Heat treatment of the bovine cytochrome c oxidase complex in the zwitterionic detergent sulfobetaine 12 (SB-12) results in loss of subunit III and the appearance of a type II copper center as characterized by electron paramagnetic resonance (EPR) spectroscopy. Previous authors (Nilsson, T., Copeland, R. A., Smith, P. A., and Chan, S. I. (1988) Biochemistry 27, 8254–8260) have interpreted this type II copper center as a modified version of the CuA site. By using electron nuclear double resonance spectroscopy, it is found that the CuA proton and nitrogen resonances remain present in the SB-12 heat-treated enzyme and that three new nitrogen resonances appear having hyperfine coupling constants consistent with histidine ligation. These hyperfine coupling constants correlate well with those recently found for the CuB histidines from the cytochrome aa3-600 quinol oxidase from Bacillus subtilis (Fann, Y. C., Ahmed, I., Blackburn, N. J., Boswell, J. S., Verkhovskaya, M. L., Hoffman, B. M., and Wikström, M. (1995) Biochemistry 34, 10245–10255). In addition, the total EPR-detectable copper concentration per enzyme molecule approximately doubles upon SB-12 heat treatment. Finally, the observed type II copper EPR spectrum is virtually indistinguishable from the EPR spectrum of CuB of the as-isolated type II copper EPR signals and diminution of the 830 nm absorption (thought to arise predominantly from the CuA site (11)) of the enzyme (12–16). A scheme which describes the perturbations to the CuA site under these various conditions has been postulated by Li et al. (14). The ligand rearrangements proposed by these workers must be re-evaluated, however, because they are based on a mononuclear structure for the CuA site. As type II signals certainly can arise from CuB under appropriate conditions, it should be noted that it has not been demonstrated definitively which copper ion is EPR-detectable as a result of these various modification procedures. The possibility exists that CuB becomes visible under one set of conditions, whereas the CuA site is modified under a different set of conditions. Also, perturbations to both copper sites can occur concurrently either by independent means or through allosteric interactions between the two copper sites. Electron nuclear double resonance (ENDOR) spectroscopy can be used to interpret ligand rearrangement reactions much more accurately than EPR and absorption spectroscopies. Thus, it was thought prudent to examine whether the type II signals that appear in the above modification experiments are accompanied by loss of the strongly coupled cysteinyl protons in the ENDOR spectrum associated with the CuA site. A change in the nitrogen ENDOR of the CuA center is certainly expected as well.

Proton and nitrogen ENDOR (35 GHz) are reported here for the bovine CeO complex after short, mild heat treatment in the zwitterionic detergent sulfobetaine 12 (SB-12), a procedure which results in a type II copper EPR signal that is very similar to that observed for the as-isolated Escherichia coli cytochrome bo complex, a structurally related ubiquinol oxidase complex. Special emphasis is placed on double integration of the first
**Derivative 9.2-GHz EPR signals obtained. Integration of EPR signals is important for two reasons. 1) The model of a mixed-valence, binuclear CuA center makes possible a scenario in which modification of this center leads to oxidation of the cuprous copper. An increase in EPR intensity would result if the magnetic coupling between the two copper ions is broken; a decrease in EPR intensity is expected if the magnetic coupling remains. 2) Uncoupling of the heme α₃-CuB binuclear center as a result of the SB-12 heat treatment procedure would make CuB EPR-detectable. The second explanation is most consistent with the data gathered in this study.**

**EXPERIMENTAL PROCEDURES**

**Enzyme Purification**—The beef heart Cco complex was isolated essentially by the Hartzell and Beinert method (17). Mitochondria were lysed with 2.5 g of Triton X-114/100 mg of protein in TEH buffer (20 mM Tris, 10 mM EDTA, 1 mM histidine, pH 8.0) supplemented with 200 mM KCl and centrifuged at 13,700 × g for 10 h. The pellet was washed three times with TEH buffer and solubilized with 2 mM potassium cholate (twice recrystallized/g of protein in TEH buffer at a protein concentration of 40 mg/ml. The enzyme was precipitated with ammonium sulfate and the pellets were redissolved in 25 mM Tris, 0.1% n-dodecyl-β-D-maltoside (DDM), 10 mM EDTA, pH 8.0 (dialysis buffer I). The modified enzyme samples, and the pyridine hemochrome method (19). This method, however, was found to be unreliable for the various modified enzyme samples, and the pyridine hemochrome method (19) was used instead. A dual wavelength extinction coefficient of 46.4 ± 1.0 mM⁻¹ cm⁻¹ (mean ± S.D.) at 588–638 nm for the reduced minus oxidized pyridine hemochrome was estimated from 10 different determinations on five different batches of as-isolated enzyme. Turnover numbers were calculated from the initial rate of ferrocytochrome c (1–80 μM initial concentration) oxidation monitored optically (550 nm or 520 nm) in 100 mM sodium phosphate, 0.1% DDM, pH 7.4. The kcat and Km for a particular enzyme sample were obtained from Eadie-Hofstee plots.

