On the Nature of Cysteine Coordination to CuA in Cytochrome c Oxidase*

Craig T. Martin‡, Charles P. Scholes¶, and Sunney I. Chan∥

(Received for publication, August 18, 1987)

The resolution of new features in the $^1$H electron nuclear double resonance (ENDOR) spectrum of the oxidized CuA site in beef heart cytochrome c oxidase is presented. In a previous study, we assigned resonances in the CuA ENDOR spectrum to hyperfine interactions of methylene protons on one or two cysteine ligands to CuA (Stevens, T. H., Martin, C. T., Wang, H., Brudvig, G. W., Scholes, C. P., and Chan, S. I. (1982) J. Biol. Chem. 257, 12106-12113). In this work, a new $^1$H ENDOR resonance in the beef heart CuA ENDOR spectrum is reported and can be assigned to either anisotropy in a previously resolved cysteine methylene proton hyperfine interaction ($A_{iso} = 12$ MHz, $A_{aniso} = 2.5$ MHz) or to a third isotropic hyperfine coupling ($A = 13.6$ MHz) to a cysteine methylene proton of a second cysteine ligand to copper. In either case, the $^1$H ENDOR results require the delocalization of approximately 50% of the unpaired spin from copper onto either one or two cysteine ligands to CuA.

To characterize further the CuA site, we have prepared yeast cytochrome c oxidase incorporating isotopically substituted [$\beta$-13C]cysteine. The CuA ENDOR spectrum of this species shows only one clearly resolved 13C hyperfine interaction ($A = 3.6$ MHz). This result confirms the assignment of at least one strongly interacting cysteine ligand to CuA and suggests that if the assignment of two cysteine ligands to CuA is correct, the two cysteines interact with copper in a highly symmetric manner.

A recent extended x-ray absorption fine structure study of native and modified forms of cytochrome c oxidase indicates the coordination of two sulfur ligands to CuA (Li, P. M., Gelles, J., Chen, S. I., Sullivan, R. J., and Scott, R. A. (1987) Biochemistry 26, 2091-2095). In light of the new possibility of two symmetrically coordinated cysteine ligands to CuA, we propose a molecular orbital description of the oxidized CuA site which is characterized by a high degree of delocalization of unpaired spin away from copper and onto a pair of symmetrically coordinated cysteine sulfur ligands. We also present a protein model for the CuA site in which two cysteine ligands derived from subunit II lie on the face of an $\alpha$-helix. This structure would allow the unprecedented stable coordination of two cysteine thiolate sulfur to copper and may provide a mechanism for the redox-linked proton pumping by cytochrome c oxidase.

The spectroscopic properties of the CuA center in oxidized cytochrome c oxidase have long been considered unusual for an isolated Cu(II) site. The copper hyperfine interaction is quite small and very nearly isotropic; the largest component of the copper hyperfine interaction does not coincide with the largest $g$-value (1). In addition, the low-field $g$-value of oxidized CuA is smaller than that found in more typical Cu(II) complexes, and the high-field $g$-value is below that of the free electron (2, 3).

CuA shows an intense optical absorption band at 830 nm. For this reason, CuA is often compared to the blue copper proteins, where the coordination of a single cysteine sulfur ligand to copper leads to a ligand-to-metal charge transfer band near 600 nm (4). The blue copper centers, whose sole function appears to be electron transfer, comprise, in addition, two histidine ligands and a weakly coordinated methionine¹ in a very distorted tetrahedral or distorted trigonal coordination. Both single crystal x-ray structural data and extended x-ray absorption fine structure (EXAFS)² spectra show that the physical environment of the blue copper site changes minimally upon reduction of Cu(II) to Cu(I), indicating only a small rearrangement energy associated with electron transfer to and from the site (5). The covalency conferred by the ligation of a single "soft" cysteine sulfur in the blue copper centers reduces the change in electron density at the copper ion upon a change in the redox state of the site. The fairly small copper hyperfine coupling (35-80 G) observed for the oxidized blue copper sites is consistent with this covalent structure (6).

¹ In at least one blue copper protein, stellacyanin, there is no methionine (34). It has been suggested that the fourth ligand to the copper in this case may be a distant cysteine sulfur (35). In any case, due to the weak nature of this interaction, the exact nature of the fourth ligand appears to be unimportant to the spectroscopy of the blue copper site.

² The abbreviations used are: EXAFS, extended x-ray absorption fine structure; ENDOR, electron nuclear double resonance; [13C]Cys, [$\beta$-13C]cysteine.
When compared to CuA, however, the copper hyperfine coupling observed in the blue copper ions is larger and considerably more anisotropic. Indeed, x-ray edge absorption studies of CuA have shown that the coordination charge at the copper ion is smaller than in blue copper centers and does not change appreciably upon reduction of the center. These and other data have prompted us and others to propose that the copper ion in the oxidized CuA site is a very covalent Cu(I) or a copper which is more formally Cu(I), with the unpaired spin residing to a large extent on an associated (cyt)l sulfur ligand(s) (8, 9). We have further proposed that this unique electronic distribution requires the strong coordination of a second cysteine sulfur ligand to copper (10, 11).

