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Chemical Modification of the Cu_A Site Affects the Proton Pumping Activity of Cytochrome *c* Oxidase[†,‡]

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ABSTRACT: Cytochrome *c* oxidase in which the Cu_A site has been perturbed by extensive modification of the enzyme with the thiol reagent *p*-(hydroxymercuri)benzoate has been reconstituted into phospholipid vesicles. The reconstituted vesicles lack respiratory control, and the orientation of the enzyme in the vesicles is similar to that of the native cytochrome *c* oxidase. In the proton translocation assay, the vesicles containing the modified enzyme behave as if they are unusually permeable to protons. When the modified and native proteins were coreconstituted, a substantial portion of the latter became uncoupled as revealed by low respiratory control and low overall proton pumping activity. These results suggest that the modified enzyme catalyzes a passive transport of protons across the membrane. When milder conditions were used for the chemical modification, a majority of the thiols reacted while the Cu_A site remained largely intact. Reconstitution of such a partially modified cytochrome *c* oxidase produced vesicles with respiratory control and proton translocating activity close to those of reconstituted native enzyme. It thus appears that the appearance of a proton leak is related to the perturbation of the Cu_A site. These observations suggest that the structure of Cu_A may be related to the role of this site in the proton pumping machinery of cytochrome *c* oxidase.

Cytochrome *c* oxidase fills a central function in the energy transduction of aerobic organisms. In eukaryotes, the enzyme, which spans the mitochondrial membrane, catalyzes the reduction of molecular oxygen to water. The reductant, cytochrome *c*, donates the electrons from the cytosolic side of the membrane while the protons consumed in the reaction are taken up from the mitochondrial matrix. In addition, one proton is actively transported from the matrix to the cytosol for each electron transferred (Wikström, 1977; Casey et al., 1979a,b). The reaction is thus electrogenic and contributes to the electrochemical potential gradient across the inner mitochondrial membrane. It has been suggested (Wikström et al., 1981) that the proton translocating activity is necessary for the full utilization of the redox span between cytochrome *c* and molecular oxygen. While a great ideal is known about the oxygen reduction in cytochrome *c* oxidase [see Malmström (1982), Naqui et al. (1986), and Wikström et al. (1981) for reviews; Blair et al., 1985], the molecular mechanism of proton translocation remains largely unknown.

The enzyme contains four redox-active metal centers, two irons in cytochromes *a* and a_3 and two copper ions which are

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usually labeled Cu_A and Cu_B. Cytochrome a_3 and Cu_B form the binuclear site where oxygen is bound and reduced, while cytochrome *a* and Cu_A are the primary acceptors of electrons from cytochrome *c*. A recent investigation of proton translocation by cytochrome oxidase in intact mitochondria (Wikström & Casey, 1985) suggests that the site of energy coupling is one of the two low-potential centers, cytochrome *a* and Cu_A. Although cytochrome *a* has been commonly assumed to be the more likely candidate (Wikström et al., 1981; Babcock & Callahan, 1983), Chan and co-workers have presented theoretical arguments that Cu_A being the site of coupling is equally consistent with current knowledge of the enzyme (Gelles et al., 1986). Furthermore, mechanistic considerations by these authors suggest that Cu_A is the center most suited for a role in proton translocation.

Recently, Gelles and Chan (1985) discovered that the Cu_A site may be chemically modified by exhaustive treatment with the thiol reagent *p*-(hydroxymercuri)benzoic acid (*p*HMB).[1] The protein product is active in electron transfer, albeit with an activity of about 20% of that of the native enzyme. Since the modified Cu_A site is not redox active under the assay conditions used, the residual activity must reflect electron transfers from cytochrome *c* to the oxygen binding site via cytochrome *a*.

Reconstitution of this modified enzyme into phospholipid vesicles allows measurement of its proton translocating activity and offers, therefore, a possibility to probe the role of Cu_A in energy transduction. We report here the characterization of reconstituted cytochrome *c* oxidase containing a modified Cu_A site with respect to energy coupling and proton translocation.

Materials and Methods

Materials. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), *p*-(hydroxymercuri)benzoic acid (*p*HMB), asolectin (from soybean, type IIs), cholic and deoxycholic acids, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and cytochrome *c* (from horse heart, type VI) were obtained from Sigma. Dodecyl maltoside and valinomycin were from Calbiochem. Sephadex G-25 was from Pharmacia and Bio-Gel P-100 from Bio-Rad. Amberlite XAD-2 was obtained from Mallinkrodt and washed (Müller et al., 1986) before use. All other chemicals were of analytical grade. The asolectin was purified by acetone–ether fractionation as described by Kagawa and Racker (1971) except that dithiothreitol (1 mM) was used as the antioxidant. Cholate and deoxycholate were recrystallized from 70% ethanol and 80% acetone, respectively.