Stock CoO concentrations and enzyme activity were calculated as described previously (18). The kinetic constants for the UQO complex used here were identical to those reported earlier (18).

**SB-12 Heat Treatment**—The SB-12 heat-treated Cco complex was prepared by a modification of the procedure of Nilsson and co-workers (12). Three parts stock enzyme was diluted with ten parts 15 mM SB-12, prepared by a modification of the procedure of Nilsson and co-workers used here were identical to those reported earlier (18).

**Subunit III Depletion**—Subunit III was removed from the Cco complex using high detergent and salt concentrations. One part stock enzyme was diluted with six parts 5% Triton X-100, 300 mM Tris-HCl, 50 mM EDTA, pH 8.5, and incubated at room temperature for 20 h. After 10-fold dilution with distilled water, the enzyme was applied to a DE52 column equilibrated with 10 mM Tris, 0.1% DDM, pH 8.0. The column was then washed with 100 ml 10 mM Tris, 0.1% DDM, pH 8.0, and the enzyme was eluted with 200 mM NaCl, 25 mM Tris, 0.1% DDM, pH 8.0. The fractions containing enzyme were pooled and concentrated to greater than 150 μM using Centricron-100s. After dialysis (50-kDa membrane) at 8°C against about 100 volumes of 25 mM Tris, 0.1% DDM, pH 8.0, for 4–10 h, the enzyme was centrifuged at 32,500 × g, 4°C for 30 min, frozen in liquid nitrogen, and stored at −80°C until use.

**Electrophoresis**—The absence or presence of subunit III in various enzyme samples was determined by SDS-polyacrylamide gel electrophoresis. Enzyme samples were dissociated using 250 mM Tris-HCl, 8 M urea, 3.3% β-mercaptoethanol, 5% SDS, 15% glycerol, pH 6.2, at room temperature for about 1 h and run on a Hoefer SE 250 vertical electrophoresis unit using a 7.2%–10.1% (acrylamide/bisacrylamide) stacking gel, a 14%–30.3% running gel, and a running buffer of 20 mM Tris, 240 mM glycine, 0.1% SDS, pH 8.4. Gels were stained with Coomassie Blue.

**Instrumentation**—Optical absorption spectra and kinetic measurements were obtained with Hewlett-Packard 8452 or 8453 diode array UV/Vis spectrophotometers. The X-band EPR spectra were recorded using a Varian E-109 spectrometer equipped with a Varian E-231 TE 102 rectangular cavity. The modulation frequency used was 100 kHz, and temperature was controlled with a helium cryostat (Oxford Instruments) or liquid nitrogen finger Dewar (Wilmad). The ENDOR spectra were recorded on a modified Varian E-109 EPR spectrometer equipped with an E-110 35-GHz microwave bridge using 100-kHz field modulation as described previously (20). To a first approximation, the ENDOR spectrum for a single orientation of a nucleus (N) of spin I consists of 2I transitions at frequencies given by:

$$\nu_i (m_i) = \nu_N/2 \pm \nu_N/2 \pm \nu_N (2m_i - 1)$$

where $$\nu_N$$ and $$\nu_i$$ are orientation-dependent hyperfine and quadrupole coupling constants and $$\nu_N$$ is the nuclear Larmor frequency (21). All ENDOR spectra were taken with a radiofrequency source mixing with a noise source generator described elsewhere to enhance the ENDOR response (22).

