Heat Treatment of Cytochrome *c* Oxidase Perturbs the Cu_A Site and Affects Proton Pumping Behavior†

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ABSTRACT: It has been previously reported that mild heat treatment (43 °C for ca. 60 min) abolishes the proton pumping activity of cytochrome *c* oxidase while leaving the oxidase activity and cytochromes *a* and a_3 unperturbed [Sone, N., & Nicholls, P. (1984) *Biochemistry 23*, 6550–6554]. We herein describe the effects of this heat treatment on the electron paramagnetic resonance (EPR) and optical absorption signatures of the redox-active metal centers in the enzyme. We find that heat treatment of the oxidized enzyme causes a local structural perturbation at the Cu_A site. After heat treatment, the enzyme sample contains three subpopulations, each of which has a different structure at Cu_A. These include (i) native Cu_A, (ii) a type 2 copper species similar to the one produced by chemical modification by *p*-(hydroxymercuri)benzoate (*p*HMB) [Gelles, J., & Chan, S. I. (1985) *Biochemistry 24*, 3963–3972], and (iii) a novel type 1 copper species. In addition to changes at the Cu_A site, we find that heat treatment results in accelerated cyanide binding and the removal of subunit III. If the cytochrome *c* oxidase is heat treated while fully reduced, none of these changes are observed except for subunit III depletion. Furthermore, partial (CO mixed-valence derivative) reduction of the enzyme as well as ligand binding to cytochrome a_3 also protects the enzyme against the heat-induced changes, indicating that the oxygen binding site plays a role in stabilizing the Cu_A site against structural perturbations. When the enzyme that was heat treated while fully oxidized is reconstituted into phospholipid vesicles, the resultant proteoliposomes behave similarly to vesicles containing the *p*HMB-modified enzyme [Nilsson et al. (1988) *Biochemistry 27*, 296–301]. These vesicles display low respiratory control despite activity and membrane orientations similar to those of vesicles containing the native enzyme. When assayed for proton pumping activity, vesicles containing the heat-treated enzyme exhibit an unusually high permeability to protons. In contrast, if the heat treatment is carried out on the fully reduced enzyme under anaerobic conditions, the Cu_A site remains intact even though subunit III is lost. Reconstitution of this enzyme derivative with phospholipids produces vesicles with respiratory control ratio, membrane orientation, and activity comparable to those obtained with the native oxidase. These vesicles exhibit proton pumping activity with an apparent H^+/e^- stoichiometry of 0.4–0.5 and a faster rate of transmembrane ΔpH dissipation as compared to vesicles containing the native enzyme. This behavior is consistent with proton pumping from a subunit III depleted enzyme. Thus, protecting the Cu_A site from modification also protects the enzyme from forming a proton-conducting pathway in the protein, suggesting that the Cu_A site plays a major role in the mechanism of proton pumping in cytochrome *c* oxidase.

It is now generally accepted that cytochrome *c* oxidase is a proton pump. This enzyme catalyzes the final electron transfer in the mitochondrial respiratory chain (from ferrocytochrome *c* to molecular oxygen) and uses part of the redox energy involved to pump protons from the mitochondrial matrix to the cytosol against a protomotive force. With the issue of proton pumping in cytochrome *c* oxidase now resolved, recent attention has been directed toward determining the site of redox-linked proton translocation. It is generally assumed that one of the four redox-active metal centers in the enzyme is the site where electron transfer is coupled to proton pumping. Because the chemistry at the oxygen binding site changes markedly during the stepwise reduction of molecular oxygen, the binuclear cytochrome a_3–Cu_B site seems ill-suited for involvement in a process that translocates one proton per electron input into the enzyme. Indeed, recent proton pumping experiments (Wikström & Casey, 1985) seem to implicate the low-potential centers, cytochrome *a* and Cu_A, as the most likely site of redox-linked proton pumping activity. These low-potential redox centers shuttle electrons from ferrocytochrome *c* to the dioxygen molecule anchored at the reduction site. Since cytochrome *c* and the low-potential centers have nearly the same reduction potential, it is unlikely that there is sufficient free energy in the intermolecular electron transfer to pump a proton against a protomotive force. It is therefore more likely that the conversion of redox energy to proton free energy is associated with the electron transfer from the low-potential centers to the dioxygen binding site.

Cytochrome *a* has received attention as the probable site of redox-linked proton translocation [for a review, see Wikström et al. (1981)] because it exhibits a pH-dependent midpoint potential of −30 mV/pH unit in mitochondria (Aratzabanov et al., 1978) and in the resting form of the enzyme (Blair et al., 1986a,b). In fact, there has been a tendency to rule out Cu_A as the site of energy transduction because its midpoint potential exhibits only a small pH dependence. Reduction of cytochrome *a*, however, is linked to the uptake of only 0.5 proton in the resting enzyme, and in the carbon monoxide inhibited enzyme, its pH dependence

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decreases to ca. −9 mV/pH unit, a proton uptake of 0.15 proton (Blair et al., 1986a). It has been often assumed that a protonation/deprotonation event should be thermodynamically linked to oxidoreduction of the proton pump site. As a result, the lack of unambiguous evidence for stoichiometric protonation/deprotonation associated with these two metal centers has been considered problematic in the identification of the site of redox-linked proton pumping in cytochrome *c* oxidase.

As recently shown by Gelles et al. (1986), a pH-dependent midpoint potential is not necessary for a viable and efficient proton pump in cytochrome *c* oxidase. Therefore, both cytochrome *a* and Cu_A deserve consideration as possible sites of proton pumping. Few structurally explicit models exist for the mechanism of proton pumping by either cytochrome *a* or Cu_A. Callahan and Babcock (1983) proposed that proton translocation is linked to electron transfer via a redox-dependent weakening of the hydrogen bond between the formyl group of cytochrome *a* and a hydrogen-donating tyrosine. The atypical structure of the Cu_A site, on the other hand, has led Chan and co-workers to hypothesize a role for Cu_A in redox-linked proton translocation (Chan et al., 1979). More recently, this group (Blair et al., 1986b; Gelles et al., 1986; Chan et al., 1988) has proposed a novel mechanism by which a redox-linked ligand substitution or rearrangement at the Cu_A site is the basis for proton pumping in cytochrome *c* oxidase.

Despite these proposals, there are few experimental studies that address the mechanistic details of proton pumping or the site of redox linkage. Several investigators have approached the study of proton pumping by selectively inhibiting this part of the enzyme's activity. Binding of DCCD[1] (Casey et al., 1980) and subunit III depletion [see Prochaska et al. (1987) for a review] have both proved effective in lowering the proton to electron stoichiometry of the proton pump without seriously affecting the oxidase activity of the enzyme. Unfortunately, neither of these perturbations has lead to unambiguous structural information about the proton pump. In a recent report, Gelles and Chan (1985) described an attempt to obtain a variant of the enzyme in which both cysteines of the Cu_A site were displaced by *p*-(hydroxymercuri)benzoate (*p*HMB)[1] treatment. Nilsson et al. (1988a) subsequently showed that the *p*HMB-modified enzyme did not sustain proton pumping activity and suggested a role for Cu_A in proton translocation.

