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Q-band Electron Nuclear Double Resonance (ENDOR) and X-band EPR of the Sulfobetaine 12 Heat-treated Cytochrome *c* Oxidase Complex*

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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 Cu_A site. By using electron nuclear double resonance spectroscopy, it is found that the Cu_A 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 Cu_B histidines from the cytochrome aa_3-600 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 Cu_B of the as-isolated cytochrome bo_3 complex from *Escherichia coli*. These data indicate that the type II copper species that appears results from a breaking of the strong antiferromagnetic coupling of the heme a_3-Cu_B binuclear center.

The aa_3-type cytochrome *c* oxidase (CcO)[1] complexes contain four redox-active metal ions, two hemes A (heme *a* and heme a_3) and two copper centers (Cu_A and Cu_B). Electrons from ferrocytochrome *c* are input into the CcO complex at the Cu_A center; electron transfer to heme *a* or the heme a_3-Cu_B binuclear site quickly follows electron input. The highly exergonic reduction of dioxygen to water at the heme a_3-Cu_B binuclear center is coupled to the pumping of protons against an electrochemical gradient (1). While for a long time the Cu_A center was thought to consist of a single copper ion coordinated by two histidines and two cysteines, the recent literature (2–7) provides a convincing argument in favor of the original hypothesis of Kroneck *et al.* (8) that the Cu_A site actually is a mixed-valence, binuclear copper center. The recent crystal structures of the CcO complex have confirmed a binuclear configuration with two bridging thiolates (9, 10).

The structural lability of the Cu_A site has been inferred from mild heat treatment, $AgNO_3$ incubation, and *p*-(hydroxymercuri)benzoate (*p*-HMB) modification experiments which resulted in the appearance of type II copper EPR signals and diminution of the 830 nm absorption (thought to arise predominantly from the Cu_A site (11)) of the enzyme (12–16). A scheme which describes the perturbations to the Cu_A 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 Cu_A site. As type II signals certainly can arise from Cu_B under appropriate conditions, it should be noted that it has not been demonstrated definitively which copper ions are EPR-detectable as a result of these various modification procedures. The possibility exists that Cu_B becomes visible under one set of conditions, whereas the Cu_A 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 Cu_A site. A change in the nitrogen ENDOR of the Cu_A center is certainly expected as well.

Proton and nitrogen ENDOR (35 GHz) are reported here for the bovine CcO 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_3 complex, a structurally related ubiquinol oxidase complex. Special emphasis is placed on double integration of the first

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[1] The abbreviations used are: CcO, cytochrome *c* oxidase, specifically the mitochondrial aa_3-type enzyme; *p*-HMB, *p*-(hydroxymercuri)benzoate; ENDOR, electron nuclear double resonance; SB-12, sulfobetaine 12; DDM, *n*-dodecyl-β-D-maltoside; UQO, ubiquinol oxidase, specifically the cytochrome bo_3 complex from *E. coli*.

derivative 9.2-GHz EPR signals obtained. Integration of EPR signals is important for two reasons. 1) The model of a mixed-valence, binuclear Cu_A 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 a_3-Cu_B binuclear center as a result of the SB-12 heat treatment procedure would make Cu_B 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/g 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 g of 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 enzyme was dialyzed (50- kDa membrane) against 50–100 volumes of dialysis buffer I and then 50–100 volumes of 25 mM Tris, 0.1% DDM, pH 8.0. After centrifugation at 32,500 × *g*, 4 °C for 30 min, the enzyme (150–250 μM) was aliquoted, frozen in liquid nitrogen, and stored at −80 °C.

Growth of *E. coli* cells and isolation of "His-tagged" bo_3-type ubiquinol oxidase (UQO) complex was accomplished as described elsewhere (18).

Concentration and Activity Assays—Stock CcO concentrations were calculated from the reduced minus oxidized difference spectrum at 605 nm ($\Delta\epsilon_{605}^{red\text{-}ox}$ = 24 mM^{-1} cm^{-1}) using sodium dithionite as the reductant. 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 k_{cat} and K_m for a particular enzyme sample were obtained from Eadie-Hofstee plots.