The proposal that the unpaired electron in CuA is delocalized onto an associated cysteine sulfur ligand (8, 9) has gained considerable support from the assignment of ligands to CuA. We have previously developed the yeast system for the incorporation of isotopically substituted amino acids into cytochrome oxidase and have used this approach in conjunction with electron nuclear double resonance (ENDOR) spectroscopy to assign nuclear hyperfine interactions from cysteine and histidine ligands to copper. The nitrogen hyperfine coupling to histidine was found to be unusually small, about one-half that observed for similar couplings in blue copper centers (12). This result might be expected if the unpaired spin in oxidized CuA is appreciably delocalized onto one or more cysteine ligand(s) to copper. Two strongly coupled proton resonances have been observed by ENDOR and in fact were assigned to methylene protons on cysteine ligand(s) to CuA (11). Whether these proton hyperfine interactions arise from couplings to methylene protons on a single cysteine ligand to the copper or, alternatively, from methylene protons of two different cysteine ligands has not been settled. In either case, the strong coordination of two cysteine ligands to CuA would be expected to result in a system with very different properties than those of the blue copper centers. In particular, the coordination of a second soft sulfur ligand would stabilize charge transfer from sulfur to copper (i.e., a transfer of unpaired spin from copper to sulfur).

A recent comparative EXAFS study of the copper sites in CuA-depleted, p-(hydroxymercuri)benzoate-modified, and native beef heart cytochrome oxidase has shown the coordination of two sulfur ligands to CuA (13). In this work, we present additional evidence for a substantial delocalization of the unpaired spin at CuA onto associated cysteine sulfur ligand(s) and articulate the case for the involvement of two cysteine sulfurs at the CuA site. Specifically, we report the resolution of new strongly coupled proton hyperfine resonances in the ENDOR spectrum of CuA. We interpret these new results in terms of two structural models, both involving the delocalization of unpaired spin from copper onto cysteine sulfur ligand(s). The first model involves the delocalization of unpaired spin onto a single cysteine sulfur ligand, whereas the second model involves a unique system in which the unpaired spin density is delocalized onto each of two cysteine thiolate ligands in a fairly symmetric fashion. The incorporation of [β-13C]cysteine into the yeast protein confirms the coordination of at least one cysteine ligand to copper. The resolution of only one hyperfine coupling to the cysteine methylone carbon(s) suggests that if two cysteine ligands are coordinated to copper, the spin distribution at the site must be symmetric as in the latter model. Finally, we suggest how the protein might provide a structure to stabilize the unusual coordination of two cysteine sulfurs to copper, and we show how the symmetric coordination of two cysteine thiolates to a Cu(I) ion could explain many of the unusual spectroscopic features of this unique copper center.

MATERIALS AND METHODS

Chemicals used in the protein purifications were of enzyme-grade when available; otherwise they were reagent-grade. Amino acids and antibiotics were the highest grades available from Sigma. Ammonium sulfate in the growth medium was reagent-grade from J. T. Baker Chemical Co. Vitamins and media for selection and crossing of yeast strains were obtained from Difco. The [β-13C]cysteine ([13C]Cys) was 95% 13C and was obtained from Cambridge Isotopes Laboratories (Boston). The isotopic composition of the [13C]Cys was verified shortly before use by 1H NMR spectroscopy and was found to be greater than 95% enriched in 13C at the methylene carbon.

Preparation and Characterization of Yeast Strains—The wild-type Saccharomyces cerevisiae haploid strain D273-10B (mating type a) was used in the growth of yeast for the isolation of unsubstituted (native) protein. This strain has been shown to respire efficiently on the nonrepressible carbon source galactose (i.e., it contains the GAL+ trait) and produces good quantities of mitochondria.

The S. cerevisiae cytochrome oxidase haploid strain JW1-2C (a CUP1) (14) was obtained from the Yeast Genetic Stock Center (Berkeley, CA). This strain is unable to utilize galactose as a carbon source (i.e., it is GAL-) and is therefore unsuitable for the respiratory growth of yeast. To correct this respiratory deficiency, the JW1-2C strain was crossed with the wild-type D273-10B strain according to standard procedures (15) in order to obtain a cysteine oxidase sufficiently sufficient in this strain. The resulting strain (designated CY1-2-1) contained both the GAL- and Cys- traits.

Large-scale Growth of Yeast—The growth of wild-type yeast was carried out as described previously in a 360-liter fermentor (11). The growth of cytochrome oxidase for the preparation of [13C]Cys-substituted protein was identical to that of the wild type, except that the media contained the following specifically added amino acids: 5 g each of His, Ser, Met, Thr, Trp, Tyr, Phe, Asn, Glu, and Arg; 20 g of Gly; 50 g of Lys; and 4.0 g of DL-[β-13C]cysteine HCl (95% 13C). Cells were allowed to grow to a density of 5.5 X 10^5 cells/ml, at which point the revertant level was less than 0.001%.

Isolation of Proteins—Yeast mitochondria were isolated according to the procedure of George-Nascimento and Poyton (16), except that the buffer used during the Dyno-Mill cell disruption was 0.4 M in sucrose. This procedure resulted in the breakage of at least 80% of the yeast cells. Yeast cytochrome oxidase was isolated from the mitochondria and purified as described previously (11). The final protein was suspended in a buffer of 50 mM potassium phosphate, pH 7.4. Protein concentration was typically 0.1 mm in 0.2-0.3-ml sample volumes.

