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Conversion of Cu_A to a Type II Copper in Cytochrome *c* Oxidase†

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ABSTRACT: When cytochrome *c* oxidase is incubated at 43 °C for ~75 min in a solution containing the zwitterionic detergent sulfobetaine 12, the Cu_A site is converted into a type II copper as judged by changes in the 830-nm absorption band and the EPR spectrum of the enzyme. SDS-PAGE and sucrose gradient ultracentrifugation indicate concomitant loss of subunit III and monomerization of the enzyme during the heat treatment. Comparison of the optical and resonance Raman spectra of the heat-treated and native protein shows that the heme chromophores are not significantly perturbed; the resonance Raman data indicate that the small heme perturbations observed are limited to the cytochrome a_3 site. Proton pumping measurements, conducted on the modified enzyme reconstituted into phospholipid vesicles, indicate that these vesicles are unusually permeable toward protons during turnover, as previously reported for the *p*-(hydroxymercuri)benzoate-modified oxidase and the modified enzyme obtained by heat treatment in lauryl maltoside. The sulfobetaine 12 modified enzyme is no longer capable of undergoing the recently reported conformational transition in which the tryptophan fluorescence changes upon reduction of the low-potential metal centers. Control studies on the monomeric and subunit III dissociated enzymes suggest that the disruption of this conformational change in the heat-treated oxidase is most likely associated with perturbation of the Cu_A site. These results lend support to the suggestion that the fluorescence-monitored conformational change of the native enzyme is initiated by reduction of the Cu_A site [Copeland et al. (1987) *Biochemistry 26*, 7311].

As a redox-linked proton pump, cytochrome *c* oxidase is expected to be an allosteric enzyme. Evidence for allosteric behavior has recently been put forth for the mitochondrial protein (Scholes & Malmström, 1986; Copeland et al., 1987, and references cited therein). Scholes and Malmström (1986) reported that electron input into the low-potential centers, namely, cytochrome *a* and Cu_A, greatly accelerates the rate of inhibitory cyanide binding at the dioxygen binding site, i.e., cytochrome a_3/Cu_B. These workers suggested that reduction of the two low-potential metal centers induces a conformational transition of the protein, opening up the dioxygen binding pocket to the ligation of exogenous ligands. This phenomenon accordingly has been termed the "open-closed transition". More direct evidence for such a protein allosteric effect has recently been provided by intrinsic tryptophan fluorescence spectroscopy (Copeland et al., 1987). These workers noted that a small number of otherwise buried tryptophan residues in the beef heart enzyme become exposed to the solvent upon reduction of Cu_A and that the fluorescence yield of the exposed tryptophans appears to be correlated with the inhibitory cyanide-binding kinetics reported by Scholes and Malmström (1986). On the basis of a detailed analysis of both the fluorescence and kinetic data, it was argued that the open-closed transition and the fluorescence-monitored transition are more likely linked to the reduction of Cu_A alone rather than

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to the reduction of both low-potential centers (Copeland et al., 1987). The kinetic and fluorescence changes are quite possibly different manifestations of the same conformational change.

Over the years, there has been increasing experimental evidence in support of a large-scale conformational change in cytochrome *c* oxidase upon reduction of the low-potential redox centers (Copeland et al., 1987, and references cited therein). Gross conformational changes are uncommon among electron-transfer proteins, presumably because such transitions would contribute to the reorganizational energy barrier to electron transfer (Marcus & Sutin, 1985). Aside from transferring electrons to dioxygen, cytochrome *c* oxidase also functions as a redox-linked transmembrane proton pump [for reviews see Blair et al. (1986), Gelles et al. (1987), and Wikström et al. (1981) and references cited therein]. This proton pumping activity has been shown to be coupled to oxidoreduction at one or both of the low-potential metal sites (Wikström & Casey, 1985). Since protein conformational transitions are obligatory in the gating of electrons and protons during the proton pumping cycle, it is tempting to implicate the proposed allosteric interaction between the low-potential redox centers and the dioxygen reduction site in the mechanism of proton pumping. Direct experimental evidence for such a correlation is, however, lacking.

In view of the potential significance of this hypothesized allosteric interaction in the mechanism of proton pumping in cytochrome *c* oxidase, we have elected to pursue the matter further. In particular, we wish to verify the role of Cu_A reduction in the allosteric transition, as several models of redox-linked proton translocation based on the Cu_A site have recently emerged (Gelles et al., 1987; Mitchell, 1987). One approach to this problem would be to study a derivative of the enzyme in which the Cu_A site has been modified without concomitant perturbation of the other redox centers. Our laboratory is in a unique position to follow up on this strategy, as we have recently been successful in preparing several enzyme derivatives in which the Cu_A site is selectively modified.