Sone and Nicholls (1984) reported earlier that heat treatment of cytochrome *c* oxidase at 43 °C results in inhibition of the enzyme's proton pumping activity while leaving its dioxygen reduction activity intact. It was also demonstrated by resonance Raman spectroscopy that cytochromes *a* and a_3 suffer only minor perturbations upon heat treatment (Sone et al., 1986). To complement these earlier studies, we have now examined this heat-treated enzyme for other structural perturbations that could account for the disruption of proton pumping. We find that incubation of cytochrome *c* oxidase at 43 °C results in a dramatic alteration of the optical and EPR signatures of the Cu_A site. In addition, vesicles containing the enzyme derivative obtained from the oxidized heat treatment (HTO enzyme) do not sustain proton pumping activity. Instead, the HTO enzyme displays an unusual permeability toward protons, similar to the *p*HMB-modified enzyme of Gelles and Chan (1985), suggesting that Cu_A modification creates a passive transmembrane proton conduction pathway through the protein. In support of this conclusion, when the Cu_A site is protected from modification by reduction, reconstitution of the resultant subunit III depleted enzyme derivative (HTR enzyme) produces vesicles capable of sustaining proton pumping activity.

Materials and Methods

Materials. *N*-2-(Hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes), tris(hydroxymethyl)aminomethane (Tris), asolectin (soybean type IIs), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and horse heart cytochrome *c* (type VI) were purchased from Sigma and used without further purification. Dodecyl β-D-maltoside and valinomycin were obtained from Calbiochem. Cholic acid from U.S. Biochemicals was purified by 3-fold recrystallization. Amberlite XAD-2 was obtained from Mallinckrodt and washed according to the procedure of Müller et al. (1986) before use. Bio-Gel P-100 was purchased from Bio-Rad and Sephadex G-125 from Pharmacia. All materials used in the electrophoresis experiments were of ultrapure electrophoresis grade unless otherwise specified. Argon for anaerobic work was made oxygen free by passing it through a manganese oxide catalyst column. Carbon monoxide from Matheson (Matheson Purity) was used without further purification.

Cytochrome *c* oxidase was isolated by the method of Harzell and Beinert (1974). Enzyme concentrations were determined by using a reduced minus oxidized $\Delta\epsilon$ of 24 mM^{-1} cm^{-1} at 605 nm. Each enzyme preparation used was stored at −80 °C until used.

Enzymatic activity was assayed by spectrophotometrically monitoring ferrocytochrome *c* oxidation (Smith, 1955). Comparisons of the activities of the heat-treated and native enzymes were in general agreement with those reported by Sone and Nicholls (1984).

Reduced cytochrome *c* oxidase was prepared as follows: The sample was placed in a spectrometer cuvette fitted with a vacuum stopcock and deoxygenated by exchanging the atmosphere with purified argon and then agitating the liquid to equilibrate it with the atmosphere. Five such cycles were usually used. Sodium dithionite (G. Fredrich Smith Co.) was then added to the solution from a side arm. The CO mixed-valence compound of cytochrome oxidase was prepared by first removing the oxygen from an enzyme sample by using argon, as above, and then replacing the argon atmosphere with carbon monoxide. The sample was then left in the dark at room temperature for several hours. Formation of these reduced forms of the enzyme was verified from their visible spectra.

Heat Treatment of Cytochrome c Oxidase. Fully oxidized cytochrome *c* oxidase samples were diluted to concentrations of 25–50 μM in 50 mM Tris/50 mM NaCl/0.5% dodecyl β-D maltoside (pH 7.7 at 25 °C) and incubated at 43 °C for 60 min in a constant-temperature water bath. Heat-treated cytochrome *c* oxidase samples were equilibrated to ice temperature before further treatment or characterization. Fully reduced and partially reduced cytochrome *c* oxidase samples were prepared in the same buffer in anaerobic optical cuvettes. Heat treatment of these sample was accomplished by immersion of the cuvette into a constant-temperature water bath.

Optical Spectroscopy. Optical spectra in the visible and near-IR region (350–900 nm) were collected at 2 °C on a Beckman Acta Model C-III dual-beam spectrophotometer,

[1] Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, dicyclohexylcarbodiimide; EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structure; Hepes, *N*-2-(hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HTO cytochrome *c* oxidase, cytochrome *c* oxidase that was heat treated while fully oxidized (resting form); HTR cytochrome *c* oxidase, cytochrome oxidase that was heat treated while fully reduced; IR, infrared; *p*HMB, *p*-(hydroxymercuri)benzoate; RCR, respiratory control ratio; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane.

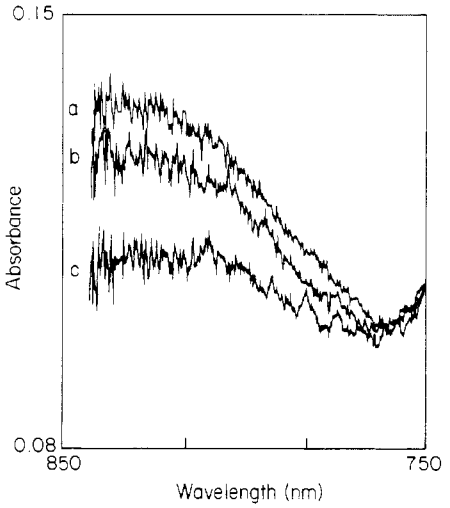

FIGURE 1: Near-IR absorption spectra of (a) native resting cytochrome *c* oxidase, (b) cytochrome *c* oxidase heat treated while reduced, and (c) cytochrome *c* oxidase heat treated while oxidized.

the digital output of which was collected by a Spex Scamp computer. Base-line spectra were digitally subtracted.

EPR Spectroscopy. EPR spectra were recorded on a Varian E-line Century Series X-band spectrometer equipped with a 12-bit analog to digital converter used for the computer digitization of the signal. Sample temperature was maintained at 77 K by immersion of the sample in liquid nitrogen or at 7 K by a liquid helium cryostat (Oxford Instruments). Oxygen was removed from EPR samples by a single equilibration with argon gas immediately prior to freezing the sample.

Cyanide Binding Assay. Cyanide binding to cytochrome a_3 was monitored at 414 nm with a Beckman Acta Model C-III dual-beam spectrophotometer. All data were collected at 2 °C with samples containing ca. 10 μM cytochrome *c* oxidase and an approximately 50-fold excess of KCN (added as a concentrated stock solution). See Figure 5 for details.

