spin signal intensities determined using a myoglobin standard were found to be independent of temperature, thus indicating that the zero-field splitting parameters (D) are nearly equal for the two high-spin ferric hemes. Since D

Table 1. The fraction of the high-spin cytochrome $\underline{a}_3$ EPR signal observed for various preparations of oxidized cytochrome $\underline{c}$ oxidase in the presence of one atmosphere NO.

| Preparation | Fraction observed |
|---|---|
| Hartzell and Beinert | 0.58 |
| Hartzell and Beinert + 100 mM $F^-$ | 0.61 |
| Hartzell and Beinert - "oxygenated" | <0.05 |
| Yu et al. | <0.05 |
| Yu et al. + 10 mM $F^-$ | 0.26 |

is known to be 9.1 $cm^{-1}$ for myoglobin[27], then D for high-spin cytochrome $\underline{a}_3$ must also be about 9 $cm^{-1}$. The broad signal centered at about g = 1.97 in the oxidized cytochrome $\underline{c}$ oxidase-NO EPR spectrum is also seen in the spectrum of the buffer plus NO sample (Fig. 4) and is presumed to be matrix-bound paramagnetic NO.

The binding curve of NO to oxidized cytochrome $\underline{c}$ oxidase (Fig. 5) demonstrates that the intensity of the high-spin cytochrome $\underline{a}_3$ signal is dependent on the NO pressure. The pressure of NO corresponding to the appearance of 50% of the observed high-spin cytochrome $\underline{a}_3$ EPR signal is about 65 mm Hg. This process of NO binding to oxidized cytochrome $\underline{c}$ oxidase is reversible, since the high-spin cytochrome $\underline{a}_3$ EPR signal disappears with removal of NO from the sample. (Fig. 4).

We have found that the optical spectrum of oxidized cytochrome $\underline{c}$ oxidase remains unchanged upon the addition of NO (Fig. 6). The lack of any effect by NO on the optical spectrum of oxidized cytochrome $\underline{c}$ oxidase indicates that no NO-heme interaction occurs. These optical results in conjunction with the EPR data strongly suggest the formation of a cytochrome $\underline{a}_3^{+3}$, $Cu_{a_3}^{+2}$-NO complex.

The addition of NO to oxidized cytochrome $\underline{c}$ oxidase prepared by the method of Yu et al. does not produce a large high-spin cytochrome $\underline{a}_3$ EPR signal. In this regard, we have found that the Hartzell and Beinert preparation of the enzyme, which exhibits a large high-spin heme EPR signal upon the addition of NO to the resting oxidized protein, <u>does not</u> exhibit a high-spin

FIGURE 4

The EPR spectra of (A) native oxidized cytochrome *c* oxidase; (B) NO added to (A) to a pressure of 723 mm Hg; (C) NO removed from (B); (D) NO added to the buffer only. The signals at g = 3.03, 2.21, and 1.5 are due to cytochrome *a* and the signals at g = 2.18, 2.03, and 1.99 are due to $Cu_a$. The temperature was 7K, microwave power was 0.2 mW, modulation amplitude was 10G, and microwave frequency was 9.16 GHz for all spectra.

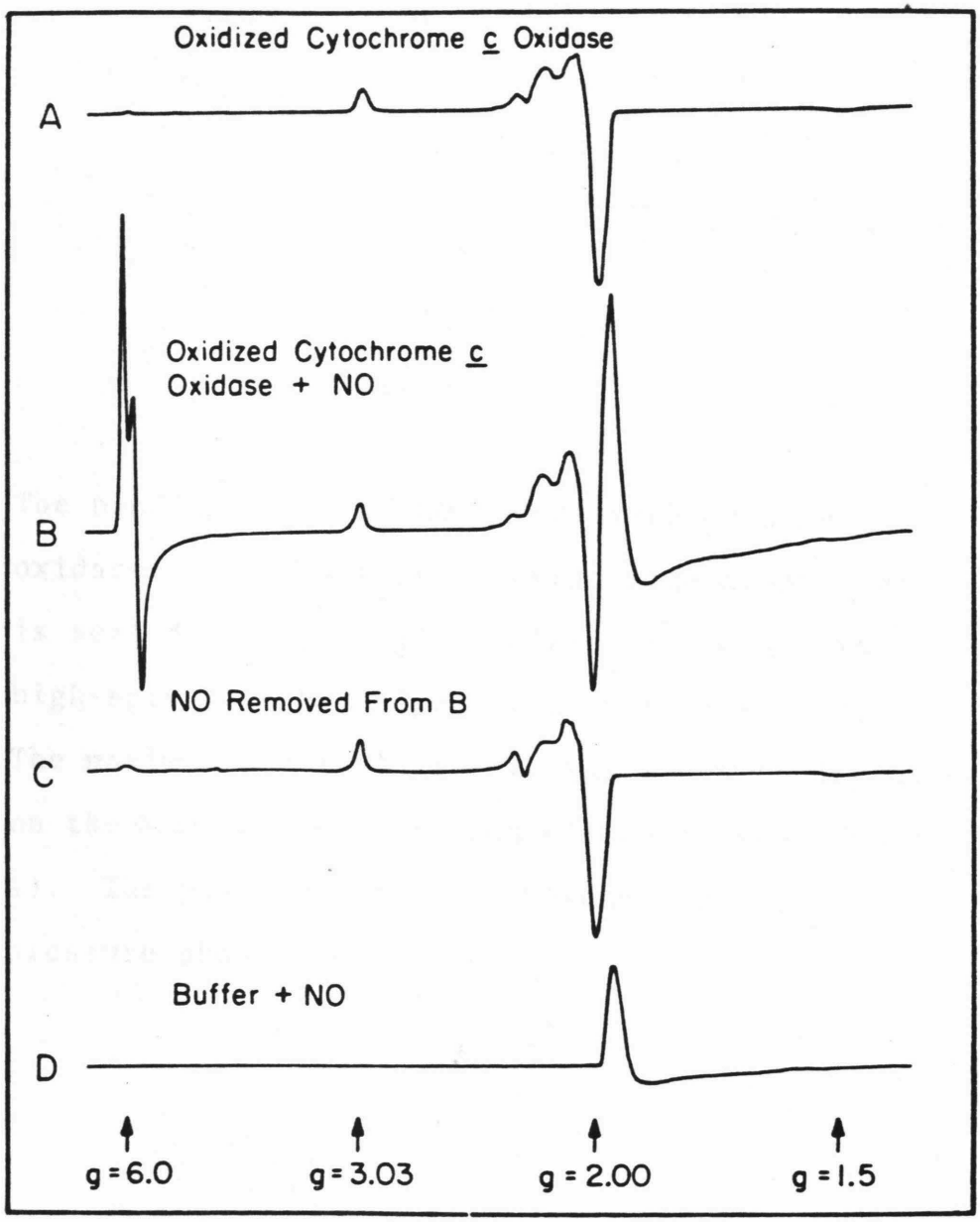

Oxidized Cytochrome c Oxidase
A
Oxidized Cytochrome c
Oxidase + NO
B
NO Removed From B
C
Buffer + NO
D
g = 6.0
g = 3.03
g = 2.00
g = 1.5

FIGURE 5

The binding curve of NO to oxidized cytochrome c oxidase. The fraction of cytochrome $a_3$ observed is scaled such that 100% refers to the maximum high-spin heme EPR signal intensity observed. The maximum signal intensity was found to depend on the method of preparation of the enzyme (Table 1). The pressure of NO represents the total pressure above the sample.

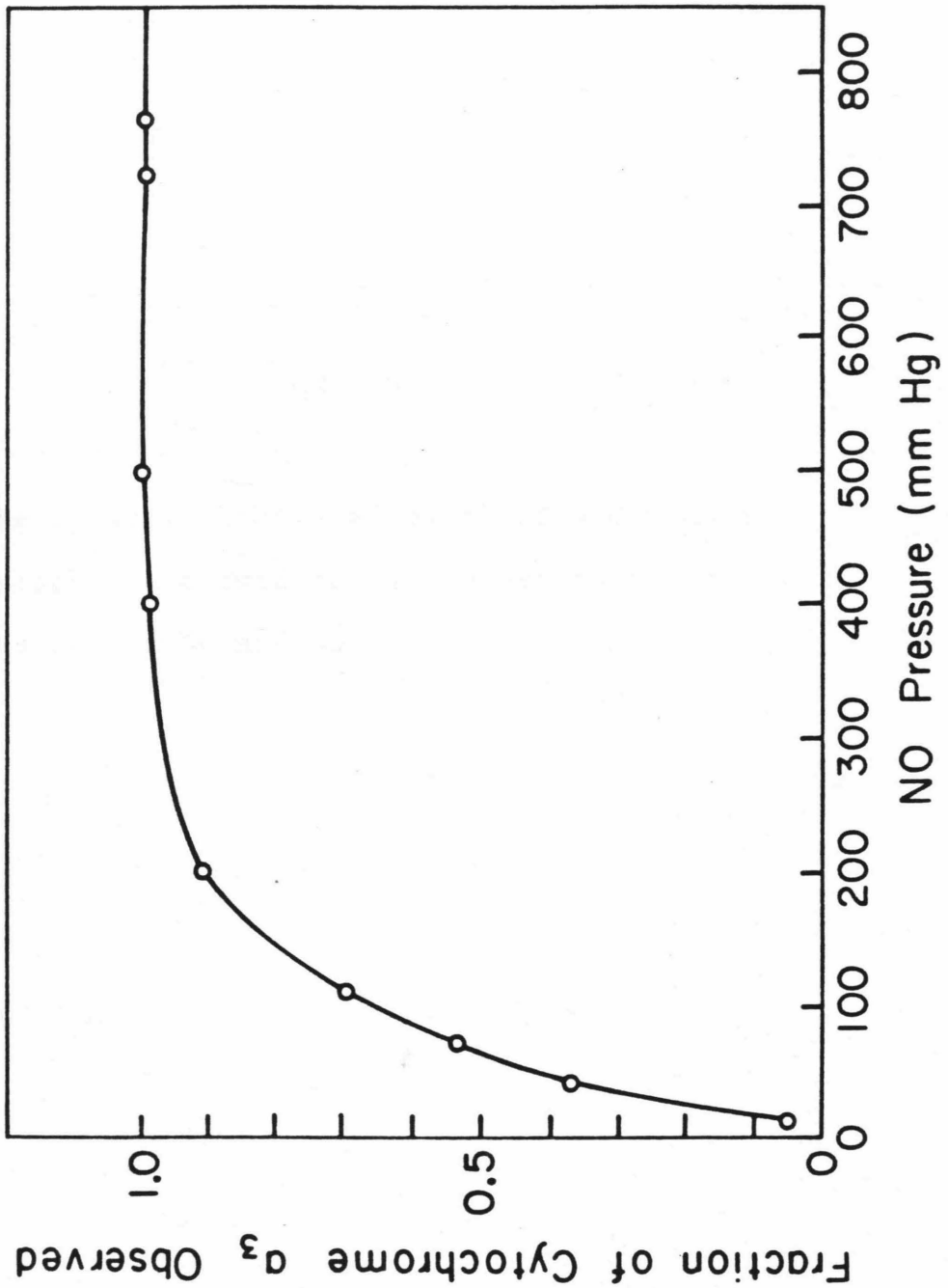
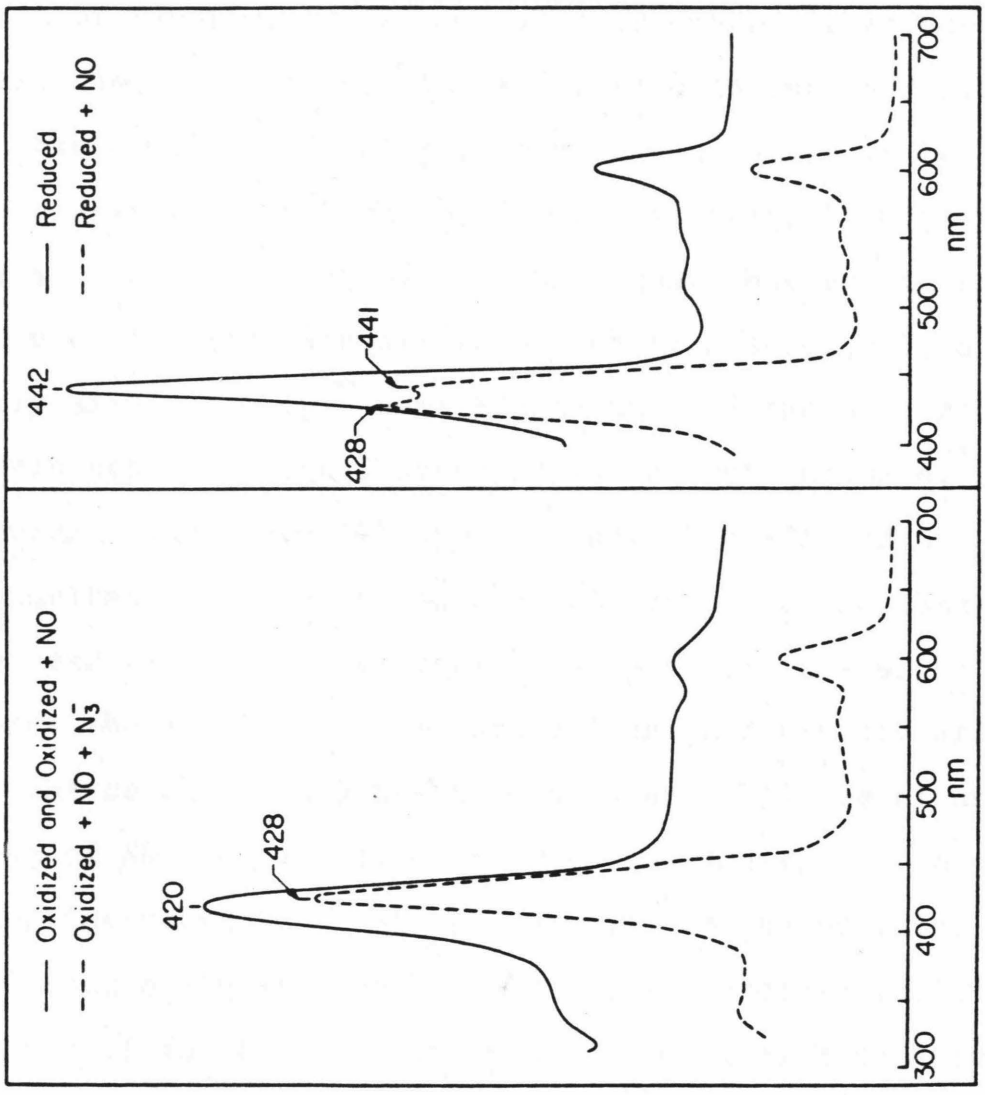

Fraction of Cytochrome $a_3$ Observed
0
0.5
1.0
0
100
200
300
400
500
600
700
800
NO Pressure (mm Hg)

FIGURE 6

The optical spectra of oxidized and reduced cytochrome c oxidase in the presence and absence of NO and $N_3^-$.


Oxidized and Oxidized + NO
Oxidized + NO + $N_3^-$
420
428
300
400
500
600
700
nm
Reduced
Reduced + NO
442
441
428
400
500
600
700
nm

EPR signal when NO is added to this enzyme in the "oxygenated" state[28].

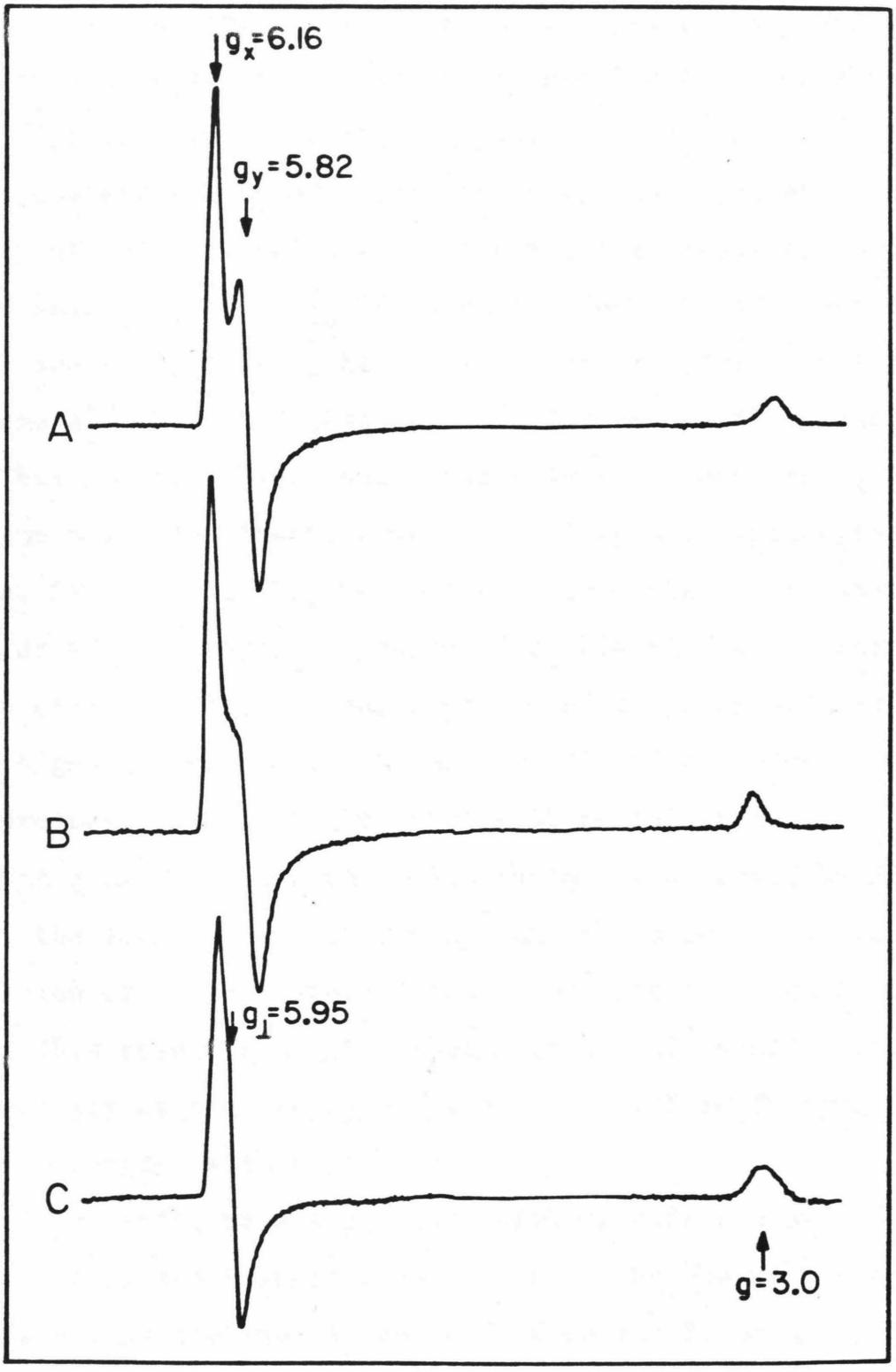
(2) Oxidized Cytochrome c Oxidase-NO plus Fluoride. In order to determine whether exogenous ligands compete with NO for the same binding site(s) in the protein, we have examined the addition of fluoride to the oxidized cytochrome c oxidase-NO complex. The addition of 100 mM fluoride to the oxidized cytochrome c oxidase-NO complex produces a change in the shape of the high-spin cytochrome $a_3$ EPR signal (Fig. 7) with no change in the total intensity. The signal observed in the presence of fluoride appears to be due to a superposition of a nearly axial high-spin heme EPR signal and the more rhombic high-spin heme EPR signal observed in the cytochrome $a_3^{+3}$, $Cu_{a_3}^{+2}$-NO complex. Therefore, it appears that fluoride and NO can bind simultaneously to the oxidized protein and the resulting complex exhibits a nearly axial high-spin heme EPR signal.