**Data Analysis**—Double integration of first derivative X-band EPR spectra was done using the baseline correction and integration capabilities of Lab Calc”. The EPR visible copper concentration was calculated from double integrals of standard solutions of CuSO4 in 100 mM imidazole, pH 8.4, or in 100 mM histidine, pH 8.0, under the same experimental conditions.
RESULTS

Development of a Type II Copper EPR Signal—As shown in Fig. 1A, SB-12 heat treatment of the CcO complex results in the development of a type II copper EPR signal. The appearance of this type II copper EPR signal is accompanied by a high-spin heme signal in the $g = 6$ ($-1100$ G) region of the spectrum. This high-spin heme signal is best seen at low temperature (Fig. 1B) where line-broadening is less of a problem. Curiously, the development of this type II EPR signal is critically dependent on the length of the first dialysis step (see "Experimental Procedures"). Double integration and correction for spectrometer gain and enzyme concentration as determined by the pyridine hemochrome technique reveals that the amount of EPR-visible copper approximately doubles after 40 h of dialysis (Fig. 1, inset). The integration error is estimated to be in the range of 10–20%; this error arises from the protein concentration estimate, the baseline correction routine, and the fact that there are small heme signals in the $g = 2$ region even at 80 K. Copper standards were used to estimate the EPR-visible copper in the native enzyme. As expected, the ratio of EPR-visible copper to enzyme complex is approximately unity for native enzyme; therefore, this was assumed to be the case, and the integration of modified enzyme was standardized to this value. There appear to be two "isosbestic points" (at 2938 and 3059 G, data not shown); these isosbestic points suggest that a single process occurs to produce the type II signal.

Various attempts were made to determine what step of the SB-12 heat treatment procedure is responsible for the perturbations to the EPR spectrum of the enzyme. Gel electrophoresis reveals that subunit III is lost during the SB-12 heat treatment procedure. Subunit III can be removed by high salt and detergent concentration (see "Experimental Procedures"). In and of itself, it is found that subunit III depletion is insufficient to yield the large type II copper signal shown in Fig. 1. However, a small amount of type II copper signal is sometimes apparent in the EPR spectrum of the subunit III-depleted CcO complex; this signal intensifies after 40 h of dialysis with respect to this same buffer results in the appearance of a small amount of the type II copper signal. A substantial type II copper signal develops after 40 h of dialysis of the CcO complex in SB-12 buffer without a 15-min heat treatment. Typical results of these experiments are tabulated in Table I. The catalytic activity ($k_{cat}$) of the enzyme decreases as a result of subunit III depletion, SB-12 heat treatment, and/or extensive dialysis, but the $K_m$ for cytochrome c binding is virtually unaffected. As the decreased $k_{cat}$ ($20–80$% of wild-type) indicates that one or more electron transfer pathways have been perturbed, it is possible that the structural modification to the copper center that gives rise to the type II signal is largely responsible for the decreased catalytic activity. However, we cannot rule out the possibility that an electron transfer path topologically distant from the modified copper site has been perturbed. For example, a structurally modified heme $α_{3}$CuB binuclear site and a disrupted CuA electron input pathway is consistent with the EPR and turnover data. The optical spectra shown in Fig. 2 reveal that there is very little perturbation to the 830 nm absorption band except in the case of the SB-12-treated samples. In general, the 830 nm absorption intensity diminishes roughly according to time in contact with SB-12. No

![Fig. 2. UV-visible absorption spectra of the six samples listed in Table I. Native, —; subunit III-depleted, —; 40-h DDM-dialyzed, —; SB-12 heat-treated, 40-h SB-12-dialyzed, —; SB-12 heat-treated, 40-h SB-12-dialyzed, —. The protein concentration in each case is 2.5 $μ$M in 25 mM Tris, 0.1% DDM, pH 8.0.](image)
correlation is found, however, between the 830 nm absorption intensity and the appearance of the type II copper signal. In fact, a substantial type II copper signal is seen for the subunit III-depleted, 40-h DDM-dialyzed enzyme, yet no diminution of the 830 nm absorption intensity is apparent. As shown in Fig. 3, the type II copper signal is characterized by a \( g \) of about 2.19, an \( A_i \) of about 190 G, and a seven-line hyperfine pattern at \( g' = 2.06 \) with a splitting of about 15 G. When analyzing the ENDOR data, it is important to note that at fields at or above that corresponding to \( g < 2.2 \), only the type II copper species can contribute to the ENDOR spectrum; at higher fields, both the native CuA center and the type II species can contribute. Spectrometer settings are the same as in Fig. 1 except for a modulation amplitude of 5 G for the inset spectrum (average of two scans).