Stock UQO 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, 100 mM sodium phosphate, 500 mM NaCl, pH 7.4, and incubated in a 40 °C water bath for 15 min. The solution was cooled on ice for 10 min and centrifuged at 32,500 × *g*, 4 °C for 30 min to remove large aggregates. After dialysis (50-kDa membrane) at 8 °C against 30–40 volumes of 5 mM SB-12, 10 mM Tris, pH 8.0, samples were applied to a short (2.5–3.5 × 2.5 cm) DE52 (Whatman) column equilibrated with 10 mM Tris, 0.1% DDM, pH 8.0. The column was then washed with 50 ml of 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 Centricon-100s (Amicon). 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.

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 Centricon-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.

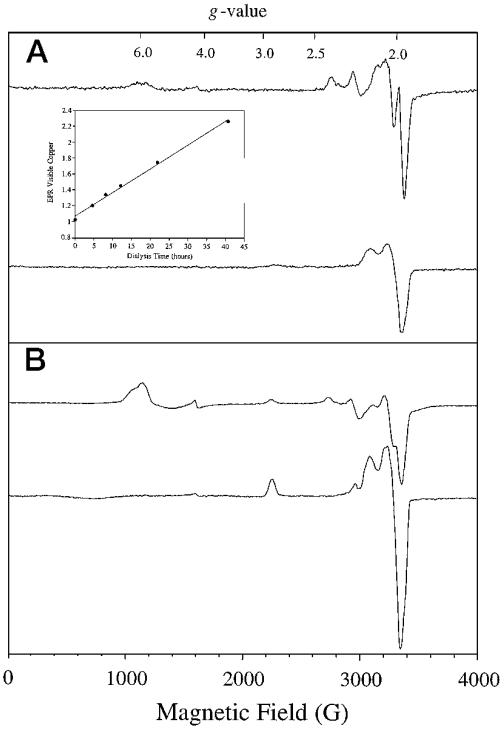

FIG. 1. *A*, the EPR spectra of the SB-12 heat-treated, 40-h SB-12-dialyzed CcO complex (*top*) and the native CcO complex (*bottom*) at 80 K (average of four scans). *B*, the EPR spectra of the same two samples at 10 K. Spectra are scaled to protein concentration based on the pyridine hemochrome method, and the buffer is 25 mM Tris, 0.1% DDM, pH 8.0. The *inset* shows the linear dependence of the integrated intensity (77 K) on dialysis time (see "Results" for details). Spectrometer settings: modulation amplitude, 10 G; time constant, 32 ms; power, 1 milliwatt; microwave frequency, 9.22 GHz; scan rate, 8.3 G/s.

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%/0.19% (acrylamide/bisacrylamide) stacking gel, a 14%/0.37% 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 2*I* transitions at frequencies given by:

$$\nu_{\pm}(m_I) = \left|A_N/2 \pm \nu_N + \frac{3}{2}P_N\,(2m_I - 1)\right| \qquad -I + 1 \le m_I \le I \qquad \text{(Eq. 1)}$$

where A_N and P_N are orientation-dependent hyperfine and quadrupole coupling constants and ν_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 $CuSO_4$ 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. 1*A*, 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. 1*B*) 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 standarized 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 against 10 mM Tris, 0.1% DDM, pH 8.0 at 8 °C. Dialysis of the native enzyme itself against 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 a_3-Cu_B binuclear site and a disrupted Cu_A 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

TABLE I
EPR-visible copper resulting from various treatments of the CcO complex

	Subunit III present?[a]	K_m	k_{cat}	EPR-visible copper (80 K)
		μM	s^{-1}	
Native	Yes	47	230	1.00
Subunit III-depleted[b]	No	56	165	1.11
40-h DDM-dialyzed[c]	Yes	52	182	1.25
40-h DDM-dialyzed,[c] subunit III-depleted[b]	No	49	152	1.49
40-h SB-12-dialyzed	No	49	128	1.81
SB-12 heat-treated, 40-h SB-12 dialyzed	No	60	47	2.25

[a] An entry of "no" implies that greater than 90% of subunit III is missing as determined by SDS-polyacrylamide gel electrophoresis.

[b] The phrase "subunit III-depleted" refers to the high detergent, high salt treatment described under "Experimental Procedures."