Since the fluoride-bound Hartzell and Beinert preparation of the enzyme exhibits a new high-spin heme EPR signal upon the addition of NO, we have also examined the addition of NO to the fluoride-bound Yu et al. preparation of the protein. In the presence of 10 mM fluoride and NO, the protein isolated by the method of Yu et al. exhibits a nearly axial high-spin heme EPR signal which accounts for 26% of one heme (Fig. 7). As was observed for the Hartzell and Beinert preparation of the protein, the NO complex in the presence of fluoride is reversible upon removal of NO from the sample.

(3) Oxidized Cytochrome c Oxidase-NO plus Cyanide. Upon

FIGURE 7

The EPR spectra of (A) oxidized cytochrome c oxidize prepared by the method of Hartzell and Beinert with NO added; (B) KF added to (A) to give a concentration of 100 mM; (C) oxidized cytochrome c oxidase prepared by the method of Yu et al. with 10 mM KF and NO. The temperature was 9 K, microwave power was 0.05 mW, modulation amplitude was 12.5 G, and microwave frequency was 9.24 GHz for all spectra.


$g_x$=6.16
$g_y$=5.82
A
B
$g_\perp$=5.95
C
g=3.0

addition of cyanide to the oxidized protein-NO complex, the high-spin heme EPR signal observed in the cytochrome $\underline{a}_3^{+3}$, $Cu_{a_3}^{+2}$-NO complex disappears. The oxidized cytochrome $\underline{c}$ oxidase-NO plus cyanide EPR spectrum exhibits normal cytochrome $\underline{a}$ and $Cu_a$ EPR signals. In addition, a signal is observed at g = 3.5 which is characteristic of a cytochrome $\underline{a}_3^{+3}$-$CN^-$ species. The intensity of this new EPR signal at g = 3.5 is equal to that of the high-spin cytochrome $\underline{a}_3$ EPR signal induced by NO. For the Hartzell and Beinert preparation of the enzyme, this low-spin cytochrome $\underline{a}_3^{+3}$-$CN^-$ EPR signal accounts for about 58% of one heme. Thus, cyanide binds quantitatively to cytochrome $\underline{a}_3$ in the enzyme molecules in which NO induces the high-spin cytochrome $\underline{a}_3$ EPR signal. Further, it was found that preincubation of cyanide with the oxidized enzyme for several hours before addition of NO resulted in the appearance of the cytochrome $\underline{a}_3^{+3}$-$CN^-$ EPR signal which now accounted for 100% of one heme. This represents the first time that 100% of both hemes in cytochrome $\underline{c}$ oxidase have been simultaneously observed by EPR. Finally, the low-spin cytochrome $\underline{a}_3^{+3}$-$CN^-$ EPR signal induced by the addition of NO completely disappeared upon the removal of the NO. This result indicates that both NO and cyanide bind simultaneously at the oxygen binding site, with NO forming a reversible complex with $Cu_{a_3}^{+2}$.

The interesting result obtained with cyanide and NO addition to the Hartzell and Beinert preparation of the enzyme led us to investigate this combined ligand effect on the Yu et al. preparation of the enzyme, in which NO does not induce a large high-

spin cytochrome $\underline{a}_3$ EPR signal. The addition of cyanide followed by NO to the oxidized Yu et al. preparation of the enzyme resulted in the appearance of a small low-spin cytochrome $\underline{a}_3^{+3}$-$CN^-$ EPR signal. Interestingly, preincubation of cyanide with the oxidized enzyme for five hours or longer followed by NO addition resulted in the observation of the cytochrome $\underline{a}_3^{+3}$-$CN^-$ EPR signal corresponding to 100% of one heme (Figure 8). Varying the preincubation time of cyanide with the oxidized enzyme between five minutes and seven hours resulted in the observation of increasingly larger amounts of the cytochrome $\underline{a}_3^{+3}$-$CN^-$ EPR signal (Figure 9). Subsequently, Brudvig et al.[29] found that the increase in the cytochrome $\underline{a}_3^{+3}$-CN EPR signal intensity upon cyanide preincubation directly paralleled the decrease in the intensity of a g' = 12 EPR signal*, as shown in Figures 8 and 9. This result taken together with the "oxygenated" oxidase result (see Chapter II, section 3.1(1)) demonstrate that at least three conformations of the oxidized protein exist.

3.3 Interaction of NO with Oxidized Cytochrome c Oxidase in the Presence of Azide. The addition of azide to the oxidized protein-NO complex results in a dramatic change in the optical spectrum (Fig. 6). The Soret band shifts 8nm to lower energy and narrows substantially, while the α-band increases two-fold in intensity. The positions of the Soret bands for various

*The g' = 12 EPR signal arises from a "forbidden" $\Delta m_s = 2$ transition from oxidized cytochrome c oxidase molecules in a conformation in which cytochrome $\underline{a}_3$ and $Cu_{a_3}$ together form an S = 2 state and cytochrome $\underline{a}_3$ has nearly axial symmetry (E is close to zero). In this case, the $\Delta m_s = 2$ transition occurs at an apparent g value of 12 at X-band, and is thus labeled g' = 12.

FIGURE 8

EPR spectra of the Yu et al. preparation of cytochrome c oxidase in the presence of both cyanide and NO. In all cases one atmosphere of NO was added, mixed with the sample for two minutes, and then the sample was immediately frozen at 77°K. The samples contained 0.2 mM cytochrome c oxidase and were preincubated with approximately 2 mM HCN at 4°C before adding NO: (A) Five-minute preincubation. Conditions: temperature, 16°K, microwave power, 0.5 mW; modulation amplitude, 16 G; microwave frequency, 9.23 GHz.

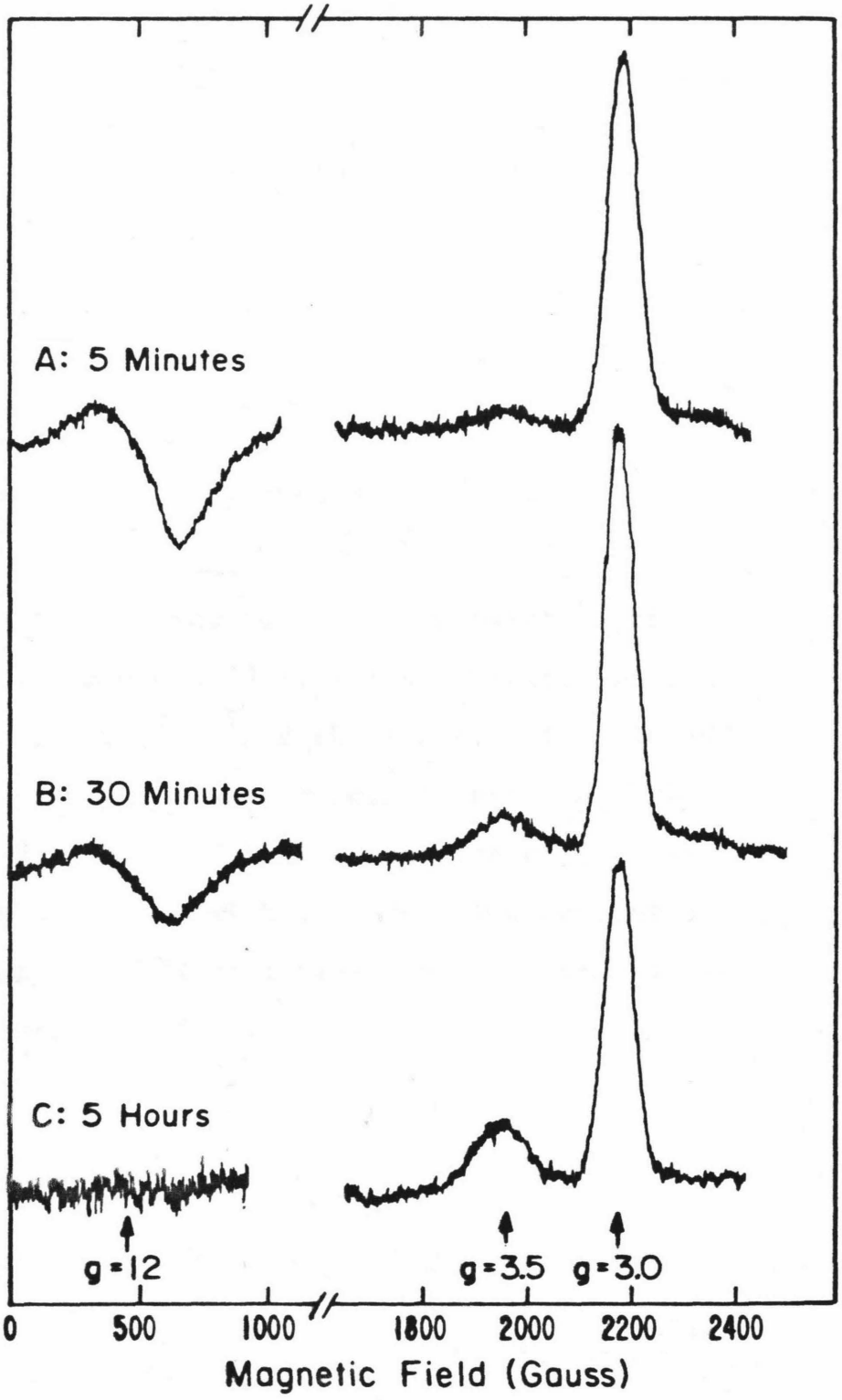
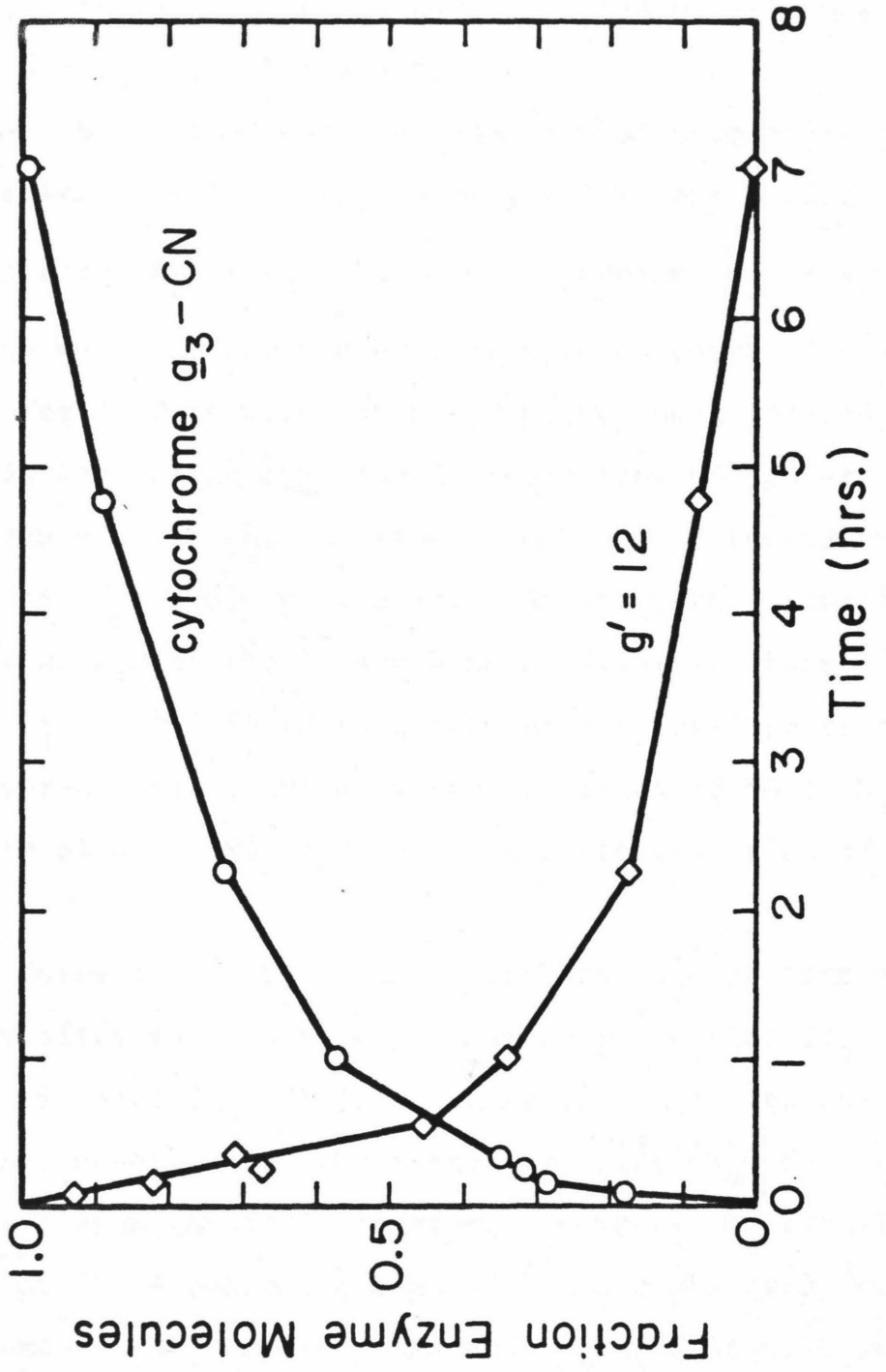

A: 5 Minutes
B: 30 Minutes
C: 5 Hours
g = 12
g = 3.5
g = 3.0
0
500
1000
1800
2000
2200
2400
Magnetic Field (Gauss)

FIGURE 9

Fraction of the Yu et al. preparation of cytochrome c oxidase which exhibited an EPR signal at g'=12 and the fraction in which NO induced a low-spin cyanocytochrome $a_3$ EPR signal at g = 3.5 as a function of time of preincubation with cyanide. The concentrations and EPR conditions were the same as in Figure 2.


Fraction Enzyme Molecules
1.0
0.5
0
0
1
2
3
4
5
6
7
8
Time (hrs.)
cytochrome $a_3$-CN
g' = 12

cytochrome c oxidase species are compared in Table 2. It is clear from this spectral comparison that the effect of azide together with NO is to reduce cytochrome $\underline{a}_3^{+3}$, followed by the binding of NO to cytochrome $\underline{a}_3^{+2}$. We have obtained no evidence that this process affects cytochrome $\underline{a}^{+3}$.

A possible scheme for the reduction of cytochrome $\underline{a}_3$ is given in Reaction 2. This scheme predicts the production of

$$\text{Cytochrome } \underline{a}_3^{+3} + N_3^- + NO \longrightarrow \text{Cytochrome } \underline{a}_3^{+2} + N_2O + N_2 \quad (2)$$

$N_2O$ which we have detected by mass spectroscopy. The substitution of $^{15}NO$ for $^{14}NO$ results in an $N_2O$ parent peak located at M/e = 45, indicating that the nitrogen from NO appears in the $N_2O$ molecule after the reaction. No $N_2O$ is detected in the absence of cytochrome c oxidase, indicating that reaction 2 probably occurs at the ligand binding site(s). Subsequently, Brudvig et al.[25] found that cytochrome c oxidase catalyzes not only reaction 2, but also the reduction of NO to $N_2O$, the oxidation of NO to $NO_2$ and the reversible oxidation of NO to $NO_2^-$.

The formation of the above cytochrome $\underline{a}_3^{+2}$-NO complex does not alter the low-spin cytochrome $\underline{a}^{+3}$ or the $Cu_a$ center EPR signals. (Fig. 10). As expected, the high-spin heme EPR signal observed for the cytochrome $\underline{a}_3^{+3}$, $Cu_{a_3}^{+2}$-NO complex disappears upon the addition of $N_3^-$. However, no EPR signals typical of NO-ferrohemoproteins [9-11] are observed for our cytochrome $\underline{a}_3^{+2}$-NO complex. Instead, <u>new</u> EPR signals appear near g = 2 and at g = 4.34. These results were observed for cytochrome oxidase c prepared by the method of Hartzell and Beinert

Table 2. The positions of the Soret bands for various cytochrome c oxidase species

| Species | Probable State | Soret position: Cytochrome a | Soret position: Cytochrome $a_3$ | Soret Observed |
|---|---|---|---|---|
| Oxidized cytochrome c oxidase | $a^{+3}$, $a_3^{+3}$ | 426 nm[a] | 414 nm[a] | 420 nm |
| Reduced cytochrome c oxidase | $a^{+2}$, $a_3^{+2}$ | 444 nm[a] | 443 nm[a] | 442 nm |
| Oxidized cytochrome c oxidase + NO | $a^{+3}$, $a_3^{+3}$ | 426 nm[b] | 414 nm[c] | 420 nm |
| Reduced cytochrome c oxidase + NO | $a^{+2}$, $a_3^{+2}$-NO | 444 nm[b] | 428 nm | 428, 441 nm |
| Oxidized cytochrome c oxidase + $N_3^-$ + NO | $a^{+3}$, $a_3^{+2}$- NO | 426 nm[b] | 428 nm | 428 nm |

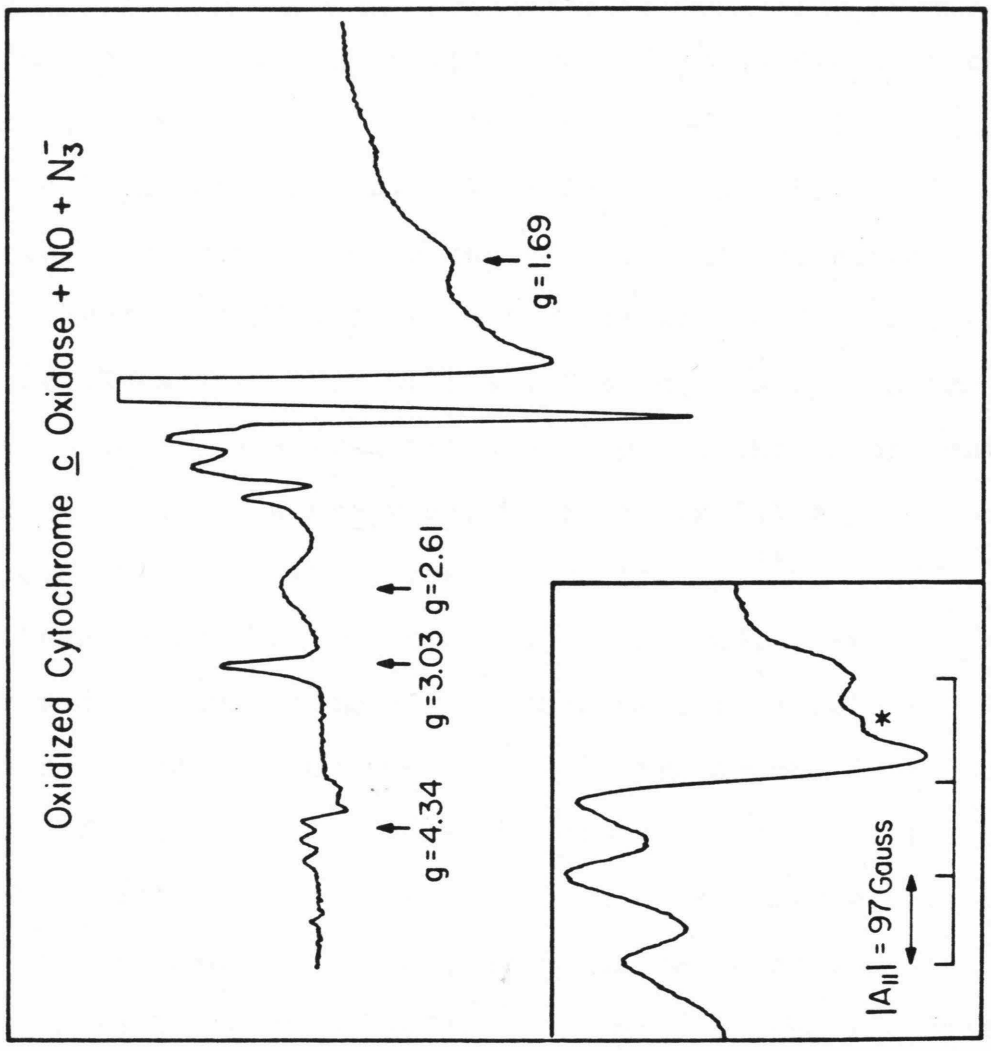
[a]Vanneste, W. H. (1966) Biochemistry 5, 838.