Cytochrome oxidase from beef heart was prepared by the method of Yu et al. (17) and was suspended in 0.5% cholate, 50 mM potassium phosphate, pH 7.4. In one experiment, beef heart protein was prepared by the method of Hartzell and Beinert (18) and was suspended in 50 mM potassium phosphate, pH 7.4. Both protein preparations were 0.3 mm in cytochrome oxidase. Sample volumes were 0.3 ml for the Hartzell and Beinert preparation, but 1.5 ml for that prepared according to Yu et al.

ENDOR Spectroscopy—ENDOR spectra were recorded as described previously (11, 19). Specific conditions are given in the figure legends. Although the sample tubes were designed to hold 1.5 ml of solution, samples were generally ~0.3 ml in volume and were placed in smaller quartz tubes mounted concentrically inside the larger ENDOR tube. This served to keep the sample near the maximum in the microwave field and resulted in an optimal cavity-filling factor.

It is important to point out that the ENDOR spectra were recorded under conditions of rapid passage (20) in order to obtain optimal signal intensities. Accordingly, the signals appear shifted somewhat in the direction of the sweep from their true positions. To obtain the true positions of the ENDOR signals, two spectra were recorded under identical conditions, but in opposite sweep directions. The true position of each ENDOR signal was then estimated as the average of the positions in these two sweeps.

ENDOR Simulations—ENDOR spectra observed at specific positions in the EPR powder patterns were simulated using first-order equations for effective g-value and hyperfine coupling at various specific powder angles (20). Allowance was made for noncoincidence of tensors by a rotation of the axes. The full powder spectrum was then obtained by summing over 200 orientations in the xy plane and 100 orientations out-of-plane.
RESULTS

In a previous report (11), we presented results identifying ligands to CuA via the direct assignment of ENDOR resonances to specific amino acid constituents in yeast cytochrome c oxidase. These assignments demonstrated conclusively the presence of at least one cysteine and one histidine as ligands to CuA. We also noted the possibility of an additional proton ENDOR signal for the yeast protein, but the signal-to-noise ratio in the ENDOR spectra available at that time was too low to allow a definitive assignment. More recently, we have demonstrated the coordination of two sulfur ligands to CuA (13). The possibility of a second cysteine sulfur ligand to CuA has prompted us to re-examine now the ENDOR spectrum of CuA from the beef heart protein. Since our earlier ENDOR work, we have succeeded in preparing larger and more concentrated beef heart protein samples. This, together with the availability of improved facilities for the signal averaging of spectra, has led to CuA ENDOR spectra of substantially improved quality.

The general features of the new beef heart ENDOR spectrum in the 31–1-MHz region (Fig. 1, upper) are similar to those reported previously for the beef heart (19) and yeast (11) proteins. However, the improved signal-to-noise of the new CuA ENDOR spectrum allows several new signals to be resolved for the beef heart protein.

New Proton Hyperfine Resonances

The two ENDOR resonances at 18.7 and 22.3 MHz, observed at $g = 2.04$ for the beef heart protein, have been previously assigned to two strongly coupled cysteine $\beta$-methylene protons from one or more cysteine ligands of CuA. The positions of these signals correspond to proton hyperfine couplings of $11.0$ and $18.6$ MHz, respectively. From these proton hyperfine couplings, we predict the Zeeman partners for each of the methylene proton signals to appear at $8.5$ and $4.2$ MHz, respectively. In the improved ENDOR spectrum obtained in this study (Fig. 1, upper), we do see a new signal at about $4.2$ MHz, which can be assigned to the Zeeman partner of the proton signal at $22.3$ MHz. However, the predicted position ($8.5$ MHz) of the Zeeman partner corresponding to the signal at $18.7$ MHz is coincident with the position of an ENDOR signal assigned to nitrogen (the peaks at $9.5$ and $7.6$ MHz were previously shown to arise from hyperfine coupling to a histidine ligand nitrogen), so that this proton signal is not expected to be clearly resolved. The weaker intensities of these lower frequency proton signals relative to their partners at the higher frequencies are given by the "ENDOR enhancement effect" (21): the ratio of intensities of the signals forming a Zeeman pair is proportional to the square of the ratio of the resonant frequencies of the two transitions.

In our ENDOR spectra of CuA for the beef heart protein, a new signal is also discerned at $19.9$ MHz. This signal, which appears as a shoulder to the proton resonance at $18.7$ MHz, has not previously been resolved. It may arise from coupling to a previously unassigned ligand nucleus (most likely a proton or nitrogen in close interaction with the unpaired spin), or it may be associated with coupling to one of the previously assigned strongly coupled protons (reflecting either the nuclear Zeeman interaction, ENDOR resonance positions for a given hyperfine coupling will move to higher frequency with increasing field strength (decreasing $g$-value).

3 Unless otherwise noted, ENDOR resonance positions are those measured with observation near the middle $g$-value in the EPR spectrum of CuA ($g = 2.04$). Note that due to the field dependence of the nuclear Zeeman interaction, ENDOR resonance positions for a given hyperfine coupling will move to higher frequency with increasing field strength (decreasing $g$-value).
yield three or four ENDOR signals in this region of the ordination schemes involving one or two cysteine ligands. On the other hand, it is quite possible that there are three or four distinct cysteine methylene protons interacting with the copper ion to account for the presence of only one cysteine ligand to CuA. These results demonstrate unambiguously that all the new signals observed in the CuA ENDOR spectrum arise from hyperfine couplings to protons.