Several methods have been developed over the years for modification of the copper sites of cytochrome *c* oxidase. Wharton and co-workers have shown that the copper ions of cytochrome *c* oxidase can be removed from the enzyme by treatment with large excesses of cyanide (Weintraub & Wharton, 1981). This method removes both Cu_A and Cu_B, and it appears to significantly affect the heme prosthetic groups as well. Gelles and Chan (1985) recently reported that the Cu_A site can be selectively converted into a type II copper upon treatment of the enzyme with *p*-(hydroxymercuri)benzoate (pHMB)[1] without gross perturbation of the heme centers or disruption of the dioxygen reduction site. Li et al. (1988) have shown that simple heat treatment of the enzyme according to the procedure of Sone and Nichols (1984) also selectively disrupts Cu_A. The resultant enzyme was heterogeneous, consisting of a mixture of three Cu_A species including (i) unperturbed Cu_A (approximately 25%), (ii) a type I (blue) Cu_A (approximately 50%), and (iii) a type II Cu_A analogous to that obtained by treatment with pHMB (approximately 25%).

In this paper we describe yet another method for disrupting the Cu_A site in cytochrome *c* oxidase. This new method converts Cu_A exclusively into a type II copper center. It is based on heat treatment of the enzyme in a zwitterionic detergent, sulfobetaine 12, rather than the nonionic detergents previously used for heat treating the enzyme (Sone & Nichols, 1984; Li et al., 1988). We shall demonstrate that in this new type II Cu_A oxidase the Cu_A center is locked into a conformation such that the tryptophan fluorescence spectrum no longer reponds to reduction of the metal centers. Although small perturbations were detected at the oxygen binding site, the other low-potential site, cytochrome *a*, was not significantly perturbed by the present treatment.

Materials and Methods

Materials

Cytochrome *c* oxidase was isolated from bovine hearts according to the method of Hartzell and Beinert (1974) as modified by Li et al. (1988). Ferrocytochrome *c* was prepared by reduction of ferricytochrome *c* (Sigma, Type VI) with sodium dithionite; residual dithionite was removed by column chromatography on Sephadex G-25. The detergents sulfobetaine 12 (Zwittergent 3-12) and dodecyl β-D-maltoside (lauryl maltoside) were purchased from CalBiochem. Triton X-100 was from Sigma. All other reagents were of the highest grades available.

Methods

Enzyme Treatments. Heat treatment of the enzyme in sulfobetaine 12 solution (SB-12) was performed by diluting a ca. 300 μM stock solution of cytochrome *c* oxidase to approximately 50 μM (per aa_3 unit) with a solution containing 15 mM SB-12, 0.05 M Hepes buffer (pH 7.2), and 0.5 M KCl and incubating for 75 min at 43 °C (Sone & Nichols, 1984; Li et al., 1988). The resulting solution was turbid and was therefore centrifuged for 60 min at 27000*g* to remove aggregated protein.

Heat treatment of the enzyme in lauryl maltoside solution was performed according to the procedure of Li et al. (1988).

Subunit III dissociated oxidase was prepared by diluting the stock oxidase to a concentration of 0.8–1.8 μM with a solution of 20 mM Tris (pH 7.8), 0.1 M NaCl, 1 mM EDTA, and 5 mg/mL lauryl maltoside and incubating at room temperature for 24 h (Hill & Robinson, 1986). No chromatography was done to separate subunit III from the sample, as some columns are known to disrupt the Cu_A site (unpublished results; Li et al., 1988). The resulting enzyme exhibited the rapid cyanide binding of the subunit III depleted protein (Hill & Robinson, 1986). The subunit III dissociated enzyme was then concentrated to ca. 5 μM by centrifugation in a Centricon protein concentrating tube with a 30-kDa cutoff membrane (Amicon) or to higher concentrations in an Amicon ultrafiltration concentrator with an XM-300 membrane.

Monomeric cytochrome *c* oxidase was prepared according to the procedure of Robinson and Talbert (1986) by dissolution of the stock enzyme into a solution of 20 mM Tris (pH 7.8), 0.1 M NaCl, 1 mM EDTA, and 10 mg/mL Triton X-100 to a concentration of ca. 5 μM.

Spectroscopic Methods. Optical spectra in the 220–900-nm region were obtained with a Beckman DU-7 UV–visible spectrophotometer interfaced to an IBM XT computer. Samples were contained in 1-cm fused silica cells, and the spectra were recorded at 25 °C.

Resonance Raman spectra were obtained by 441.6-nm excitation with a helium–cadmium laser (Liconix). Samples were flowed through a capillary tube at a rate of ca. 0.5 mL/s by a peristaltic pump (Masterflex) (Copeland et al., 1985). Throughout the experiments the samples were maintained anaerobic by flowing N_2 gas over the sample reservoir. The

[1] Abbreviations: EPR, electron paramagnetic resonance; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; pHMB, *p*-(hydroxymercuri)benzoate; RR, resonance Raman; SB-12, sulfobetaine 12; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

excitation laser beam was focused onto the capillary, and scattered light was collect at 90° to the incident beam. The scattered light was focused onto the entrance slit of a Spex 1401 double monochromator, which was driven by a Spex Scamp computer, and signal was detected with a Hamamatsu R955 photomultiplier tube. All spectra were obtained at a scan rate of 1 cm^{-1}/s and a spectral resolution of 5 cm^{-1}. The reported spectra are each the sum of three to five consecutive scans. Optical spectra were recorded for all samples before and after acquisition of the Raman data to ensure sample integrity.