SDS–PAGE and Gel Filtration. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed with a gel system and conditions similar to that described by Rigell et al. (1987), except that samples were typically denatured for 4 h at ice temperature before loading. The gels shown were stained with Coomassie blue. The enzyme samples were allowed to stand overnight before centrifugation in order to allow the subunit III to aggregate. Scanning densitometry data were collected with a Beckman Acta Model C-III dual-beam spectrophotometer and gel scanning attachment.

Gel filtration (Bio-Gel P-100 or Sephadex G-125) was performed at room temperature (23 °C) with a ca. 10 × 2 cm column. The enzyme was eluted with 50 mM NaCl/50 mM Tris/0.5% dodecyl β-D-maltoside, pH 7.7.

Proton Pumping Measurements. Heat-treated enzyme derivatives were reconstituted into phospholipid vesicles by the cholate dialysis method and characterized in terms of their respiratory control ratio (RCR) and membrane orientation, as well as H^+/e^- stoichiometry as outlined in Nilsson et al. (1988) for the *p*HMB-modified enzyme. Proton pumping was assayed by monitoring the extravesicular pH changes following the addition of ferrocytochrome *c*. Assay conditions and buffers were the same as described in Nilsson et al. (1988a).

RESULTS

Effects of Heat Treatment on the Spectroscopic Properties of Cu_A. Heat treatment of cytochrome *c* oxidase causes significant structural changes to Cu_A, which are evident from both

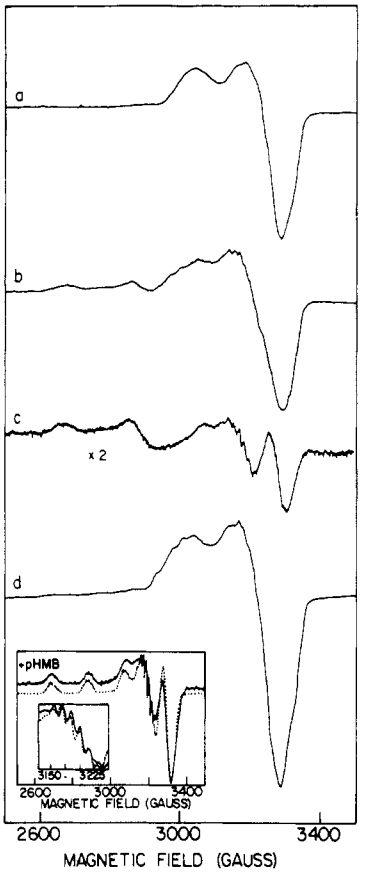

FIGURE 2: EPR signal from the Cu_A site in cytochrome *c* oxidase. (a) Native resting enzyme; (b) heat-treated resting enzyme; (c) heat-treated enzyme reduced with ascorbate and a catalytic amount of cytochrome *c*; (d) spectrum b minus spectrum c. Microwave frequency, 9.159 GHz; microwave power, 10 mW; modulation frequency, 100 KHz; modulation amplitude, 10.0 G, sample temperature, 77 K. Inset: (solid line) *p*HMB-modified cytochrome *c* oxidase; (dashed line) simulated powder EPR spectrum calculated for *p*HMB-modified Cu_A. Data taken from Gelles and Chan (1985).

near-IR and EPR spectra. In the near-infrared, the native oxidized Cu_A center gives rise to a broad, weak (ϵ = 2 mM^{-1} cm^{-1}) absorption at 830 nm (Figure 1). This band has been attributed to a charge-transfer transition between Cu^{2+} and one or more cysteine ligands (Beinert et al., 1980; Blair et al., 1983). Upon heat treatment the intensity of the 830-nm absorption diminishes by approximately 75%, indicating that a large fraction of the Cu_A centers in the sample has undergone a structural change.

The nature of this perturbation can be seen more clearly in the EPR spectrum. Figure 2a shows the EPR spectrum of native oxidized cytochrome *c* oxidase at 77 K. (Under these conditions, the spectrum arises exclusively from the Cu_A site.) This Cu_A EPR spectrum is unique among biological copper centers because it is unusually isotropic and does not exhibit resolved hyperfine splittings (Aasa et al., 1975; Hoffman et al., 1980; Stevens et al., 1982). As seen in Figure 2b, heat treatment alters the Cu_A spectrum substantially. The most notable difference is the appearance of resolved copper hyperfine lines not present in the native Cu_A signal. Since Cu^{2+} EPR line shapes reflect both the nature of the ligands and the ligation geometry of a copper center (Peisach & Blumberg, 1974), the observed spectral changes indicate that major structural alterations occur at the Cu_A site upon heat treatment.

The new EPR spectrum appears to contain contributions from at least two new copper species in addition to a signal

Table I: EPR Parameters for Cu_A in Various Cytochrome *c* Oxidase Species

copper species	$g_\parallel$	$A_\parallel$ (cm^{-1})
native Cu_A	2.18	
type 2 (heat treatment)	2.19	0.020
type 2 (*p*HMB modification)	2.21	0.019
type 1 (heat treatment)	2.16	0.004
type 2 coppers[a]	2.18–2.25	0.017–0.020
type 1 coppers[a]	2.19–2.287	0.0035–0.009

[a] Data for typical copper EPR parameters taken from Boas et al. (1978).

from residual Cu_A. We have been able to resolve some of these spectral contributions by exploiting differences in the redox potentials of the species from which they arise. Reduction of heat-treated cytochrome *c* oxidase under mild conditions (ca. 10-fold excess of ascorbate and a catalytic amount of cytochrome *c*) results in the disappearance of approximately 75% of the copper EPR signal. What remains is an EPR signal (Figure 2c) closely resembling the type 2 species produced by the chemical modification of the Cu_A site by *p*HMB (Gelles & Chan, 1985). [For a review of copper protein classifications, see Gray and Solomon (1986).] Although this type 2 copper species is not reduced by ferrocytochrome *c*, the enzyme's physiological reductant, it can be reduced by the addition of solid sodium dithionite (data not shown).

The EPR spectrum of the ferrocytochrome *c* reducible component can be obtained by subtracting spectrum 2c from 2b. This difference spectrum, shown in Figure 2d, appears to consist of two signals: native Cu_A and a new copper signal with a Cu^{2+} hyperfine coupling constant (0.004 cm^{-1}) characteristic of the type 1 copper centers of blue copper proteins. The fraction of EPR-visible copper represented by this "blue" copper species can be estimated as follows: The type 2 species accounts for 25% of the total copper signal. Quantitation of the 830 band (Figure 1) shows that 25% residual native Cu_A remains in the sample. Thus, another 25% of the EPR signal intensity can be assigned to native Cu_A. The remaining 50% appears to be made up by the type 1 (blue) copper species. Table I summarizes the measured *g* values ($g_\parallel$) and hyperfine coupling constants ($A_\parallel$) for native Cu_A as well as the type 1 and type 2 copper species produced upon heat treatment of the enzyme.