[b]It is assumed that the cytochrome a Soret band is not dependent on the oxidation or ligation state of cytochrome $a_3$.

[c]Cytochrome $a_3^{+3}$ is assumed to be unperturbed by NO since the optical spectrum is virtually unchanged upon the addition of NO to oxidized cytochrome c oxidase.

FIGURE 10

The EPR spectrum of oxidized cytochrome c oxidase in the presence of $N_3^-$ and NO. The inset is a blowup of the half-field transition region. The peak labeled with an asterisk (*) is not part of the triplet signal and is probably due to extraneous ferric iron. The temperature was 7K, microwave power was 2 mW (200 mW for the spectrum shown in the inset), modulation amplitude was 10G, and the microwave frequency was 9.16 GHz.


Oxidized Cytochrome c Oxidase + NO + $N_3^-$
g=4.34
g=3.03
g=2.61
g=1.69
$|A_{II}|$ = 97 Gauss
*

as well as that prepared by the method of Yu et al.

EPR transitions near g = 2 ($\Delta M_s$ = 1) and g = 4 ($\Delta M_s$ = 2) are characteristic of a triplet species with a small zero-field splitting. Accordingly, we have attributed the new EPR signals which we observe near g = 2 and at g = 4.34 to a triplet species. The $\Delta M_s$ = 2 transition which we observe exhibits a four-line hyperfine pattern with a splitting of 97 gauss due to a copper nucleus ($|A_{11}|$ = 0.020 $cm^{-1}$). This value of $|A_{11}|$ is indicative of a Type 2 copper ion (30). We propose that the triplet signals originate from magnetic coupling of the unpaired electron on the cytochrome $\underline{a}_3^{+2}$-NO site with that of the $Cu_{a_3}^{+2}$. This represents the first EPR signal ever observed for $Cu_{a_3}$ in cytochrome $\underline{c}$ oxidase. The $\Delta M_s$ = 1 transition near g = 2 indicates that $|D| \approx |3E| \approx 0.07$ $cm^{-1}$, where D and E are the axial and rhombic zero-field splitting parameters respectively(31).

We have found that the process of NO binding in this one-quarter reduced enzyme complex cannot be immediately reversed by either the removal of the NO atmosphere or the subsequent addition of $O_2$. This is in strong contrast to the oxidized enzyme-NO complex where NO binding can be readily reversed, and in the reduced enzyme-NO complex where removal of the NO atmosphere followed by $O_2$ addition results in the immediate displacement of the bound NO. A very slow reversal of NO binding in the one-quarter reduced enzyme, induced by removal of the NO atmosphere and subsequent $O_2$ addition, results from the known(25) oxidation of NO to $NO_2^-$ in the one-quarter reduced complex. Thus, the stability of this one-electron reduced

complex taken together with the EPR results suggest that NO bridges between cytochrome $\underline{a}_3^{+2}$ and $Cu_{a_3}^{+2}$.

## 4. DISCUSSION

We have studied the interaction of NO with oxidized and reduced cytochrome c oxidase. Different results have been obtained depending on whether NO interacts with the reduced protein or the oxidized protein in either the presence or absence of azide. In the reduced-NO complex with the yeast protein, it has been possible to identify the endogenous axial ligand to cytochrome $\underline{a}_3$. For the oxidized beef heart protein in the absence of azide, we have obtained evidence for a cytochrome $\underline{a}_3^{+3}$, $Cu_{a_3}^{+2}$-NO complex. In the presence of azide, the observations suggest that a bridged cytochrome $\underline{a}_3^{+2}$-NO-$Cu_{a_3}^{+2}$ complex is formed. In both of these oxidized protein complexes, the antiferromagnetic coupling between cytochrome $\underline{a}_3$ and $Cu_{a_3}$ observed in the native oxidized protein is broken.

### 4.1 The Reduced-NO Yeast Cytochrome c Oxidase Complex.

The substitution of $^{15}N_2$-histidine for $^{14}N$-histidine in a histidine yeast auxotroph has allowed the isolation of $^{15}N$-his isotopically labeled yeast cytochrome c oxidase. Nitric oxide binding studies with this labeled protein have allowed identification of histidine as the endogenous axial ligand to cytochrome $\underline{a}_3$. The original suggestion [9] of histidine as the axial ligand to cytochrome $\underline{a}_3$ arose from evidence that a nitrogen atom was bound axially to cytochrome $\underline{a}_3$. This suggestion led to the logical hypothesis [32] of an imidazole bridge between cytochrome $\underline{a}_3$ and $Cu_{a_3}$ facilitating

the strong antiferromagnetic interaction between these two metal centers.

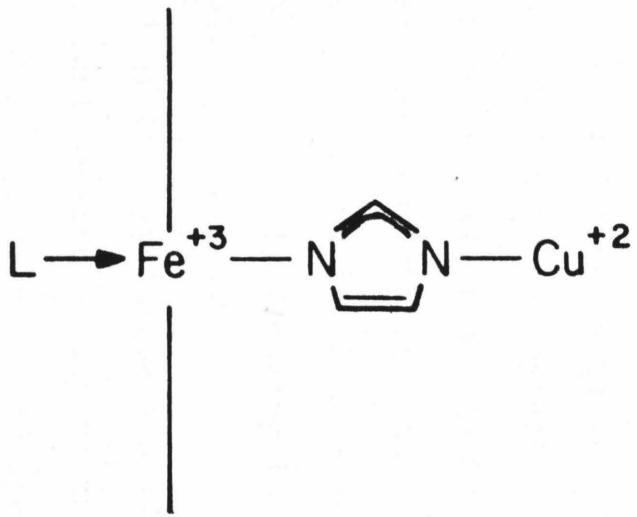
With the present definitive assignment of histidine as the axial ligand, two models for the oxygen binding site are possible (Figure 11). In model A, a strongly-bound imidazole bridges the iron and copper metal centers[32], with the ligand binding site being the free axial position of the heme iron. In model B, the ligand binding site is between the two metal centers[33]. However, since the results of the interaction of NO with the oxidized protein in the presence of azide provide the most discriminating evidence for the two models, the relative merits of the two models will be discussed in section 4.4.

4.2 Interaction of NO with Oxidized Cytochrome c Oxidase. We have shown that NO interacts with oxidized cytochrome c oxidase and breaks the antiferromagnetic couple between the iron and copper. The resulting complex exhibits a new high-spin heme EPR signal which corresponds to as much as 58% of cytochrome $a_3$. The optical spectrum of the oxidized protein remains unchanged upon the addition of NO. Taken together, these observations indicate that there is no NO-heme interaction. Moreover, no EPR signals are observed for $Cu^{+2}_{a_3}$ in the presence of NO. Therefore, it appears that NO interacts with $Cu^{+2}_{a_3}$ to form a cytochrome $a_3^{+3}$, $Cu^{+2}_{a_3}$-NO complex. This represents the first solid evidence that $Cu_{a_3}$ binds exogenous ligands.

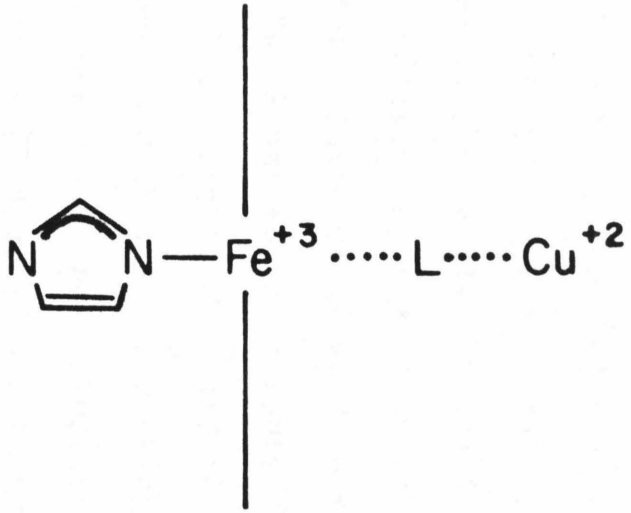
There are two ways in which NO might interact with $Cu^{+2}$

FIGURE 11

Two proposed structures for the cytochrome $\underline{a}_3$-$Cu_{a_3}$ couple in oxidized cytochrome $\underline{c}$ oxidase. L stands for a possible ligand.




A




B

to produce an EPR silent species: (i) that NO reduces the copper or (ii) that NO coordinates to the copper to form an exchange coupled complex. We feel that the situation (ii) above is more likely, since the formation of our cytochrome $\underline{a}_3^{+3}$, $Cu_{a_3}^{+2}$-NO complex is easily reversible upon the removal of NO. We have observed no signal which can be assigned to a $Cu_{a_3}^{+2}$-NO triplet. Inasmuch as we do observe a high-spin cytochrome $\underline{a}_3$ EPR signal which accounts for as much as 58% of one heme, we believe that $Cu_{a_3}^{+2}$ and NO spins are antiferromagnetically coupled with a large exchange interaction relative to kT. In support of the contention that NO forms a strongly exchange coupled complex with $Cu_{a_3}^{+2}$, it is worth noting that the temperature dependence of the high-spin cytochrome $\underline{a}_3$ signal can be fit by taking only the Boltzman factor into account. This indicates that this metal center is magnetically isolated from cupric $Cu_{a_3}$, necessitating that the $Cu_{a_3}^{+2}$-NO complex be diamagnetic.

When NO is added to oxidized cytochrome c oxidase, we do not observe a signal from 100% of cytochrome $\underline{a}_3$. This could arise because of a conformational heterogeneity of the enzyme molecules which renders only a subpopulation of the enzyme capable of binding NO. However, it is also possible that NO does bind to all of the cytochrome c oxidase molecules but that a conformational heterogeneity of the protein allows only a fraction to be observed by EPR spectroscopy. In order to observe a high-spin cytochrome $\underline{a}_3$ EPR signal the interaction of NO with $Cu_{a_3}^{+2}$ must dominate the exchange interaction

between $Cu^{+2}_{a_3}$ and cytochrome $\underline{a}_3^{+3}$. It is probable that a change in conformation of the cytochrome $\underline{a}_3$-$Cu_{a_3}$ site will alter the magnitude of the exchange interaction between these two metal centers. Thus, it is likely that more than one conformation of NO bound oxidized cytochrome $\underline{c}$ oxidase exists: one in which the cytochrome $\underline{a}_3$-$Cu_{a_3}$ interaction is large with respect to the $Cu_{a_3}$-NO interaction resulting in an EPR silent cytochrome $\underline{a}_3$ and another in which the $Cu_{a_3}$-NO interaction dominates resulting in an EPR visible cytochrome $\underline{a}_3$.

The binding studies with fluoride and cyanide to the oxidized protein-NO complex demonstrated the existence of at least three distinct conformations of the oxidized protein: (i) the conformation giving rise to the rhombic high-spin cytochrome $\underline{a}_3$ EPR signal upon NO addition, (ii) the conformation in the Yu et al. preparation which gives rise to the axial high-spin cytochrome $\underline{a}_3$ EPR signal immediately upon the addition of NO and fluoride, and (iii) the conformation which slowly binds cyanide to give the low-spin cytochrome $\underline{a}_3^{+3}$-$CN^-$ EPR signal upon NO addition (40% of the enzyme molecules isolated by the method of Hartzell and Beinert). These studies laid the ground work for the much more extensive conformational study of cytochrome $\underline{c}$ oxidase of Brudvig et al.[29]. In this study[29], it was concluded that there are at least four distinct conformations and the sequence of their formation upon reoxidation of the reduced enzyme was elucidated. The sum of these conformations accounted for 100% of the enzyme molecules in both the Hartzell and Beinert and the Yu et al. preparations of the protein.

4.3 Interaction of NO with Oxidized Cytochrome c Oxidase in the Presence of Azide. We have shown that $N_3^-$ addition to the cytochrome $\underline{a}_3^{+3}$, $Cu_{a_3}^{+2}$-NO complex results in reduction of cytochrome $\underline{a}_3$ to the ferrous state, followed by the binding of NO to the cytochrome $\underline{a}_3$ moiety. Furthermore, EPR signals typical of a triplet species appear, originating from the thermally accessible S = 1 excited state of the antiferromagnetically coupled spins of the NO-bound site and $Cu_{a_3}^{+2}$. Magnetic coupling between the cytochrome $\underline{a}_3^{+2}$-NO complex and $Cu_{a_3}^{+2}$ could arise if NO bridges the two metal sites.

Our proposal for the NO bridge is consistent with the known affinity of NO for ferrous cytochrome $\underline{a}_3$[9,10] as well as the affinity for cupric $Cu_{a_3}$ established here[12]. The evidence (EPR and optical) that neither the removal of NO from the sample nor the subsequent addition of $O_2$ results in the immediate displacement of NO in the cytochrome $\underline{a}_3^{+2}$-NO-$Cu_{a_3}^{+2}$ complex, indicates that this bridge is a stable one. In contrast, the binding of NO in the case of the fully reduced cytochrome c oxidase-NO complex ($Fe_{a_3}^{+2}$-NO) and in the case of cytochrome $\underline{a}_3^{+3}$, $Cu_{a_3}^{+2}$-NO ($Cu^{+2}$-NO) is reversible.

The value of $|A_{11}|$, 0.020 $cm^{-1}$, obtained from the hyperfine splittings observed for the $\Delta M_s = 2$ transition of the cytochrome $\underline{a}_3^{+2}$-NO-$Cu_{a_3}^{+2}$ triplet species is indicative of a type 2 copper site. In this regard, recent resonance Raman studies[34,35] have demonstrated that neither copper center in native oxidized cytochrome c oxidase is a type 1 copper site. In addition, $Ag^+$ binding studies by Brudvig and Chan[33]

demonstrated that $Cu_{a_3}$ is not a type 1 copper site. Taken together, these results suggest that the structure of $Cu_{a_3}$ in the native oxidized protein may be similar to a type 2 copper center. It has recently been found by Malmström et al.[35] that a $Cu_{a_3}$ EPR signal can be induced from the partially reduced enzyme in the presence of both CO and $O_2$. This allows a state of the protein to be prepared in which cytochrome $\underline{a}_3$-CO is reduced and $Cu_{a_3}$ is oxidized. The EPR signal observed from $Cu_{a_3}$, with g values of 2.28, 2.11, 2.05, and $A_{11} = 0.010\ cm^{-1}$, is quite similar to that of copper in superoxide dismutase[36] (g = 2.26, 2.10, 2.03; $A_{11}$ = 0.013 $cm^{-1}$).

It is reasonable to assume that the structure of $Cu_{a_3}$ is similar to that of the superoxide dismutase copper, for which three histidine imidazole ligands form a plane with the copper, while a fourth imidazole is bent slightly out-of-plane[38]. Furthermore, one axial position on the superoxide dismutase copper was found to be available for the coordination of exogenous ligands, analogous to the ability of $Cu_{a_3}$ to bind NO.

4.4 The Structure of the Cytochrome $\underline{a}_3$-$Cu_{a_3}$ Site in Cytochrome c Oxidase. The findings reported here permit us to make some definite conclusions regarding the structure of the cytochrome $\underline{a}_3$-$Cu_{a_3}$ site. Two models which have been proposed for the cytochrome $\underline{a}_3$-copper site are shown in Figure 11. Our evidence that NO bridges the two metal centers in the cytochrome $\underline{a}_3^{+2}$-NO-$Cu_{a_3}^{+2}$ complex strongly argues in favor of model B.

Model B would allow formation of a bridged "peroxy" species as an intermediate in the reduction of molecular oxygen to water by cytochrome c oxidase. In this regard, the distance between cytochrome $a_3$ and $Cu_{a_3}$ may be estimated from the magnitude of D, the zero-field splitting in the cytochrome $a_3^{+2}$-NO-$Cu_{a_3}^{+2}$ complex. A value for |D| of 0.07 $cm^{-1}$ has been obtained from the breadth of the $\Delta M_s = 1$ transition for this triplet state. A distance of 3.4 Å between the spin centers can be calculated assuming a purely dipolar interaction[38]. However, spin-orbit and exchange interactions can also contribute to the zero-field splitting in triplets. These contributions to the zero-field splitting in the cytochrome $a_3^{+2}$-NO-$Cu_{a_3}^{+2}$ complex have been estimated[39] and appear to be quite small. Taking these considerations into account and the fact that the spin on cytochrome $a_3^{+2}$-NO lies primarily on the nitrogen of NO, we estimate that the distance between cytochrome $a_3$ and $Cu_{a_3}$ is about 5 Å. This distance is close to that expected if dioxygen bridges between the two metals. This close proximity is likely important in the stabilization and anchoring of reactive intermediates which are formed during the reduction of dioxygen to water.