Fluorescence spectra (290–420 nm) were obtained by 280-nm excitation as described previously (Copeland et al., 1987). Samples were contained in fused silica fluorescence cells equipped with screw-on airtight septum caps. For the reduced enzyme spectra the samples were purged with either N_2 or Ar for at least 15 min before the addition of a slight excess of aqueous sodium dithionite or ferrocytochrome *c*. The spectra shown in Figure 8 are each the sum of three consecutive scans and are not corrected for inner filter effects. Such a correction was applied to the data (Copeland et al., 1987) but was found to affect only the absolute fluorescence intensity and not the bandshape or emission maxima of the spectra.

Electron paramagnetic resonance (EPR) spectra were obtained at 77 K on a Varian E-line Century Series X-band spectrometer. The samples were degassed and equilibrated with 1 atm of argon before freezing in liquid nitrogen.

SDS–PAGE and Sucrose Gradient Ultracentrifugation. SDS–PAGE was performed according to the procedure of Falke et al. (1985) except that 4% acrylamide/0.05% bis(acrylamide) stacking and 15% acrylamide/0.2% bis(acrylamide) separatory gels were used. One volume of 20 μM enzyme was added to 1 volume of sample buffer (20% glycerol, 2% mercaptoethanol, 4% SDS, 0.13 M Tris, and 10 mg/100 mL of bromophenol blue). The sample was mixed well by vortexing and incubated at room temperature for 15 min. Twenty microliters of the mixture was applied to the top of the stacking gel. Gels were stained with Coomassie Blue as described by Falke et al. (1985). Molecular weights were determined from a plot of migration (distance from the stacking gel edge) vs log M_r for a Pharmacia low molecular weight calibration kit containing the following protein standards: rabbit muscle phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), egg white ovalbumin (43 kDa), bovine erythrocyte carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and bovine milk α-lactalbumin (14.4 kDa). Gels were scanned optically at 560 nm with a Beckman Acta CIII spectrophotometer equipped with a Beckman Gel Scanner 2.

The degree of monomerization of the enzyme was determined by sucrose density gradient ultracentrifugation as described by Finel and Wikström (1986).

Enzyme Assays. Enyme activity was monitored by O_2 consumption using an oxygen electrode (Nilsson et al., 1988) and by spectrophotometrically following ferrocytochrome *c* oxidation (Smith, 1955). Proton pumping assays were performed on enzyme samples reconstituted into phospholipid vesicles as described by Nilsson et al. (1988).

RESULTS

Characterization of the SB-12 Oxidase. Incubating cytochrome *c* oxidase at 43 °C for 75 min in sulfobetaine 12 detergent results in precipitation of about 50% of the enzyme originally in solution (as judged optically at the Soret maximum). Centrifugation of the oxidase yielded a sample with an optical spectrum similar to that seen in previous heat

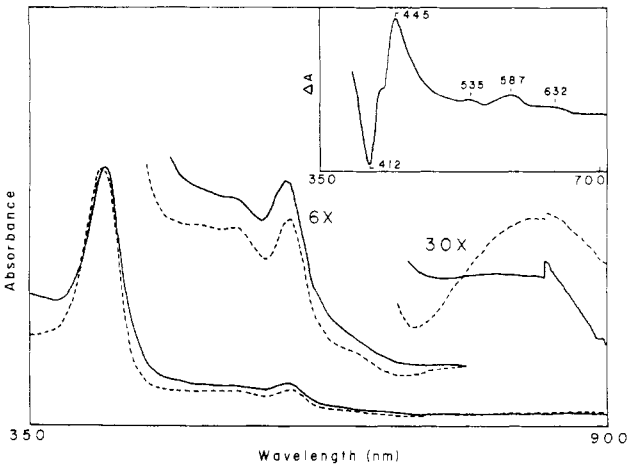

FIGURE 1: Optical spectra of native (---) and SB-12 heat-treated (—) cytochrome *c* oxidase. Inset: Difference spectrum of the native minus SB-12 heat-treated enyzme.

treatments (Sone & Nichols, 1984; Li et al., 1988) and chemical modifications (Gelles & Chan, 1985) of the enzyme; we observe a 2–4-nm red shift of the Soret maximum, an intensification of the α-band region, and a diminution of the Cu_A absorption at ca. 830 nm (Figure 1). The observed Soret red shift is similar to that seen for subunit III depleted oxidase and may be the result of heat-induced dissociation of subunit III from the SB-12 oxidase (vide infra). From the difference spectra between the resting and the dithionite-reduced SB-12 enzyme we estimate that between 15 and 35% of the original 830-nm absorptivity remains after heat treatment, depending on the protein sample. Thus, a major fraction of the enzyme has been converted into a form where Cu_A has been disrupted.

