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The Nature of Cu_A in Cytochrome *c* Oxidase*

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The isolation and purification of yeast cytochrome *c* oxidase is described. Characterization of the purified protein indicates that it is spectroscopically identical with cytochrome *c* oxidase isolated from beef heart. Preparations of isotopically substituted yeast cytochrome *c* oxidase are obtained incorporating [1,3-$^{15}N_2$] histidine or [β,β-$^2H_2$]cysteine. Electron paramagnetic resonance and electron nuclear double resonance spectra of the isotopically substituted proteins identify unambiguously at least 1 cysteine and 1 histidine as ligands to Cu_A and suggest that substantial spin density is delocalized onto a cysteine sulfur in the oxidized protein to render the site Cu(I)—Ṡ.

When EPR spectra were first recorded from cytochrome *c* oxidase, it was recognized that only one of the two coppers in the fully oxidized enzyme exhibited an EPR signal (1, 2). The observed EPR spectrum was not like those normally observed for Cu(II), however, and a number of explanations were forwarded. These included the proposal of an interaction between the two copper atoms in the enzyme, rendering a site with unusual EPR characteristics and only one-half the expected EPR intensity of two coppers (1). Subsequently it was shown (3, 4) that the EPR visible copper was an isolated $S = 1/2$ site. Thus, the unusual EPR spectrum of the EPR visible copper (which will be referred to as the Cu_A center) should be explained on the basis of the geometry and/or ligation of an isolated copper ion. A considerable interest in the structure of the Cu_A center has derived from efforts to explain the unusual EPR properties of this site.

The EPR signal from the Cu_A center is atypical of Cu(II) in that no copper hyperfine splittings are clearly resolved and the *g* values are quite small; in fact, one *g* value is below the free electron *g* value. Only two mechanisms, consistent with an isolated copper site, can be invoked to explain the unusual EPR properties. One possibility is that the unpaired electron spin resides primarily on an associated ligand (5–8). This mechanism calls for extensive charge delocalization from the involved associated ligand onto the copper ion. The *g* values for the Cu_A center are, in fact, typical of those in thiyl radicals, and it has been suggested that the Cu_A center EPR signal might be due to a disulfide interacting with a copper ion (8) or due to a sulfur radical (5). A second possibility is that the orbital containing the unpaired electron is a hybrid 3*d* copper orbital with strong admixtures of 4*s* and 4*p* character (9, 10). In this case, the unpaired electron would reside primarily on the copper. The unusual EPR properties would result from distorting the copper into a near tetrahedral geometry which allows mixing of copper 4*s* and 4*p* orbitals with the 3*d* ground state.

With regard to the second possibility, a class of copper proteins is known in which the copper ion is forced into a distorted tetrahedral geometry. These copper sites are referred to as type 1 or blue coppers and have been extensively studied because they also exhibit unusual spectroscopic properties (11). In particular, the EPR spectra of type 1 coppers are unusual in that the copper hyperfine interaction is considerably reduced from that normally observed from Cu(II) complexes, although the *g* values are *normal*. The optical spectrum, from which the name blue copper originated, also is unusual in that a very strong charge transfer absorption ($\epsilon \simeq 5000\ M^{-1}\ cm^{-1}$) is observed near 600 nm. Normally, Cu(II) has only very weak absorption bands in the visible spectrum.

The Cu_A center in cytochrome *c* oxidase has been associated with the type 1 coppers for two reasons. First, the EPR spectrum of the Cu_A center superficially resembles that of type 1 coppers; second, cytochrome *c* oxidase has a very intense absorption near 600 nm, part of which has been believed to be due to copper (12, 13). Therefore, if the unusual EPR properties of the Cu_A center result from a distortion of the copper ion into a near tetrahedral geometry that allows mixing of 3*d*, 4*s*, and 4*p* orbitals, then type 1 coppers may be appropriate models for the Cu_A center.

A number of studies have been carried out in which the Cu_A center has been compared to type 1 coppers. The EPR parameters of the Cu_A center were compared to those of the type 1 coppers (5). It was found that the type 1 coppers fall within a well defined region in a plot of $A_\parallel$ *versus* $g_\parallel$, whereas the Cu_A parameters were markedly different. Comparison of the x-ray absorption data on the Cu_A center with those of the type 1 coppers plastocyanin (14) and stellacyanin (15) has also shown that the type 1 coppers do not closely resemble the Cu_A

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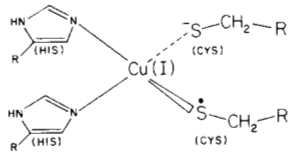

FIG. 1. **Structure for the Cu_A center in cytochrome *c* oxidase proposed by Chan *et al.* (6, 7, 16).**

center. In particular, the x-ray absorption edge studies indicate that the type 1 copper in plastocyanin is in the cupric oxidation state when oxidized and in the cuprous oxidation state when reduced. However, the x-ray absorption edge of the Cu_A center does not change substantially upon reduction; in both the oxidized and reduced states the Cu_A center appears as either a highly covalent Cu(II) or a Cu(I). Taken together, these results argue against a type 1 copper for the Cu_A center. The unusual *g* values and associated Cu hyperfine interaction observed by EPR, together with the absorption edge results obtained in x-ray spectroscopy experiments, are in fact more indicative of a copper ion in a ligand environment where there has been considerable electron delocalization onto the copper from an associated ligand.

On the basis of the above considerations, sulfhydryl-binding experiments, and well known copper cysteine chemistry, we proposed some time ago (6, 7, 16) that the Cu_A center consisted of a Cu(I) ion ligated by two cysteine sulfurs, one a cysteinate and the other a sulfur radical, and two histidine nitrogens (Fig. 1). We have now undertaken experiments to test this model by incorporating cysteine substituted with ^2H at the methylene carbon ([2H]Cys)[1] or histidine substituted with ^{15}N at both imidazole ring positions ([^{15}N]His) into yeast cytochrome *c* oxidase. The EPR and ENDOR properties of these two samples of isotopically substituted cytochrome *c* oxidase are described in this work. The results of these experiments together with the available physical data on this site will be discussed in the light of the model for the Cu_A center shown in Fig. 1.