Additional evidence for the formation of a blue copper species during heat treatment comes from the visible absorption difference spectrum between the heat-treated and native cytochrome *c* oxidase (Figure 3), which reveals a broad band with ϵ ca. 1.5 mM^{-1} cm^{-1} in the 610–615 nm region and a smaller feature at approximately 740 nm. These optical features are remarkably similar to the cysteine to copper charge-transfer transitions of blue copper proteins, which give them their characteristic blue color [see Gray and Solomon (1986) for a review]. It should be noted, however, that this absorption difference lies directly beneath the heme A α-band absorption. While the spectra of the hemes do not appear significantly altered upon heat treatment (see below), even small changes in a band of such large extinction (24 mM^{-1} cm^{-1}) may produce significant features in the difference spectrum. *p*HMB-modified cytochrome *c* oxidase (Gelles & Chan, 1985) also shows small changes in this spectral region compared to the native enzyme (P. Smith, personal communication).

Effects of Heat Treatment on Cytochromes a and a_3 and Cu_B. Resonance Raman studies of heat-treated cytochrome *c* oxidase (Sone et al., 1986) have shown the hemes to be largely unperturbed. To verify these conclusions, we have used

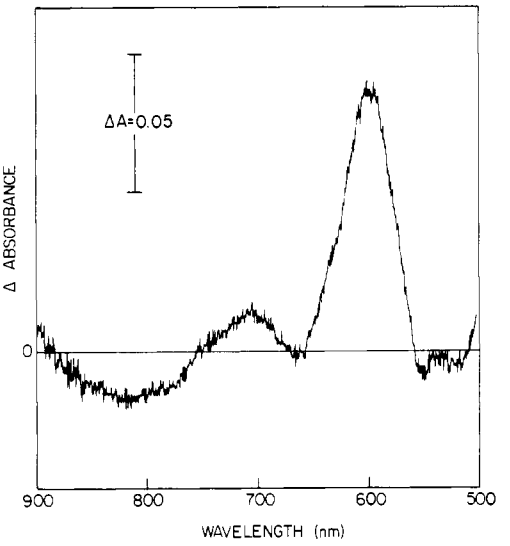

FIGURE 3: Visible absorption difference spectrum of heat-treated resting minus native cytochrome *c* oxidase in the 500–900-nm region.

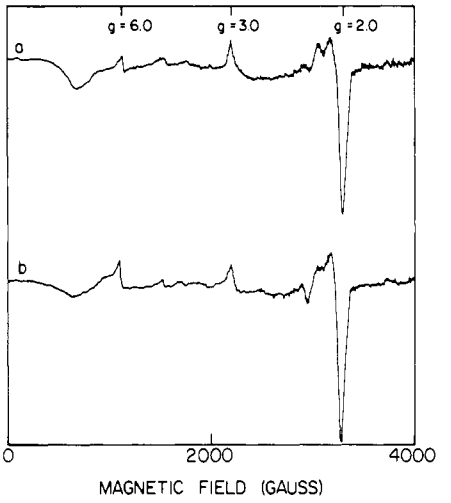

FIGURE 4: EPR spectra of (a) native resting and (b) heat-treated cytochrome *c* oxidase. Microwave frequency, 9.16 GHz; microwave power, 10 mW; modulation frequency, 100 KHz; modulation amplitude, 10.0 G; sample temperature, 10 K. Spectra shown are normalized to enzyme concentration.

low-temperature EPR spectroscopy to monitor the effects of heat treatment on cytochromes *a* and a_3 and Cu_B. Spectra of native and heat-treated cytochrome *c* oxidase are compared in Figure 4. At 7 K, only minor changes are observed in the EPR signatures for cytochromes *a* and a_3. The EPR signal at $g = 3.0$, which has been assigned to the low-spin ferric heme of cytochrome *a*, shows no significant changes in spectral shape, intensity, or position. The broad signal at $g = 12$, assigned to the antiferromagnetically coupled cytochrome a_3–Cu_B site, decreases in intensity by 10–30% compared to native enzyme. This signal, however, varies dramatically in intensity and shape from preparation to preparation. The sharp feature at $g = 6$ arises either from partial reduction or uncoupling of the binuclear cytochrome a_3–Cu_B site or from denaturation of cytochrome *a* (Brudvig et al., 1981). Quantitation of the $g = 6$ signal shows that it arises from less than 2% of the heat-treated enzyme molecules. Taken together, these observations indicate that, following heat treatment, cytochrome *a* remains structurally intact and that the coupling between cytochrome a_3 and Cu_B remains unbroken.

Oxidase Activity of the Heat-Treated Enzyme. Although the blue copper species can be reduced by ferrocytochrome

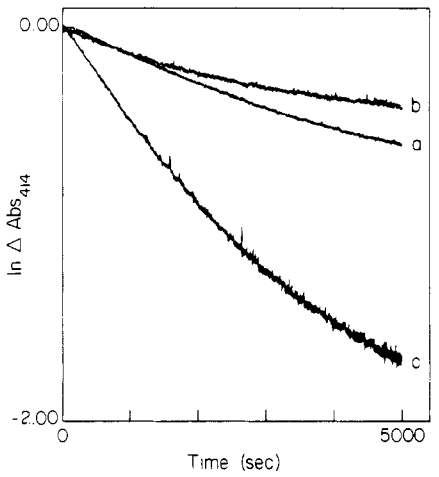

FIGURE 5: Cyanide binding curves for (a) native resting enzyme, (b) cytochrome *c* oxidase heat treated while reduced, and (c) cytochrome *c* oxidase heat treated while oxidized. Cyanide binding was observed by monitoring the absorbance at 414 nm (A_{414}). The quantity ln ΔA_{414} is ln $[[A(\text{final}) - A(t)]/[A(\text{initial}) - A(\text{final})]]$.

c, the type 2 copper cannot be and, therefore, cannot be expected to participate in electron transfer during turnover. In fact, *p*HMB-modified cytochrome *c* oxidase, which has been shown to contain almost exclusively a type 2 copper A site, has only 20–30% of the activity of the native enzyme (Gelles & Chan, 1985). In contrast, 70–80% of the native activity remains after heat treatment, suggesting that the type 1 copper is still active in electron transfer.