Spectral Comparisons with the Yeast Protein—The ENDOR spectrum of CuA in the yeast protein also shows indications of more than one resonance in the region of the strongly coupled protons. Although the spectral resolution here is not as good as for the beef heart protein because the two strongly coupled proton resonances have shifted closer together, the spectrum in Fig. 1 (lower) does display a shoulder toward the high frequency side of the resonance near 21 MHz. Certainly the shape of the spectrum in this region is consistent with the presence of additional ENDOR signals for the yeast enzyme similar to those presently observed for the beef heart protein.

One Versus Two Cysteine Ligands

The resolution of a new strongly coupled proton signal in the ENDOR spectrum of CuA raises the interesting question of whether the signals in the 18–24-MHz region can be totally accounted for by the presence of only one cysteine ligand to CuA. The additional ENDOR signals could be accounted for by sample or conformational heterogeneities. On the other hand, it is quite possible that there are three or four distinct cysteine methylene protons interacting with the copper ion to yield three or four ENDOR signals in this region of the spectrum. This latter interpretation would indicate a unique coordination for CuA in which two different cysteine ligands are strongly coordinated to the copper center. We now present spectroscopic results directed at discriminating between coordination schemes involving one or two cysteine ligands.

ENDOR Spectra of CuA in Two Different Beef Heart Preparations—In an effort to clarify the nature of the newly resolved proton signals in the 18–24-MHz region of the CuA ENDOR spectrum, we have compared the ENDOR spectra of oxidized CuA in two different preparations of beef heart cytochrome oxidase that had formerly been shown to exhibit different conformational heterogeneities at the oxygen reduction site (22). ENDOR spectra of CuA from beef heart cytochrome oxidase isolated according to the procedures of Yu et al. (17) and Hartzell and Beinert (18) are compared in Fig. 3. These spectra for the two preparations are identical, indicating that if conformational heterogeneity were the cause of the additional methylene proton couplings within each sample, the nature and extent of this conformational heterogeneity would be independent of the nature of the preparation, a conclusion which is contrary to earlier findings for the oxygen reduction site (22).

ENDOR Spectra of CuA Observed at Different g-Values—It is possible that the new features in the strongly coupled proton region of the CuA ENDOR spectrum are merely the corresponding anisotropic components of the previously assigned resonances at 18.7 and 22.3 MHz. When the ENDOR spectrum is observed at the middle g-values, many crystal orientations contribute to the powder ENDOR spectrum, and a slightly different nuclear hyperfine interaction may be associated with each orientation due to the anisotropy in this interaction. In order to investigate this possibility, ENDOR spectra of oxidized CuA in the beef heart protein were recorded with the magnetic field at various positions along the EPR powder spectrum. Comparison of the experimental ENDOR spectra shown in Fig. 4 does reveal variations as the magnetic field is varied from one g-value extremum to the other. As predicted by the field dependence of the nuclear Zeeman interaction, all the resonances in the strongly coupled proton region of the spectrum shift to higher frequency as the magnetic field is increased to observe spectra at smaller g-values. However, the resolution of the two components at 19–20 MHz depends critically on the g-value of observation. In particular, the new feature at 19.9 MHz (observed at $g = 2.04$) becomes better resolved at $g$-values near 2.08 and is not at all resolved at the $g$-value extrema. One possible interpretation of these observations is that the newly resolved feature is an aniso-

![Fig. 2. ENDOR spectra of CuA in beef heart cytochrome oxidase at two different microwave frequencies: 9.08 and 9.34 GHz. A, a wide sweep range showing both the region of the strongly coupled protons and the histidine nitrogens; B, an enlarged view showing only the region of the strongly coupled protons. Conditions were: temperature, 2.1 K; microwave power, 10 microwatts; field modulation, 4.0 G; sweep rate, 5.2 MHz/s; and instrumental time constant, 0.02 s.](image)

![Fig. 3. ENDOR spectra of CuA in two different preparations of beef heart cytochrome oxidase. Trace A, protein purified according to the procedure of Yu et al. (17); trace B, protein prepared according to the procedure of Hartzell and Beinert (18). Conditions were: temperature, 2.1 K; microwave power, 10 microwatts; microwave frequency, 9.08 (trace A) and 9.11 (trace B) GHz; field modulation, 4.0 G; sweep rate, 5.2 MHz/s; and instrumental time constant, 0.01 s.](image)
tropic component of the previously resolved resonance at 18.7 MHz.

In order to determine the anisotropy of the proton nuclear hyperfine interaction required to account for these ENDOR features of the strongly coupled protons, we have attempted to simulate ENDOR spectra at various observation g-values. In these simulations, the EPR g-values (g_x = 1.998, g_y = 2.04, g_z = 2.17; A_x = 11.5, A_y = 14.5, and A_z = 10.0 MHz; EPR line width, 0.03 g-value units; ENDOR line width, 0.2 MHz; and microwave frequency, 9.112 MHz. The hyperfine tensor y' axis was rotated 35° toward the g tensor z axis.