Cytochrome *c* oxidase was purified from beef heart mitochondria as described by Hartzell and Beinert (1974) and stored at −80 °C. Enzyme concentrations were calculated from ΔA_{red-ox} at 605–630 nm using an extinction coefficient of 27 mM^{-1} cm^{-1} (Hill & Greenwood, 1984).

Cytochrome *c* was reduced by the addition of solid sodium dithionite and then gel filtered on a short Sephadex G-25 column. The concentration of reduced cytochrome *c* was determined as described by Casey (1986).

Exhaustive Modification of Cytochrome c Oxidase with pHMB. The reaction conditions used by Gelles and Chan (1985) were used except that the Tween-20 in the reaction buffer was replaced by 10 mM dodecyl maltoside. After 24 h at room temperature, the mixture was centrifuged (25000*g*, 15 min) to remove solid *p*HMB. The supernatant was concentrated by ultrafiltration (Amicon XM-300 membrane) to a volume of ca. 0.5 mL. To remove the remaining *p*HMB, the solution was passed down a Bio-Gel P-100 gel filtration column (0.8 × 15 cm) equilibrated with 0.1 M Hepes/K^+, pH 7.4, containing 0.3% deoxycholate. The protein fraction was collected, kept on ice, and used for reconstitution within a few hours.

Partial Modification of Cytochrome c Oxidase with pHMB. Cytochrome *c* oxidase was diluted to a concentration of 7 μM (calculated from the absorption at 420 nm and ϵ = 141 mM^{-1} cm^{-1}; Blair et al., 1982) with 50 mM Tris-HCl and 50 mM NaCl, pH 7.7, containing 0.5% dodecyl maltoside. Then 156 μL of the same buffer saturated with *p*HMB (41.6 mM *p*HMB as judged from the absorption at 232 nm and ϵ = 16.9 mM^{-1} cm^{-1}; Boyer, 1954) was added, giving a final concentration of *p*HMB of 0.98 mM. The extent of modification was monitored via the absorbance change at 250 nm (Boyer, 1954; Benesch & Benesch, 1962). Following partial modification, the absorbance change at 250 nm was 0.35 after correction for the contribution from free *p*HMB. Since $\Delta\epsilon$ = 7.6 mM^{-1} cm^{-1} (Boyer, 1954), this corresponds to the modification of on the average six to seven thiols per enzyme. To quench the reaction, cysteine (final concentration 1 mM) was added, and the reaction mixture was concentrated to ca. 0.5 mL by ultrafiltration. EPR spectroscopy of a sample prepared this way showed that the Cu_A signal is essentially unperturbed. If the reaction was allowed to proceed further (4 h at room temperature), a larger absorbance change, corresponding to the modification of approximately nine thiols per enzyme, was obtained. However, in this case, the EPR spectrum of Cu_A becomes perturbed, showing the appearance of hyperfine structure in the $g_{\parallel}$ region.

Reconstitution of Cytochrome c Oxidase. The enzyme was reconstituted into phospholipid vesicles by cholate dialysis as described by Casey (1986) with minor modifications. The second and third dialysis buffers were supplemented with 20 g/L Amberlite XAD-2, and a fourth dialysis (15 h) versus 200 volumes of 0.1 mM Hepes/K^+, 22 mM K_2SO_4, and 55 mM sucrose, pH 7.4, was added. After dialysis, the vesicles were centrifuged (25000*g*, 30 min) to remove large particles. This step was found to improve the respiratory control of the vesicles significantly.

Two different batches of cytochrome *c* oxidase were used in the present work. One, which was used for the majority of the experiments, produced well-coupled vesicles when it was reconstituted. The other, however, gave vesicles with poor respiratory control (RCR = 2.6–2.8). Following Finel and Wikström (1986), we found that this could be much improved by sucrose gradient ultracentrifugation of the enzyme prior to reconstitution. Consequently, an ultracentrifugation step was routinely included in reconstitutions using this batch of enzyme.