This conclusion is corroborated by low-temperature EPR spectroscopy as shown in Figure 2; the SB-12 oxidase exhibits a copper EPR spectrum that is dominated by features characteristic of a type II Cu^{2+} species, with some residual contribution from unperturbed Cu_A. This EPR spectrum is strikingly similar to that obtained after chemical modification of the Cu_A center with pHMB (Gelles & Chan, 1985). In contrast, cytochrome *c* oxidase that has been heat treated in the presence of other detergents (e.g., lauryl maltoside) exhibits an EPR spectrum with only a 25% contribution from a type II Cu_A species, the majority of the signal being due to a blue copper species (Li et al., 1988). As with pHMB modification (Gelles & Chan, 1985), the type II Cu_A in the SB-12 oxidase is not reducible by ferrocytochrome *c*, but is reduced by sodium dithionite, as determined by the diminution of the Cu_A EPR signal upon addition of these reductants.

The heme chromophores do not appear to be significantly perturbed in the SB-12 oxidase as judged by optical and resonance Raman spectroscopy. The resonance Raman (RR) spectrum of cytochrome *c* oxidase is known to contain distinct spectroscopic signatures for each of the two hemes of the enzyme (Choi et al., 1983, and references cited therein). The RR spectrum of the reduced SB-12 enzyme is very similar to that of reduced native cytochrome *c* oxidase (Figure 3) except for intensity decreases of two bands associated with the cytochrome a_3 chromophore; the iron–histidine stretch at ca. 215 cm^{-1} and the cytochrome a_3 formyl stretch at ca. 1665 cm^{-1}. Each of these bands decreases in intensity by ~43%. In contrast, none of the vibrations assigned to the cytochrome *a* chromophore are perturbed by heat treatment of the enzyme. A similar intensity diminution for the iron–histidine stretch, but not for the formyl band, was reported for the reduced enzyme heat-treated in Tween 20 detergent (Sone et al., 1986);

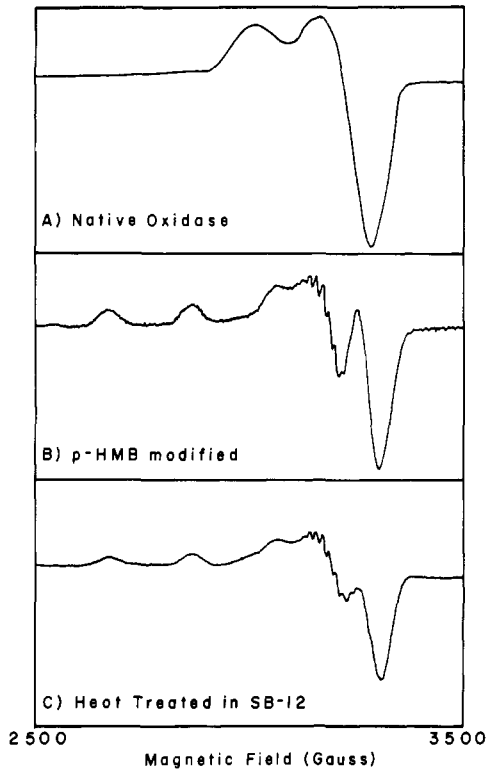

FIGURE 2: EPR spectra of Cu_A for (A) native, (B) pHMB-modified, and (C) SB-12 heat-treated cytochrome *c* oxidase. The pHMB-modified enzyme was prepared by the method of Gelles and Chan (1985). Sample temperature, 77 K; microwave frequency, 9.16 GHz; microwave power (A) 10 mW, (B) 0.2 mW, (C) 5 mW; modulation amplitude, 10 G.

we likewise find that heat treatment in lauryl maltoside affects the iron–histidine stretch but not the cytochrome a_3 formyl vibration (data not shown). Interestingly, Ondrias and co-workers find similar changes in the RR spectrum of pHMB-modified cytochrome *c* oxidase; these workers additionally see some small changes in cytochrome *a* vibrations (Ondrias et al., personal communication). RR spectra of the reduced subunit III dissociated enzyme (Hill & Robinson, 1986) and reduced monomeric oxidase (Robinson & Talbert, 1986) show no changes relative to the native reduced enzyme. A detailed discussion of the RR features of the heat-treated cytochrome *c* oxidases will be deferred to a later publication.

Recently Freire and co-workers (Rigell et al., 1985; Rigell & Freire, 1987) have studied the thermal denaturation of cytochrome *c* oxidase by differential scanning calorimetry and SDS–PAGE and shown that a transition occurs at ca. 47 °C, which corresponds to dissociation of subunit III from the enzyme. Likewise, Li et al. (1988) found that heat treatment of the enzyme in lauryl maltoside solution led to dissociation of subunit III. Since it has been proposed that this subunit plays a role in cytochrome *c* oxidase proton pumping, it was important to determine whether the SB-12 enzyme is missing subunit III. When native and SB-12 oxidase were subjected to column chromatography on Sephadex G-100 followed by SDS–PAGE, the results (Figure 4) clearly indicated loss of subunit III in the case of the SB-12 enzyme. The relative absorbance (at 560 nm) for subunit III/subunit II between native and SB-12 oxidase indicates that at least 60% of subunit III is lost in the SB-12 enzyme.