**FIG. 4.** Experimental and simulated ENDOR spectra of Cu_A observed at several different g-values. Experimental conditions were as described for Fig. 3 (lower). Simulation parameters for the features from 18 to 21 MHz were: g_x = 1.998, g_y = 2.04, and g_z = 2.17; A_x = 11.5, A_y = 14.5, and A_z = 10.0 MHz; EPR line width, 0.03 g-value units; ENDOR line width, 0.2 MHz; and microwave frequency, 9.112 MHz. The hyperfine tensor y' axis was rotated 35° toward the g tensor z axis.

so severe over a limited range of observation g-values (e.g. g ~ 20.8) that the 18.7- and 19.9-MHz signals do not merge into one apparent resonance when the Cu_A ENDOR spectrum is monitored near these g-values.

**13C Cys-substituted Yeast Protein**—In an attempt to resolve the ambiguity over the number of cysteine ligands to Cu_A and to determine the extent to which unpaired spin density is delocalized onto the coordinating sulfur(s), we prepared yeast cytochrome oxidase substituted with 13C at the cysteine methylene carbon. The EPR spectra of oxidized Cu_A in cytochrome oxidase isolated from wild-type yeast and yeast substituted with [13C]Cys (not shown) reveal no significant differences and are quite similar to the spectrum of oxidized Cu_A from the beef heart protein. These spectral similarities indicate that incorporation of [13C]Cys into the Cu_A site results in no additional hyperfine couplings greater than about 30 MHz.

The ENDOR spectra of [13C]Cys-substituted and native yeast cytochrome oxidase are compared in Fig. 5 (upper). At frequencies above 7 MHz, the ENDOR spectra of oxidized Cu_A in the native and 13C-substituted yeast proteins are essentially identical. However, careful examination of the low frequency region (1–10 MHz) reveals a new ENDOR signal near 5 MHz in the spectrum of the 13C-substituted protein. Scanning only this region and optimizing ENDOR conditions for this signal, we see in Fig. 5 (lower) that there is clearly a new signal at 5.0 MHz which is not present in the native protein. This signal can only be due to a coupling to 13C of a cysteine ligand. We predict a Zeeman partner for this signal at either 1.6 or 11.8 MHz. Examination of spectra such as those in Fig. 5 (upper) reveals no new features near 12 MHz. The base-line drift at frequencies below 2 MHz does not allow us to verify the existence of a Zeeman partner at 1.6 MHz; however, this assignment seems most likely. Averaging narrow sweeps in both directions yielded a true peak position of 5.2 MHz for the new signal, which corresponds to a 13C coupling of 3.6 MHz for the cysteine methylene carbon.

Weakly coupled 13C hyperfine signals should occur centered at the 13C nuclear Zeeman frequency. Under the conditions used in Fig. 5 (lower), such signal will appear centered at 13.4 MHz. The loss of other resolved signals in the ENDOR spectrum of the [13C]Cys-substituted protein suggests either that there are no other weakly coupled cysteine methylene 13C nuclei at the Cu_A site or, if there is another weakly coupled methylene 13C (from a second cysteine ligand), that its coupling is approximately 3.6 MHz as well. These results on the [13C]Cys-substituted yeast protein can be interpreted in terms of either a lone cysteine at the Cu_A site or an essentially symmetrical interaction of two cysteine thiolate sulfurs with the copper ion.

**DISCUSSION**

In our previous study (11) of isotopically substituted yeast cytochrome c oxidase, we demonstrated conclusively the coordination of one histidine and at least one cysteine ligand to Cu_A. The interpretation of the data from [δ,δ,δ-3H_2]cysteine-substituted protein was, however, ambiguous as to the coordination of one or two cysteine ligands. A more recent EXAFS study of native and Cu_A-depleted forms of cytochrome oxidase presents strong evidence for the coordination of two sulfur ligands to Cu_A (13). In this work, we have discerned new features in the ENDOR spectrum of Cu_A in beef heart cytochrome c oxidase that in principle can clarify the issue of cysteine sulfide coordination. We now show how these new results, together with the observation of only one apparent coupling to 13C in Cu_A from [δ-13C]Cys-substituted yeast...
Cysteine Coordination to CuA

1H Hyperfine Interactions—The newly resolved signal near 20 MHz in the CuA ENDOR spectrum of beef heart cytochrome oxidase may be interpreted in several ways. First, it may be that there exists some structural heterogeneity in the CuA centers which causes a subset of them to have slightly different methylene proton hyperfine couplings. As will be discussed below, this change in hyperfine coupling could occur by a slight rotation of the β-CH₂ group relative to the π-orbital on the sulfur atom containing the unpaired spin density. Although small rotations of this sort are not unexpected in a protein, this interpretation would require two distinct preparations of CuA centers within the isolated protein with very precise, yet different rotational orientations. Moreover, it would require this heterogeneity to be a property of the oxidized protein independent of the method of preparation. Although conformational heterogeneity has been observed for the oxygen reduction site in cytochrome oxidase, this heterogeneity depends on the method of isolation and varies among different preparations obtained by the same procedure (22).

The heterogeneity observed at the oxygen-binding site is probably related to the different modes of ligand coordination necessary for catalysis. CuA is not involved in substrate binding and is unlikely to show similar effects.