Model B also suggests the possibility of the formation of a μ-oxo bridge between these two metal centers after the complete reduction of $O_2$. It might be that the "oxygenated" enzyme produced by reaction of the reduced enzyme with $O_2$ consists of such a μ-oxo bridge at the cytochrome $a_3$-$Cu_{a_3}$ site. It is possible that the "oxygenated" enzyme differs

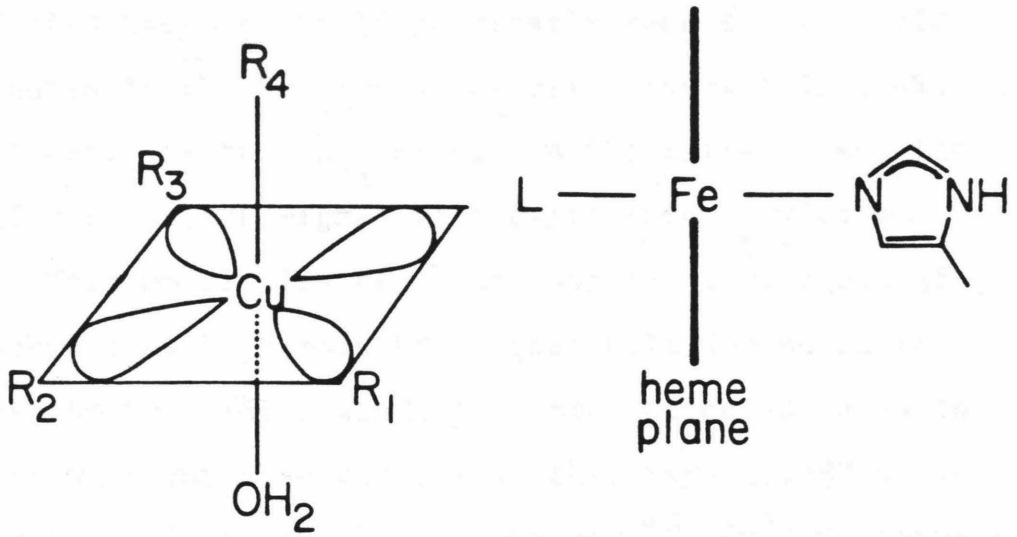
from the oxidized "resting" enzyme only in terms of the nature of the bridging ligand. If this is the case, then the conformational difference normally associated with these two states of the enzyme might be understood. In any case, model B would seem to confer some degree of conformational flexibility on this part of the enzyme, inasmuch as cytochrome $\underline{a}_3$ apparently can accommodate a number of exogenous ligands of varying sizes as its sixth ligand.

Our finding that $Cu_{a_3}$ is similar to a type 2 copper in the cytochrome $\underline{a}_3^{+2}$-NO-$Cu^{+2}_{a_3}$ complex suggests that $Cu_{a_3}$ has a similar ligand environment in the native oxidized enzyme, i.e., square-planar coordination or octahedral coordination with a strong tetragonal distortion. For a $d^9$ copper involved in square-planar coordination, it is well known that the unpaired electron is in a $d_{x^2-y^2}$ orbital, with the orbital lobes directed towards the ligands in the square plane. The strong antiferromagnetic coupling between cytochrome $\underline{a}_3$ and $Cu_{a_3}$ in the oxidized enzyme would be facilitated through a bridging ligand bound equatorially to $Cu_{a_3}$ as is illustrated in Figure 12a. This model raises the exciting possibility of modulating the interaction of $Cu_{a_3}$ with the bridging ligand, and hence cytochrome $\underline{a}_3$, via the binding of strong exogenous ligands above or below the square plane of $Cu_{a_3}$. The attachment of a strong field ligand to the axial position of $Cu_{a_3}$ should place the unpaired electron in a square plane containing the stronger ligand (Fig. 12b). When this occurs, the interactions of the copper ion with cytochrome $\underline{a}_3$ will be modified.

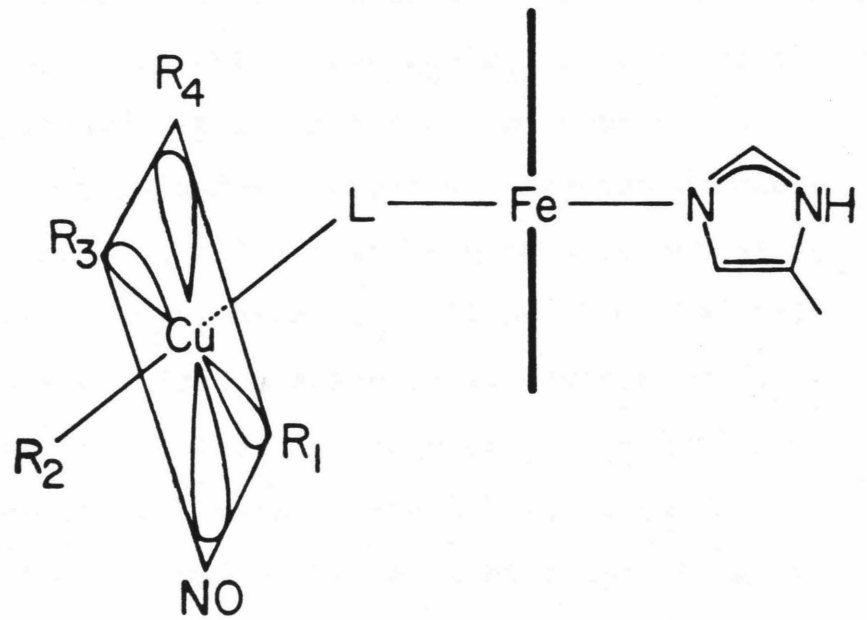
FIGURE 12

Proposed structure of the cytochrome $\underline{a}_3$ - $Cu_{a_3}$ site in (A) native oxidized cytochrome $\underline{c}$ oxidase and (B) NO-bound oxidized cytochrome $\underline{c}$ oxidase. $R_1$, $R_2$, $R_3$, and $R_4$ denote endogenous ligands and L denotes the bridging ligand (which may or may not be endogenous).

A




B



This model explains the results of our EPR studies of the NO-bound oxidized enzyme. If NO is a stronger field ligand than $R_2$ and L (Fig. 12), then binding of NO to $Cu_{a_3}$ in the axial position would shift the axes of the ligand field. In this manner, the antiferromagnetic exchange interaction between $Cu_{a_3}$ and cytochrome $\underline{a}_3$ would be greatly reduced; it would be eliminated if the $Cu_{a_3}$-NO interaction results in a diamagnetic center at the $Cu_{a_3}$ site. In the latter case, the observation of an EPR signal from cytochrome $\underline{a}_3$ will be allowed. This model also predicts that the appearance of the cytochrome $\underline{a}_3$ high-spin EPR signal will depend on the nature of the bridging ligand, L, a prediction which is in accordance with our observations on the "oxygenated" enzyme and the effect of $CN^-$ on the cytochrome $\underline{a}_3^{+3}$, $Cu_{a_3}^{+2}$-NO complex.

Finally, we consider the implications of our NO experiments on the mechanism by which cytochrome $\underline{a}_3$-$Cu_{a_3}$ site reduces oxygen. In this regard, it is important to compare the reduction of the protein under aerobic and anaerobic conditions. When cytochrome $\underline{c}$ oxidase is reduced anaerobically, $Cu_{a_3}$ is reduced before cytochrome $\underline{a}_3$. Also, when the fully reduced enzyme is reoxidized anaerobically, cytochrome $\underline{a}_3$ is oxidized before $Cu_{a_3}$. These differences in reduction potentials between the two metal centers have allowed cytochrome $\underline{a}_3$ to be observed by EPR as a high-spin heme in a state where cytochrome $\underline{a}_3$ is oxidized while $Cu_{a_3}$ is reduced[5]. However, it is clear from this work that in the presence of NO, the reduction potential of cytochrome

$\underline{a}_3$ becomes higher than that of $Cu_{a_3}$. This raises the question of whether or not the reduction potentials measured on the anaerobic enzyme have any direct bearing on the enzyme during its reaction with dioxygen. Presumably the coordination of dioxygen to the enzyme would also greatly perturb the reduction potential of cytochrome $\underline{a}_3$. In fact, it is possible that the cytochrome $\underline{a}_3^{+2}$-NO-$Cu_{a_3}^{+2}$ triplet state, which is stabilized by NO, resembles a state formed during the reaction of the enzyme with dioxygen. Thus, the role of cytochrome $\underline{a}_3$ may be to anchor dioxygen to the enzyme while remaining in the ferrous state, and the role of $Cu_{a_3}$ may be to receive electrons from the cytochrome $\underline{a}/Cu_a$ centers and sequentially transfer them to dioxygen.

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# CHAPTER III: STRUCTURE OF THE $Cu_a$ CENTER

## 1. INTRODUCTION

The $Cu_a$ center of cytochrome c oxidase was first characterized by EPR by Beinert et al.[1]. They noted that only half of the copper in the protein was accounted for by the EPR spectrum. That observation is consistent with the subsequent determination[2,3] that the second copper ($Cu_{a_3}$) was strongly exchange coupled with cytochrome $a_3$ to produce an EPR silent coupled site. In addition, Beinert et al.[1,4] found the $Cu_a$ EPR signal to be unlike any EPR signal previously recorded for copper proteins or copper complexes. This latter observation has led to a considerable number of spectroscopic investigations aimed at elucidating the unique nature of the $Cu_a$ center.

The EPR signal of the $Cu_a$ center is unique in two respects. First, one of the g values is below that of the free electron value of 2.0023. Second, copper hyperfine splittings are not observed with X-band EPR spectroscopy. The mechanisms most commonly used to explain the unique $Cu_a$ EPR properties are through extensive spin delocalization from $Cu_a$ to its ligands[5,6] or through the mixing of 4s and 4p orbitals into the 3d ground state brought about by distortion of copper into a near tetrahedral geometry[7].

The spin delocalization mechanism for explaining the unique $Cu_a$ EPR signal suggested the presence of a sulfur radical[5] rather than a Cu(II) ion. The proposal of a sulfur radical in cytochrome c oxidase did not receive considerable

attention until the x-ray absorption study of Hu et al.[8]. They interpreted the copper x-ray absorption edge as arising from one Cu(II) ($Cu_{a_3}$) and a Cu(I) ($Cu_a$). The x-ray absorption edge studies together with the unique $Cu_a$ EPR properties and the $Ag^+$ titrations of cytochrome c oxidase led to the model for the $Cu_a$ center of Chan et al.[9,10] shown in Figure 1. In this model, $Cu_a$ is ligated to two cysteines and two histidines, with the delocalization of an electron from one of the cysteinyl sulfurs to $Cu_a$, resulting in a $Cu_a(I)$-$\dot{S}$ spin system.

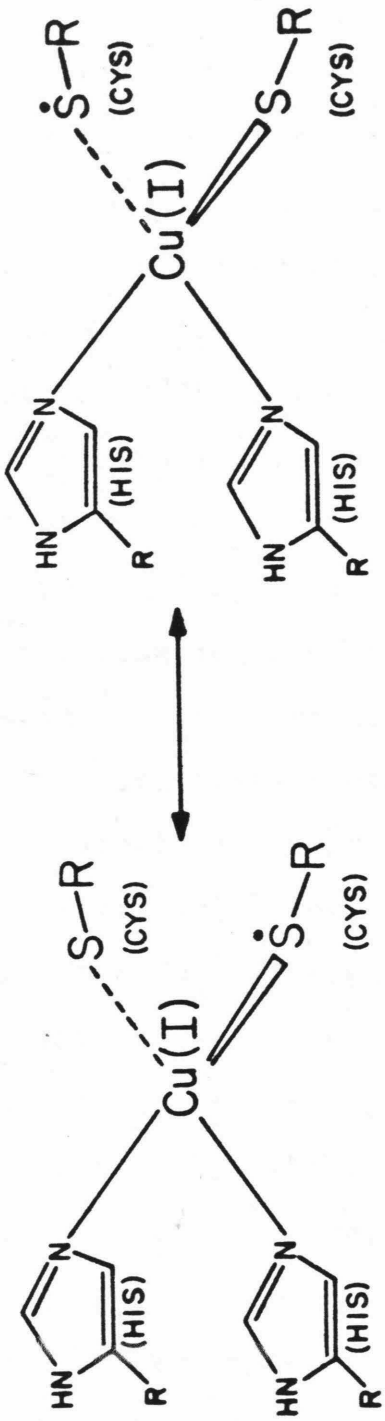
Following the presentation of this model for the $Cu_a$ center, there has been a flurry of spectroscopic investigations aimed at determining the validity of a sulfur radical model. Some of the more enlightening studies will be described here.

The measurement of the EPR signal of $Cu_a$ at S-band (3 GHz) allowed the resolution of copper hyperfine for the X and Z orientations[11]. The copper hyperfine couplings are 45 and $\lesssim$40 G in the Y and Z directions, respectively, both of which are small for copper. In addition, a 25 G hyperfine coupling due to a S = 1/2 site was resolved only in the Y orientation. This 25 G coupling could either be due to a strongly coupled proton or a static dipolar splitting of $Cu_a$ by cytochrome a[12], if these two centers are sufficiently close (10-13 Å). Recent ENDOR studies have lent some insight into the origin of this 25 G coupling in the $Cu_a$ EPR spectrum[13,14].

The ENDOR spectra revealed at least one nitrogen coupling

FIGURE 1

Proposed structure for the $Cu_a$ center in cytochrome c oxidase.


S—R
(CYS)
S—R
(CYS)
Cu(I)
N
(HIS)
HN
R
N
(HIS)
HN
R
S—R
(CYS)
S—R
(CYS)
Cu(I)
N
(HIS)
HN
R
N
(HIS)
HN
R

and two strong proton couplings in the $Cu_a$ center EPR signal. However, the proton couplings were small, 4 and 6 G, and the ENDOR spectra did not reveal a 25 G proton coupling which could account for the 25 G coupling observed in the S-band EPR spectrum. These studies thus indicated that this 25 G coupling might arise from a dipolar splitting of $Cu_a$ by cytochrome a. In addition, the most recent ENDOR investigation of cytochrome c oxidase by Hoffman et al.[14] observed copper hyperfine couplings to $Cu_a$ EPR signal that were essentially isotropic ($|A_x|$ = 68 MHz, $|A_y|$ = 98 MHz, $|A_z|$ = 90 MHz). This observation led the authors to conclude that the best interpretation of the $Cu_a$ EPR spectrum is that it arises from a Cu(II), in support of the proposal[7] that the admixture of 4s and 4p orbitals into the 3d ground state could account for this EPR spectrum. However, calculations taking into account the g anisotropy and the very small nearly isotropic copper hyperfine interaction of the $Cu_a$ EPR signal[12], indicate it is necessary to mix three times as much 4p orbital character as 3d in order to fit the EPR properties of the $Cu_a$ center. Thus, it appears that the best description of the $Cu_a$ center is that of a Cu(I)-$\dot{S}$ complex.

The exact nature of the ligands to $Cu_a$ has been more difficult to ascertain. The presence of at least one nitrogen at the $Cu_a$ center was implicated in the ENDOR studies and in the $Ag^+$ titrations[9,10]. The possibility that cysteine was a ligand to $Cu_a$ was supported by the similarity of the EPR properties of $Cu_a$ and sulfur radicals. In addition, cysteine

would provide methylene protons adjacent to sulfur that could strongly couple to the electron spin. We thus initiated investigations with the yeast enzyme in an attempt to obtain isotopically labeled cytochrome c oxidase so as to determine the ligands to $Cu_a$ and the nature of its unique EPR properties. From our model shown in Figure 1, it was felt that isotopically labeled histidine and cysteine proteins would likely yield the most information. In this chapter, we report the incorporation of $^{15}N$-histidine and $^{12}CD_2$-cysteine into histidine and cysteine yeast auxotrophs, respectively. The isotopically labeled cytochrome c oxidase molecules were isolated from these auxotrophs and the EPR spectra were obtained. These studies have lent considerable insight into the nature of the ligands to $Cu_a$ and origin of this metal center's unique EPR properties.

## 2. MATERIALS AND METHODS

Beef heart cytochrome c oxidase was isolated by the method of Hartzell and Beinert[15]. The purified protein was dissolved in 0.5% Tween 20/50 mM Tris-$HNO_3$, pH 7.4 to a concentration of 56 mg/ml. This preparation of the protein contained 9 nmoles heme a/mg protein.

All the chemicals used in the enzyme purifications were of enzyme grade when available, and otherwise they were reagent grade. All the chemicals used in the growth of yeast such as vitamins, amino acids and galactose were the highest grade available from Sigma. The labeled histidine used for yeast growth was 95% $^{15}N$ in both histidine ring nitrogen

positions and was obtained from VEB BERLIN-CHEMIE, BERLIN-ADLERSHOF.