In order to characterize the changes in the cytochrome a_3 site of the SB-12 enzyme more fully, we have examined the effects of heat treatment on the binding of exogenous ligands to this site. As depicted in the inset of Figure 5, the binding of CN^- to the SB-12 enzyme is much slower than that for the native protein. In contrast, the lauryl maltoside heat-treated (Li et al., 1988) and subunit III dissociated (Hill & Robinson, 1986) enzymes show significantly accelerated CN^- binding relative to the native enzyme. Nevertheless, the cyanide binding is complete within 24 h for both the SB-12 and native enzymes as judged by a shift of the Soret maxima to 427 nm. When the cyanide-bound mixed-valence species are formed upon reduction with dithionite, slightly different Soret spectra are observed for the native and SB-12 oxidase. In the native enzyme this mixed-valence cyanide species shows a split Soret

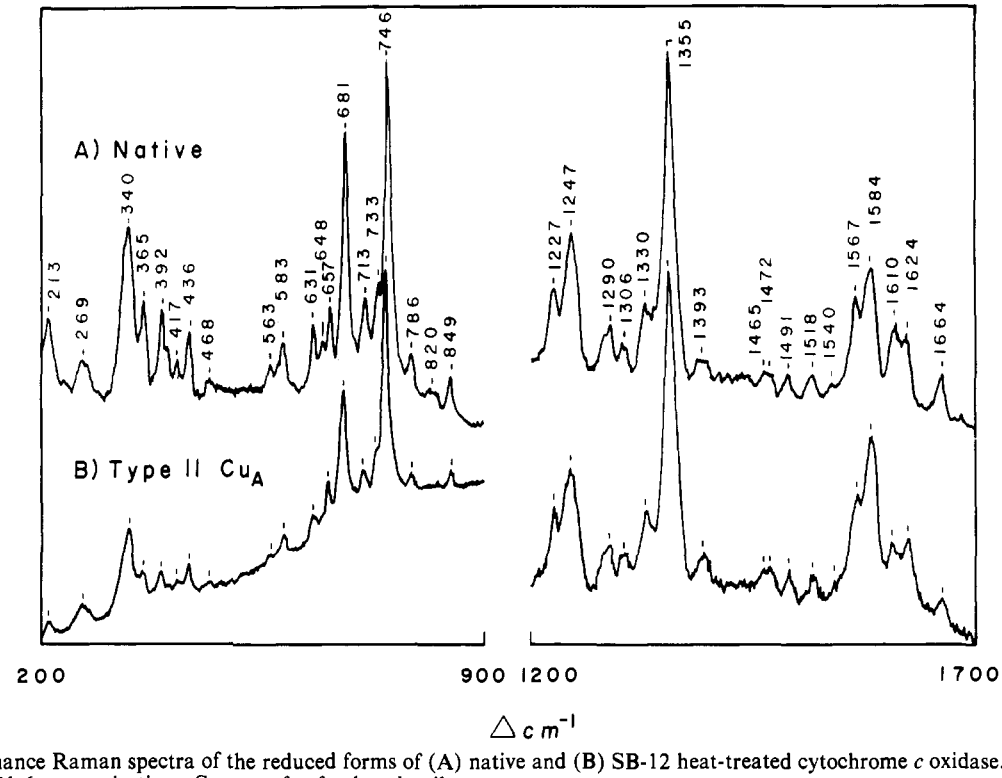

FIGURE 3: Resonance Raman spectra of the reduced forms of (A) native and (B) SB-12 heat-treated cytochrome *c* oxidase. The spectra were obtained with 441.6-nm excitation. See text for further details.

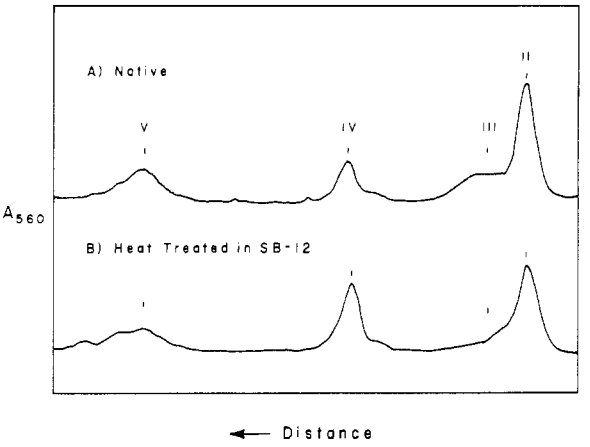

FIGURE 4: SDS–PAGE gel scans for (A) native and (B) SB-12 heat-treated cytochrome *c* oxidase in the region of subunits II–V.