Cyanide Binding to Heat-Treated Cytochrome c Oxidase. Recently, it has been shown that reduction of Cu_A is involved in the closed-to-open transition of the oxygen binding site, leading to rapid binding of cyanide (Scholes & Malmström, 1986; Copeland et al., 1987). These results suggest that the heat treatment, which causes a large perturbation at the Cu_A site, might also have consequences at the oxygen binding site. The kinetics of cyanide binding in the native and heat-treated enzymes are compared in Figure 5. Heat treatment of the enzyme accelerates the binding of cyanide more than 10-fold. Because the binding is not monophasic, the relative rates were estimated by comparing initial slopes of the curves.

Subunit III Depletion in the Heat-Treated Enzyme. The heat treatment described also depletes subunit III from the enzyme. Figure 6 shows densitometer scans from denaturing SDS–polyacrylamide electrophoretic gels of cytochrome *c* oxidase. Subunit III is clearly absent in the trace for the heat-treated enzyme.

Heat Treatment of Fully Reduced Cytochrome c Oxidase. The preceding results were all obtained by heat treating cytochrome *c* oxidase in its oxidized, resting state. The same heat treatment, carried out on the fully reduced enzyme, produces almost none of these effects. Subunit III is still lost (Figure 6c), but otherwise, the reduced form of the enzyme is protected against all of the effects of heat treatment documented above. As shown in Figure 1b, the 830-nm absorption, indicative of native Cu_A, retains 80% of its intensity when the enzyme is heat treated in its reduced form and then reoxidized. (Reoxidation is necessary since the 830-nm feature arises from oxidized Cu_A only.) The actual fraction of native Cu_A remaining was probably larger than 80% because the sample was centrifuged to remove traces of denatured enzyme before this spectrum was taken. Subsequent experiments with other batches of enzyme show as much as 97% protection of the Cu_A site from modification. Copper EPR spectra (data not shown) support the conclusion that Cu_A is largely unperturbed.

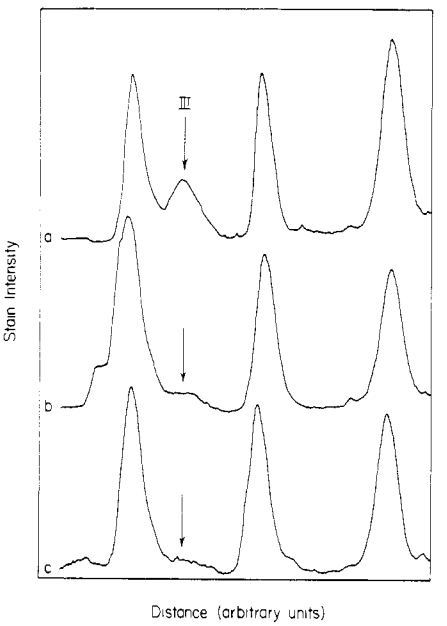

FIGURE 6: Densitometric traces of SDS–PAGE gels showing (a) native enzyme, (b) enzyme heat treated while oxidized, and (c) enzyme heat treated while reduced. Subunit III is indicated by the arrow.

Heat treatment of reduced cytochrome *c* oxidase does not produce the accelerated cyanide binding observed with heat treatment of the oxidized enzyme. When a sample of the reduced enzyme was heat treated and then reoxidized overnight at ice temperature to allow it to revert completely to the resting state, the kinetics of cyanide binding to this sample, observed the next day, were even slower than those of the resting enzyme (Figure 5b). This discrepancy is probably explained by the fact that freshly prepared resting enzyme contains small subpopulations of more active forms of the enzyme that could bind cyanide quickly (Brudvig et al., 1981). Presumably, the overnight incubation allowed the enzyme to assume a more homogeneous resting state.

Heat Treatment of Mixed-Valence Derivatives. Cytochrome *c* oxidase contains four redox-active metal centers, each of which accepts an electron when the enzyme becomes reduced. The observed protection effects could arise from the reduction of any one of the metal centers or a combination thereof. In order to localize the protection phenomenon, we performed heat treatments on several mixed-valence and ligand-bound forms of the enzyme. Heat treatment of the carbon monoxide mixed-valence compound (in which cytochrome a_3 and Cu_B are reduced and cytochrome *a* and Cu_A are oxidized) results in no greater loss of intensity at 830 nm than heat treatment of the reduced enzyme. This is strong evidence that it is the reduction of the oxygen binding site which is responsible for the protection observed in the fully reduced enzyme. Heat treatment of the cyanide-bound, fully oxidized enzyme results in some loss of intensity at 830 nm, but still much less than that in the case of native enzyme. Thus, ligand binding to the oxygen binding site can confer some protection even in the absence of reduction. Finally, the cyanide mixed-valence compound of the enzyme (in which cytochrome *a* and Cu_A are reduced) is no more protected against heat treatment than the fully oxidized cyanide compound. This suggests that, surprisingly, reduction of Cu_A does not protect the Cu_A site from heat-induced modification.

Gel Filtration of the Reduced Heat-Treated Enzyme. Heat treatment of both the oxidized and the fully reduced enzyme leads to the dissociation of subunit III. In the experiments reported here, the dissociated subunit III polypeptide was

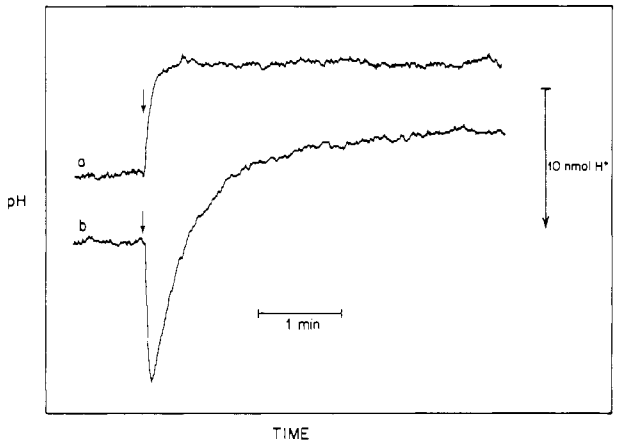

FIGURE 7: Comparison of (a) HTO and (b) native cytochrome *c* oxidases in the proton pumping assay. The reaction mixtures in each case contained 0.25 mL of vesicles (0.63 nmol of enzyme), 1.1 mL of medium (44 mM KCl/56 mM choline chloride), and valinomycin to a final concentration of 11 μM. The pH of the mixture was adjusted to that of the cytochrome *c* stock solution. At the times indicated by the arrow, 15.9 nmol of ferrocytochrome *c* was added.