MATERIALS AND METHODS

All chemicals used in the enzyme purifications were of enzyme grade when available; otherwise, they were reagent grade. All the chemicals used in the growth of yeast such as vitamins, amino acids, and galactose were the highest grades available from Sigma. The [1,3-$^{15}N_2$]histidine used for yeast growth was 95% ^{15}N in both histidine ring nitrogen positions and was obtained from Veb Berlin-Chemie, Berlin-Adlershof.

The [β,β-$^2H_2$]cysteine was synthesized according to the malonate condensation procedure of Crawhall and Elliot (17) as modified by Beilan (18) for use without the isolation and purification of intermediates. This procedure afforded the easiest approach to specific substitution at the β-carbon of cysteine. [$^2H_2$]formaldehyde (98% $^2H_2$, Stohler) was used to introduce the deuterated methylene group of the amino acid. The initial cysteine product was oxidized to cystine and then recrystallized. Immediately prior to use, the purified cystine was reduced to DL-cysteine with tin and HCl.

Preparation of the Yeast Enzyme

Preparation and Isolation of Yeast Auxotrophs—The wild type *Saccharomyces cerevisiae* haploid strain D273-10B was mutagenized with ethylmethanesulfonate as described by Fink (19). Cells that grew on minimal medium plus cysteine were then checked for growth in the absence of 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$^-$ and 10B His$^-$, respectively.

Large Scale Yeast Growth—For the isolation of yeast mitochondria and cytochrome *c* oxidase, the yeast cells were grown in a 350-liter fermenter which was interfaced to a Sharples continuous flow centrifuge. For growth of the wild type yeast cells in the 350-liter fermenter, the media contained yeast nitrogen-base components (20), 1% galactose, 1 kg of casamino acids (Difco), 11 g each of uracil and adenine, 11 g of penicillin, and 17 g of 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 fermenter to a level of about 10^6 cells/ml, and growth was allowed to proceed to about 5×10^8 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 g of each uracil and adenine, 17 g of all L-amino acids except *either* histidine *or* cysteine, 11 g of penicillin, and 17 g of streptomycin. In the case of the histidine auxotroph, 2 g of DL-[1,3-$^{15}N_2$]histidine·HCl (95% $^{15}N_2$) were added to the media. For the cysteine auxotroph, 6 g of DL-[β,β-$^2H_2$]cysteine·HCl (98% $^2H_2$) were added to the growth media. The starter cultures of the two auxotrophs used for inoculation produced starting cell densities of about 3×10^6 cells/ml. The cultures were allowed to grow for 2–3 days, with monitoring for revertants and contaminants every 12 h. The cells were harvested when the increase in cell density began to level off (~3–5 $\times$ 10^7 cells/ml). 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%.

Isolation of Yeast Mitochondria—Mitochondria were isolated from yeast by the method of Tzagoloff (21) as modified by Shakespeare and Mahler (22). The yeast cells were frozen in liquid nitrogen (~500 g, wet weight) and transferred to a precooled 1-gallon steel Waring blender. This frozen pellet was blended on high speed until all chunks were reduced to a very fine powder. About 1,400 ml 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 blended for 2 min alternating between low and high speeds. After adjusting the pH to about 7.5 with KOH, the suspension was centrifuged at 2,000 $\times$ *g* for 15 min. The supernatant was adjusted to pH 5.2 with acetic acid and centrifuged at 54,000 $\times$ *g* 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 2,000 $\times$ *g* pellet. The mitochondria were resuspended in the phosphate/KCl/EDTA buffer to a protein concentration of 20 mg/ml, as determined by the method of Lowry *et al.* (23) and stored at −85 °C until use.

Isolation of Yeast Cytochrome c Oxidase—The isolated mitochondria at 20 mg/ml of protein concentration were solubilized by the slow addition of 3 mg of cholate (Calbiochem A grade)/mg of protein as a 20% solution. All steps in the isolation were carried out at 0–4 °C. To this suspension was added 176 g of solid ammonium sulfate/liter (to 30% of saturation) with stirring. After ammonium sulfate addition, the pH was adjusted to 7.4 and the suspension stirred for 4 h. This suspension was centrifuged at 27,000 $\times$ *g* for 30 min and the supernatant brought to 45% of saturation with the addition of 94 g of solid ammonium sulfate/liter. After stirring for 15 min, the suspension was centrifuged at 27,000 $\times$ *g* for 20 min. 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 for 15 min followed by centrifugation at 27,000 $\times$ *g* for 20 min. The supernatant was adjusted to 39% of ammonium sulfate saturation followed by centrifugation at 27,000 $\times$ *g* for 20 min. 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 for 24 h against a 100-fold larger volume of 0.5% cholate, 10 mM Tris-acetate, 1 mM EDTA, pH 7.4. This dialysis resulted in some precipitation of protein which was removed by centrifugation at 200,000 $\times$ *g* for 30 min.

[1] The abbreviations used are: [^2H]Cys, [β,β-$^2H_2$]cysteine; [^{15}N]His, [1,3-$^{15}N_2$]histidine; [^2H]Cys cytochrome *c* oxidase, the protein specifically substituted with [^2H]Cys; [^{15}N]His cytochrome *c* oxidase, the protein specifically substituted with [^{15}N]His; ENDOR, electron nuclear double resonance.

Finally, the clarified high speed supernatant was applied to a cytochrome *c* affinity column (1.5 × 30 cm, 25-cm bed height) previously equilibrated with the above dialysis buffer. This cytochrome *c* affinity column was prepared by linking 1 g of cytochrome *c* (Sigma Type VI) to 15 g of CNBr-activated Sepharose 4B (Sigma) according to published procedures (24, 25). After all the green protein solution had passed onto the column, the column was washed first with 100 ml of dialysis buffer followed by 100 ml of dialysis buffer containing 2% cholate. Very little green color eluted with these wash buffers; it was mostly brownish gold proteins that eluted under these conditions. The cytochrome *c* oxidase was eluted with 0.2 M KCl, 10 mM Tris-acetate, 1 mM EDTA, pH 7.4 buffer which contained either 0.5% cholate or 0.5% Tween-20. The elution of protein from the cytochrome *c*-Sepharose 4B column was monitored continuously at 280 nm with a Gilson HM UV-Visible dual beam spectrophotometer. The eluted fractions were checked for cytochrome *c* oxidase by measuring the absorbance difference between 603 and 630 nm in the presence of dithionite (26). The green fractions were collected, and cholate was added (as a 20% solution) to a final concentration of 1.5%. This solution was brought to 25% ammonium sulfate saturation and centrifuged at 20,000 × *g* for 10 min. The supernatant was adjusted to 38% ammonium sulfate saturation, and the resulting pellet was readily dissolved in a small volume of 0.5% cholate or Tween-20 buffer. The protein solution was then stored at −85 °C until use.