[c] The phrase "DDM-dialyzed" denotes dialysis against 10 mM Tris, 0.1% DDM, pH 8.0, at 8 °C followed by the short DE52 column treatment described for the SB-12 heat-treated enzyme.

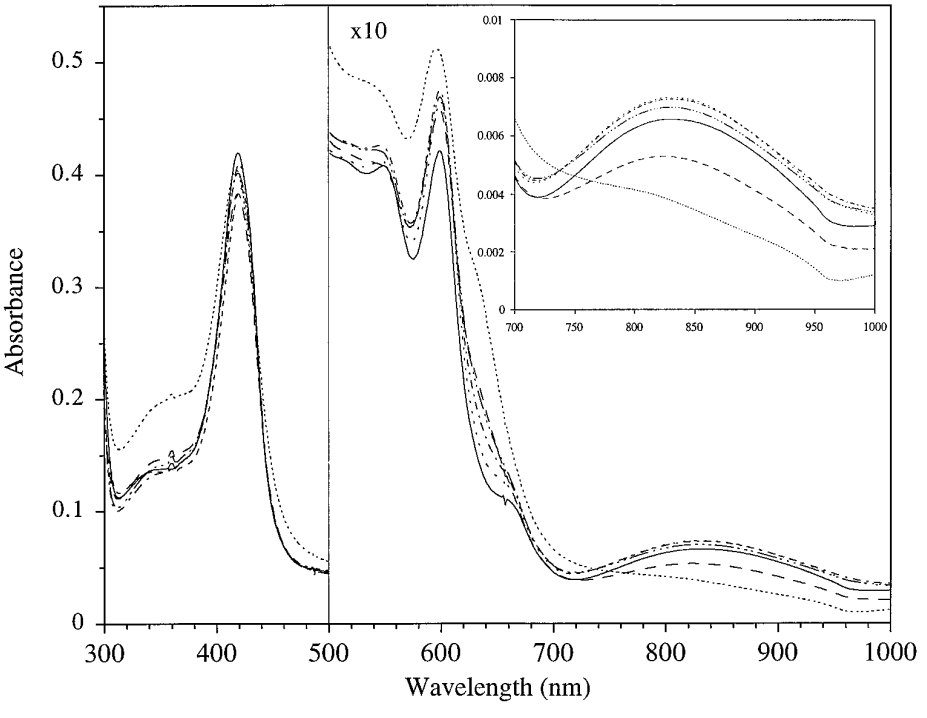

FIG. 2. **UV-visible absorption spectra of the six samples listed in Table I.** Native, ——; subunit III-depleted, – · – · –; 40-h DDM-dialyzed, - - - - -; subunit III-depleted, 40-h DDM-dialyzed, —···—; 40-h SB-12-dialyzed, – – –; SB-12 heat-treated, 40-h SB-12-dialyzed, ·····. The protein concentration in each case is 2.5 μM in 25 mM Tris, 0.1% DDM, pH 8.0.