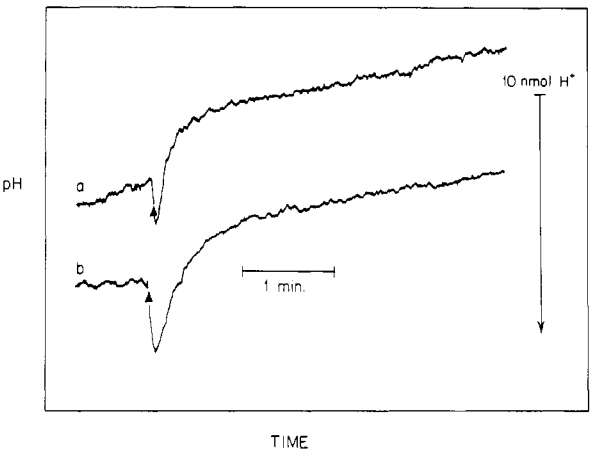

FIGURE 8: Comparison of (a) HTR and (b) native cytochrome *c* oxidases in the proton pumping assay. Reaction conditions were the same as for Figure 7 except that a different batch of enzyme was used.

removed from solution by incubating the sample overnight to allow the subunit to aggregate and then centrifuging the sample to pellet the aggregate. In our early experiments, we attempted to remove subunit III from the heat-treated enzyme solutions by gel filtration. Samples that had been heat treated in the reduced form and then reoxidized displayed an intact 830-nm absorption. However, when these samples were passed down a Bio-Gel P-100 or Sephadex G-25 column, the 830-nm band largely disappeared. This result suggests that some aspect of the reduced heat treatment, perhaps the removal of subunit III, renders the enzyme more susceptible to structural perturbations.

Proton Pumping in Heat-Treated Oxidized (HTO) Cytochrome c Oxidase. Figure 7 compares the proton pumping behavior of heat-treated oxidized (HTO) and native enzymes that were reconstituted into phospholipid vesicles. We observe that rapid alkalinization accompanies the oxidation of ferrocytochrome *c* by the heat-treated enzyme (Figure 7a). In contrast, the addition of ferrocytochrome *c* to well-coupled vesicles containing the native enzyme (Figure 7b) results in a transient acidification followed by a slow decay of the transmembrane ΔpH to a new equilibrium value. The rapid rise in the extravesicular pH observed for vesicles containing the heat-treated enzyme is quite similar to the result obtained with cytochrome *c* oxidase modified by *p*HMB (Nilsson et al., 1988a).

In contrast to the results of Sone and Nicholls (1984), our reconstitution of heat-treated cytochrome *c* oxidase produces vesicles with a low respiratory control ratio (RCR). Typically, RCR values of ca. 1.2–1.5 are obtained for the HTO enzyme while values of 4.0–6.0 are obtained for the native enzyme. As with the native enzyme, the orientation assay shows that between 75% and 85% of the HTO enzyme is reconstituted with the cytochrome *c* binding site facing the external medium.

As discussed by Nilsson et al. (1988a), the rapid alkalinization accompanying ferrocytochrome *c* oxidation by reconstituted *p*HMB-modified enzyme as well as the low RCR values may be accounted for either by scalar proton consumption by unreconstituted enzyme in the external medium or by a transmembrane proton conduction pathway in the modified enzyme. Since the HTO enzyme displays normal vesicle orientations, it is unlikely that unreconstituted enzyme is the cause of the observed low RCR values and the rapid alkalinization behavior. The most likely explanation for our observation is increased proton permeability in these vesicles resulting from creation of a transmembrane proton conductance through the enzyme during heat treatment.

Proton Pumping in Heat-Treated Reduced (HTR) Cytochrome c Oxidase. When reduced cytochrome *c* oxidase is heat treated under anaerobic conditions, subunit III becomes dissociated, but the Cu_A site remains unperturbed. If the formation of a transmembrane proton conduction pathway in the heat-treated enzyme is linked to modification of the Cu_A site, vesicles containing enzyme that is heat treated while in the reduced form should remain coupled and display proton pumping activity. Indeed, vesicles containing the HTR enzyme display RCR values of 2.5–3.0. For the enzyme preparation used in this experiment, native enzyme gives RCR values of 2.4–3.0. As expected, the HTR enzyme is competent in the proton pumping assay (Figure 8). However, in contrast to the native enzyme, H^+/e^- stoichiometries obtained for the HTR enzyme are 0.4–0.5. Typical stoichiometries obtained for this batch of native enzyme are 0.6–0.7. In addition, the acidification decay rate is slightly faster with the HTR enzyme than for the native enzyme. These results agree with proton pumping measurements on the reconstituted subunit III depleted enzyme [see Prochaska et al. (1987) for a review].

Discussion

Sone and Nicholls (1984) reported that incubation of cytochrome *c* oxidase at 41–43 °C results in the inhibition of proton pumping activity with only minor perturbations of either the heme A centers or the oxidase activity. We have extended the earlier study to examine the metal centers of heat-treated cytochrome *c* oxidase by EPR spectroscopy. We observe no significant changes in the spectra of the hemes, but the EPR spectrum of Cu_A is appreciably altered, indicating pronounced structural changes at that site. Concomitant with the change in the Cu_A EPR spectrum is a 75% decrease in the intensity of the Cu_A 830-nm absorption band. Heat treatment of our enzyme preparation also results in a 20–30% loss of oxidase activity, a 10-fold acceleration of cyanide binding to cytochrome a_3, and the removal of subunit III. We further find that reduction of, or ligand binding to, the oxygen binding site of the enzyme prevents the effects of heat treatment except for the loss of subunit III.

Heat treatment of cytochrome *c* oxidase results in a dramatically altered Cu_A EPR spectrum. The new spectrum appears to consist of three components: a type 2 copper signal, a type 1 copper signal, and a contribution from residual native Cu_A. The type 2 copper signal corresponds to $\sim$25% of the

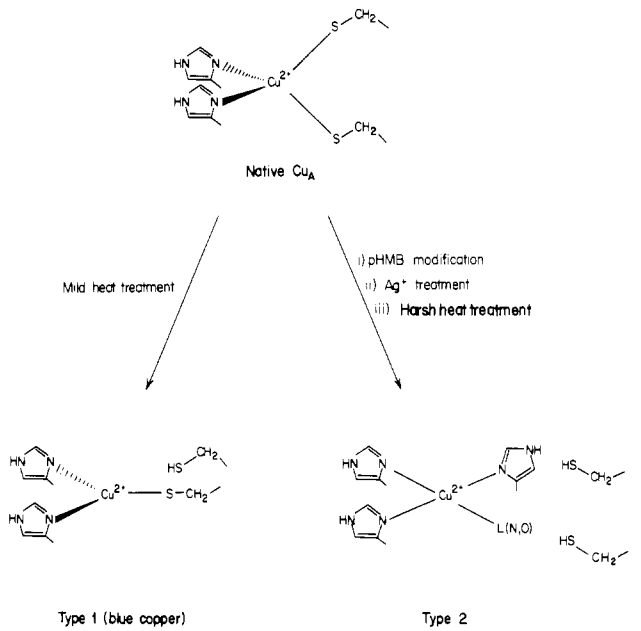

FIGURE 9: Proposed scheme for the production of observed forms of modified Cu_A. Displacement of one thiolate ligand during heat treatment as shown in the left pathway results in the formation of a species with spectroscopic properties similar to a type 1 (blue) copper center. Displacement of two thiolate ligands, as in *p*HMB modification, in Ag^+ treatment, and partially during heat treatment, results in the formation of a type 2 copper center as shown in the right pathway. In the case of chemical modification by *p*HMB or Ag^+, the free cysteine sulfhydryls shown for the type 2 copper may exist as thiolate ligands to Hg^+ or Ag^+, respectively.