Protein Assays

Protein concentrations were determined by the method of Lowry *et al.* (23). Heme a concentrations were determined by two methods: 1) pyridine hemochromogen assay (27), assuming an extinction coefficient of 26.0 mM^{-1} cm^{-1} at 587 nm, and 2) the difference in absorbance between 603 and 630 nm after reduction with sodium dithionite (26), assuming an extinction coefficient of 16.5 mM^{-1} cm^{-1}. The results from these two procedures are in good agreement. The activity of the cytochrome *c* oxidase so isolated was measured polarographically with a YSI model 53 oxygen electrode in a pH 7.4 medium containing 50 mM phosphate buffer, 0.5% Tween-80, 0.2 mg/ml of cytochrome *c*, and 30 mM ascorbate at 30 °C.

Absorption spectra of yeast cytochrome *c* oxidase were recorded on a Cary 219 spectrophotometer at room temperature. All EPR spectra were recorded at 10–20 K on a Varian E-line Century Series X-band spectrometer equipped with an Air-Products Heli-Trans low temperature system.

ENDOR Spectroscopy

ENDOR spectra were recorded at SUNY, Albany, with a spectrometer described elsewhere (28, 29). Spectra were obtained at 2.1 K in the dispersion (*x'*) mode under rapid passage conditions with microwave powers of about 10 microwatts (30). The amplitude of the ENDOR radiofrequency was typically 0.5 G peak to peak. Field modulation at 100 KHz was used for detection. A peak to peak field modulation amplitude of the order of 5 G is most effective in obtaining nitrogen and strongly coupled proton ENDOR; a modulation amplitude of the order of 0.5 G is most effective in obtaining well resolved ENDOR spectra from the weakly coupled protons near the free proton frequency (30).

RESULTS

Purification and Characterization of Yeast Cytochrome c Oxidase

The progress of a typical preparation of yeast cytochrome *c* oxidase is summarized in Table I. We note a substantial enhancement in heme a content after the purification of the protein on the cytochrome *c* affinity column (24, 25). Fig. 2 shows the specific affinity of this column toward yeast oxidase; here the elution profiles of protein and oxidase are compared. While a substantial amount of protein is eluted at low salt concentrations, most of the cytochrome oxidase elutes at higher (0.2 M KCl) salt concentrations. The final heme a content of 8.7 nmol of heme a/mg of protein compares well with typical values of 9–11 reported for preparations of beef heart cytochrome *c* oxidase. Although this ratio is a useful indicator of biochemical purity, a low number implying the presence of protein contaminants and a high number suggesting partial proteolysis or loss of subunits, the value to be expected for cytochrome *c* oxidase based on the true molecular weight of the monomer is still not available for either the yeast or the beef heart protein (for a critical review, see Wikström *et al.* (31)).

TABLE I

Purification of yeast cytochrome c oxidase

	Total protein	Specific activity	Heme a/protein
	mg	*nmol O_2/mg/sec*	*nmol/mg*
Mitochondrial particles	18,800	1.04	ND[a]
First ammonium sulfate fractionation (30 to 45%)	2,200	4.96	0.48
Second ammonium sulfate fractionation (28 to 39%)	680	2.62	0.96
Combined peak fractions of cytochrome oxidase with affinity column eluate	43	11.5	5.9
Purified yeast cytochrome *c* oxidase	30	11.9	8.7

[a] ND, not determined.

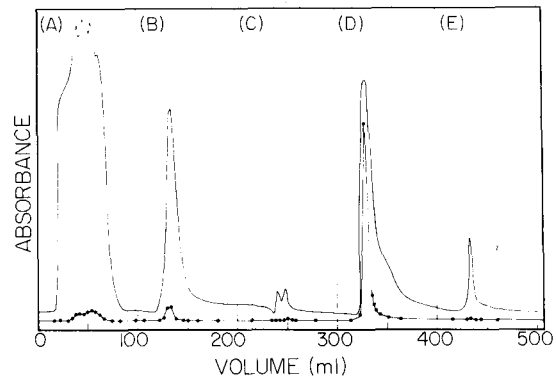

FIG. 2. **Elution profiles of yeast cytochrome *c* oxidase (*bottom, solid circles*) and total protein (*top*).** Elution buffers were 10 mM Tris-acetate, 1 mM EDTA, pH 7.4, containing 0.5% cholate, *A*; 2% cholate, *B*; 0.5% Tween-20, 0.02 M KCl, *C*; 0.5% Tween-20, 0.2 M KCl, *D*; or 0.5% Tween-20, 1.0 M KCl, *E*. Methods of monitoring concentrations of protein and yeast cytochrome *c* oxidase are described in the text.

Optical spectra of the yeast protein thus purified are shown in Fig. 3. These spectra reveal that this preparation of cytochrome *c* oxidase is free of detectable contamination from cytochromes *b*, *c* and c_1, as evidenced by the A_{red}(602 nm)/A_{red}(550 nm) ratio of 2.4. The A_{red}(423 nm)/A_{red}(442 nm) ratio is 0.40, thus the amount of nonreducible oxidase (31) is low as well. Other relevant spectral ratios are: A_{red}(442 nm)/A_{ox}(423 nm) = 1.24 and A_{red}(602 mm)/A_{ox}(598 nm) = 2.09. All ratios are close to the ratios reported by Lemberg for beef heart cytochrome *c* oxidase (32).