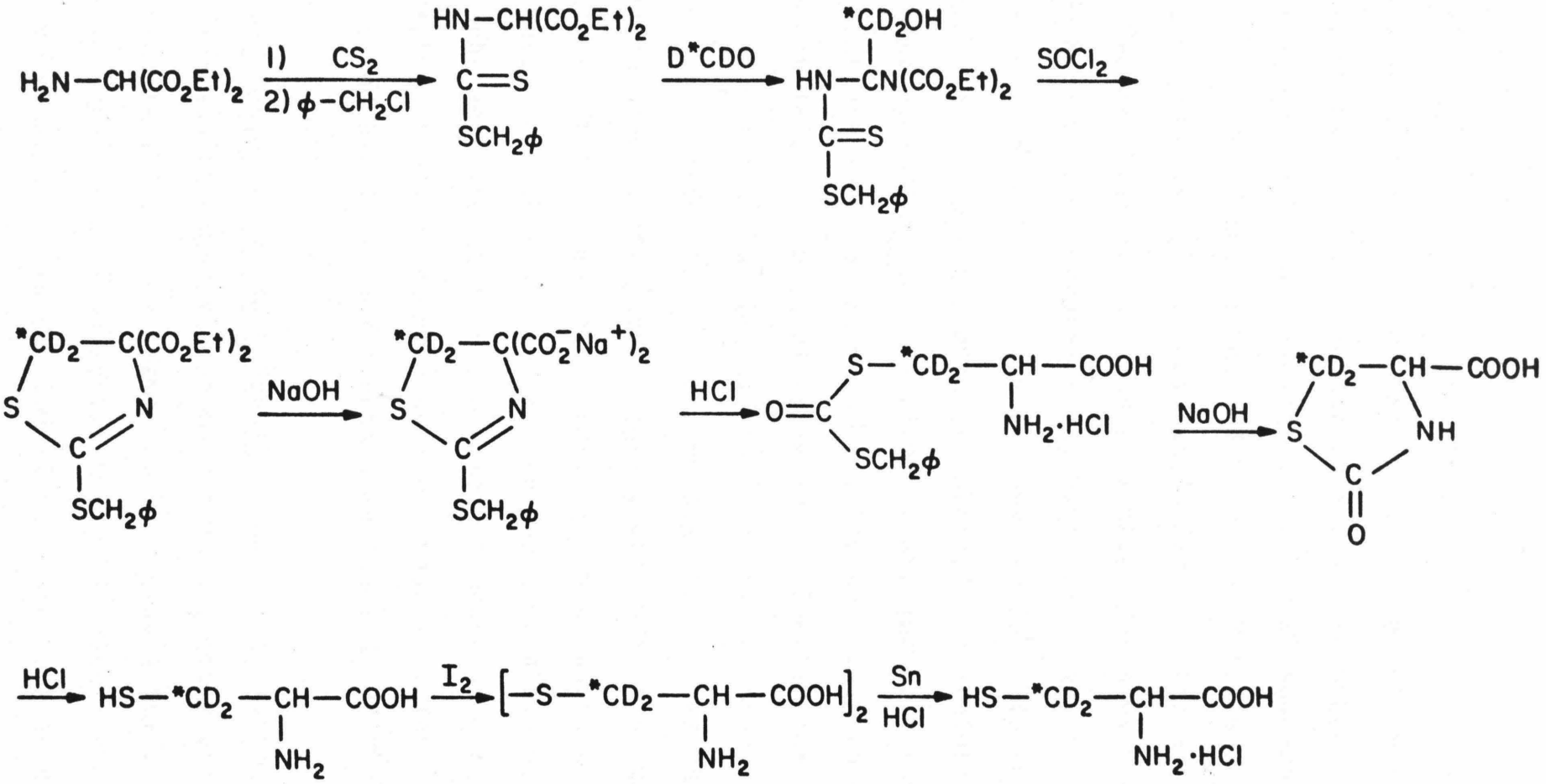
2.1 Synthesis of $^{12}CD_2$-Cysteine. Cysteine was synthesized according to the malonate condensation procedure of Crawhall and Elliot[16] as modified by Beilan[17] for use without the isolation and purification of intermediates. This procedure afforded the easiest approach to the specific labeling of the β carbon. Figure 2 outlines the synthesis. This procedure involved the condensation of formaldehyde with diethyl ((benzylthio)thiocarbonylamino) malonate (DTBM), followed by cyclization with thionyl chloride to give thiazoline. Saponification, decarboxylation and hydrolysis yields S-((benzylthio)carbonyl)) cysteine, which was cyclicized to 2-ketothiazolidine-4-carboxylic acid and finally hydrolyzed in acid to cysteine. Cysteine was oxidized to cystine, which was then isolated and recrystallized. Purified cystine was reduced to D,L-cysteine with tin and HCl. The expected yield was 48%.

DBTM was synthesized from diethylaminomalonate in three batches, the average yield of which was 87%. $^{12}CD_2$-cysteine was synthesized from DBTM and $D_2$-formaldehyde (98% D, Stohler). $D_2$-formaldehyde was prepared by refluxing paraformaldehyde ($-CD_2-O-$) as a 0.5 M solution. The $^{12}CD_2$-cysteine was prepared in six batches, the average yield of which was 28%.

2.2 Preparation and Isolation of Yeast Auxotrophs. The wild-type haploid Saccharomyces cerevisiae strain D273-10B was mutagenized with ethylmethanesulfonate as described by

FIGURE 2

Outline of the conversion of diethylamino malonate to cysteine with the isotopic label being introduced as formaldehyde.



Fink[18]. Freshly grown yeast cells were suspended in 3% ethylmethanesulfonate at 30°C for 50 min. The cells were then washed three times by centrifuging and resuspension in sterile water, each time transferring the cell suspension to a new tube. The final washed and resuspended cells were diluted tenfold into liquid minimal media with the desired growth factor (histidine if one is looking for a histidine auxotroph), which was cysteine and histidine together. These cells were allowed to grow for two days at 30°C, followed by plating out on petri plates (to about 100 colonies/plate) containing minimal media plus either histidine or cysteine. Cells that grew on minimal plus cysteine were then checked for growth in the absence of cysteine and checked for growth on defined media containing all amino acids except cysteine. Colonies that proved to be cysteine auxotrophs were then finally checked for respiratory-deficient mutations, characteristic of "petite" mutants. Colonies that passed all the above tests were then stored on rich media at 4°C. Histidine auxotrophs were isolated by the same procedure as outlined above except the supplemented nutrient was histidine. The cysteine and histidine auxotrophs were labeled 10B $Cys_1^-$ 10B $His_1^-$, respectively.

2.3 Large Scale Yeast Growth. For the isolation of yeast mitochondria and cytochrome c oxidase, the yeast cells were grown in a 350 liter fermentor which was interfaced to a Sharples continuous flow centrifuge. For growth of the wild-type yeast cells in the 350 liter fermentor the media

contained yeast nitrogen base components[19], 1% galactose, 1 Kg casamino acids (Difco), 11 g each uracil and adenine, 11 g penicillin and 17 g streptomycin. In addition, 95% ethanol was added as an additional carbon source at a rate of about 1 gallon/day. A freshly grown yeast culture was used to inoculate the 350 liter fermentor to a level of about $10^6$ cells/ml, and growth was allowed to proceed to about $5x10^8$ cells/ml. All yeast growth was carried out at 30°C. The yield of yeast cells after 3 days of growth was about 5-6 Kg wet weight.

The yeast auxotrophs were grown as described for the wild-type cells except the media contained yeast nitrogen base components, 1% galactose, 11% each uracil and adenine, 17 g of all L-amino acids except either histidine or cysteine, 11 g penicillin and 17 g streptomycin. In the case of the histidine auxotroph 2 g D,L-histidine·HCl (95% $^{15}N_2$) were added to the media. For the cysteine auxotroph 6 g D,L-cysteine·HCl (98% $^{12}CD_2$) were added to the growth media. The starter cultures of the two auxotrophs were added to cell densities of about $3x10^6$ cells/ml. The cultures were allowed to grow for about 2 - 3 days, with monitoring for revertants and contaminants every 12 hours. The cells were harvested when the increase in cell density began to level off (~$3-5x10^7$ cells/ml), and at this point a sample of the culture was removed to determine the level of revertants. For both the cysteine and histidine auxotrophs the level of revertants at the completion of growth was less than 0.004%.

2.4 Isolation of Yeast Mitochrondria. Mitochondria were isolated from yeast by the method of Tzagoloff[20] as modified by Shakespeare and Mahler[21]. The yeast cells were frozen in liquid nitrogen (~500 g, wet weight) and transferred to a pre-cooled one gallon steel Waring blender. This frozen pellet was blended on high-speed until all chunks were reduced to a very fine powder. About 1400 mls of room temperature buffer, 0.4 M sucrose, 50 mM Tris/acetate, 2 mM EDTA, pH 7.4 were added to the blender. This suspension was then homogenized for 2 minutes alternating between low and high speed. After adjusting the pH to about 7.5 with KOH the suspension was centrifuged at 2000 xg for 15 min. The supernatant was adjusted to pH 5.2 with acetic acid and centrifuged at 54,000 xg for 30 min, and the mitochondrial pellets were then resuspended in 50 mM phosphate, 1% KCl, 1 mM EDTA, pH 7.4. Since each liquid nitrogen treatment resulted in breakage of only between 15 - 20% of the yeast cells, this procedure was repeated until almost all cells were broken, as evidenced by the size of the light fluffy layer of broken cell debris in the 2000 xg pellet. The mitochondria were resuspended in the $PO_4$/KCl/EDTA buffer to a protein concentration of 20 mg/ml, as determined by the method of Lowry et al.[22].

2.5 Isolation of Yeast Cytochrome c Oxidase. Many of the published procedures for the isolation and purification of yeast cytochrome c oxidase were attempted, but none were particularly satisfactory. This probably results from the

fact that most yeast oxidase procedures were not designed to yield a pure preparation by EPR criteria, as was needed for this work. For this reason various portions of several procedures were combined to yield a new method for the isolation of the yeast protein which produced samples free from contaminating EPR signals.

The isolated mitochondria at 20 mg/ml protein concentration were solubilized by the slow addition of 3 mg cholate (Calbiochem A grade) per mg protein as a 20% solution. All steps in the isolation were carried out at 0-4°C. To this suspension was added 176 g solid ammonium sulfate/liter (30% A.S.) with stirring. After ammonium sulfate addition the pH was adjusted to 7.4 and the suspension stirred for 4 hours. This suspension was centrifuged at 27,000 xg for 30 minutes, and the supernatant brought to 45% of saturation with the addition of 94 g solid ammonium sulfate/liter. After stirring for 15 minutes the suspension was centrifuged at 27,000 xg for 20 minutes. The pellets were resuspended in 15 ml/g of original mitochondrial protein with 0.25 M sucrose/50 mM Tris·acetate/0.5% cholate/pH 7.4 buffer and adjusted to 28% of saturation with a saturated ammonium sulfate solution (saturated at 4°C). All subsequent additions of ammonium sulfate were from a saturated solution. This solution was stirred 15 minutes followed by centrifugation at 27,000 xg for 20 min. The supernatant was adjusted to 39% of ammonium sulfate saturation, followed by centrifugation at 27,000 xg for 20 minutes. These pellets were

resuspended in 2 ml/g of original mitochondrial protein with 1% Tween-80/20 mM phosphate/1 mM EDTA/pH 7.0 buffer. This solution was then dialyzed 24 hours against a 100-fold larger volume of 0.5% cholate/10 mM Tris•acetate/1 mM EDTA/ pH 7.4. This dialysis usually resulted in some precipitation of protein which was removed by centrifugation at 200,000 xg for 30 min. The clarified high-speed supernatant was applied to a cytochrome c affinity column (1.5x30 cm, 25 cm bed height) previously equilibrated with the above dialysis buffer. This cytochrome c affinity column was prepared by the linking of cytochrome c (Sigma Type VI) to CNBr-activated sepharose 4B (Sigma) by published procedures[23,24]. After all the green protein solution had passed onto the column, the column was washed first with 100 mls of dialysis buffer followed by 100 mls of dialysis buffer containing 2% cholate. Very little green color eluted with these wash buffers, as it was mostly brownish-gold proteins that eluted under these conditions. The cytochrome c oxidase was eluted with 0.5% cholate/10 mM Tris•acetate/1 M KCl/1 mM EDTA/pH 7.4 buffer. The green fractions were collected and cholate was added (as a 20% solution) to a final concentration of 1.5%. This solution was then brought to 25% of ammonium sulfate saturation and centrifuged at 20,000 xg for 10 minutes. The supernatant was brought to 38% of ammonium sulfate saturation and centrifuged at 20,000 xg for 10 minutes. The 38% A.S. pellet should be dark green and was dissolved in a small volume of dialysis buffer, so as to keep the protein concentrated for

EPR experiments. The protein solution was then stored at -85°C until use.

2.6 EPR Spectroscopy. EPR spectra were typically recorded on .3-.4 ml samples of the enzyme at a protein concentration of 0.1-0.2 mM. The EPR spectra were recorded on a Varian E-line century series X-band spectrometer equipped with an Air-Products Heli-Trans low temperature system.

2.7 NMR Spectroscopy. The $^{13}C$ NMR spectra for $^{15}N$-histidine and $^{12}CD_2$-cysteine were run on a JEOL FX90 and Varian XL-100 NMR spectrometers, respectively. In both cases $D_2O$ was used as an internal lock. Both samples were run under conditions of proton decoupling. The sample and instrumental conditions are given in the figure legends.

## 3. RESULTS

3.1 NMR of Labeled Amino Acids. The $^{13}C$-NMR spectra of $^{15}N_2$-histidine and $^{12}CD_2$-cysteine are shown in Figures 3 and 4, respectively. The $^{13}C$-NMR spectrum of $^{15}N_2$-histidine very clearly shows the splitting of the ring carbons by the I=1/2 $^{15}N$ nucleus. The $^{3}C$-carbon of labeled cysteine (Figure 4) is seen as the most upfield carbon, and it is split into a quintet as expected. The intensity ratio for this quintet is 1:2:3:2:1, precisely as expected for ~100% deuterium substituted. If the deuterium had dropped to a level as low as 90% during the synthesis of cysteine then the quintet intensity ratio would be expected to be about 1:2:6:2:1. due to the large nuclear overhauser enhancement in proton-decoupled

FIGURE 3

$^{13}C$-NMR spectra of (A) 1M $^{14}N$-histidine in $D_2O$ and (B) 0.1 M $^{15}N_2$-histidine in $D_2O$. Spectra (A) and (B) were obtained with 1000 and 40,000 scans, respectively. Both spectra were obtained with a 4,000 Hz spectral width and a 1 Hz line broadening.

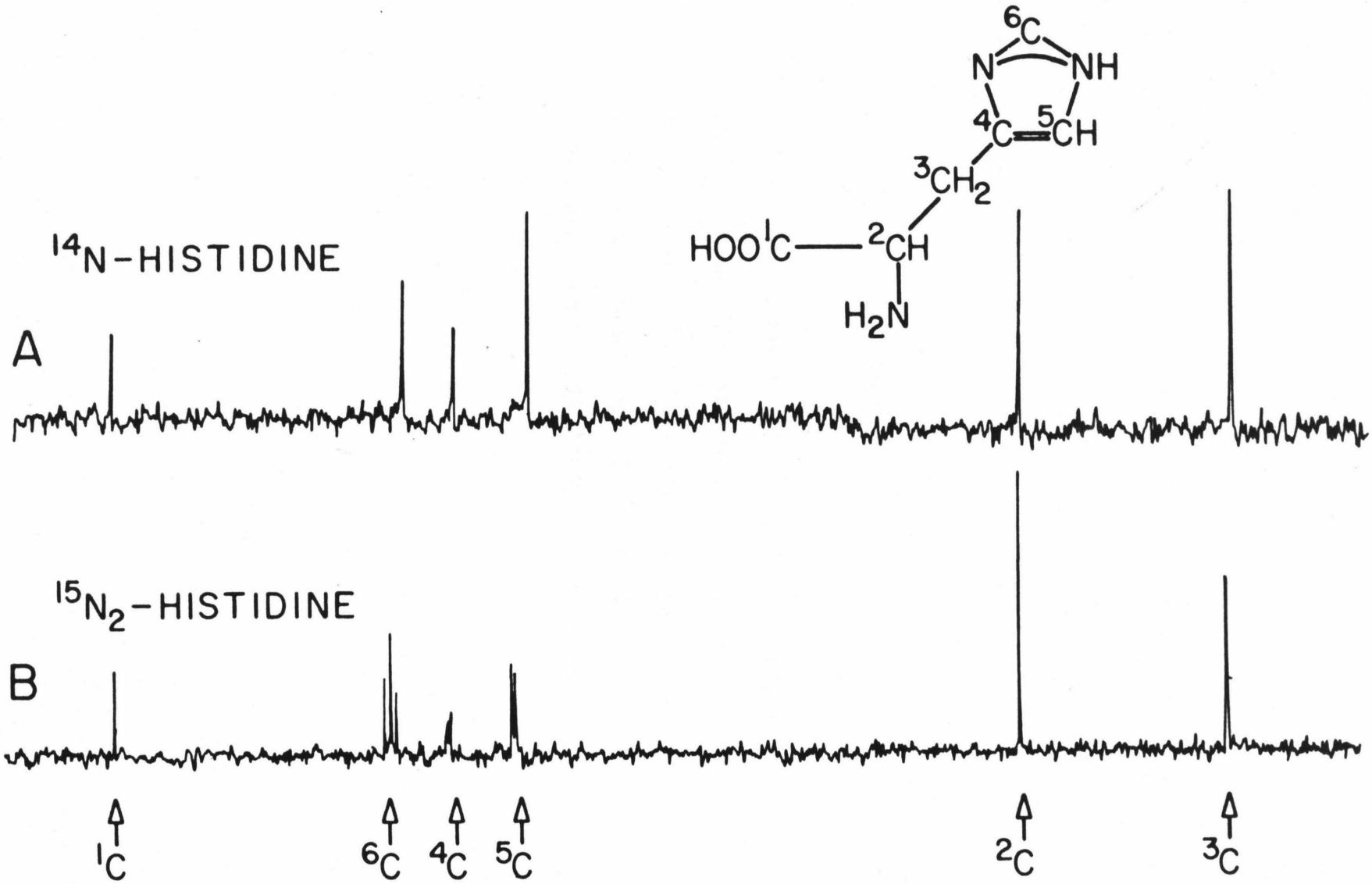
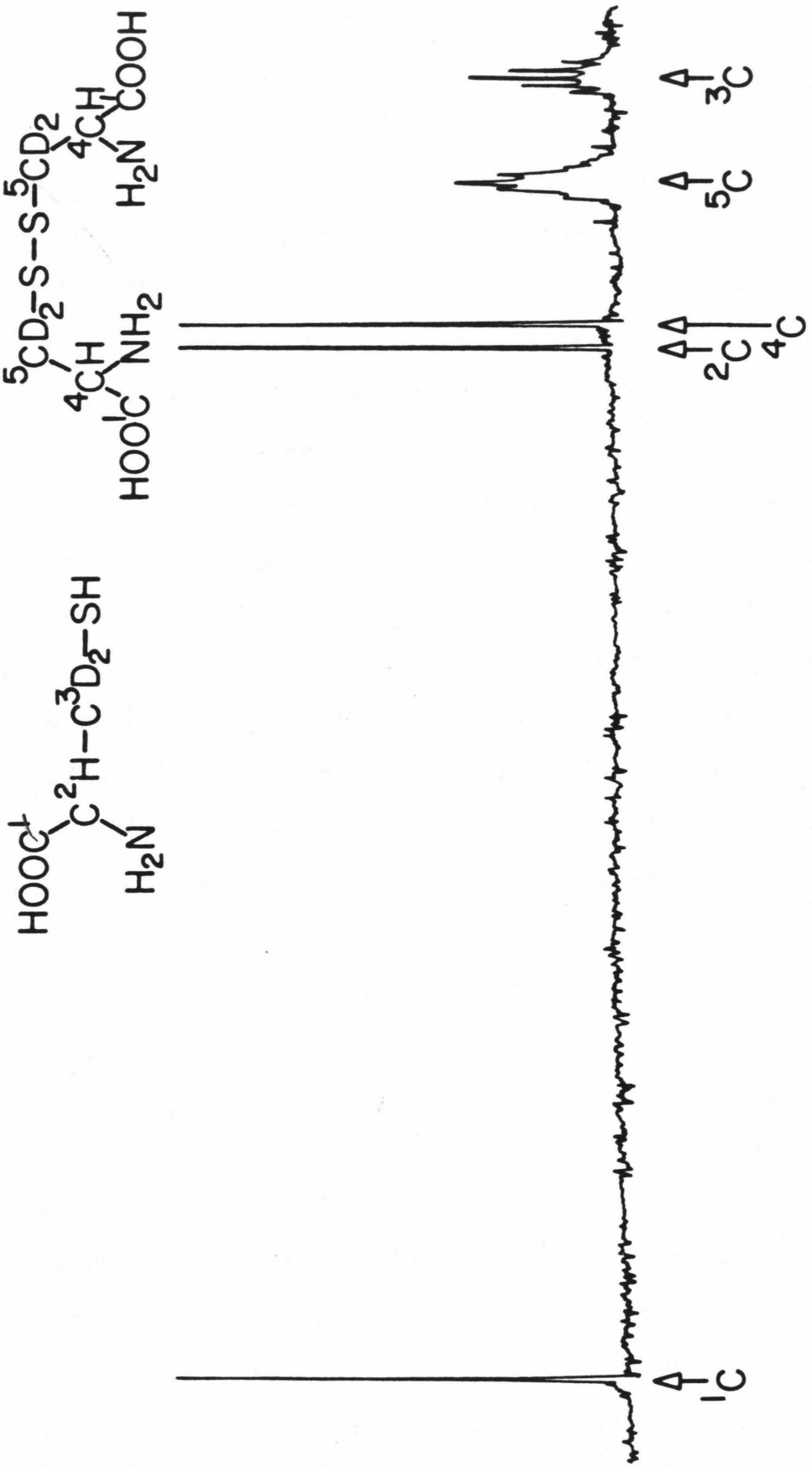

$^{14}$N-HISTIDINE
A
$^{15}N_2$-HISTIDINE
B
$^{1}C$
$^{6}C$
$^{4}C$
$^{5}C$
$^{2}C$
$^{3}C$

FIGURE 4

$^{13}$C-NMR spectrum of 2M $^{12}CD_2$-D,L-cysteine in $D_2O$. The spectrum was obtained with 45,000 scans, 4,200 Hz spectral width and 1 Hz line broadening. This sample contained about 50% $^{12}CD_2$-D,L-cysteine.