Fig. 4 shows the EPR spectrum of the as-isolated UQO complex. The type II copper signal in this spectrum is characterized by a \( g \) of 2.19, an \( A_i \) of about 190 G, and a seven-line hyperfine pattern at \( g_i = 2.06 \) with a splitting of about 15 G. At liquid helium temperatures, a substantial high-spin heme signal is present (data not shown) indicating that the heme o_o-CuB binuclear center is magnetically uncoupled.

No attempt was made to reverse the structural changes that give rise to the type II copper EPR signal, and no diminution of this EPR signal was ever observed once it appeared. However, extensive efforts in the past to reverse the type II modification were unsuccessful.\(^2\)

\(^1\)H ENDOR—The 35-GHz continuous-wave \(^1\)H ENDOR spectrum of the SB-12 heat-treated, 40.5-h SB-12-dialyzed CcO complex obtained in the \( g_i \) region of the type II copper species at fields low enough that the CuA site cannot contribute shows a doublet centered at the proton Larmor frequency that vanishes upon exchange into \( \text{D}_2\text{O} \) buffer (shown here for the modified CcO complex, demonstrated previously for the cytochrome \( \text{aa}_{3-600} \) complex (23)). Experimental conditions: modulation amplitude, 0.6 G; scan rate, 1 MHz/s; temperature, 2 K (average of 50 scans). The cytochrome \( \text{aa}_{3-600} \) ubiquinol oxidase spectrum is reproduced from Fann et al. (23).

\(^2\) P. M. Li and S. I. Chan, unpublished observations.
II copper EPR envelope. In addition, the proton ENDOR signals associated with the CuA center are still present and remain unperturbed at all fields that fall within the CuA EPR envelope indicating this site remains intact under the experimental conditions (data not shown). The proton ENDOR spectrum is very similar to that reported by Fann et al. (23) for the CuB site of the cytochrome aa₃-600 ubiquinol oxidase complex isolated from Bacillus subtilis (Fig. 5C). As is the case with the Bacillus enzyme, this strongly coupled proton (ν₁ ~ 10 MHz) is solvent-exchangeable (Fig. 5B) and is consistent with a bound hydroxide anion.

\[ 4^{14}N \text{ENDOR} - \text{Fig. 6, top, shows the ENDOR spectrum of the SB-12 heat-treated, 40-h SB-12 dialyzed CeO complex (35.04 GHz, } g_1 = -2.22 ("low field"), g_2 = 2.04 ("high field")), the native CeO complex (35.1 GHz, } g_1 = 2.04), \] the cytochrome aa₃-600 ubiquinol oxidase complex (35.2 GHz, } g_1 = -2.23 ("low field"), g_2 = 2.05 ("high field"))\). Experimental conditions are the same as for Fig. 5 except: modulation amplitude, 2.5 G (CcO)/1.25 G (Cy); scan rate, 2 MHz/s. The cytochrome aa₃-600 spectra are reproduced from Fann et al. (23).}

The appearance of a type II copper species as a consequence of SB-12 heat treatment of the CeO complex has been reported previously by Nilsson et al. (12). In addition, p-HMB treatment or incubation with AgNO₃ has been found to cause the appearance of type II species (13, 15, 16). In terms of EPR characterization, the type II copper species we report here appears to be identical to the one described by these authors. This type II species has been interpreted to result from modification of the CuA site of the CeO complex. In the scheme of Li et al. (14), CuA is assumed to be mononuclear with two nitrogens and two cysteine ligands. Mild heat treatment in DDM results in loss of one of the cysteine ligands thereby yielding a type I (blue) copper center. Heat treatment with SB-12 or treatment with p-HMB or AgNO₃ causes deligation of the second cysteine and the ligation of at least one more histidine to produce a type II copper center. The multifrequency EPR work of Kronke and co-workers (2–10), biophysical data on overexpressed subunit II fragments, and the recent crystal structures of the CeO complex indicate, however, that the CuA center is actually a mixed-valence, binuclear copper center with two bridging thiolates (Fig. 7). The CuA modification scenario of Li et al. (14) obviously conflicts with these more recent data.