Gradients of 5–30% sucrose (w/v) in 0.5 M potassium phosphate buffer, pH 7.4, containing 2% cholate in a total volume of 4.5 mL were prepared in 5-mL centrifuge tubes as described by Luthe (1983). Samples (approximately 60 nmol of cytochrome *c* oxidase in 0.5 mL) were applied, and the tubes were centrifuged for 16 h at 40000 rpm in a Beckman SW50.1 rotor. The green band of cytochrome *c* oxidase was collected manually from the top, concentrated by ultrafiltration, and reconstituted.

Characterization of Reconstituted Vesicles. Respiratory control was measured polarographically at 25 °C using an oxygen electrode from Yellow Springs Instruments. The re-

[1] Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; *p*HMB, *p*-(hydroxymercuri)benzoic acid; RCR, respiratory control ratio(s); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

action medium contained 50 mM potassium phosphate, pH 6.8, 50 mM KCl, 0.1 mM EGTA, 25 μM cytochrome *c*, and 15 mM ascorbate. Reactions were initiated by the addition of reconstituted vesicles. The respiratory control ratio (RCR) was calculated by dividing the rate obtained in the presence of 0.2 μM CCCP and 0.02 μM valinomycin with that obtained in the absence of the uncouplers.

The orientation of the enzyme in the reconstituted vesicles was determined essentially as described by Thelen et al. (1984), except that anaerobic conditions, instead of cyanide, were used to prevent turnover of the enzyme. The sample, in a Thunberg-type spectrophotometric cell equipped with a septum port and a sidearm, was made anaerobic by repeated evacuation and flushing with purified argon. After a spectrum of the fully oxidized enzyme was taken, the fraction facing outward in the active orientation was reduced by the addition of ascorbate. Another spectrum was then taken, and the remaining oxidized enzyme (the fraction facing the vesicular lumen) was reduced by solid sodium dithionite added from the sidearm. The fraction of enzyme with the cytochrome *c* binding site facing the external medium was calculated as A_{red-ox} at 605–630 nm obtained with ascorbate divided by the corresponding value obtained with dithionite.

Proton Translocation Assay. Extravesicular pH changes were measured with a Radiometer PHM 82 pH meter equipped with a GK 2401C electrode. The instrument was interfaced to an IBM PC AT for data collection and storage. The data acquisition rate was 2 s^{-1}, and the time constant of the system (including mixing time) was ca. 2 s. Reaction mixtures contained 1.1 mL of medium (44 mM KCl and 56 mM choline chloride), 0.25 mL of vesicles (0.8–1.5 nmol of cytochrome *c* oxidase), and valinomycin to a final concentration of 11 μM. The pH of the reaction mixture was carefully adjusted to that of the cytochrome *c* stock solution with 0.5 mM H_2SO_4 or dilute, carbonate-free NaOH. The stirred reaction vessel was thermostated at 20 °C, and air depleted of CO_2 by scrubbing with 10% KOH was blown over the surface of the solution. Reactions were initiated by the addition of reduced cytochrome *c* in an approximately 15-fold excess over the reconstituted cytochrome *c* oxidase. The external and total buffering capacities were obtained by adding a known amount of dilute standard H_2SO_4 (Casey, 1986). For the determinations of proton translocation stoichiometry, the initial acidifications were obtained from extrapolation of the decays to zero time (Casey, 1986; Müller et al., 1986). Under these assay conditions, we did not find any appreciable dependence of the number of protons transported per electron on the excess cytochrome *c* in the range 10–30-fold excess.

Results

Reconstitution of the modified cytochrome *c* oxidase produced vesicles with a very low degree of coupling. Typically, respiratory control ratios (RCR) of 1–1.2 were obtained. Control samples treated identically except *p*HMB was omitted gave RCR values of 2.5–3.2 while native oxidase that had been incubated with 0.3% deoxycholate before reconstitution gave values between 4.5 and 6. The orientation assay showed that 75–85% of the enzyme was oriented with the cytochrome *c* binding site facing the external medium in all three cases.

Figure 1 shows the behavior of the reconstituted native and control enzymes in the proton translocation assay. With the native enzyme (trace A), a stoichiometry of 0.85 proton ejected per electron transferred was obtained after extrapolation to zero time. Other vesicle preparations with the native enzyme gave values between 0.7 and 0.9 H^+/e^-. In the presence of 4 μM CCCP, addition of cytochrome *c* resulted in rapid alkalinization corresponding to ca. 1 H^+/e^- (not shown).