HOOC
C²H–C³D₂–SH
H₂N
5CD₂–S–S–5CD₂
4CH
HOOC
NH₂
4CH
H₂N
COOH
1C
2C
4C
5C
3C

$^{13}C$-NMR spectra. This observation demonstrates that the cysteine had retained the high isotopic enrichment purchased in the formaldehyde.

3.2 EPR of Isolated Unlabeled Yeast Cytochrome c Oxidase. The EPR spectra of the protein fractions that eluted from the cytochrome c affinity column with 0.5% cholate and with 2% cholate are shown in Figure 5. It can be seen that very little cytochrome c oxidase eluted in either case. The optical spectra (not shown) bore out this conclusion. The main components eluting under low-salt conditions are iron-sulfur proteins and the mitochondrial cytochrome c reductase. The EPR spectrum of the cytochrome c oxidase that eluted with 1M KCl and precipitated at 38% of ammonium sulfate saturation is shown in Figure 6. This EPR spectrum compared very favorably with some of the best beef heart cytochrome c oxidase EPR spectra. Therefore, the procedure for isolating yeast cytochrome c oxidase outlined in this work is very satisfactory for preparing protein samples for EPR spectroscopy. The overall yield of purified oxidase from 500 grams of yeast cells is about 15 - 30 mg protein.

3.3 EPR of Isotopically Labeled Yeast Cytochrome c Oxidase. The EPR spectra of $^{15}N$-his and $^{12}CD_2$-cys yeast cytochrome c oxidase are shown in Figure 7. The EPR spectra for both of the isotopically labeled proteins are qualitatively similar to the EPR spectrum of the unlabeled yeast protein. However, differences are observed in the g=2 region of the $Cu_a$ signal. These differences are accentuated in Figure 8

FIGURE 5

EPR spectra of (A) 0.5% cholate and (B) 2% cholate washes of the cytochrome c affinity column. Conditions: temperature, 15K; microwave power, 1.0 mW; modulation amplitude, 16 G; microwave frequency, 9.23 GHz; scan width, 4000 G.

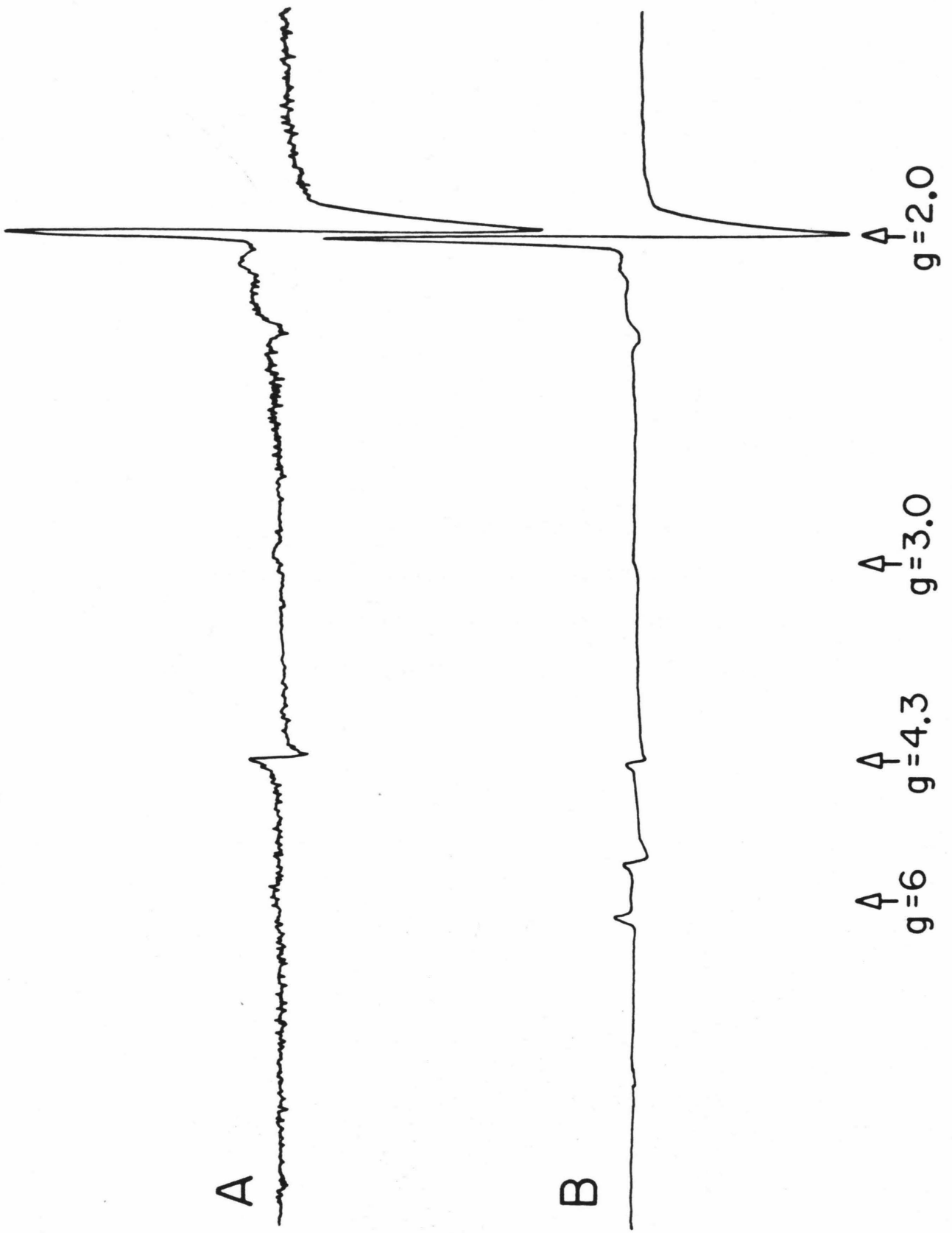

A
B
g=6
g=4.3
g=3.0
g=2.0

FIGURE 6

EPR spectrum of purified unlabeled yeast cytochrome c oxidase. The instrumental conditions were the same as for Figure 5 except that the microwave power was 0.2 mW.

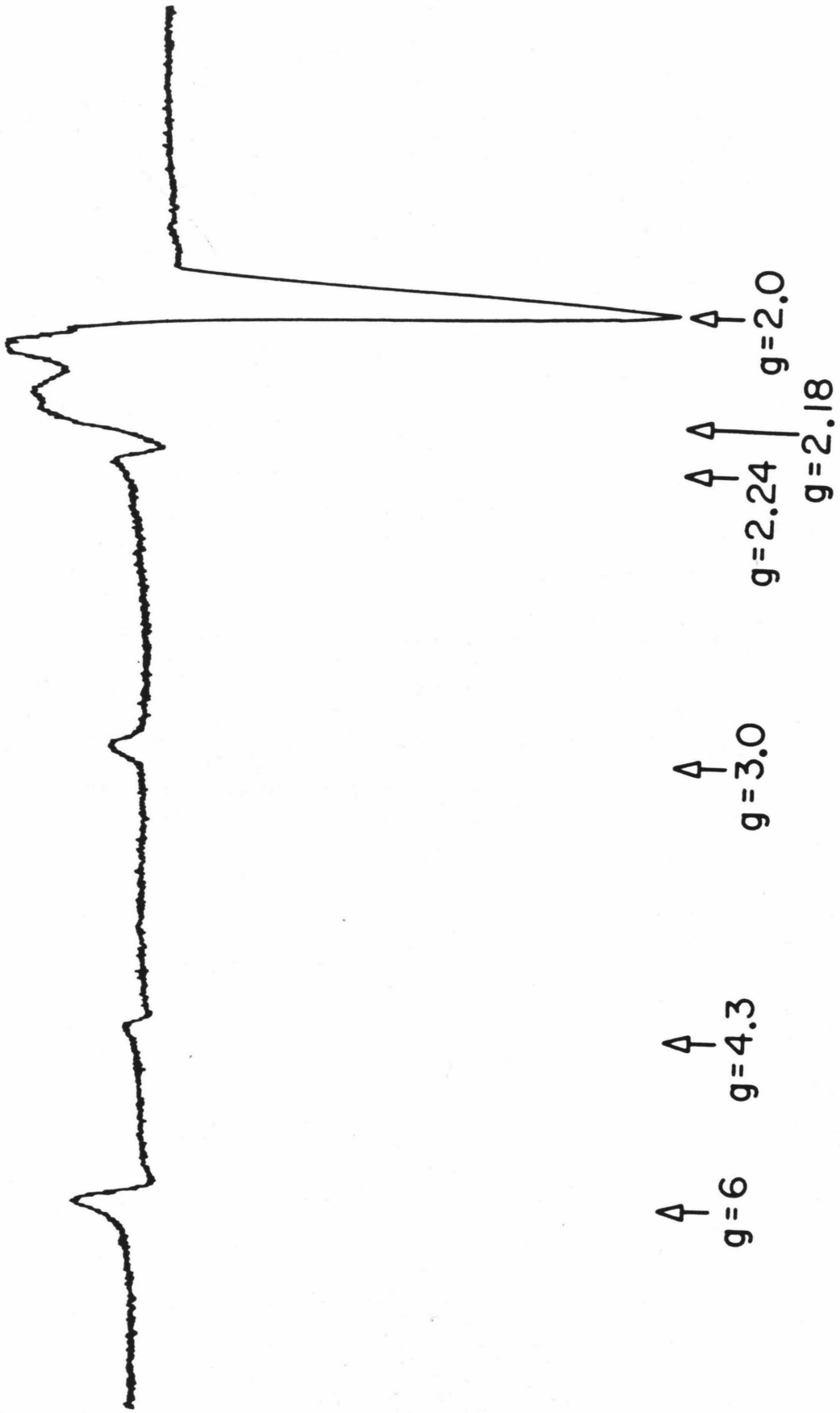
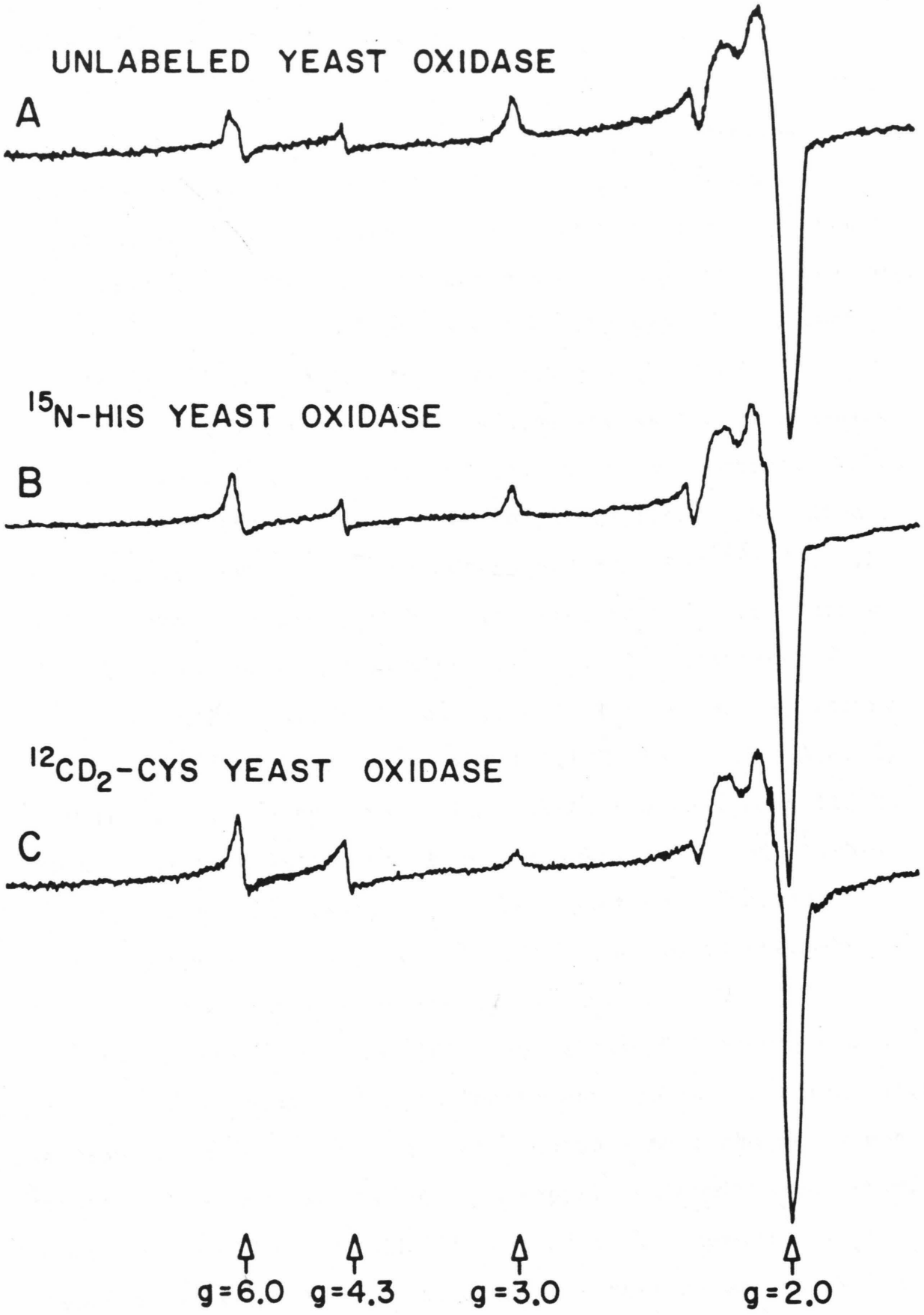

g=2.0
g=2.18
g=2.24
g=3.0
g=4.3
g=6

FIGURE 7

EPR spectra of (A) unlabeled yeast oxidase, (B) $^{15}N$-his yeast oxidase and (C) $^{12}CD_2$-cys yeast oxidase. The instrumental conditions were the same as for Figure 5.


UNLABELED YEAST OXIDASE
A
$^{15}$N-HIS YEAST OXIDASE
B
$^{12}CD_2$-CYS YEAST OXIDASE
C
g=6.0
g=4.3
g=3.0
g=2.0

wherein the g=2 regions have been expanded. The major difference is that the g=2 region has sharpened up for the labeled proteins, allowing the resolution of previously obscured hyperfine structure. In the $^{15}$N-his oxidase EPR spectrum the hyperfine features near g=2.03 have sharpened slightly relative to the unlabeled protein spectrum. However, in the $^{12}CD_2$-cys oxidase EPR spectrum the hyperfine structure in the g=2.03 region is almost completely resolved.

The origin of the spectral sharpening is the elimination of small hyperfine couplings to the unpaired electron. Table 1 summarizes the various hyperfine interactions associated with the $Cu_a$ EPR center, as determined by ENDOR[13,14]. The 17 MHz isotropic nitrogen hyperfine interaction could arise from a histidine nitrogen bound to $Cu_a$. Substitution of $^{15}$N-his would result in a reduction in the overall splitting of the I=1 $^{14}$N nucleus of 34 MHz (2A,12G) to 24 MHz (A,8.6G) for the I=1/2 $^{15}$N nucleus. Thus, if histidine were a ligand to $Cu_a$ (or two histidines) then the substitution of $^{15}$N-his should cause a narrowing of the EPR spectrum. Therefore, from the spectra in Figure 8 it can be concluded that there is at least one histidine ligand to $Cu_a$.