A second interpretation is that the signal seen at 19.9 MHz is due to hyperfine coupling to a [15N]nitrogen ligand. The ENDOR spectra recorded at different microwave frequencies (Fig. 2) rule out this possibility. The new signal shifts with a change in the field position (at constant g-value) in the manner expected for a proton. Due to the uniquely large magnitude of the proton nuclear magnetic moment relative to all other nuclei, this signal can be unambiguously assigned to a proton hyperfine coupling.

A third explanation of the data is that the features at 18.7 and 19.9 MHz are separate ENDOR resonances arising from couplings to methylene protons from two distinct cysteines coordinated to copper. Within this interpretation, the resonances would be largely isotropic, with small anisotropic interactions leading to small changes in the line widths of the ENDOR resonances observed at different g-values. At the g-value extrema, the two broad resonances could overlap to give the appearance of a single resonance. The assignment of the signal seen at 19.9 MHz to a separate ENDOR resonance would indicate hyperfine coupling (A = 13.6 MHz) to a third methylene proton on a second cysteine ligand to CuA. The other proton on this second cysteine could give rise to a resonance which overlaps one of the strongly coupled proton signals, or it may be at an angle relative to the orbital containing the unpaired spin (see below) such that its coupling is very small. Regardless, this interpretation would predict a fourth methylene proton coupling. Careful analysis of the beef heart protein CuA ENDOR spectrum in the region near 23–24 MHz does reveal a shoulder on the high frequency side of the major signal at 23 MHz, consistent with the presence of an underlying signal near 24 MHz. ENDOR spectra taken in opposite sweep directions (not shown) demonstrate that this distortion is not caused by rapid passage or similar effects. This high frequency shoulder is even more pronounced in the ENDOR spectra of the yeast protein (the new signal being centered at about 22.5 MHz). Thus, there are good reasons to suspect four different strongly coupled proton signals in the 19–24-MHz region of the CuA ENDOR spectra. If this interpretation proves correct, these signals must originate from two sets of methylene protons belonging to two almost symmetrically coordinated cysteine ligands to CuA. The assignment of individual peaks in the spectrum to specific methylene proton pairs, however, is not possible with the available data.

We cannot rule out completely the possibility that the feature at 19.9 MHz is an anisotropic component of the previously identified hyperfine resonance at 18.7 MHz. ENDOR spectra recorded at different EPR g-values show a distinct dependence on the g-value of observation. In particular, the experimental spectra presented in Fig. 4 show good resolution of the features at 18.7 and 19.9 MHz when recorded at EPR g-values near 2.08, but show a merging of these features at both g-value extrema. The ENDOR resonance(s) near 22 MHz show much less variation with g-value. In order to assess this possibility, we have attempted spectral simula-
Estimation of the Unpaired Spin Density on Cysteine Sulfur

In the light of the above considerations, we allow for two explanations for the origin of the newly resolved proton ENDOR features: either they arise from anisotropic interactions with the previously assigned cysteine methylene protons or they arise from a third (and possibly fourth) cysteine methylene proton. The possibility of more than two strongly coupled protons to Cu₄ in the beef heart protein (and presumably also in the yeast protein) would require that there are two cysteine ligands to Cu₄. The comparable magnitude of these proton couplings, combined with the apparent measurement of only one resonance for $^{13}$C hyperfine interaction(s) in the $[^{13}$C]Cys sample, would indicate that, if there are two cysteine ligands to Cu₄, each has very similar unpaired spin density. We now proceed to estimate the extent of delocalization of unpaired spin density from copper onto the cysteine sulfur ligand(s). Although precise determinations require a knowledge of which signals arise from couplings to protons on the same cysteine ligand, the qualitative arguments are similar for all protons with resonances in the 19–24-MHz region of the ENDOR spectra.

Isotropic hyperfine interactions from methylene protons adjacent to a $\sigma$-type sulfur radical arise primarily from hyperconjugation (24–26). The magnitude of the interaction is expected to depend on the dihedral angle $\phi$ between the C–H bond and the sulfur $3p$ orbital containing the unpaired electron according to Equation 1.

$$A = A_0^d \cos^2 \phi$$  (1)

We have discussed previously how this relationship and the measured hyperfine couplings (or their isotropic components) can be exploited to solve for $\rho^d$, the spin density in a $p$-orbital on sulfur, and the proton dihedral angles (11). Assuming that the two proton hyperfine couplings of 11.0 and 18.6 MHz arise from protons on the same cysteine ligand, two possible solutions are found: either 25 or 70% of the unpaired spin on Cu₄ is localized in a $\sigma$-type orbital on sulfur. For either of these two solutions, the dihedral angles of the methylene protons would be in a region of the function wherein the predicted hyperfine couplings are very sensitive to angle. As an example, for the solution predicting 25% of the unpaired spin on sulfur, the dihedral angle for the proton giving rise to the 11.0-MHz hyperfine coupling is calculated to be 43°. The slope of the function at this point is such that a rotation of only 0.7° will result in a 1-MHz change in the hyperfine coupling (or a 0.5-MHz change in the observed signal position). Given the relatively narrow line widths of the strongly coupled proton signals for Cu₄, (more than 0.5–1 MHz), this analysis would indicate a rather narrow distribution of dihedral angles for each of the methylene protons at the low temperature of the ENDOR experiments, i.e. there is very little conformational freedom of the cysteine sulfur ligands in the protein.