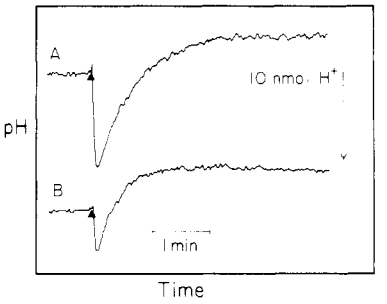

FIGURE 1: Proton pumping by the native and control enzymes. (A) The reaction mixture contained 1.3 nmol of reconstituted native cytochrome *c* oxidase. (B) The reaction mixture contained 1.4 nmol of reconstituted control enzyme (treated as described in the text). At times indicated by the triangles, 15.9 nmol of ferrocytochrome *c* was added. The external buffering capacities, indicated by the vertical arrow, were determined in both samples after the cytochrome *c* induced pH changes had decayed completely. The vertical scales are adjusted so that the initial acidifications brought about by adding 10 nequiv of H_2SO_4 correspond to the same vertical displacement for the two traces.

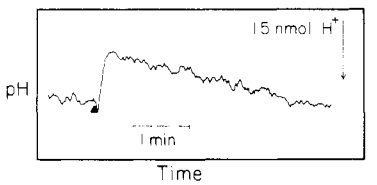

FIGURE 2: Behavior of the vesicles containing modified enzyme. The reaction mixture contained 0.5 mL of vesicles (0.9 nmol of enzyme) in a final volume of 1.35 mL, and 15.9 nmol of ferrocytochrome *c* was added at the time indicated by the triangle. The vertical arrow reflects the total buffering capacity.

Reconstituted control enzyme (trace B) gave stoichiometries around 0.5, which is significantly lower than that obtained with the native enzyme. This is similar to what has been observed earlier with cytochrome *c* oxidase depleted of subunit III (Puettner et al., 1984) or treated with dicyclohexylcarbodiimide (Casey et al., 1979a,b). However, at the protein:detergent ratio used in the present work, subunit III is not expected to dissociate from the enzyme (Hill & Robinson, 1986). Another explanation for the low stoichiometry is that prolonged incubation at room temperature may partially monomerize the enzyme. Finel and Wikström (1986) have shown that the apparent stoichiometry of proton translocation is lower with monomeric cytochrome *c* oxidase.

The vesicles containing the modified enzyme behaved dramatically different. Figure 2 shows an almost instantaneous alkalinization of the external medium upon the addition of cytochrome *c*. These vesicles clearly do not display any proton pumping activity. Moreover, the scalar protons consumed in the vesicular lumen are replenished from the external medium much faster than the permeability of the bilayer would allow. This increased membrane permeability toward protons could be the result of a leak introduced in the enzyme by the chemical modification or due to the presence of a soluble uncoupler (traces of *p*HMB?) in the vesicle preparation. Alternatively, the modified enzyme is not reconstituted at all and only present in solution in the external medium. Any one of these explanations is consistent with the low respiratory control ratio obtained for the reconstituted modified enzyme mentioned earlier. However, we find that the orientation of the reconstituted modified enzyme falls in the same range as those of the native and control samples, indicating that it is incorporated in a similar manner. We also have no evidence that residual *p*HMB is acting as an uncoupling agent (see

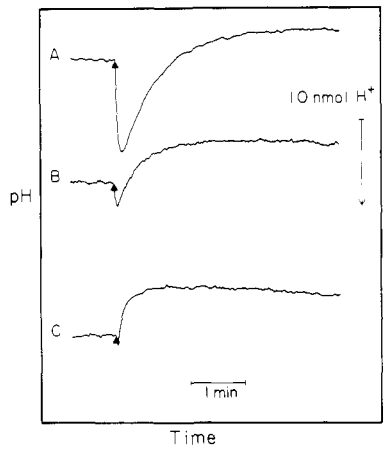

FIGURE 3: Comparison of proton pumping by reconstituted mixtures of native and modified enzymes with that of native enzyme alone. (A) The reaction mixture contained 1.3 nmol of reconstituted native enzyme. (B) The reaction mixture contained 1.3 nmol (total) of a reconstituted mixture containing 80% native enzyme and 20% modified enzyme. (C) The reaction mixture contained 1.2 nmol (total) of a reconstituted mixture containing 60% native enzyme and 40% modified enzyme. At the times indicated by the triangles, 15.9 nmol of ferrocytochrome *c* was added. External buffering capacities were determined as in Figure 1 and are displayed in the same way.

below) in the vesicle preparation. This leaves the existence of a transmembrane proton-conducting pathway in the modified cytochrome *c* oxidase as the most likely explanation for the observed behavior in the proton pumping assay.