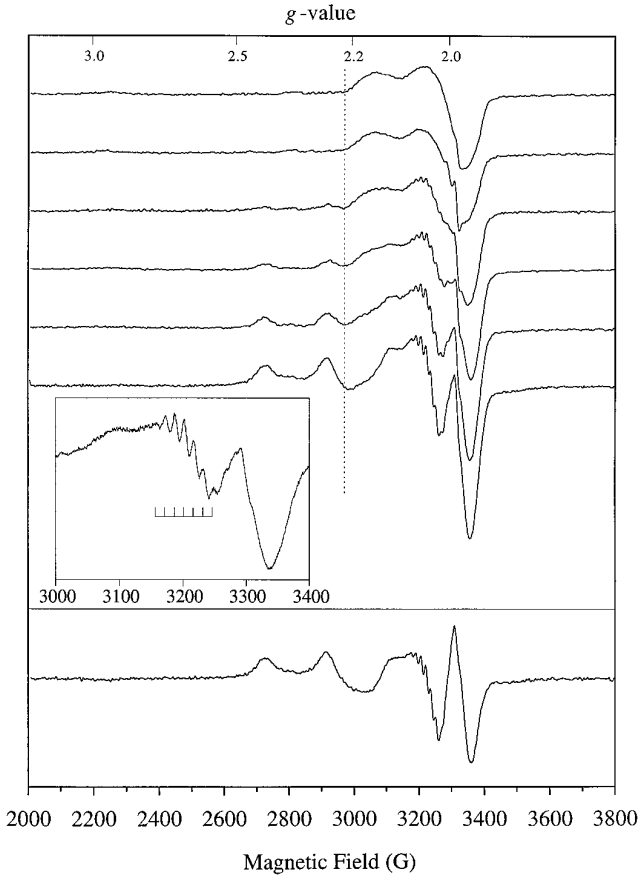

FIG. 3. **The EPR spectra at 80 K of the six samples listed in Table I.** From *top* to *bottom*: native; subunit III-depleted; 40-h DDM-dialyzed; subunit III-depleted, 40-h DDM-dialyzed; 40-h SB-12-dialyzed; SB-12 heat-treated, 40-h SB-12 dialyzed. Spectra are scaled to protein concentration based on the pyridine hemochrome method. The *inset* shows the seven-line hyperfine pattern for the SB-12 heat-treated, 40-h SB-12 dialyzed enzyme. The *bottom panel* shows the difference spectrum between the SB-12 heat-treated, 40-h SB-12 dialyzed and the native samples. Note that at lower fields ($<g \sim 2.2$), only the type II copper species can contribute to the ENDOR spectrum; at higher fields, both the native Cu_A 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).

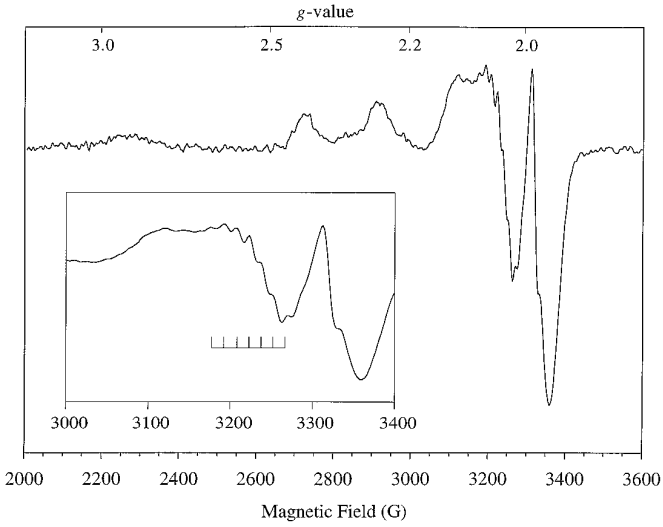

FIG. 4. **The EPR spectrum at 80 K of the as-isolated cytochrome bo_3 complex.** Spectrometer settings are the same as in Fig. 1 except for a time constant of 64 ms (average of 16 scans). Compare with Fig. 3.

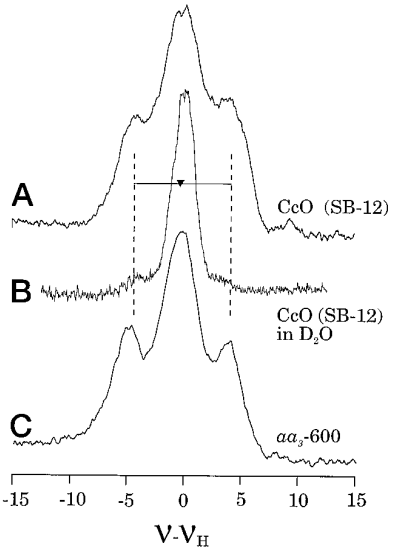

FIG. 5. **Comparison of ^1H ENDOR spectra of the SB-12 heat-treated, 40-h SB-12 dialyzed CcO complex (35.04 GHz, $g_\parallel \approx 2.20$) and the cytochrome aa_3-600 ubiquinol oxidase complex (35 GHz, $g_\parallel \approx 2.23$).** The spectra from both enzymes show a hyperfine-split doublet centered at the proton Larmor frequency that vanishes upon exchange into D_2O buffer (shown here for the modified CcO complex, demonstrated previously for the cytochrome 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 aa_3-600 ubiquinol oxidase spectrum is reproduced from Fann *et al.* (23).