Similarly the EPR spectrum of the purified yeast protein (Fig. 4) shows less than 1% high spin heme and no evidence of the adventitious copper or a contaminant signal around g = 2, which we have often seen in the spectra of the protein purified on the DEAE-column (33). Integrations of the EPR signals reveal a Cu_A to cytochrome *a* ratio of 0.81, again very close to typical values reported for the beef heart protein. Thus, we conclude that the yeast cytochrome *c* oxidase which we have isolated and purified compares favorably, on the basis of the spectroscopic criteria, with some of the best beef heart cytochrome *c* oxidase preparations.

EPR of Labeled Proteins

The EPR spectra of native, [^{15}N]His, and [^{2}H]Cys yeast

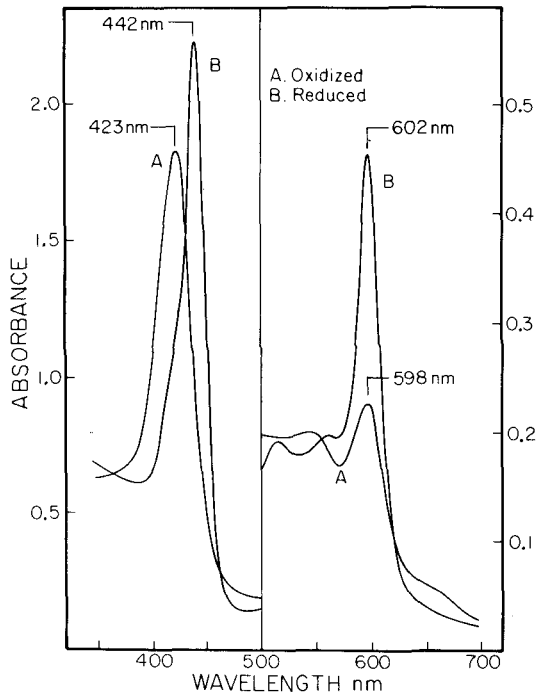

FIG. 3. **Optical spectra of yeast cytochrome *c* oxidase (21 μM).** *A*, oxidized enzyme, *B*, enzyme reduced with 20 mM ascorbate and 0.2 mM *p*-phenylenediamine.

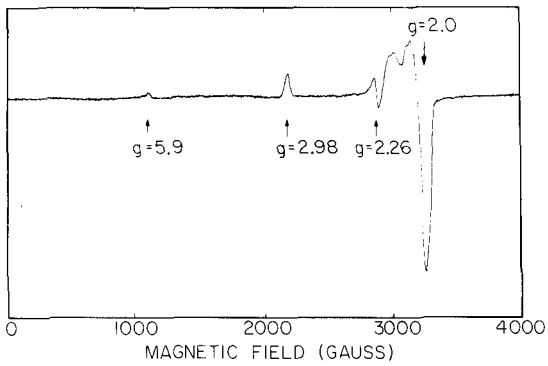

FIG. 4. **EPR spectrum of yeast cytochrome *c* oxidase.** Conditions: temperature, 10 K; microwave frequency, 9.10 GHz; microwave power, 0.02 milliwatt; modulation amplitude, 16 G.

cytochrome *c* oxidase are compared in Fig. 5. The EPR spectra for both of the isotopically substituted proteins are qualitatively similar to the EPR spectrum of the native yeast protein. However, differences are noted in the $g = 2$ region of the Cu_A signal. The important observation is that this part of the Cu_A signal has sharpened up somewhat for both of the isotopically substituted proteins, allowing the resolution of previously obscured hyperfine structure. This is most evident in the EPR spectrum of the [^{2}H]Cys protein; here, the hyperfine structure in the $g = 2.03$ region, seen previously only at S-band (34), is almost completely resolved. We note that this structure, which has been assigned to a copper hyperfine interaction of 45 G that is further split by interaction with either an $I = 1/2$ nuclear or $S = 1/2$ electron spin, remains unchanged on deuterium substitution of the cysteinyl β-protons. Thus, the spectral sharpening seen in the isotopically substituted proteins arises from the elimination or modification of smaller hyperfine couplings associated with the Cu_A center, such as those reported in ENDOR studies on beef heart cytochrome *c* oxidase (30).

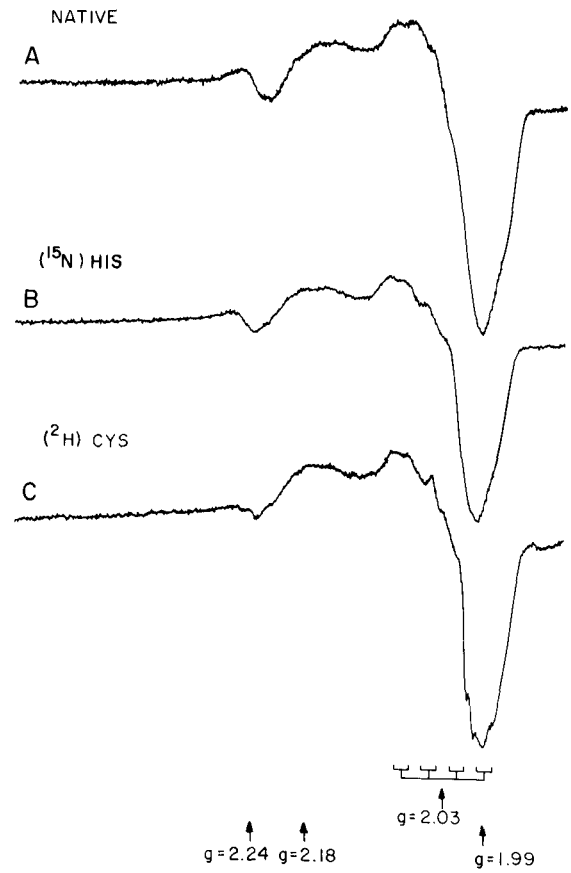

FIG. 5. **EPR spectra of native, *A*; [^{15}N]His, *B*; and [^{2}H]Cys yeast cytochrome *c* oxidase, *C*.** Conditions: temperature, 15 K; microwave frequency, 9.23 GHz; microwave power, 1.0 milliwatt; modulation amplitude, 16 G.