We have assumed hyperconjugation as the principal contribution to the spin density at the methylene protons. Another mechanism that can contribute to the observed hyperfine interaction is spin polarization (26). In model studies of sulfur-centered radicals, Equation 1 has proven adequate to explain the measured proton hyperfine couplings (25). The extremely small $^{13}$C hyperfine coupling observed for the cysteine methylene carbon (3.6 MHz) is consistent with a negligible spin polarization of the $\sigma$-system by the unpaired electron spin in the sulfur $\sigma$-orbital.

The use of Equation 1 predicts that there is substantial unpaired spin density distributed away from the copper ion in oxidized Cu₄. At the very least, 25% of the spin density is localized on a sulfur ligand to Cu₄. If the new ENDOR features resolved at 19.9 and ∼24 MHz are to be assigned to the methylene protons of a second cysteine ligand, then we may conclude that the cysteine coordination is only slightly inequivalent and that in excess of 50% of the unpaired spin may be delocalized away from copper.

Finally, if the correct assignment of the new ENDOR features is anisotropic proton hyperfine interaction, one can use the magnitude of the anisotropic interaction to obtain an independent estimate of the unpaired spin density at the sulfur atom, irrespective of the number of cysteines associated with the Cu₄ site. In carbon-centered radicals, the distributed dipole interaction for $\beta$-protons interacting with spin on the $\alpha$-carbon 2.2 Å away is about 7 MHz (25). In cysteine, the distance between sulfur and its $\beta$-proton is somewhat larger, 2.4 Å (27), so that the distributed dipole interaction should decrease to about 5 MHz. This value can be compared with the anisotropic interaction of 2.5 MHz obtained from simulations of the ENDOR spectra at the different $g$-values. This analysis predicts an unpaired spin density near 50% on a single cysteine sulfur ligand to Cu₄, which is a factor of 2 higher than the unpaired spin density estimated from the hyperfine coupling constants using Equation 1. Since the anisotropic interaction predicts the delocalization of approximately 50% of an unpaired spin away from copper onto a cysteine sulfur ligand, we prefer assignment of the new ENDOR features to a second cysteine ligand. A recent comparative EXAFS study of the copper sites in Cu₄-depleted, p-(hydroxymercuri)benzoate-modified, and native cytochrome oxidase indicates that the structure for the Cu₄ site is consistent with two NO ligands and two equivalent sulfur ligands.

In any case, the delocalization of a substantial amount of unpaired spin density onto cysteine sulfur ligand(s) must confer unique electronic properties to Cu₄. We present in the Miniprint\* a molecular orbital description of the Cu₄ site based on symmetric coordination of two cysteine ligands to Cu₄.

\* Portions of this paper (including “Appendix: A Coordination Model for Cu₄,” and Figs. A1–A3) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
Cysteine Coordination to Cu₄₅

A Protein Coordination Model for Cu₄₅

The possibility of two cysteine ligands to Cu₄₅ prompts an analysis of the compiled evolutionary sequence data available for cytochrome oxidase to provide evidence on the identity of the cysteine ligands in the primary sequence of the protein. In the more than a dozen species for which the sequences of subunits I-III are known, no cysteines are conserved in either subunit I or III (28). In the 22 known sequences of all but one species, only two cysteine ligands (corresponding to positions 196 and 200 of the beef heart sequence) are conserved in subunit II. For the sequence of subunit II from wheat, it appears that one of these two cysteines (at position 200 in the beef heart sequence) is not conserved (29). These data clearly show that Cys¹⁹⁶ must be a ligand to Cu₄₅. However, the EXAFS data for the beef heart protein combined with the ENDOR data for protein from beef heart and yeast suggest two cysteine ligands. The arguments presented here predict that if the second cysteine (Cys²⁰⁰) is a ligand to Cu₄₅ in most forms of cytochrome oxidase, then the enzyme from wheat will have very different spectroscopic properties. Furthermore, if Cu₄₅ is involved in proton pumping (30), we predict that the wheat form may not pump protons and may only function in transferring electrons to oxygen.

An important feature of the molecular orbital description proposed for Cu₄₅ in the Miniprint is the coordination of copper by two cysteine sulfur ligands which are close enough to each other to form a partial bond between them. However, it is essential that the two cysteine ligands not be able to approach each other too closely, as copper can be an efficient catalyst of the oxidation of cysteine to cystine (31). The two conserved cysteine residues implicated as ligands to Cu₄₅ are separated by only three amino acids in the primary sequence. A Corey-Pauling-Koltun model showing such an arrangement of two cysteine residues 4 amino acid residues apart on an α-helix is shown in Fig. 6. Such a model allows one to put upper and lower limits on the distance attainable between the two cysteine sulfurs. We estimate the smallest center-to-center distance between these two sulfur atoms to be ~3 Å. This distance is too large for stable disulfide bond formation (the disulfide bond length in cystine is 2.04 Å (27)) and thus precludes the oxidation of the two cysteine ligands to cystine. However, we note that tetrahedral ligation of the two cysteine sulfurs to a copper ion in this configuration of the peptide would impart essentially symmetric copper-cysteine coordination with a copper-sulfur distance of ~2.30 Å, in accordance with recent EXAFS results (13) as well as the earlier data of Scott et al. (32).