**DISCUSSION**

The explanation for the appearance of the type II signal upon SB-12 heat treatment must be re-evaluated in light of the binuclear nature of the CuA center and the data presented here. There are two possibilities. The first is that the SB 12 heat treatment procedure causes disruption of the CuA site; the approximate doubling of the copper EPR intensity arises from...
full oxidation of the two copper ions. The second possibility is that the Cu\textsubscript{A} site remains in a native configuration and the magnetic coupling of the heme a\textsubscript{3}-Cu\textsubscript{B} binuclear center is broken making Cu\textsubscript{A} EPR-visible. In this scenario, the extra EPR-visible copper, all of which appears to be in a type II configuration, is Cu\textsubscript{B}. The strongest evidence in support of the first explanation is the diminution in the 830 nm absorption intensity, which is believed to arise predominantly from the Cu\textsubscript{A} center (11), upon SB-12 heat treatment of the enzyme. It has been found here, however, that the type II EPR signal can be created without any apparent loss in the 830 nm absorption intensity (Fig. 2). In addition, \textsuperscript{3}H and \textsuperscript{14}N ENDOR spectroscopy reveals that the Cu\textsubscript{A} resonances are present when a stoichiometric amount of the type II copper species is EPR-detectable. Instead, three additional \textsuperscript{14}N ENDOR resonances are detected (Fig. 6). The seven-line hyperfine pattern in the g\textsubscript{5} region of the EPR spectrum (Fig. 3) is consistent with three approximately equivalent nitrogen ligands. The excellent agreement between the hyperfine coupling constants of the type II center described here and those for the Cu\textsubscript{A} center of the cytochrome aa\textsubscript{3}-600 ubiquinol oxidase complex of \textit{B. subtilis} (Table II), which does not contain a Cu\textsubscript{A} center, allows confident assignment of the three new nitrogen ENDOR resonances as Cu\textsubscript{A} histidines. Finally, the EPR spectrum of the as-isolated UQO complex contains the identical type II copper species that results upon SB-12 heat treatment of the CcO complex. As the UQO complex does not contain a Cu\textsubscript{A} center but is otherwise structurally similar to the CcO complex (1), and, in this particular sample, the heme a\textsubscript{3}-Cu\textsubscript{B} binuclear center is magnetically uncoupled, these data provide strong confirmation that the type II signals that appear as a result of the various CcO treatments discussed here do indeed arise from Cu\textsubscript{B}. The changes in the resonance Raman spectrum of heme a\textsubscript{3} when the type II copper signal is apparent in the CcO EPR spectrum are now more easily understood (12, 24). The perturbations that uncouple the heme a\textsubscript{3}-Cu\textsubscript{B} binuclear center and make Cu\textsubscript{B} EPR-detectable also disrupt the heme a\textsubscript{3} pocket.

Given that the type II EPR signal seen arises predominantly from Cu\textsubscript{B}, it is important to question to what extent the structural integrity of the Cu\textsubscript{B} redox site is preserved. Cline and co-workers (25) report an EPR spectrum of Cu\textsubscript{B} obtained by full reduction of the enzyme, flushing with O\textsubscript{2}, and quick freezing in liquid N\textsubscript{2}. This spectrum is characterized by a g\textsubscript{5} of about 2.28 and an A\textsubscript{5} of about 100 G. Photoysis and freezing of the fully reduced CO-bound CcO complex in the presence of oxygen has been found to yield a type II copper signal with a g\textsubscript{5} of 2.26–2.28 and an A\textsubscript{5} of 102–137 G (26, 27). High pH causes the appearance of a type II species with a g\textsubscript{5} of 2.30 and and A\textsubscript{5} of 136 G (28). Addition of cyanide to the \textit{Thermus thermophilus} cytochrome \textit{ba}\textsubscript{3} complex produces a type II copper species with a g\textsubscript{5} of 2.28 and an A\textsubscript{5} of about 140 G (29, 30). These spectra contrast with the type II signal obtained by SB-12 heat treatment which has a larger A\textsubscript{5} (~190 G) and a smaller g\textsubscript{5} (~2.19). As harsher treatments tend to result in larger A\textsubscript{5} values, the indication is that the structural integrity of the Cu\textsubscript{B} site has been perturbed in the experiments described here. A small perturbation to the heme a\textsubscript{3}-Cu\textsubscript{B} site clearly must have occurred to break the magnetic coupling in this binuclear center. Both the SB-12 heat-treated CcO and the cytochrome \textit{aa}\textsubscript{3}-600 complexes contain a proton with an A\textsubscript{5} of about 10 MHz (Fig. 5). In the case of the cytochrome \textit{aa}\textsubscript{3}-600 complex, this proton is solvent-exchangeable and has been tentatively assigned as arising from a bound hydroxide anion (23). A hydroxide anion could certainly be the fourth ligand for the type II copper site reported here.