ENDOR Studies of Yeast Cytochrome c Oxidase

The Cu_A ENDOR spectrum of *native* yeast cytochrome *c* oxidase observed at $g = 2.04$ with a modulation amplitude of 4.0 G is shown in Fig. 6*A*. This spectrum is very similar to that of the beef heart protein. The two signals seen at 21.7 and 19.7 MHz are due to strongly coupled protons and correspond to proton hyperfine couplings of 16.2 and 12.2 MHz, respectively. These proton couplings compare with couplings of 19.2 and 12.0 MHz seen for the beef heart protein (30). The two signals seen at 7.1 and 9.2 MHz are split by twice the characteristic ^{14}N Zeeman energy ($2\nu_{^{14}N} = 2.00$ MHz). They can accordingly be assigned to a ^{14}N hyperfine coupling of 16 MHz. A similar nitrogen coupling of 17 MHz is seen for the beef heart protein. ENDOR spectra taken at $g = 2.00$ and $g = 2.10$ (not shown) confirm that, as in the beef heart protein, both the nitrogen and the proton couplings are isotropic.

ENDOR spectra of *isotopically substituted* and *native* yeast cytochrome *c* oxidase are compared at two different modulation amplitudes in Fig. 6. Note that due to the availability of only small amounts of isotopically substituted proteins having approximately 2 orders of magnitude fewer spins than the beef heart samples of Ref. 30, it was necessary to make use of extensive signal averaging and moderately high modulation amplitudes in order to obtain spectra with reasonable signal to noise. The amplitude of the field modulation can affect the line shapes and intensities of ENDOR peaks differently (28). In order to ensure that our conclusions are independent of field modulation, we present in Fig. 6 ENDOR spectra taken at field modulation amplitudes of 4.0 and 6.4 G. Note that comparisons should only be made between spectra obtained

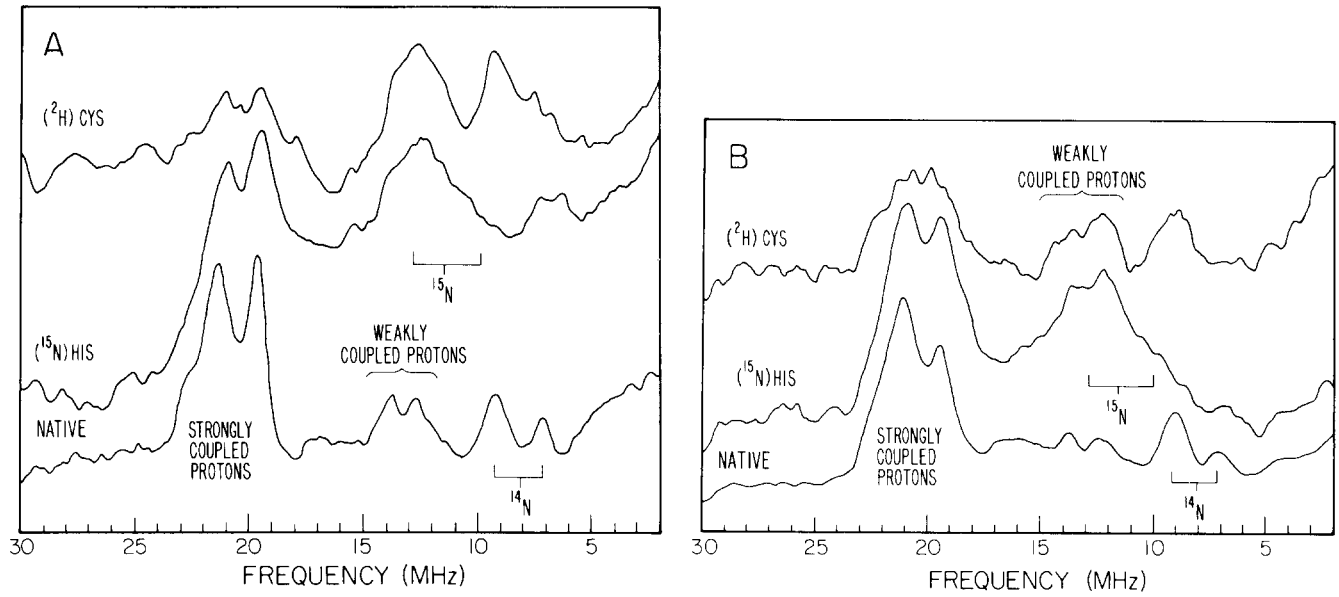

FIG. 6. ENDOR spectra of native, [^{15}N]His, and [^{2}H]Cys yeast cytochrome *c* oxidase observed at $g = 2.04$ using two field modulations. *A*, 4.0 G; *B*, 6.4 G. Conditions: temperature, 2.1 K; microwave frequency, 9.12 GHz; microwave power, 10 microwatts; sweep rate, 3.1 MHz/sec. In *A* the instrumental time constants for native, [^{15}N]His, and [^{2}H]Cys were 0.05, 0.10, and 0.15 s, respectively; and in *B* it was 0.10 s throughout.

with the same field modulation amplitude.

[^{2}H]Cys Substituted Protein—Comparison of the [^{2}H]Cys-substituted and native protein ENDOR spectra in Fig. 6 reveals a significant change in the 18–24 MHz region. As approximate indicators of intensity for changes in the 18–24 MHz region, we have used internal comparisons with the ^{14}N ENDOR resonance at 9 MHz and with the weakly coupled proton ENDOR resonances near 14 MHz. Both methods indicate that the intensity of the signals in the 18–24 MHz region decreases by more than 50% on substitution of [^{2}H]Cys for native cysteine in the yeast protein. We note that within the signal to noise, the line shapes in this region of the ENDOR spectra appear qualitatively different between the [^{2}H]Cys protein and the native protein, suggesting that there may be other interfering signals here. If this is the case, the strongly coupled protons have decreased in intensity by more than 50% upon deuterium substitution. Unfortunately, the small deuteron nuclear magnetic moment precludes observation of the deuterium ENDOR directly (1–3 MHz region). In any case, these ENDOR results together with the EPR data presented earlier demonstrate unambiguously that there is at least 1 cysteine ligand associated with Cu$_A$.