EPR-visible copper. Its *g* values and hyperfine coupling constants are almost identical with those of the *p*HMB-modified Cu_A reported by Gelles and Chan (1985) (see Table I). Like the *p*HMB-modified copper, this species cannot be reduced by ferrocytochrome *c* but can be reduced by dithionite. It is therefore likely that these two type 2 modified Cu_A derivatives are similar in structure. We note that a similar type 2 copper signal is also observed upon treatment of cytochrome *c* oxidase with Ag^+ (Chan et al., 1978, 1979). The existence of such varied routes to this copper species suggests that the structure of the modified Cu_A center is not determined by the modification agent. Apparently, the tendency toward modification is an inherent property of the protein and ligand structure of the Cu_A site, and the same modification can be induced in several different ways.

The remainder of the copper EPR spectrum can be assigned to native Cu_A and a type 1 blue copper species. We estimate that 25% of the Cu_A remains in its native form since the 830-nm absorption retains ~25% of its intensity after heat treatment. The remaining EPR contribution, which accounts for ~50% of the enzyme, displays a hyperfine coupling constant of 0.004 cm^{-1} (Figure 2d; Table I) and bears a striking resemblance to the EPR signatures of the blue copper proteins.

Although our data do not allow for the umambiguous determination of structures, one possible model for the structures of these Cu_A derivatives and the routes to their formation is shown in Figure 9. Native Cu_A has been shown to have at least one histidine ligand and two sulfur ligands, of which one is from a cysteine side chain (Stevens et al., 1982; Li et al., 1987; Martin et al., 1988). In our model, the displacement of one cysteine ligand would lead to the formation of a type 1 blue copper site and displacement of both cysteines would lead to the formation of a type 2 copper site. Gelles and Chan (1985) have shown that the type 2 copper EPR signal is likely to arise from a site with at least three nitrogenous ligands, and a recent EXAFS study of the *p*HMB-modified enzyme has implicated a fourth nitrogen or oxygen ligand (Li et al., 1987). We propose that the harsh conditions under which the type 2 copper signal is produced results in the displacement of both cysteine ligands. With milder conditions, such as incubation at 43 °C, the displacement of one cysteine could predominate, leading to a type 1 copper species with one cysteine and two nitrogenous ligands.

Sone and Nicholls (1984) reported that the oxidase activity of their heat-treated enzyme is the same as that of the native enzyme. We observe a 20–30% decrease in activity. By comparison, *p*HMB modification of the enzyme results in an 80% loss of activity (Gelles & Chan, 1985). The activity of our heat-treated enzyme is therefore consistent with the presence of a 25% subpopulation bearing a type 2 copper having the same low level of turnover as the *p*HMB-modified enzyme. This raises the possibility that the Sone and Nicholls heat treatment, which was performed on a different enzyme preparation, produces the type 1 blue copper species without significant formation of a type 2 copper species.

Gelles and Chan (1985) explained the diminished activity of the *p*HMB-modified enzyme in terms of a decreased reduction potential of the type 2 copper center. They reasoned that since the type 2 copper cannot be reduced by ferrocytochrome *c*, a key step in the enzyme's electron-transfer pathway is disrupted. Enzyme bearing the type 1 blue copper center appears to have nearly full activity, presumably because the type 1 copper can accept electrons from ferrocytochrome *c*.

Our data show that reduction of, or ligand binding to, the oxygen binding site stabilizes the Cu_A site with respect to heat treatment. This is clear evidence for a conformational link between the oxygen binding site and Cu_A. We have also demonstrated a correlation between Cu_A modification and the acceleration of cyanide binding. Copeland et al. (1987) recently demonstrated that reduction of Cu_A is associated with the triggering of the "closed-to-open" transition at the oxygen binding site. Although our heat-treated enzyme does not have the extremely rapid cyanide binding properties of the "open" conformation, the parallel between these findings is of some interest since both involve communication between Cu_A and the oxygen binding site.

Recently, Hill and Robinson (1986) showed that cytochrome *c* oxidase, which had been depleted of subunit III by extensive incubation in lauryl maltoside, displayed fast cyanide binding kinetics. In the present study, we find that enzyme which is heat treated while oxidized displays loss of subunit III as well as fast cyanide binding in addition to a modified Cu_A site. In contrast, enzyme that is heat treated while reduced exhibits normal Cu_A spectroscopic signatures and cyanide binding kinetics similar to that of the resting enzyme following reoxidation even though subunit III is lost. Thus, with this method, subunit III removal alone causes neither the observed structural perturbations at the Cu_A site nor the elevated cyanide binding rates. However, the ease with which Cu_A is modified when heat-treated enzyme is passed down a gel filtration column suggests that the enzyme is more susceptible to modification at the Cu_A site when subunit III is lost.

Protection of the Cu_A site from heat-induced modification also protects the enzyme from displaying a heat-induced proton conductance. This observation suggests that it is a perturbation of the Cu_A site that causes a transmembrane proton leak in the enzyme. Nilsson et al. (1988a) obtained evidence for a similar transmembrane leak in the *p*HMB-modified enzyme and suggested that it is linked to disruption of the Cu_A site.

It is therefore likely that these two enzyme derivatives contain similar proton conduction pathways. Although the EPR spectrum for the HTO enzyme is complicated, nearly 25% of the copper appears to be converted to a form almost spectroscopically identical with *p*HMB-modified Cu_A. The similarity of these two copper sites suggests that it is the type 2 form of the Cu_A site that is associated with the transmembrane leak. As noted earlier (Nilsson et al., 1988a), the presence of a small fraction of leaky enzymes may mask proton pumping activity from the remaining fraction of the enzyme in the vesicle preparation because a substantial portion of the reconstituted vesicles will contain both intact and leaky enzyme molecules in the same vesicle.