To investigate the proton permeability of the modified enzyme further, we have coreconstituted it with native cytochrome *c* oxidase. Under the reconstitution conditions used here, a substantial portion of the enzyme is expected to become incorporated into vesicles containing more than one enzyme molecule (Casey et al., 1984). If the modified enzyme were responsible for the enhanced proton permeability, all vesicles that contain at least one molecule of modified enzyme would behave as if completely leaky and give rise to traces similar to that in Figure 2. Only vesicles that contain native oxidase exclusively would yield ordinary pumping traces (Figure 1A). The net effect would thus be the uncoupling of that fraction of the native enzyme located in vesicles also containing a modified enzyme.

Native and *p*HMB-modified cytochrome *c* oxidases were mixed in ratios of 0.8:0.2 and 0.6:0.4 (concentrations based on oxidized − reduced difference spectra) and reconstituted. Characterization of the reconstituted vesicles gave RCR of 1.7 and 1.3, respectively. The outward orientations of the enzyme in the two vesicle preparations were 71% and 77%, respectively. A reconstitution of native enzyme alone carried out in parallel resulted in vesicles with an RCR of 6. In the coreconstituted samples, the modified enzyme contributes only a small fraction of the total activity. Accordingly, the low RCR values obtained can most probably be attributed to uncoupling of part of the native enzyme rather than merely the presence of uncoupled, modified enzyme.

In Figure 3, proton translocation of the reconstituted mixtures (traces B and C) is compared to that obtained from the vesicles containing native enzyme only (trace A). The appearances of traces B and C show that the inclusion of even a small amount of modified enzyme exerts a drastic effect on the net proton translocation. To evaluate the effect of coreconstitution more precisely, we estimated the proton translocation expected if the native and modified enzymes behaved independently. With the specific activity of the modified enzyme being ca. 20% of that of the native, it may be calculated that the initial acidification obtained from the 0.8:0.2 mixture would be 90% of that obtained from the native enzyme alone.[2]. The corresponding estimate for the 0.6:0.4 mixture is 75%.

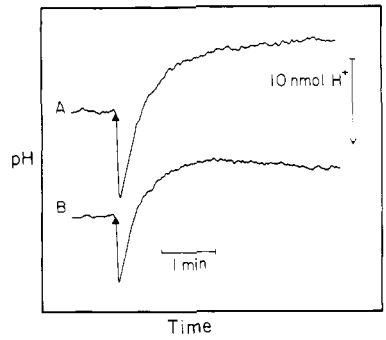

FIGURE 4: Comparison of proton pumping by a mixture of native and modified enzymes that had been reconstituted separately with that of the native enzyme alone. (A) The reaction mixture contained 1.3 nmol of reconstituted native enzyme. (B) The reaction mixture contained 0.63 nmol of reconstituted native enzyme and 0.22 nmol of reconstituted modified enzyme. Ferrocytochrome *c* additions (15.9 nmol) are indicated by the triangles. External buffering capacities were determined as in Figure 1 and are displayed in the same way.

To verify the validity of this treatment, the experiment was repeated with a mixture of native and modified enzymes that had been reconstituted separately. Figure 4 shows that the calculation of expected acidification is correct within 15%. This result also excludes the presence of a soluble uncoupler in the vesicle preparation containing the modified enzyme as the source of its enhanced proton permeability.

A comparison between the experimental and expected initial acidifications obtained from the reconstituted mixtures clearly shows that a substantial part of the native cytochrome *c* oxidase becomes uncoupled when it is reconstituted together with the *p*HMB-modified enzyme. The low RCR found for the mixtures are in accord with this explanation. These results strongly support the notion that the modified cytochrome *c* oxidase contains a pathway for the transmembrane transport of protons. On the other hand, totally different results would have been obtained had the low RCR and proton pumping activity been due to impaired insertion of the modified enzyme into the vesicles.