In order to ascertain whether there are weaker proton hyperfine interactions from cysteines associated with Cu$_A$, we have compared in Fig. 7 the ENDOR spectra between native and [^{2}H]Cys cytochrome *c* oxidase in the region of the weakly coupled protons. It appears that there are no detectable changes in this region when [^{2}H]Cys is substituted for native cysteine in the yeast protein, although the signal to noise may not allow us to rule out this possibility completely.

[^{15}N]His Substituted Protein. Upon comparison of the [^{15}N]His and native protein ENDOR spectra (Fig. 6), we see that only the intensities of the ^{14}N ENDOR signals at 7 and 9 MHz are substantially reduced in the spectrum of the [^{15}N]His protein relative to that of the native protein. Due to the difference in the nuclear magnetic moments between the two nitrogen isotopes, substitution of the ^{14}N nucleus by ^{15}N would replace the ^{14}N ENDOR signals at 7 and 9 MHz by ^{15}N ENDOR signals at 10 and 13 MHz, respectively. We do, in fact, observe increased intensity in the weakly coupled proton region in the ENDOR spectrum of the [^{15}N]His protein,

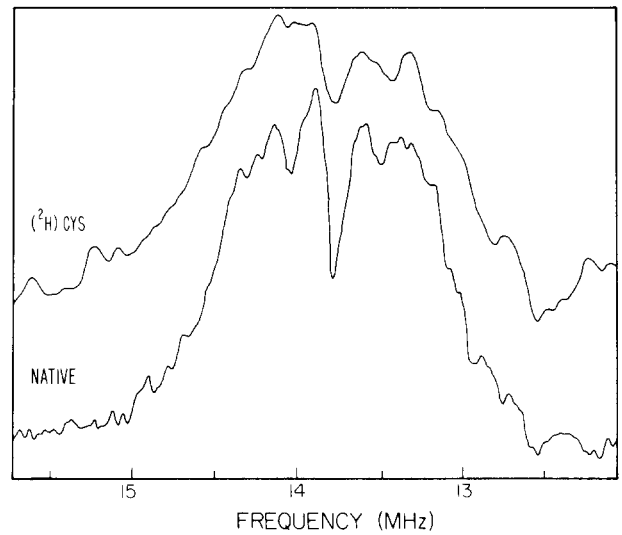

FIG. 7. ENDOR spectrum showing the weakly coupled proton region of native and [^{2}H]Cys yeast cytochrome *c* oxidase observed at $g = 2.00$ using a field modulation of 0.5 G. Conditions are as in Fig. 6 except that the sweep rate was 0.4 MHz/sec and time constants were 0.05 and 0.10 s for native and [^{2}H]Cys proteins, respectively.

consistent with the appearance of resonances at 10 and 13 MHz. From these observations, it is clear that there is at least 1 histidine ligand to Cu$_A$ in cytochrome *c* oxidase.

DISCUSSION

In this paper we report the successful isolation and purification of cytochrome *c* oxidase from the yeast *S. cerevisiae*. The protein thus isolated is shown to have spectroscopic properties very similar to those of cytochrome *c* oxidase isolated from beef heart, suggesting that the active sites of the two proteins are identical. Inasmuch as the yeast system can be manipulated to provide direct incorporation of isotopically substituted amino acids, we have used this system to probe the structure of the metal centers of this protein by preparing

[^{2}H]Cys- and [^{15}N]His-substituted cytochrome *c* oxidase. These studies, together with those described in a recent report (35), represent the first concrete information regarding the ligands to the metal centers in cytochrome *c* oxidase.

Analysis of EPR Spectra

The X-band EPR spectra of the [^{15}N]His and [^{2}H]Cys proteins are very similar to those of the native protein, the only difference being that in the spectra of the isotopically substituted proteins, there is some narrowing of the spectral components that make up the region around $g = 2$. In the case of the [^{15}N]His protein, substitution of ^{15}N for ^{14}N at both histidine ring positions results in some spectral sharpening in this region. Assuming the dominant nitrogen interaction is the 17-MHz hyperfine seen in the ENDOR spectrum of the native protein, substitution of [^{15}N]His would result in a reduction in the overall splitting from the $I = 1$ ^{14}N nucleus of 34 MHz ($2A$ = 12 G) to 24 MHz (A = 8.6 G) for the $I = 1/2$ ^{15}N nucleus. The sharpening of spectral features in the Cu_A signal (Fig. 5*B*) is consistent with at least this change in hyperfine interaction, although it is possible that there are other (smaller) nitrogen couplings from histidine ligands that are also modified upon this isotopic substitution.

Similarly, the substitution of ^{2}H for ^{1}H at the methylene carbon of cysteine eliminates the proton hyperfine interactions from the methylene protons of cysteine and results in some sharpening of the spectral components around $g = 2$. This permits the resolution of hyperfine structure not seen previously in the X-band spectrum. This EPR spectrum of the [^{2}H]Cys protein may be compared with the S-band (2–4 GHz) EPR spectrum of beef heart cytochrome *c* oxidase which was recently reported (34). The latter spectrum shows a resolved eight-line hyperfine pattern along $g_y = 2.03$. The splittings observed for the [^{2}H]Cys protein at X-band can, in fact, be fitted to this eight-line hyperfine pattern very well. The eight-line pattern observed in the S-band EPR spectrum was interpreted in terms of a 45 G copper hyperfine interaction ($I = 3/2$) and an additional 25 G interaction with either an $I = 1/2$ or an $S = 1/2$ spin. Inasmuch as the substitution of deuterons for the methylene protons in cysteine does not alter the observed eight-line hyperfine pattern, specifically, substitution does not eliminate the 25 G interaction but merely allows it to be resolved at X-band, we can rule out the cysteinyl methylene protons as the origin of a possible 25 G proton ($I = 1/2$) hyperfine interaction.