Several consequences of this protein coordination model of Cu₄₅ are immediately apparent. First, between the 2 conserved cysteine residues in the primary sequence, there is a conserved glutamic acid residue that has been shown to be labeled by water-soluble carbodiimides (33). The helical model for Cu₄₅ coordination places this glutamic acid residue at the extreme opposite face of the α-helix relative to the copper-binding site. This prediction would explain how the 2 cysteine residues can both be coordinated to a well-buried copper site, while a residue between them can be exposed to the aqueous phase at the surface of the protein. The structure shown in Fig. 6 places the Cu₄₅ center approximately 10 Å from the carboxyl group of the glutamic acid and, presumably, from the surface of the protein.

A second consequence of this structure concerns the rigidity of the Cu₄₅ coordination, in particular the side chain rotational mobility of the cysteine sulfur ligands. The molecular orbital description of the oxidized Cu₄₅ site assumes some bonding character directly between the two sulfur atoms. For optimization of this interaction, the two cysteine sulfur atoms must be able to approach as close as possible. As discussed above, the helix model for Cu₄₅ coordination allows a closest approach of approximately 3 Å. To attain this minimal separation, however, the amino acid side chains must orient in a specific fashion. Significant rotation of the cysteine side chains about the α- or β-carbons would likely disrupt this interaction considerably. However, we expect any bonding interaction between the sulfur orbitals to be diminished or abolished when the Cu₄₅ site is reduced. The prediction then is that in the reduced Cu₄₅ site (only), at least one of the cysteine ligands to Cu₄₅ may be able to rotate somewhat to facilitate the donation of an electron to a nearby acceptor or to allow the protonation of one of the cysteine sulfurs during an intermediate step of proton pumping. A model for proton pumping by Cu₄₅ which incorporates many of these features has recently been proposed (30).

In summary, the measurement of a ¹³C hyperfine coupling to cysteine methylene carbon(s) in the ENDOR spectrum of Cu₄₅ from [¹³C]Cys-substituted cytochrome oxidase confirms our previous assignment of at least one cysteine ligand to Cu₄₅. The identification of new ENDOR resonances arising from cysteine methylene protons provides support for the assignment of a second cysteine ligand to copper and provides further information on the electronic distribution at the Cu₄₅ site. In either case, the magnitudes of the proton hyperfine couplings and the methylene ¹³C hyperfine coupling indicate that the unpaired spin in the oxidized Cu₄₅ center is extensively delocalized away from copper and onto the cysteine sulfur ligand(s). We show how a symmetric coordination of
two cysteine sulfur ligands to Cu₄ can account for many of the unique spectroscopic features of the site. We further propose how the protein may achieve such an unusual coordination and how this coordination may be involved in the pumping of protons by cytochrome c oxidase.

REFERENCES

Cysteine Coordination to Cu₄₈

orbits in the resulting Si⁺⁺ molecular orbital. Similarly, depending on the extent to which the copper 3d orbitals (specifically the t₂g orbitals) are lowered in energy by back-bonding interactions (see below), the sulfur radial density at the S⁻⁻⁻ orbital will vary.

The molecular orbital energy diagram shown in Fig. 3 delineates several other intermolecular interactions between the copper core and its ligands. These interactions are more difficult to estimate quantitatively and we will merely note their qualitative features. First, the unoccupied 3d orbitals of sulfur can overlap with the occupied t₂g and t₂g orbitals on copper to delocalize charge that are otherwise localized on the sulfur atom. This overlap of sulfur orbitals with t₂g orbitals on copper is responsible for the lowering of the energy of the copper t₂g and t₂g orbitals specifically, but also lowers the intermolecular repulsion (bond formation) because of the entire copper 3d shell. Hence, the relatively hard sulfur 3d orbitals, which were formerly at a higher energy than the CuS(t₂g) orbitals, gain some back-bonding energy from charge delocalization into the sulfur 3d orbitals. The unoccupied terminal 3d orbitals, on the other hand, can interact with the CuS(t₂g) orbitals to stabilize a back-bonding interaction and to promote some unoccupied state into the unoccupied 3d orbitals.

Finally, our new model for Cu₄₈ explains the unusual optical spectrum of the oxidized center. From the electron configuration derived from Fig. 3, one may expect the ligand-to-metal charge transfer transition (0000) with characteristic of the blue copper centers with the corresponding transition (B₁ - B₃) in Cu₄₈. This energy of the sulfur 3d orbitals in the new model for Cu₄₈ means both the ground and excited state orbitals in precise alignment with both ligands and metal atom core character. The sulfur-to-metal interaction also means the energy of the ground state orbitals so that the resonant energy of the orbital transition is lower. Indeed, the relatively weak optical band observed for Cu₄₈ does occur at lower energy, measured at about 350 nm.

The main features of the molecular description of the Cu₄₈ site in cytochrome oxidase are the mixing in energy of the sulfur 3d orbitals and a concomitant lowering in energy of the Cu₄₈ oscillator strength with respect to Cu₄₈. This favors a more complex mixing of the sulfur orbitals with the copper 3d orbitals in the higher filled molecular orbitals (containing the unpaired electron). The result is an oxidized copper center with substantial CuS(t₂g) sulfur radical character.

Figure A1. Representation of the molecular orbitals originating from the direct interaction between two sulfur ligands in Cu₄₈.