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 $g_\parallel$ of about 2.19, an $A_\parallel$ of about 190 G, and a seven-line hyperfine pattern at $g_\perp = 2.06$ 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 $g \sim 2.2$, EPR signals of the Cu_A site and the type II copper species overlap (see Fig. 3). At lower fields, only the type II copper species can contribute to the ENDOR spectrum.

Fig. 4 shows the EPR spectrum of the as-isolated UQO complex. The type II copper signal in this spectrum is characterized by a $g_\parallel$ of 2.19, an $A_\parallel$ of about 190 G, and a seven-line hyperfine pattern at $g_\perp = 2.04$ 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_3-Cu_B 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]

^1H ENDOR—The 35-GHz continuous-wave ^1H ENDOR spectrum of the SB-12 heat-treated, 40.5-h SB-12-dialyzed CcO complex obtained in the $g_\parallel$ region of the type II copper species at fields low enough that the Cu_A site cannot contribute shows a doublet centered at the proton Larmor frequency ($\nu_H \approx 46.3$ MHz) and split by a hyperfine coupling constant $A_\parallel \approx 10$ MHz (Fig. 5*A*) as interpreted with the relation: $\nu_\pm (^1H) = |\nu(^1H) \pm A(^1H)/2|$. This proton, which is not seen for the native enzyme, is not associated with the hemes because the hyperfine-split doublet vanishes when the spectrum is taken outside the type

[2] P. M. Li and S. I. Chan, unpublished observations.

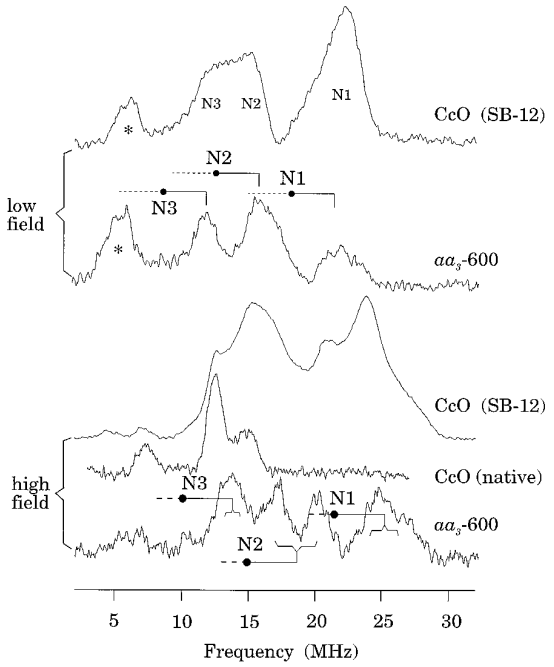

FIG. 6. **Comparison of ^{14}N ENDOR spectra of the SB-12 heat-treated, 40-h SB-12 dialyzed CcO complex (35.04 GHz, $g_\parallel \approx 2.22$ ("low field"), $g_\perp = 2.04$ ("high field")), the native CcO complex (35.1 GHz, $g_\perp = 2.04$), and the cytochrome aa_3-600 ubiquinol oxidase complex (35.2 GHz, $g_\parallel \approx 2.23$ ("low field"), $g_\perp = 2.05$ ("high field")).** Experimental conditions are the same as for Fig. 5 except: modulation amplitude, 2.5 G (CcO)/1.25 G (aa_3-600); scan rate, 2 MHz/s. The cytochrome aa_3-600 spectra are reproduced from Fann *et al.* (23).

II copper EPR envelope. In addition, the proton ENDOR signals associated with the Cu_A center are still present and remain unperturbed at all fields that fall within the Cu_A 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 Cu_B site of the cytochrome aa_3-600 ubiquinol oxidase complex isolated from *Bacillus subtilis* (Fig. 5*C*). As is the case with the *Bacillus* enzyme, this strongly coupled proton ($A_\parallel \approx 10$ MHz) is solvent-exchangeable (Fig. 5*B*) and is consistent with a bound hydroxide anion.