A dipolar interaction between Cu_A and cytochrome *a* ($S = 1/2$) has been proposed by Greenaway *et al.* (9) and by Froncisz *et al.* (34) which could lead to a dipolar splitting of the Cu_A signal of the order of 25 G (36). The manifestation of such a static dipolar splitting would be strongly temperature-dependent and would occur only at low temperatures when the relaxation of cytochrome *a* is sufficiently slow. Accordingly, we have studied the temperature dependence (10–95 K) of this splitting in the X-band EPR spectra of [^{2}H]Cys cytochrome *c* oxidase. We have observed no change in the Cu_A hyperfine pattern even at temperatures as high as 95 K, at which temperatures the cytochrome *a* signal at $g \simeq 3$ is broadened almost beyond detection due to rapid relaxation of the heme center. Scholes *et al.*[2] have also noted that the splitting observed in the S-band EPR spectrum of beef heart cytochrome *c* oxidase remains unchanged at temperatures as high as 77 K. These results argue against a dipolar interaction between Cu_A and cytochrome *a* as the origin of the 25-G interaction. The nature of this interaction thus remains elusive.

[2] C. P. Scholes, W. Froncisz, and J. S. Hyde, private communication.

It is well known that, even for the best preparations of beef heart cytochrome *c* oxidase where the stoichiometric copper to iron molar ratio has been established to be unity, the total measured integrated intensity of the EPR signal from Cu_A is only 80% of that expected from the integrated intensity of the cytochrome *a* EPR signal (37). We have obtained the same result for the yeast protein. No satisfactory explanation of this Cu_A EPR intensity anomaly has yet been offered; it seems possible, however, that the apparent reduced intensity of the Cu_A EPR signal may arise from anisotropic spectral broadening resulting from magnetic interaction of Cu_A with another paramagnetic center in the protein, *e.g.* cytochrome *a*.

Analysis of ENDOR Spectra

Comparison of the ENDOR spectra of native, [^{2}H]Cys, and [^{15}N]His cytochrome *c* oxidase reveals even more clearly that there is at least 1 cysteine and 1 histidine as ligands to Cu_A.

[^{15}N]His-substituted Protein—The assignment of a 16-MHz coupling to ^{14}N of a histidine in the native protein is confirmed by the observed changes in the 7–15 MHz region on substitution of [^{15}N]His for [^{14}N]His in cytochrome *c* oxidase. Although the signal to noise of the ENDOR spectrum of the [^{15}N]His protein is low, two observations are unambiguous: 1) the strong peak at 9 MHz is no longer present in the spectrum of the [^{15}N]His protein; and 2) the changes in intensity and line shape of the 10–15-MHz region are consistent with two new signals arising from ^{15}N substitution. There appear to be no other ENDOR signals that might be ascribed to histidine ring nitrogens. It is interesting to note that a hyperfine coupling of 16–17 MHz is unusually low for a histidine nitrogen ligand to Cu(II) (38, 39). However, it is not unexpected for a system where there has been a large delocalization of unpaired spin from Cu to a sulfur ligand.

[^{2}H]Cys-substituted Protein—The existence of at least 1 cysteine as a ligand to Cu_A is clearly demonstrated by the ENDOR of [^{2}H]Cys-substituted cytochrome *c* oxidase. The two resonances seen at 19.7 and 21.7 MHz decrease in intensity by greater than 50% on substitution of [^{2}H]Cys for native cysteine. The incomplete elimination of intensity in this region can be rationalized as follows. It is possible that some proton exchange may have occurred at the methylene carbon during the biosynthesis of the enzyme. Alternatively, there may exist other signals in this region which obscure the actual decrease in intensity of the signals due to the strongly coupled protons from the cysteine. This second possibility raises the question of what other centers could give rise to ENDOR signals in this region. The presence of a distinct shoulder at 24 MHz in the ENDOR spectrum of native, [^{2}H]Cys, and [^{15}N]His cytochrome *c* oxidase indicates that this signal could not be due to hyperfine from a second histidine nitrogen or associated with methylene protons of a cysteine ligand to Cu_A. We plan further study on this signal, particularly with respect to its field dependence, so as to ascertain its identity.

The observed proton hyperfine couplings of 16 and 12 MHz (19 and 12 MHz for the beef heart protein) from the cysteinyl methylene protons can be interpreted in two ways. The couplings may arise from two methylene protons on the same cysteine, or alternatively, they may be due to two methylene protons on two *different* cysteine ligands. Proton hyperfine interactions from methylene protons adjacent to a sulfur radical arise from hyperconjugation (40). They are essentially isotropic and their magnitude depends on the dihedral angle, ϕ, between the sulfur $3p_z$ orbital containing the unpaired electron and the C—H bond (41). The two isotropic proton coupling constants are given by

$$A_1 = A_0 \rho_S^{\pi} \cos^2 \phi \quad (1)$$

$$A_2 = A_0 \rho_S^{\pi} \cos^2 (\phi - 125°) \quad (2)$$

where A_0 is a constant (A_0 = 88 MHz for the *N*-acetyl-L-cysteine neutral sulfur radical (41)) and ρ_S^{π} is the π-spin density on the sulfur. Knowing A_1 and A_2 for a given sulfur center, one can then solve for ρ_S^{π} and the dihedral angle ϕ.

If we assume that the methylene proton hyperfine values of 12 and 19 MHz observed for the beef heart protein arise from protons on two *different* cysteine ligands to Cu$_A$, a range of angles may be chosen such that the other proton on each methylene carbon exhibits a negligible (less than 3 MHz) hyperfine coupling. For this situation, one cysteine sulfur can be assigned an unpaired spin density (ρ_S^{π}) ranging from 0.14 to 0.37, while the other cysteine sulfur will have a spin density ranging from 0.23 to 0.52.

A simpler situation is that the hyperfine couplings of 12 and 19 MHz arise from protons on the *same* cysteine ligand. In this case, only two distinct solutions are possible. In one, ϕ = 164° and ρ_S^{π} = 0.23; and in the other, ϕ = 60° and ρ_S^{π} = 0.83. The latter solution is more in agreement with other spectroscopic data on the Cu$_A$ site as will be discussed in the next section. It is important to note that the assignment of the strong proton hyperfine couplings to methylene protons on the same cysteine ligand does not preclude the existence of a second cysteine ligand to Cu$_A$. In fact, if ρ_S^{π} is as high as 0.83, a second cysteine ligand is necessary (6) to facilitate this large delocalization of spin from copper to the first cysteine (*i.e.* a delocalization of charge from cysteine to copper). If this were so, the magnitude of the hyperfine coupling to these more distant protons would be expected to be very small.