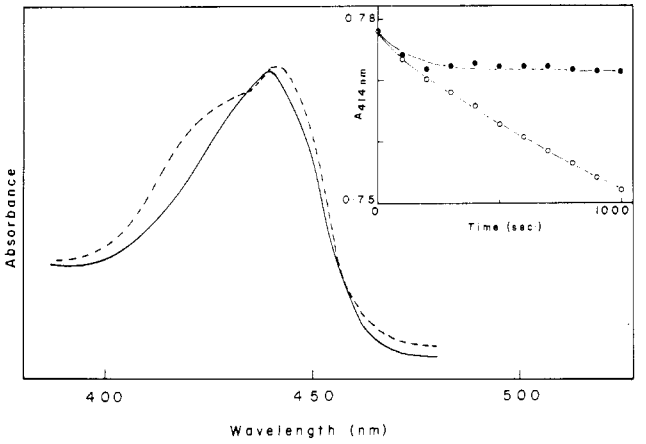

FIGURE 5: Optical spectra, in the Soret region, for the mixed-valence CN^--bound forms of native (- - -) and SB-12 heat-treated (—) cytochrome *c* oxidase. Inset: Time course of CN^- binding to the resting forms of native (○) and SB-12 heat-treated (●) cytochrome *c* oxidase.

band with maxima at 427 and 442 nm (Figure 5). In the SB-12-mixed-valence cyanide adduct, however, the Soret bands of the two hemes are not resolved, showing single maximum at ca. 440 nm. That the cytochrome a_3 site remains oxidized in this state of the SB-12 enzyme is confirmed by the oxidation state marker band (ν_4) in the RR spectra (Figure 6). For both the native and heat-treated enzyme one observes two oxidation-state marker bands at 1355 cm^{-1} (for ferrous cytochrome *a*) and at 1365 cm^{-1} (for ferric, cyanide-bound cytochrome a_3) in the mixed-valence cyanide-bound state (Ching et al., 1985). We thus conclude that the wavelength maximum and extinction coefficient of the cyanide-bound ferric cytochrome a_3 are altered by the heat treatment in SB-12 detergent.

Despite the changes in the Cu_A and cytochrome a_3 sites, the SB-12 enzyme retains about 30% of its cytochrome *c* oxidation activity (after correcting for residual unmodified enzyme) on the basis of O_2 consumption (Nilsson et al., 1988) and cytochrome *c* oxidation (Smith, 1955) assays. The proton pumping activity of the reconstituted enzyme, on the other hand, is severely altered by heat treatment in sulfobetaine 12 detergent. Figure 7 compares proton pumping traces of the native and heat-treated enzymes. As previously noted for cytochrome *c* oxidase heat-treated in other detergents (Li et al., 1988) or modified by pHMB (Nilsson et al., 1988), vesicles reconstituted with the SB-12 heat-treated enzyme show significant leakiness toward protons during turnover. In the cases of the lauryl maltoside and SB-12 heat-treated enzymes, it is not clear

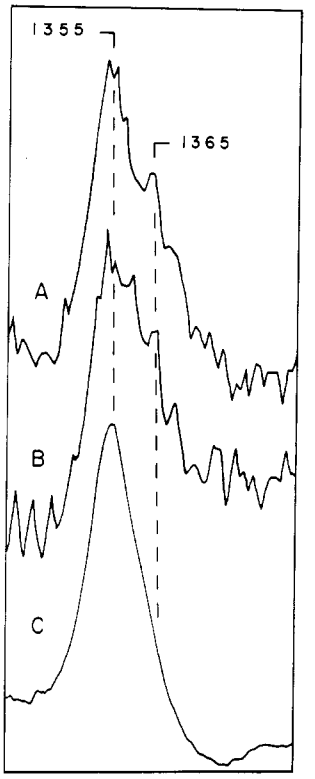

FIGURE 6: Oxidation-state marker band (ν_4) in the resonance Raman spectra of (A) mixed-valence CN^--bound native cytochrome *c* oxidase, (B) mixed-valence CN^--bound SB-12 heat-treated cytochrome *c* oxidase, and (C) fully reduced native cytochrome *c* oxidase.

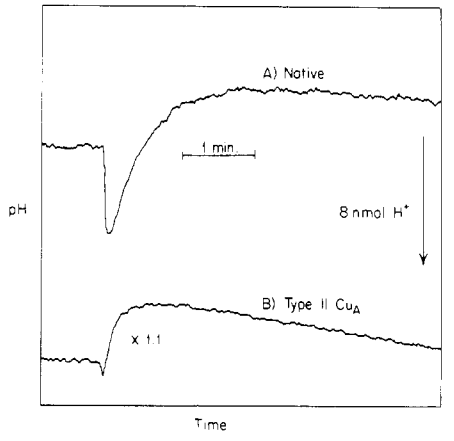

FIGURE 7: Proton pumping traces for (A) native and (B) SB-12 heat-treated cytochrome *c* oxidase reconstituted into phospholipid vesicles. The triangle indicates the time of addition of 15 nmol of ferrocytochrome *c* to a reaction mixture of 1.1 mL of pump medium, 0.25 mL of vesicles, 11 μM valinomycin, and 1.5 nM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) that had been adjusted to the same pH (7.15) as the cytochrome *c*. After the ferrocytochrome *c* induced pH changes had decayed, a pulse of 15 nequiv of H_2SO_4 was added to the reaction mixtures to determine the external and total buffering capacities. The external buffering capacity of (A) is indicated by the arrow. On the basis of total buffering capacities, the scale of (B) has been expanded by a factor of 1.1 relative to (A). See Nilsson et al. (1988) for further details.