The finding that SB-12 heat treatment makes Cu\textsubscript{B} EPR-detectable raises the issue of which copper ions give rise to the type II signals seen upon AgNO\textsubscript{3} or p-HMB treatment (13, 15, 16). Since ENDOR experiments have not been attempted on such enzyme samples, a definitive conclusion cannot be made here. It is certainly possible that these treatments affect the Cu\textsubscript{A} site exclusively since they are thiol-specific reagents. Gelles and Chan (13) observed an approximate 40% increase in copper EPR intensity upon p-HMB treatment of the enzyme. This observation is consistent with a scenario where the type II copper signals arise from Cu\textsubscript{B} due to perturbations similar to those caused by SB-12 heat treatment. On the other hand, disruption of the Cu\textsubscript{A} center and oxidation of the cuprous copper would also result in a similar increase in the integrated EPR intensity.

While the ENDOR data reveal the presence of native Cu\textsubscript{A} resonances in the presence of stoichiometric amounts of type II copper, the possibility that a substoichiometric population of Cu\textsubscript{A} sites has been perturbed cannot be ruled out. The creation of a small amount of a type II copper species from severe disruption of the Cu\textsubscript{A} ligation structure would be undetectable in the EPR and ENDOR spectra due to the strong signals arising from the Cu\textsubscript{B} center. The diminution in the 830 nm absorption intensity and the slightly larger than stoichiometric increase in the integrated intensity of the copper EPR signals are consistent with this possibility.

On the other hand, less drastic perturbations to the Cu\textsubscript{A} site could occur. Li et al. (14) found that heat treatment in DDM results in substoichiometric populations of both type I and type II copper species. Zickermann et al. (31) have recently found that mutation of the weakly coordinating methionine of the binuclear CcO center to isoelucine results in a type I copper EPR spectrum (g\textsubscript{5} = 2.18, A\textsubscript{5} = 61 G) and loss of 830 nm absorption intensity. This type I species could arise in the native enzyme upon dissociation of the weak Met-Cu bond and lengthening of one of the Cu-S bonds as shown in Fig. 7. As a result of this conformational rearrangement, one of the copper atoms would have a type I-like structure (one His, one Cys, one carbonyl, and a long Cu-S bond). The other copper atom would have a high reduction potential due to the two strongly coordinating cysteines and, therefore, would become completely reduced with the electron initially shared by the two atoms. As only the type I-like copper would be EPR-visible, the EPR integration would remain constant. The loss in 830 nm absorption intensity reported here for some samples may result from a Cu\textsubscript{A} rearrangement of this type. The associated type I EPR signals are likely to be difficult to detect in the presence of strong type II signals and need not be identical to those resulting from the methionine mutation structure (31). The well-known interaction potentials in the CcO complex (1) indicate the presence of conformational interactions between redox centers. Therefore, the disruption of the heme a\textsubscript{3}-Cu\textsubscript{B} binuclear site that produces a type II signal could potentially, under some conditions, perturb the methionine of the Cu\textsubscript{A} center resulting in loss of 830 nm absorption intensity. In fact, during enzyme turnover, the Cu\textsubscript{A} reduction potential could be modulated through allosteric interactions between the heme a\textsubscript{3}-Cu\textsubscript{B} binuclear site and the Cu\textsubscript{A} methionine.

To summarize, the Cu\textsubscript{A} center undergoes a small structural modification and becomes EPR-detectable as a type II copper species upon SB-12 heat treatment. The thiol-specific reagents AgNO\textsubscript{3} and p-HMB, both of which cause the appearance of a type II copper species, are not expected to affect the CcO complex in the same manner. However, it is feasible that the type II species resulting from treatment with these reagents also is a perturbed heme a\textsubscript{3}-Cu\textsubscript{B} center and not a drastically modified Cu\textsubscript{A} site. It remains possible that SB-12 heat treat-
ment perturbs a small population of CuA sites since this procedure results in a decrease in the 830 nm absorption intensity of the enzyme, perhaps through allosteric interactions with the methionine of the CuA site. It has been demonstrated, however, that, under some conditions, CuB of the fully oxidized CcO complex is EPR-detectable in the form of a type II copper center.

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