The narrowing of the $^{12}CD_2$-cys oxidase EPR spectrum must arise from elimination of the methylene proton hyperfine interactions with $Cu_a$. It is quite reasonable that the methylene protons of a cysteine bound to a copper ion would have hyperfine couplings of 12 and 19 MHz (4.3 and 6.8 G, respectively), especially if substantial spin density resided on the cysteine

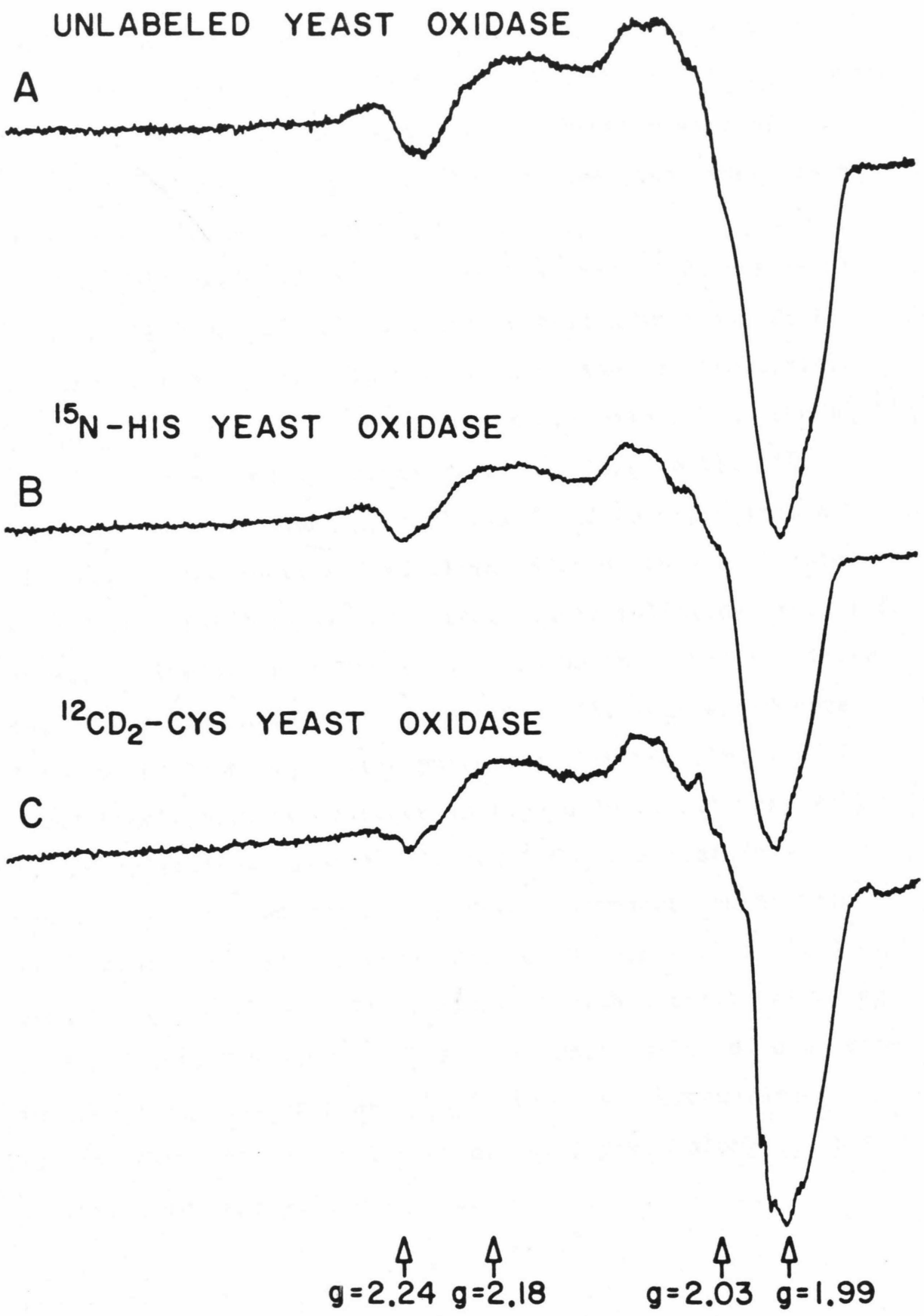
Table 1. Hyperfine Interactions* Associated with the $Cu_a$ Center

| Nuclei | Magnitude of Hyperfine Coupling (MHz) | | |
|---|---|---|---|
| | X | Y | Z |
| Copper | 68 | 98 | 90 |
| Proton | | | |
| a) weakly coupled | - | 2.3 | 2.0 |
| | - | 1.2 | 1.3 |
| | - | 0.8 | 1.3 |
| | - | 0.3 | 0.4 |
| b) strongly coupled | | 12,19 (isotropic) | |
| Nitrogen | | 17 (isotropic) | |

*From references 13 and 14

FIGURE 8

EPR spectra of (A) unlabeled yeast oxidase, (B) $^{15}N$-his yeast oxidase and (C) $^{12}CD_2$-cys yeast oxidase. The instrumental conditions were the same as for Figure 5 except that the scan range was 1000 G.


UNLABELED YEAST OXIDASE
A
$^{15}$N-HIS YEAST OXIDASE
B
$^{12}CD_2$-CYS YEAST OXIDASE
C
g=2.24
g=2.18
g=2.03
g=1.99

sulfur. The elimination of these strongly coupled proton hyperfine interactions would be expected to lead to substantial narrowing of the EPR spectrum. Thus, the narrowing of the $^{12}CD_2$-cys oxidase EPR spectrum demonstrates that there is at least one cysteine sulfur ligand to $Cu_a$.

The resolved hyperfine structure in the $^{12}CD_2$-cys yeast oxidase EPR spectrum allows a more detailed analysis of the $Cu_a$ center EPR signal. In this regard, the low-temperature S-band (3GHz) EPR spectrum of beef heart oxidase was shown[11] to exhibit resolved hyperfine structure (Figure 9). The hyperfine pattern along $g_y$=2.03 was found to arise from a 45 G copper interaction (I=3/2) in addition to a 25 G interaction from a S=1/2 (or I=1/2) site. These splittings result in an eight line hyperfine pattern as shown in Figure 9. These magnetic field independent hyperfine splittings would give rise to the same eight line pattern at X-band (9GHz). This eight line hyperfine is shown in Figure 10 to fit very well to the splittings observed for the $^{12}CD_2$-cys protein at X-band. The two outermost hyperfine components emphasized with arrows in this spectrum (Figure 10) are each split 80 G from the $g_y$ value of 2.03, consistent with a total splitting of (3x45G)+(1x25G)=160G. Thus, the substitution of deuterons for the methylene protons in cysteine yeast cytochrome c oxidase does not change the hyperfine pattern along $g_y$, but allows it to be resolved at X-band.

FIGURE 9

S-band (3 GHz) EPR spectrum of Beef heart cytochrome c oxidase taken from Froncisz et al.[11]. The features labeled I thru VII are the newly resolved features. The eight line hyperfine stick diagram has been added assuming A=45 G for a I=3/2 copper and A=25 G for a S or I=1/2 center. Modulation amplitude was 4 G.

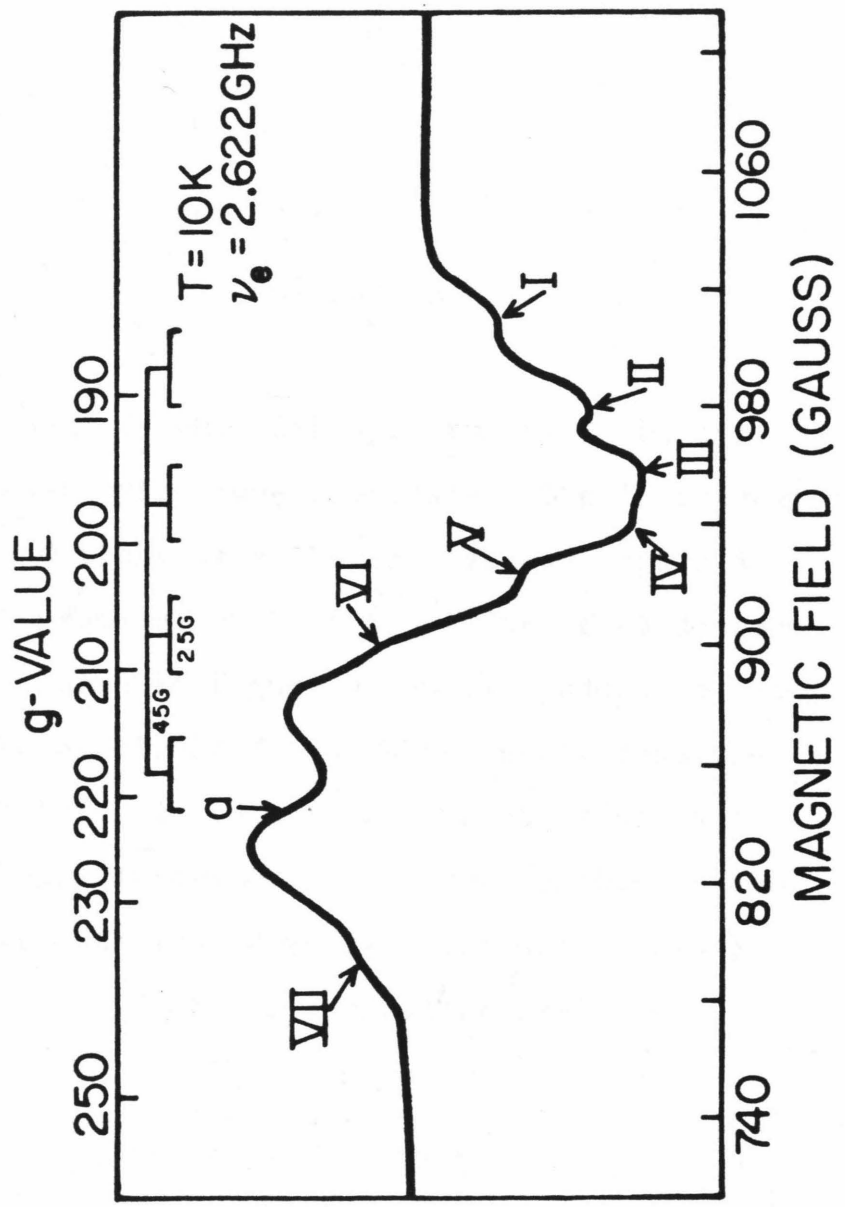
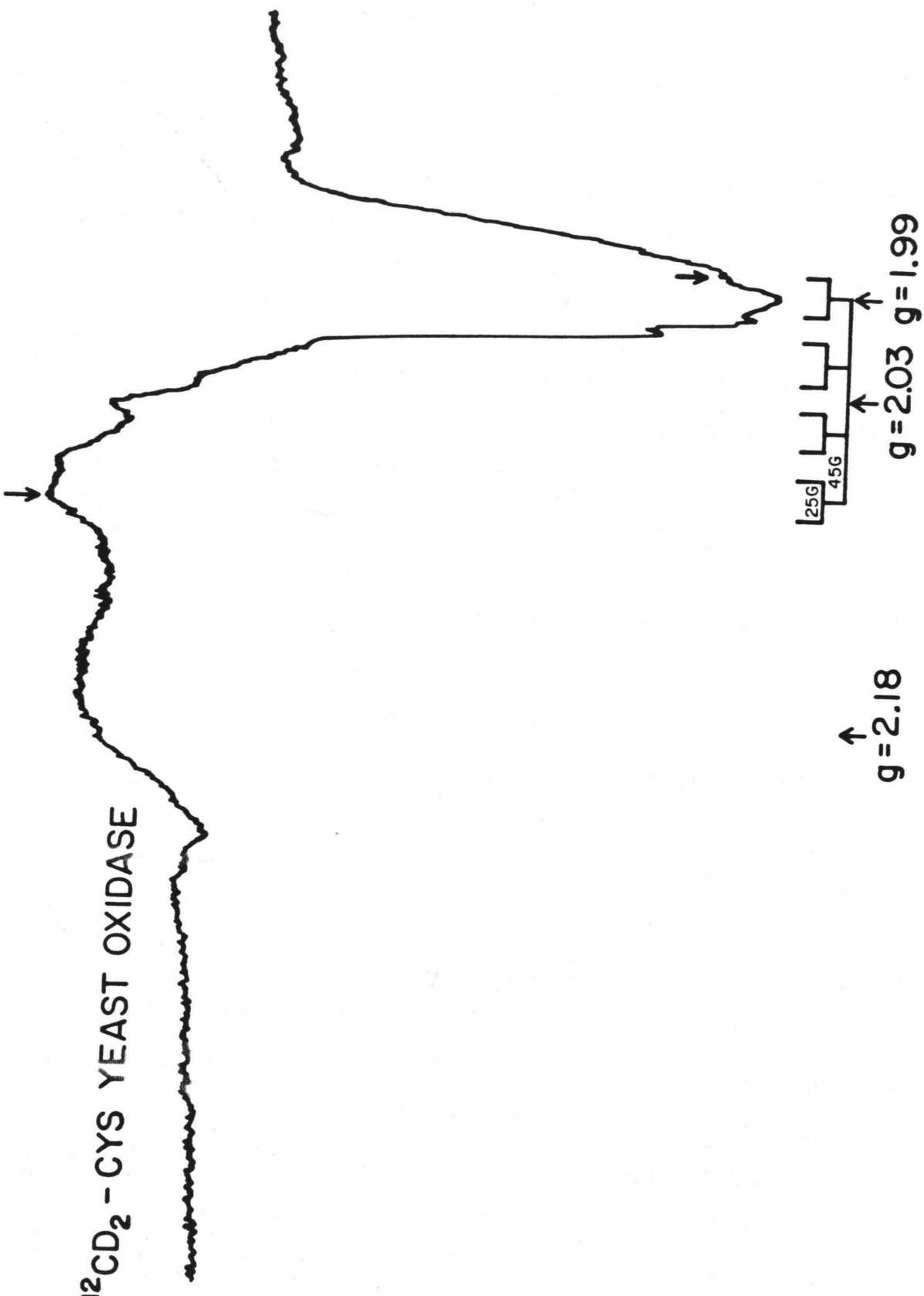

g-VALUE
250
230
220
210
200
190
740
820
900
980
1060
MAGNETIC FIELD (GAUSS)
T=10K
$\nu_e$=2.622GHz
45G
25G
g
I
II
III
IV
V
VI
VII

FIGURE 10

X-band (9 GHz) EPR spectrum of $^{12}CD_2$-cys yeast cytochrome c oxidase. The instrumental conditions were the same as for Figure 8. The same eight line hyperfine stick diagram as shown in Figure 9 has been added to show the excellent fit with the newly resolved features in this $^{12}CD_2$-cys yeast oxidase X-band spectrum. The arrows emphasize the two outermost hyperfine components along $g_y$ = 2.03 with a separation of 160 G.


$^{12}CD_2$ - CYS YEAST OXIDASE
25G
45G
g=2.18
g=2.03
g=1.99

## 4. DISCUSSION

The substitution of $^{15}N$-his and $^{12}CD_2$-cys into yeast cytochrome c oxidase has demonstrated the involvement of at least one histidine and one cysteine as ligands to $Cu_a$. These studies together with the results presented in Chapter II of this work represent the first concrete information regarding the ligands to the metal centers in cytochrome c oxidase. The determination of the involvement of cysteine and histidine as ligands to $Cu_a$ now allows a more detailed analysis of the $Cu_a$ center EPR signal, in additon to providing clues as to this metal center's role in the protein. We now consider certain features of the $Cu_a$ center EPR signal in more detail.

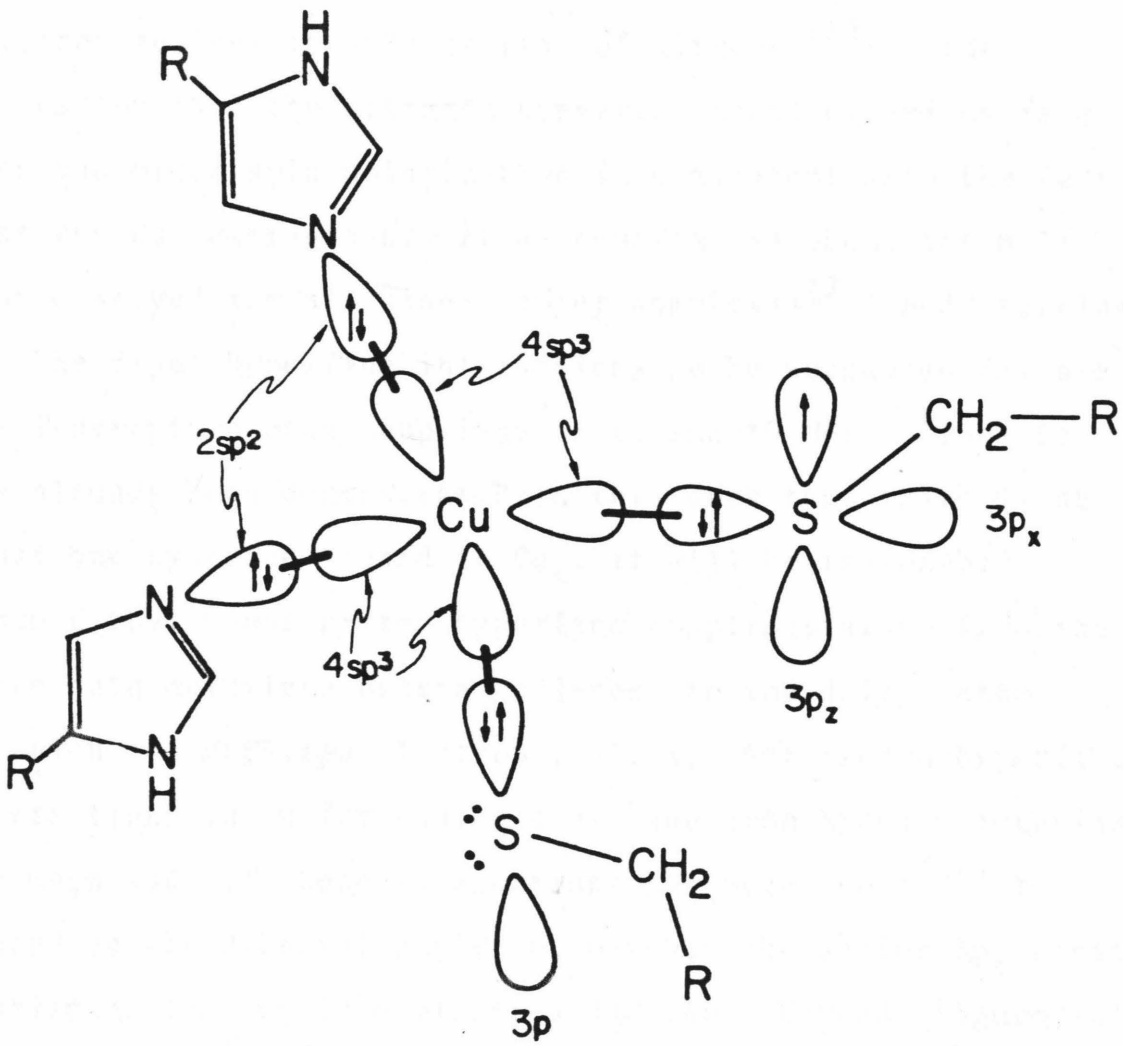
The hyperfine interactions observed along $g_y$ at S-band[11] and for the $^{12}CD_2$-cys yeast protein at X-band in this work correspond to a 45G coupling due to I=3/2 copper and a second coupling of 25G, resulting in the observed eight line pattern. The 25G hyperfine interaction is thought to arise from either a very strongly coupled proton or the dipolar splitting of $Cu_a$ by cytochrome a. The only reasonable candidate for a proton interaction of this magnitude would be a cysteine methylene proton. Thus, this work has ruled out the possibility that a nuclear spin causes the 25G splitting. Therefore, the most reasonable interpretation is that $Cu_a$ is split by the S=1/2 cytochrome a metal center. This interpretation is consistent with the EPR saturation work of Greenaway et al.[7] and Brudvig[12] that suggested that the

unusual saturation behavior of the $Cu_a$ EPR signal could best be explained by a weak dipolar interaction between $Cu_a$ and cytochrome a. Taken together these results strongly support the calculation of Brudvig[12] which showed that cytochrome a at a distance of 10-13 Å from $Cu_a$ could result in an orientation dependent 25G (0.0025 $cm^{-1}$) static dipolar splitting of the $Cu_a$ center EPR signal.