A Model for the Cu$_A$ Site

The incorporation of [^{15}N]His and [^{2}H]Cys into yeast cytochrome *c* oxidase has clearly shown the existence of at least 1 histidine and 1 cysteine as ligands to Cu$_A$. Furthermore, our ENDOR measurements on the [^{2}H]Cys-substituted protein suggest that there is substantial spin delocalization onto a cysteinyl sulfur ligand. We will now examine these results, along with EPR and other pertinent physical data amassed on the Cu$_A$ site, and show how these data can be rationalized in terms of the model (Fig. 1) proposed for the site by Chan *et al.* (6, 7, 16).

The EPR spectrum for Cu$_A$ is unique with respect to other copper complexes in that one of its *g* values is less than that of the free electron. It is important to note that Cu(II) complexes always exhibit *g* values greater than or equal to the free electron *g* value because the spin-orbit coupling parameter is negative for a $3d^9$ system (42). In order to observe *g* values less than the free electron *g* value, it is necessary to mix the 3*d* orbital containing the unpaired electron with an orbital that will exert a positive spin-orbit coupling. There are only two reasonable possibilities: either the 3*d* orbital is mixed with the 4*p* orbital of copper (9, 10) or it is mixed with an orbital of an associated sulfur ligand (5–8).

Similarly, the observed copper hyperfine values for the Cu$_A$ center are unique among Cu(II) complexes. Recent ENDOR studies have revealed a small copper hyperfine interaction associated with the Cu$_A$ center, but this copper hyperfine interaction is small and nearly isotropic (10) ($|A_x|$ = 68 MHz, $|A_y|$ = 98 MHz, $|A_z|$ = 90 MHz). As discussed recently (16), this unusually isotropic copper hyperfine interaction for the Cu$_A$ center indicates that the distributed dipole interaction between the unpaired electron and the copper nucleus is small. A number of investigators have invoked the mixing of a copper 4*p* orbital with the 3*d* orbital in order to reduce the distributed dipole interaction (9, 10). However, recent calculations have shown that in order to eliminate the anisotropic copper hyperfine interaction, it is necessary to include about 3 times as much 4*p* character as $3d_{x^2-y^2}$ or $3d_{xy}$ character (16). We feel that such a large mixing is unreasonable in view of the fact that the $3d^8 4p$ configuration lies about 125,000 cm^{-1} above the $3d^9$ configuration for divalent copper (14). Another way to reduce the distributed dipole interaction of the electron spin with the copper nucleus is the delocalization of the unpaired electron spin density onto an associated ligand sufficiently removed from the copper ion, *i.e.* onto an associated sulfur ligand.

Extensive delocalization of spin onto sulfur in the oxidized state would predict that copper remains mainly in the cuprous state both in the oxidized and the reduced forms of the Cu$_A$ center. X-ray absorption edge data from the Cu$_A$ center in both the oxidized and reduced forms appear to be consistent with this prediction (14, 15). These studies indicate that Cu$_A$ is either a Cu(I) or a *very* covalent Cu(II) in the oxidized protein. More important, the energy of the 1*s*-4*s* transition of Cu$_A$ does not change substantially when the enzyme is reduced. This latter finding is readily rationalized in terms of our model since, upon reduction, an associated ligand would be the actual electron acceptor, rather than the Cu ion itself.

Recently, *extended* x-ray absorption fine structure experiments have been undertaken on the copper K absorption edge of cytochrome *c* oxidase. The preliminary results (43) suggest that two sulfur atoms are associated with Cu$_A$. In the oxidized enzyme, the smaller copper-sulfur distance is 2.27 Å. When the enzyme is reduced,[3] the bond length is 2.31 Å. The Cu—S(Cys) bond length for Cu$_A$ in both oxidized and reduced cytochrome *c* oxidase is close to the Cu—S(Cys) bond length in reduced plastocyanin of 2.25 Å (44). In contrast, the Cu—S(Cys) bond length in oxidized plastocyanin is 2.1 Å. This similarity between the Cu—S(Cys) bond length in oxidized cytochrome *c* oxidase and reduced plastocyanin, as well as the lack of significant change of the Cu—S bond length upon reduction of cytochrome *c* oxidase, is strong evidence that Cu$_A$ is ligated by two cysteinyl sulfurs and is formally Cu(I) in the oxidized enzyme, in accordance with the main features of the Chan model.

In search of an appropriate model for the Cu$_A$ site, we have recently noted with interest the properties of a substituted horse liver alcohol dehydrogenase wherein the catalytic zinc has been replaced by copper. X-ray crystallographic studies of native horse liver alcohol dehydrogenase show that the catalytic zinc ion is coordinated in a distorted tetrahedral geometry by two cysteinyl sulfurs, one histidyl imidazole nitrogen, and a water or hydroxyl oxygen, and that this center is buried in a deep hydrophobic pocket of the protein (45). This catalytic Zn(II) can be reversibly removed and Cu(II) inserted into the site, resulting in a copper center with EPR and optical properties similar to those of the blue coppers (46, 47). Interestingly, when the NADH cofactor is bound to the copper-substituted protein (presumably at its specific binding site near to, but separate from, the catalytic site (45)), the EPR spectrum exhibited by the copper center is very similar to the EPR spectrum exhibited by Cu$_A$ of oxidized cytochrome *c* oxidase, both with respect to observed *g* values and with respect to the unusually low copper hyperfine coupling discussed earlier. More detailed EPR, ENDOR, and extended x-ray absorption fine structure studies of this model copper protein will prove useful in further testing the Chan model of the Cu$_A$ center in cytochrome *c* oxidase.

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[3] R. A. Scott, S. P. Cramer, R. W. Shaw, and H. Beinert, unpublished results.

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