how much of the observed vesicle leakiness is to be associated with modification of the Cu_A site and how much is due to coincidental changes in the protein matrix (i.e., dissociation of subunit III). The observed leakiness cannot be entirely attributed to subunit III loss, however, since we and other workers have shown that subunit III depleted oxidase does continue to pump protons, albeit at a reduced stoichiometry relative to the native enzyme (Sarti et al., 1985; Peuttner et

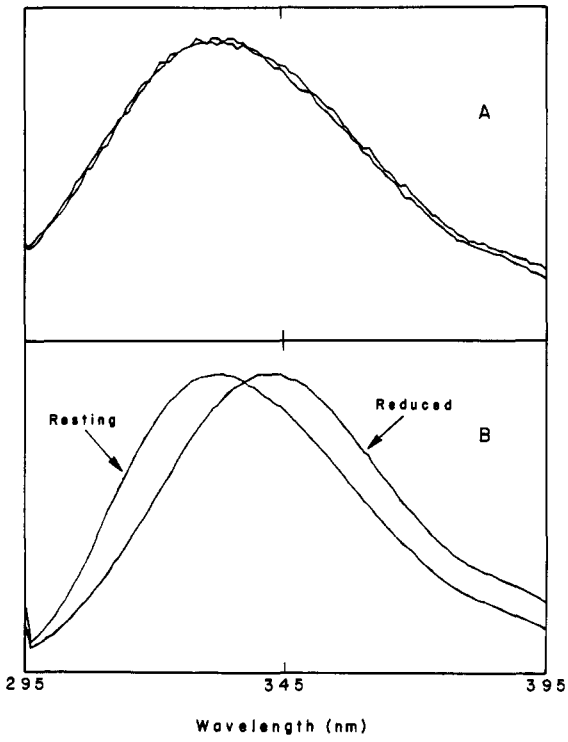

FIGURE 8: Comparison of the 280-nm-excited tryptophan fluorescence spectra of resting and reduced cytochrome *c* oxidase: (A) after heat treatment in sulfobetaine 12 detergent; (B) native enzyme.

al., 1985). Likewise, the isolated cytochrome *c* oxidase from *Paracoccus dentrificans*, which lacks subunit III, also pumps protons when reconstituted into phospholipid vesicles (Solioz et al., 1982).

Sucrose gradients run on the SB-12 and native enzymes indicate that a majority of the enzyme is monomerized upon heat treatment in SB-12. Wikström and co-workers (Finel & Wikström, 1986) have shown that monomeric cytochrome *c* oxidase pumps protons at a reduced efficiency as compared to the native dimeric molecules. However, the observed monomerization of the SB-12 enzyme would not account for the unusual proton permeability seen in the proton pumping assays (Figure 7). Nilsson et al. (1988) have proposed disruption of the proton pumping machinery and opening up of an unobstructed transmembrane proton channel in the pHMB-modified enzyme. Disruption of the proton pumping mechanism and opening of a proton channel during turnover may occur in the SB-12 enzyme also.

Conformational Transitions in the SB-12 Enzyme. Recently we showed that reduction of the Cu_A site of cytochrome *c* oxidase results in a rapid conformational change of the protein that is manifested by changes in the emission of several of the enzyme's tryptophan residues (Copeland et al., 1987). Since the Cu_A site is significantly altered in the SB-12 enzyme, we hypothesized that the enzyme might be locked into one or the other conformational state here. This turns out to be the case. Figure 8 compares the tryptophan fluorescence of the oxidized and dithionite-reduced states of the native and SB-12 enzymes. Upon reduction of the native enzyme one typically observes a ca. 16-nm red shift, from 329 to 345 nm, of the tryptophan emission maximum (Copeland et al., 1987). In contrast, the SB-12 enzyme exhibits an emission maximum at 330 nm regardless of the redox states of the various metal centers of the enzyme. We therefore conclude that the conformation exhibiting the red-shifted tryptophan fluorescence spectrum in the native enzyme is no longer accessible by Cu_A reduction after heat treatment in SB-12 detergent. The conformational change monitored by fluorescence spectroscopy is not significantly affected by the dissociation of subunit III or by heat treatment in lauryl maltoside, which primarily produces a blue copper species (Li et al., 1988). Thus, it appears that the loss of the fluorescence-monitored transition is associated with the formation of the type II Cu_A species, although other perturbations of the protein matrix cannot be ruled out as affecting the conformational transition. We were unable to examine the conformational transition for monomer oxidase by fluorescence spectroscopy because of the large absorbance from Triton X-100 in the 280-nm region.

Previously we suggested that the conformational change monitored by tryptophan fluorescence was associated with the open-closed transition of the enyzme (Copeland et al., 1987). The open-closed transition is characterized by extremely rapid cyanide binding at cytochrome a_3 upon electron input to the low-potential metal centers of cytochrome *c* oxidase (Scholes & Malmström, 1986). If our suggestion is correct, then the SB-12 oxidase should be incapable of undergoing this transition. Preliminary experiments on the native SB-12 heat-treated, pHMB-modified, monomeric, lauryl maltoside heat-treated, and subunit III dissociated oxidases indicate that disruption of the Cu_A is correlated with a perturbation of the open-closed transition.