It has already been concluded, from data presented in this chapter's Introduction and by Brudvig[12], that this metal center is best described as a Cu(I)-$\dot{S}$ system. However, it seems necessary at this time to consider the origin of the copper and nitrogen hyperfine couplings in the context of a Cu(I)-$\dot{S}$ complex. It is thus necessary to discuss the polarization of the inner shell paired as well as bonding electrons by the outer shell unpaired electron. The orbitals involved as formulated by Brudvig[12] are shown in Figure 11 in the limit where all the unpaired electron spin density is localized in the sulfur $3p_z$ orbital. In order to get the observed isotropic copper hyperfine coupling of 85 MHz, Brudvig[12] has shown that it is only necessary to have 1.7% of one unpaired electron in the copper 4s orbital. Similarly, to account for the extremely small anisotropic copper hyperfine coupling of $|2B| = 17$ MHz it is necessary to mix 7% of one unpaired electron into a copper 4p orbital[12]. These numbers (1.7% and 7%) agree well with the notion that copper obtains unpaired electron spin density from the donation of a spin polarized sulfur lone pair into a copper $4sp^3$ hybrid

FIGURE 11

Proposed model for spin polarization of the sulfur $3p_x$, copper $4sp^3$ and nitrogen $2sp^2$ orbitals by the unpaired electron in a $3p_z$ orbital. Taken from Brudvig[12].


R
H
N
N
4sp3
2sp2
CH2—R
Cu
S
3px
N
4sp3
R
N
H
3pz
S
CH2
R
3p

orbital.

The origin of the nitrogen hyperfine coupling of 17 MHz can also be accounted for by a spin polarization model. However, in this case it is a second order effect since the approximately 10% spin density in the copper $4sp^3$ orbital will further polarize the other paired electrons of copper. The 17 MHz hyperfine coupling implies that 1% of one unpaired electron resides in a 2s orbital of nitrogen[12]. The suggestion that the nitrogen hyperfine coupling arises from a second order spin polarization is consistent with the fact that the $Cu_a$ nitrogen hyperfine coupling is about one half that observed for all other copper complexes[25] and proteins[26].

The final hyperfine interactions to be accounted for are the isotropic proton couplings of 12 and 19 MHz. Since it has already been demonstrated in this work that there is at least one cysteine ligand to $Cu_a$, it will be reasonably assumed that these proton hyperfine couplings arise from the cysteinate methylene protons adjacent to the sulfur atom on which the unpaired electron resides. For proton hyperfine interactions in sulfur radicals arising from hyperconjugation[27], the magnitude of these interactions has been shown[28] to depend on the dihedral angle, $\phi$, between the sulfur $3p_z$ orbital containing the unpaired electron and the C-H bond (Figure 12) according to equations (1) and (2). In equations (1) and (2) $A_1$ and

$$A_1 = A_o \rho_s^{\pi} \cos^2 \phi \qquad (1)$$

$$A_2 = A_o \rho_s^{\pi} \cos^2 (\phi - 125^o) \qquad (2)$$

$A_2$ are the isotropic proton couplings observed, $A_o$ is a constant ($A_o$=88 MHz for the N-acetyl cysteine neutral sulfur radical[28]) and $\rho_s^\pi$ is the $\pi$-spin density on the sulfur. The proton hyperfine couplings of 12 and 19 MHz require[12] that $\phi$ be close to 60° (Figure 12). With $\rho_s^\pi$=0.84, $A_o$=88 MHz and $\phi$=59.5° the proton couplings $A_1$ and $A_2$ are calculated to be 19 and 12 MHz, respectively. The remaining 16% of one unpaired electron is delocalized onto protons, nitrogen and copper through spin polarization. Thus, it has been shown that all the hyperfine interactions associated with the $Cu_a$ center EPR signal can be quantitatively as well as qualitatively accounted for by our Cu(I)-sulfur radical model.

The unusual nature of the $Cu_a$ center as envisioned in our model (Figure 1) must certainly be linked to its unique role in cytochrome c oxidase. In addition to this metal center's role of electron transfer, we have proposed[9,10] that the $Cu_a$ center is involved in the pumping of protons across the inner mitochondrial membrane. In this regard, it has been shown that the $Cu_a$ center is probably deeply buried within the protein matrix[10] and thus reduction of this center would result in an isolated negative charge within a region of low dielectric constant. This would be expected to result in a large increase in potential energy of the $Cu_a$ center which could then be coupled to the conservation of energy in cytochrome c oxidase. Our proposed scheme for this process is depicted in Figure 13. This scheme involves pulling a proton from the mitochondrial matrix solution to

FIGURE 12

Geometry of the sulfur $3p_z$ orbital with respect to the adjacent methylene group for the $Cu_a$ center. This corresponds to the solution to equations (1) and (2) obtained from the 12 and 19 MHz isotropic proton hyperfine couplings. The diagram is drawn such that the methylene carbon lies behind sulfur.

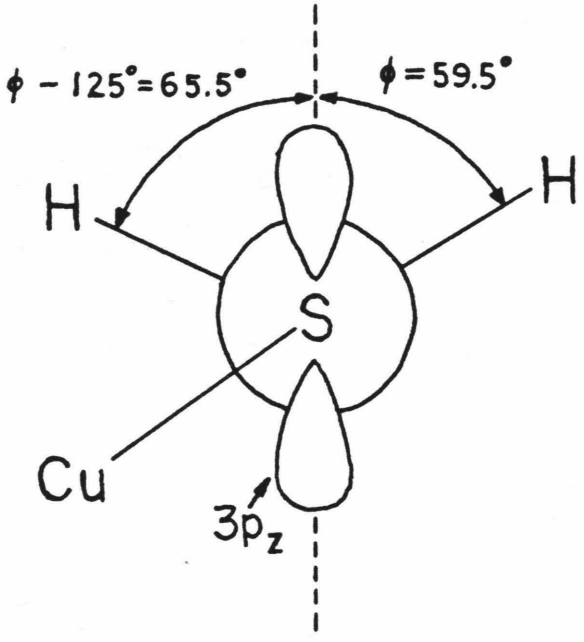
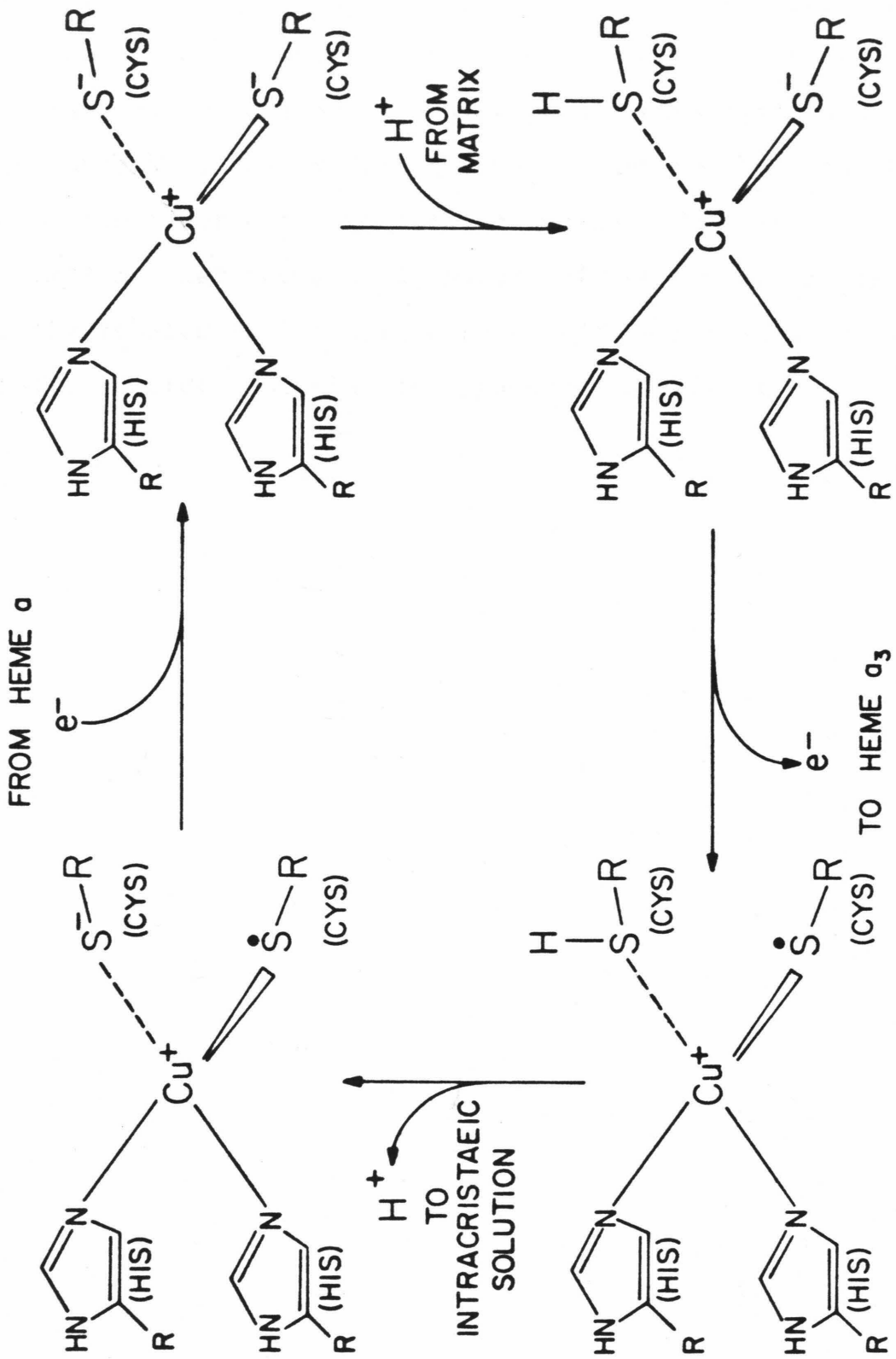

φ - 125°=65.5°
φ=59.5°
H
H
S
Cu
3p_z

FIGURE 13

A possible proton pumping mechanism in cytochrome c oxidase.


FROM HEME a
e⁻
H⁺
FROM MATRIX
e⁻
TO HEME a₃
H⁺
TO INTRACRISTAEIC SOLUTION
Cu⁺
(CYS)
(HIS)
HN
R
H
N

balance the negative charge. Upon transfer of an electron away from the $Cu_a$ center, this proton would now leave an isolated positive charge in a hydrophobic environment, which could be expelled from the $Cu_a$ center to the opposite side of the inner mitochrondrial membrane. Therefore, it may very well be that the unusual nature of the $Cu_a$ center is directly related to its unique role of coupling electron transfer to proton pumping in cytochrome c oxidase.

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# CHAPTER IV: SUMMARY

The original goal of this research was to investigate the metal centers of cytochrome c oxidase in an attempt to understand their respective roles in the enzymatic functioning of the protein. When this work was initiated some three and one half years ago, the x-ray absorption edge spectrum of oxidized cytochrome c oxidase had just been obtained. This x-ray absorption edge study found that one of the coppers appeared to be Cu(I) ($Cu_a$) even in the oxidized protein. The isotopic substitution studies reported in this work were designed to ascertain the ligands to $Cu_a$, in addition to probing the nature of the electronic structure of the $Cu_a$ metal center. The x-ray absorption edge study together with the isotopic labeling studies reported in this work and all the other physical chemical measurements strongly support the contention that the best description of the $Cu_a$ center is that of Cu(I)-$\dot{S}$. In particular, the isotopic substitution studies indicate that there is at least one cysteine and one histidine as ligands to $Cu_a$. Perhaps the most important result of the isotopic labeling studies was that a careful analysis of the newly resolved nuclear hyperfine interactions associated with the $Cu_a$ EPR signal in $^{12}CD_2$-cys yeast oxidase indicates that cytochrome a is most likely interacting with $Cu_a$ through a dipolar mechanism. This has allowed an estimate of the distance between the two metal centers in cytochrome c oxidase whose function is the transfer of electrons to the oxygen reduction site.

The oxygen reduction site of cytochrome c oxidase has been known to consist of the cytochrome $a_3$ metal center. The involvement of $Cu_{a_3}$ in oxygen reduction had been much more speculative, however. The model receiving the most favor when this work began, envisioned an imidazole bridge between $Cu_{a_3}$ and cytochrome $a_3$. There had not been any conclusive data to suggest that $Cu_{a_3}$ was capable of binding exogenous ligands. Nitric oxide binding studies of cytochrome c oxidase have greatly increased our understanding of the structure of the two metal centers constituting the ligand binding site, in addition to their respective roles in the reduction mechanism.

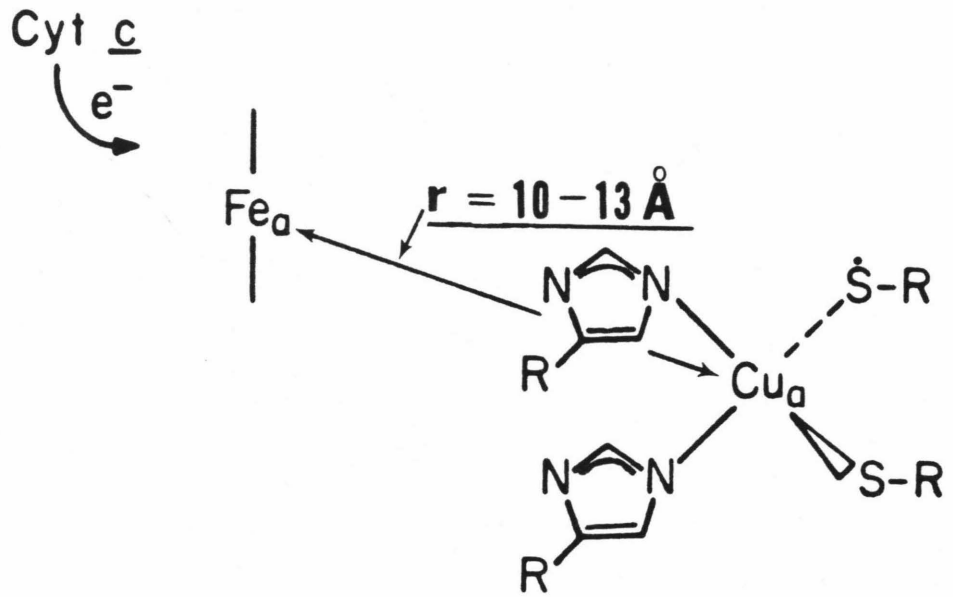
Nitric oxide binding studies of the isotopically labeled reduced yeast protein have demonstrated conclusively that histidine is the axial ligand to cytochrome $a_3$. However, these studies did not address the question of the position of the imidazole vis-a-vis the $Cu_{a_3}$ center. The nitric oxide binding studies involving the oxidized protein in the presence of azide did address this question. This one electron reduced NO-bound protein complex gave rise to a triplet state whose EPR spectrum revealed new information about the oxygen binding site. The EPR parameters together with the chemical stability of this NO-bound complex led to the conclusion that nitric oxide bridges the two metal centers in this one electron reduced protein complex. This conclusion carried with it the implication that the already identified histidine axial ligand to cytochrome $a_3$ was distal vis-a-vis $Cu_{a_3}$.

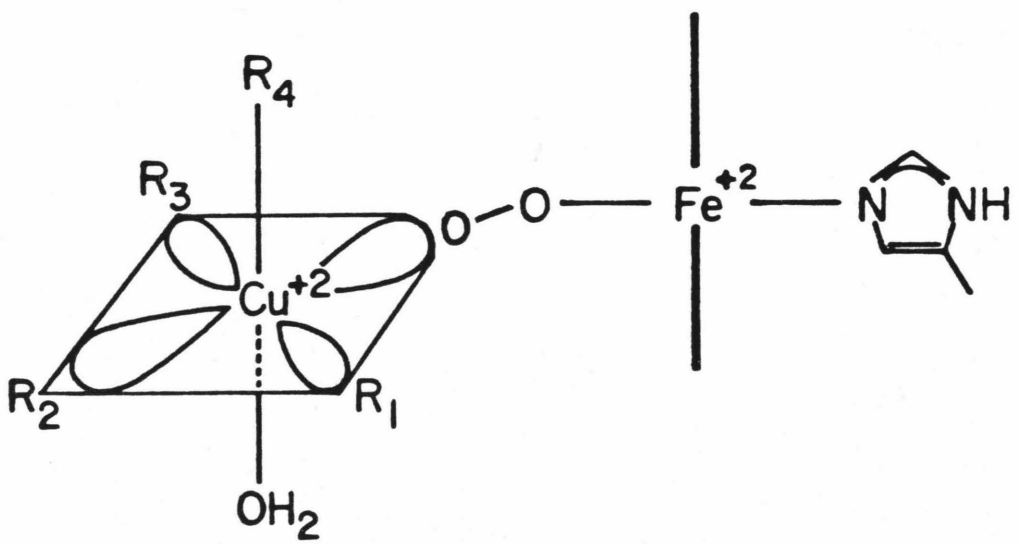
The EPR spectrum of the triplet state represented the first time that $Cu_{a_3}$ had been observed by EPR, allowing substantial structural information to be extracted. This EPR spectrum allowed a distance between $Cu_{a_3}$ and cytochrome $\underline{a}_3$ of 5 Å to be calculated. In this triplet state $Cu_{a_3}$ was also found to be square planar or octahedral with a strong tetragonal distortion, ruling out the suggestion that $Cu_{a_3}$ is a Type 1 or blue copper center. Furthermore, these nitric oxide binding studies demonstrated for the first time that $Cu_{a_3}$ was capable of binding exogenous ligands. It was also found that it is possible to modulate the exchange interaction between $Cu_{a_3}$ and cytochrome $\underline{a}_3$ by varying the exogenous ligands.

The results presented in this work represent a contribution to the understanding of the overall role of the metal centers in the structure and enzymatic function of cytochrome $\underline{c}$ oxidase. An overall picture of the metal centers in the protein is depicted in Figure 1. Further studies will be necessary to completely elucidate the structure of the metal centers in the absence of high resolution x-ray crystal data.

FIGURE 1

The four metal centers in cytochrome c oxidase.


Cyt c
e⁻
Fe$_a$
r = 10 − 13 Å
N
N
Ṡ-R
R
Cu$_a$
N
N
S-R
R


R$_4$
R$_3$
O
O
Fe$^{+2}$
N
NH
Cu$^{+2}$
R$_2$
R$_1$
OH$_2$