^{14}N ENDOR—Fig. 6, *top*, shows the ENDOR spectrum of the SB-12 heat-treated, 40.5-h SB-12-dialyzed CcO complex obtained in the "$g_\parallel$" region of the type II copper species where the Cu_A center does not resonate. Also shown in Fig. 6, *bottom*, is the ENDOR spectrum obtained near $g \sim 2.04$, which is near $g_\perp$ for both the type II copper species and the Cu_A site; thus, each copper center can contribute to the ENDOR spectrum at this higher field. The observed resonance frequencies are those expected for ^{14}N ligands coordinated to copper where each ligand yields an ENDOR pattern as described by Equation 1. In the $g_\parallel$ spectrum (Fig. 6, *top*), only resonances arising from the type II copper species are expected; ENDOR peaks with frequencies greater than 10 MHz can be assigned to ν_+ branches of three ^{14}N ligands, denoted as N1, N2, and N3, with $A_\parallel$ hyperfine couplings of 38, 24, and 17 MHz, respectively. No quadrupole splittings were resolved. The resonance at 7 MHz (marked with an *asterisk*) is associated with heme nitrogens as the signal persists outside the type II copper EPR envelope. The ^{14}N ENDOR peaks from the three distinct type II copper nitrogens can be unambiguously tracked in the field-dependent ENDOR spectra taken as the field increases from $g_\parallel$ into the Cu_A region ($2.2 < g < 1.94$), where the ^{14}N ENDOR pattern becomes more complicated due to the overlap of the ^{14}N signals

TABLE II

^{14}N hyperfine coupling constants (in MHz) for the type II copper center in the SB-12 heat-treated CcO complex and for Cu_B of the B. subtilis cytochrome aa_3-600 ubiquinol oxidase complex

Uncertainty in A: ±3 MHz. Values for the cytochrome aa_3-600 ubiquinol oxidase complex were originally reported by Fann *et al.* (23).

		N1	N2	N3
SB-12 heat-treated CcO complex	$A_\parallel$	38	24	17
Cytochrome aa_3-600 complex	$A_\parallel$	37	25	17
	$A_\perp$	42	31	20

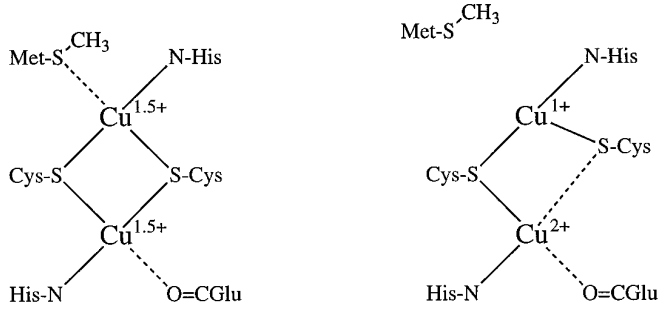

FIG. 7. *Left*, schematic of the binuclear nature of the Cu_A site as revealed by x-ray crystallography (9, 10). The fourth ligand for the lower copper is the main chain carbonyl of a glutamic acid residue. *Right*, possible perturbation to the Cu_A site that results in the appearance of a type I EPR signal (lower copper atom) and loss in 830 nm absorption intensity. See "Discussion" for details. *Solid lines* denote strong bonds; *dashed lines* denote weak ligation.

from the native Cu_A center and the type II copper species (Fig. 6, *bottom*). Although the $A_\perp$ values for N1, N2, and N3 are not precisely measurable, they fall within the range of those from ^{14}N ligands to Cu_B of the cytochrome aa_3-600 ubiquinol oxidase complex (23). The ^{14}N ENDOR spectra from this ubiquinol oxidase complex and those reported here for the SB-12 heat-treated CcO complex are compared in Fig. 6; hyperfine coupling constants are summarized in Table II.

DISCUSSION

The appearance of a type II copper species as a consequence of SB-12 heat treatment of the CcO complex has been reported previously by Nilsson *et al*. (12). In addition, *p*-HMB treatment or incubation with $AgNO_3$ has been found to cause the appearance of this 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 Cu_A site of the CcO complex. In the scheme of Li *et al.* (14), Cu_A is assumed to be mononuclear with two nitrogen 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 $AgNO_3$ 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 Kroneck and co-workers (2–10), biophysical data on overexpressed subunit II fragments, and the recent crystal structures of the CcO complex indicate, however, that the Cu_A center is actually a mixed-valence, binuclear copper center with two bridging thiolates (Fig. 7). The Cu_A modification scenario of Li *et al.* (14) obviously conflicts with these more recent data.