Given that a vesicle containing at least one molecule of modified cytochrome *c* oxidase becomes completely uncoupled, the fraction of enzyme reconstituted into vesicles containing more than one enzyme molecule may be estimated from the net acidifications obtained. The trace obtained from the 0.8:0.2 mixture may be deconvoluted into a sum of two contributions: 63% from "pumping" vesicles and 37% from "leaking" vesicles. Assuming a Poisson distribution for the number of enzymes per vesicle (Apell & Läuger, 1986), we estimate that ca. 35% of the enzyme is in vesicles containing at least two enzyme molecules. This is in reasonable agreement with the distribution found by Casey et al. (1984).

Under the reaction conditions used to carry out the *p*HMB modification, all thiols of the protein are expected to become modified by the reagent. However, it has been shown earlier (Tsudzuki et al., 1967) that a majority of the thiols react very

[2] If f is the fraction of modified enzyme and a its specific activity relative to that of the native enzyme, the fractions of the ferrocytochrome *c* consumed by the native and modified enzymes are $(1-f)/(1-f+fa)$ and $fa/(1-f+fa)$, respectively. Since the fraction consumed by the modified enzyme causes *alkalinization*, the initial acidification obtained from the mixture relative to that obtained from the native enzyme alone is $(1-f-fa)/(1-f+fa)$.

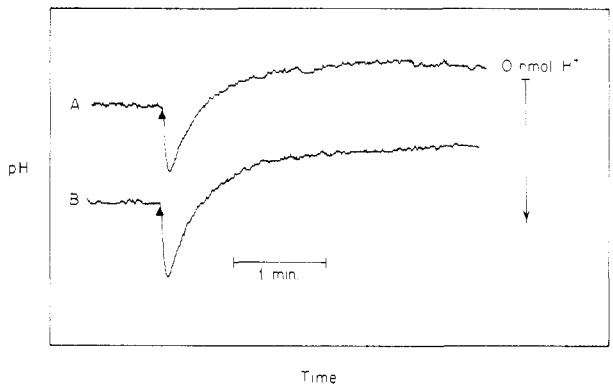

FIGURE 5: Comparison of proton pumping by native and partially modified enzymes. (A) The reaction mixture contained 1.3 nmol of reconstituted native enzyme. (B) The reaction mixture contained 1.2 nmol of reconstituted, partially modified enzyme. At the times indicated by the triangles, 15.1 nmol of ferrocytochrome *c* was added. External buffering capacitites were determined as in Figure 1 and are displayed the same way.

rapidly with *p*HMB at a much lower reagent concentration. Gelles and Chan (1985) found that Cu_A is not affected under these conditions. To assess whether the modification of thiols not associated with Cu_A is responsible for the leakiness of the *p*HMB-modified cytochrome *c* oxidase, we prepared and reconstituted a short-time modified sample.

When a sample that had been exposed to *p*HMB for only a short time was layered on a sucrose gradient, ultracentrifuged, and reconstituted, we obtained vesicles with an RCR of 3.8. A sample of native cytochrome *c* oxidase that had been ultracentrifuged and reconstituted in parallel gave vesicles with RCR = 3.5. The outward orientations were 74% and 71%, respectively. Proton translocation by these vesicles is shown in Figure 5. Clearly, modification of the rapidly reacting thiols has not impaired the proton pumping activity. However, the apparent stoichiometry for proton translocation for both vesicle preparations is significantly lower (ca. 0.5) than the vesicles used for the experiment shown in Figure 1A. The reason for this discrepancy is not clear but seems to be related to the use of two different batches of cytochrome *c* oxidase.

DISCUSSION

Taken together, the proton translocation results obtained with partially and exhaustively modified enzymes indicate that the disruption of the Cu_A site is necessary for the appearance of the proton leak. There are, however, a total of 11 free thiols in cytochrome *c* oxidase (Buse et al., 1985), 2 of which are most probably ligands to Cu_A (Darley-Usmar et al., 1981; Li et al., 1987). Since we were only able to modify six to seven thiols without affecting the Cu_A site, it cannot be entirely excluded that the modification of the remaining two to three nonligand thiols is causing the appearance of the proton leak. However, we have also found that heating of cytochrome *c* oxidase according to Sone and Nicholls (1984) induces a change in the Cu_A EPR signal as well as the loss of proton pumping activity (P. M. Li, T. Nilsson, and S. I. Chan, unpublished results). Although the resulting EPR spectrum is complex, a significant portion of the copper has been converted to a form with an EPR signal very similar to that of the *p*HMB-modified enzyme. Furthermore, we obtain poorly coupled vesicles that display leaky behavior in the proton pumping assay when the heat-treated enzyme is reconstituted. The result that a leaky enzyme can be obtained without the use of *p*HMB strongly argues against covalent modification of thiols not coordinated to Cu_A being the cause for the leakiness observed in the present work.