DISCUSSION

The results presented here indicate that the Cu_A center of cytochrome *c* oxidase can be readily converted to a type II copper species by gentle heating in a zwitterionic detergent, sulfobetaine 12. This approach has considerable advantage over the previous methods of quantitatively converting Cu_A to a type II center, particularly chemical modification with pHMB (Gelles & Chan, 1985). First, the present method requires comparatively little time to effect the conversion, 75 min as compared to 24 h. Also, the heat treatment does not lead to nonspecific modification of surface amino acid residues as is the case with pHMB treatment. Finally, the present method does not require the introduction of exogenous aromatic groups to the protein solution that interfere with certain types of spectroscopic and exogenous ligand-binding studies. However, the yield of type II Cu_A is lower here than upon pHMB treatment, and the conversion is not completely quantitative. The heat treatment also results in undesired alterations of the protein matrix: monomerization and subunit III dissociation.

In all other aspects, however, the enzyme species resulting from SB-12 heat treatment and pHMB modification appear to behave identically. The optical, RR, and EPR spectra of the two forms are virtually indistinguishable, both showing the same perturbations relative to the native enzyme. Likewise, the proton pumping behaviors of the reconstituted enzymes are strikingly similar. Both forms of the enzyme also show reduced electron-transfer activity relative to the native enzyme. Thus, the SB-12 heat treatment should prove useful for future studies of the type II Cu_A cytochrome *c* oxidase.

Our tryptophan fluorescence studies indicate that the type II Cu_A form of the enzyme is incapable of undergoing the conformational change previously reported for the native enzyme (Copeland et al., 1987). Scholes and Malmström (1986) have argued that the open-closed transition is triggered by reduction of the low-potential metal centers of the enzyme, cytochrome *a* and Cu_A. Our own work on the transition monitored by fluorescence spectroscopy specifically implicates the reduction of Cu_A only (Copeland et al., 1987). In the case of the SB-12 enzyme, the EPR and optical data indicate significant perturbation of the Cu_A site, while the RR spectra

suggest that the cytochrome *a* chromophore is *not* significantly altered by this modification of the enzyme. In light of this, the observation that SB-12 cytochrome *c* oxidase does not undergo the fluorescence-monitored transition is consistent with our earlier conclusion that Cu_A reduction is responsible for the onset of this conformational transition.

The open-closed transition has been implicated in the mechanism of proton pumping in cytochrome *c* oxidase (Jensen et al., 1984; Scholes & Malmström, 1986), although it remains to be established whether or not this transition is in fact associated with proton pumping. If a role in proton pumping can be demonstrated for the conformational transition monitored by tryptophan fluorescence, the present results would strongly implicate Cu_A as the site of coupling between electron-transfer and proton pumping activities in cytochrome *c* oxidase, as has recently been suggested by this laboratory (Gelles et al., 1987). Of course, such a demonstration would by no means exclude a role for cytochrome *a* in the proton pumping mechanism of the enzyme.

The cyanide binding and RR studies presented here indicate that the perturbations resulting from SB-12 heat treatment are not limited to the Cu_A site of the enzyme, but include small changes at the oxygen binding site as well. The intensity changes seen in the RR spectra for the iron–histidine and formyl stretches of cytochrome a_3 are indicative of a change in relative geometry of the heme, leading to reduced π overlap between the porphyrin system and the axial histidine and peripheral formyl carbonyl, thus lowering the Franck–Condon factors for these vibrations. The diminished rate of cyanide binding to cytochrome a_3 likewise suggests a change in the ligand binding site of the enzyme, although the nature of the change is not obvious from these studies.

The changes seen at the cytochrome a_3 site upon heat treatment could very well be independent of the changes seen at the Cu_A site. However, control studies indicate that these effects are not related to either monomerization or subunit III dissociation, the other major perturbations associated with heat treatment in SB-12. Since it is already well established that significant redox interaction exists between the low-potential metal centers and the oxygen binding site (Wikström et al., 1981; Blair et al., 1982), it seems likely that the changes seen at the two metal centers are linked to one another through a small-scale conformational change of the intervening protein matrix. The conversion of the native Cu_A center to a type II copper requires a significant rearrangement of the ligands bound to this metal center (Li et al., 1988). It is easy to envision how such a large-scale rearrangement around the Cu_A site could be translated along the polypeptide chain and between subunits to effect changes at the oxygen binding site. Such an allosteric interaction would also explain the apparent changes in spectral interaction between cytochromes *a* and a_3 in the mixed-valence cyanide-bound SB-12 enzyme (Figure 5).

In summary, we have presented a new method for converting the Cu_A center of cytochrome *c* oxidase into a type II Cu_A species without the addition of exogenous reagents. The modified enzyme resulting from this treatment does not undergo the conformational transition that is detectable by tryptophan fluorescence. These results support our previous suggestion that reduction of Cu_A is necessary to initiate the fluorescence-monitored conformational change in the native oxidase.

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Registry No. SB-12, 14933-08-5; Cu, 7440-50-8; cytochrome *c* oxidase, 9001-16-5.

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