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 Cu_A center and the data presented here. There are two possibilities. The first is that the SB 12 heat treatment procedure causes disruption of the Cu_A 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_A site remains in a native configuration and the magnetic coupling of the heme a_3-Cu_B binuclear center is broken making Cu_B EPR-visible. In this scenario, the extra EPR-visible copper, all of which appears to be in a type II configuration, is Cu_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_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, ^1H and ^{14}N ENDOR spectroscopy reveals that the Cu_A resonances are present when a stoichiometric amount of the type II copper species is EPR-detectable. Instead, three additional ^{14}N ENDOR resonances are detected (Fig. 6). The seven-line hyperfine pattern in the $g_\perp$ 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_B center of the cytochrome aa_3-600 ubiquinol oxidase complex of *B. subtilis* (Table II), which does not contain a Cu_A center, allows confident assignment of the three new nitrogen ENDOR resonances as Cu_B 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_A center but is otherwise structurally similar to the CcO complex (1), and, in this particular sample, the heme o_3-Cu_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_B. The changes in the resonance Raman spectrum of heme a_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_3-Cu_B binuclear center and make Cu_B EPR-detectable also disrupt the heme a_3 pocket.

Given that the type II EPR signal seen arises predominantly from Cu_B, it is important to question to what extent the structural integrity of the Cu_B redox site is preserved. Cline and co-workers (25) report an EPR spectrum of Cu_B obtained by full reduction of the enzyme, flushing with O_2, and quick freezing in liquid N_2. This spectrum is characterized by a $g_\parallel$ of about 2.28 and an $A_\parallel$ of about 100 G. Photolysis 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 $g_\parallel$ of 2.26–2.28 and an $A_\parallel$ of 102–137 G (26, 27). High pH causes the appearance of a type II species with a $g_\parallel$ of 2.30 and and $A_\parallel$ of 136 G (28). Addition of cyanide to the *Thermus thermophilus* cytochrome ba_3 complex produces a type II copper species with a $g_\parallel$ of 2.28 and an $A_\parallel$ of about 140 G (29, 30). These spectra contrast with the type II signal obtained by SB-12 heat treatment which has a larger $A_\parallel$ (~190 G) and a smaller $g_\parallel$ (~2.19). As harsher treatments tend to result in larger $A_\parallel$ values, the indication is that the structural integrity of the Cu_B site has been perturbed in the experiments described here. A small perturbation to the heme a_3-Cu_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 aa_3-600 complexes contain a proton with an $A_\parallel$ of about 10 MHz (Fig. 5). In the case of the cytochrome aa_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_B EPR-detectable raises the issue of which copper ions give rise to the type II signals seen upon $AgNO_3$ 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_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_B due to perturbations similar to those caused by SB-12 heat treatment. On the other hand, disruption of the Cu_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_A resonances in the presence of stoichiometric amounts of type II copper, the possibility that a substoichiometric population of Cu_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_A ligation structure would be undetectable in the EPR and ENDOR spectra due to the strong signals arising from the Cu_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_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 Cu_A center to isoleucine results in a type I copper EPR spectrum ($g_\parallel$ = 2.18, $A_\parallel$ = 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_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_3-Cu_B binuclear site that produces a type II signal could potentially, under some conditions, perturb the methionine of the Cu_A center resulting in loss of 830 nm absorption intensity. In fact, during enzyme turnover, the Cu_A reduction potential could be modulated through allosteric interactions between the heme a_3-Cu_B binuclear site and the Cu_A methionine.

To summarize, the Cu_B 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 $AgNO_3$ 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_3-Cu_B center and not a drastically modified Cu_A site. It remains possible that SB-12 heat treat-

ment perturbs a small population of Cu_A 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 Cu_A site. It has been demonstrated, however, that, under some conditions, Cu_B of the fully oxidized CcO complex is EPR-detectable in the form of a type II copper center.

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