The type 2 copper sites produced by heat treatment and *p*HMB modification do not participate in electron transfer from ferrocytochrome *c*. In contrast, the type 1 copper site, which is present in ca. 50% of the heat-treated enzyme molecules, is electron-transfer competent. Because even 25% of the type 2 copper species is sufficient to uncouple a majority of the reconstituted vesicles, whether the modified enzyme with the type 1 Cu_A is capable of redox-linked proton translocation or behaves as an uncoupler is an issue that cannot be addressed at present.

In addition to the present heat treatment and *p*HMB modification (Gelles & Chan, 1985), it also has been recently found that heating cytochrome *c* oxidase in the detergent sulfobethaine 12 yields a Cu_A-modified type 2 copper site in high yields (Nilsson et al., 1988b). Treatment of the enzyme with Ag^+ also modifies the Cu_A site in a similar manner (Chan et al., 1978, 1979). Such strikingly similar type 2 copper sites resulting from Cu_A modification by methods as diverse as chemical thiol reagents and localized heat denaturation suggest that the protein architecture about the Cu_A site is essential to its structural integrity. The fact that all Cu_A modifications we have observed [including heat treatment in sulfobethaine 12 (Nilsson et al., 1988b)] result in the same kind of transmembrane proton permeability strongly implicates a role for Cu_A in proton transport within the enzyme.

Gelles et al. (1986) have proposed a theoretical model for proton pumping based on Cu_A as the site of energy transduction and proton pumping. In this model, the pump site was required to be in contact with protons from *both* the matrix *and* the cytosol via appropriate proton channels. Proton gating was achieved by allowing protonation–deprotonation steps to occur either on the matrix side or on the cytosolic side of the pump but not both sites at once. This is tantamount to a modified alternating access model (Wyman, 1979; Wikström et al., 1981). One possible interpretation for the results observed here is that Cu_A is associated with proton gating via an alternating access mechanism. If Cu_A is the proton gate, disruption of the site may destroy the alternate access of the pump site to protons, forming a contiguous proton channel through the enzyme. Since protons must travel to and from the pump site on a time scale comparable to electron transfer within the enzyme, the fast alkalinizations observed are consistent with such a disruption of proton gating. An alternate explanation for the observed proton permeability is that *p*HMB and heat modifications cause a protein conformational change, which opens up a proton conduction pathway within the enzyme stretching from the matrix side to the cytosolic side of the enzyme. This possibility seems less likely, however, since it is inconceivable that such a gross conformational change can take place without also disrupting cytochrome *a* or the oxygen binding site. In fact, removal of subunit III, a 30-kDa membrane-bound polypeptide, induces a large change in protein architecture without leading to significant uncoupling. The HTR enzyme species, which has been protected from Cu_A modification by reduction, is also protected from the formation of a proton leak through the enzyme. These observations all argue for a picture in which a transmembrane proton leak is specifically associated with disruption of the Cu_A site.

The work described here suggests that Cu_A plays an important role in proton conduction through cytochrome *c* oxidase. While it appears that Cu_A may participate in proton gating, it is also enticing to speculate that Cu_A is the site of energy transduction since Cu_A functions as an electron shuttle between cytochrome *c* and the oxygen binding site. As discussed by Chan et al. (1988), the Cu_A site may be amenable to redox-linked structural changes that make it a good candidate for the site of energy transduction. This hypothesis proposes that the two cysteine sulfurs coordinated to oxidized Cu_A (Cu^{2+}) interact with one another in such a way that reduction of the site (to Cu^{1+}) may cause a ligand substitution or rearrangement. It is interesting to speculate that this ligand rearrangement actually involves a local protein conformational change that also gates protons by alternating access. This model predicts that the Cu_A-modified derivatives of cytochrome *c* oxidase are incapable of pumping protons. However, since the formation of an efficient proton leak in the Cu_A-modified enzyme derivatives would effectively mask proton pumping activity, it is impossible to determine whether the proton pumping machinery is intact in the Cu_A-modified enzymes. Finally, since, in addition to proton gating, the free energy transducer must also gate electrons, it is important to experimentally demonstrate electron gating before Cu_A can be identified as the site of redox-linked proton translocation.

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Preparation of a One-Subunit Cytochrome Oxidase from *Paracoccus denitrificans*: Spectral Analysis and Enzymatic Activity†

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ABSTRACT: Cytochrome *c* oxidase was isolated from *Paracoccus denitrificans* as a two-subunit enzyme. Chymotrypsin-catalyzed proteolysis reduced the molecular weight of each subunit by about 8000. The spectral properties of this preparation, as well as its K_m for cytochrome *c* (1.7 μM), remained unchanged with respect to the native enzyme. V_{max} was reduced by about 55% when assayed in Triton X-100 or in Triton X-100 supplemented with asolectin. Following further proteolysis by *Staphylococcus aureus V8* protease, subunit I remained unchanged as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, whereas subunit II was split into small peptides. These were removed by ion-exchange high-performance liquid chromatography. The one-subunit enzyme had an apparent molecular weight of 43 000. The reduction of molecular weight was also confirmed by the diminution of the ultraviolet/Soret absorption ratio. This value was 1.8-2.1 for the native enzyme and 1.3-1.5 for the one-subunit enzyme. The spectral properties (including the spectrum CO reduced minus reduced) were not modified by the proteolytic treatment, indicating that cytochromes *a* and a_3 were present in equal amounts. The lack of spectral alteration and the known close association of the copper B atom with cytochrome a_3 suggest that copper B is also contained within the one-subunit enzyme. The K_m of the one-subunit oxidase was similar to that of the two-subunit enzyme; V_{max} was decreased by about 50%. The activity of the one-subunit oxidase had a salt-dependent maximum at 30 mM KCl, almost identical with that of the undigested enzyme, and was inhibited by micromolar concentrations of KCN.

In the large family of cytochrome *c* oxidases the bacterial enzymes have been studied with increasing frequency during the last 10 years, mainly because of their structural analogy with respect to the eukaryotic type (Fee et al., 1986; Ludwig, 1987). Although they contain only 1-3 subunits relative to the 9-13 present in eukaryotic cells, their enzymatic and spectral properties are almost identical. The cytochrome *c* oxidase of *Paracoccus denitrificans* was isolated as two-subunit enzyme (Ludwig & Schatz, 1980), and its main physicochemical characteristics have been analyzed (Ludwig et al., 1982; Solioz et al., 1982; Nalecz et al., 1985; Bolli et al., 1986). The minimum requirement for cytochrome *c* oxidase activity seems to be a two-subunit structure. The only known exception is *Thermus thermophilus* (Yoshida et al., 1984), which was isolated in an active form consisting of a single subunit. The gene sequences of *P. denitrificans* subunits I-III have been recently published (Steinrücke et al., 1987; Raitio et al., 1987). Despite the fact that a gene for subunit III was identified,

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