It thus appears that it is the perturbation of the Cu_A site that is responsible for the present results. This would support the proposal by Gelles et al. (1986) that Cu_A constitutes the site of redox-linked proton translocation in cytochrome *c* oxidase. It must, however, be noted that in the event proton translocation activity were unaffected by the modification, the protons ejected into the extravesicular medium would escape detection because of the leakiness of the modified enzyme. An alternative interpretation is therefore that the Cu_A modification introduces an adventitious transmembrane leak while the proton translocating apparatus remains intact.

The disruption of the pumping site remains nevertheless an attractive explanation for the high leak rates observed. Clearly, the proton translocating site must be accessible to protons in the aqueous bulk phases on both sides of the membrane. Since the metal centers in cytochrome *c* oxidase are all located inside the protein, the enzyme must contain provisions to facilitate intramolecular proton transfers. It has thus been demonstrated that the site of oxygen binding and reduction, which requires the exchange of both water and protons with a bulk phase, is specifically connected to the matrix side of the membrane (Konstantinov et al., 1986). Although the mechanism for proton conduction remains obscure, similar proton pathways can be envisioned linking the site of energy coupling to both sides of the membrane.

A minimum requirement for the pumping site is that a bound proton should not have access to both sides of the membrane simultaneously (Wikström et al., 1981; Tanford, 1983). A structural perturbation that removes this property of alternating access to the pump site, and consequently interconnects the two channels, would result in the kind of proton leak observed in the *p*HMB-modified cytochrome *c* oxidase described here. For example, in the model proposed by Gelles et al. (1986), the cupric ion serves as an electrostatic barrier against adventitious proton transfers. A structural perturbation of the site can easily be imagined, removing this function of the metal ion.

The finding that *p*HMB-modified cytochrome *c* oxidase "uncouples" coreconstituted native enzyme in the respiratory control measurements suggests that the leak is capable of turning over at a rate approaching that of the electron transfer reaction in the native enzyme (ca. 200 s^{-1}). The proton transfer mechanism involved is thus clearly kinetically competent for a role in the normal catalysis.

In conclusion, we have demonstrated that exhaustive modification of cytochrome *c* oxidase with *p*HMB is accompanied by the creation of a facile proton leak. This result is most likely due to the perturbation of the Cu_A site and implies a role for the latter in the control of proton transfers in the enzyme. To qualify as a site for energy transduction, a metal center must, however, be capable of controlling both proton and electron transfers (DeVault, 1971; Wikström et al., 1981; Blair et al., 1986). A plausible mechanism for the control of electron transfers by Cu_A has been put forward by Gelles et al. (1986). Here, it was shown how structural changes at the site can be exploited to "gate" electrons as required for energetic coupling. Although the proposed electronic structure of the Cu_A site indicates that it is conducive to redox-linked structural changes (P. M. Li, J. Gelles, T. Nilsson, and S. I. Chan, unpublished results), little direct evidence for this exists at present. Thus, although Cu_A is an attractive candidate for the proton translocating site in cytochrome *c* oxidase, much further work is needed to place the proposal on a more solid foundation. Specifically, the experimental demonstration of electron gating remains an important objective.

Registry No. Cu, 7440-50-8; hydrogen ion, 12408-02-5; cytochrome *c* oxidase, 9001-16-5.

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Intermediates in the Refolding of Ribonuclease at Subzero Temperatures. 1. Monitoring by Nitrotyrosine Absorbance†

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ABSTRACT: Derivatives of ribonuclease A in which tyrosines-73, -76, and -115 were nitrated have been synthesized, purified to homogeneity, and characterized by NMR, isoelectric points, absorbance spectra, and catalytic activity. The positions of their reversible thermal unfolding transitions were determined in 35% methanol at pH* 3.0 and 6.0. In the present study the kinetics of the refolding of these nitrotyrosine derivatives were measured at −15 °C at pH* 3.0 and 6.0 by using a cryosolvent composed of 35% aqueous methanol. The rates of folding of different regions of the molecule were determined by using the nitrotyrosines as environmentally sensitive probes. Multiphasic kinetics were observed for the refolding of the nitro-Tyr-115, -73, and -76 derivatives. The native environment about Tyr-115 was formed more rapidly than that about Tyr-73 and -76, and the native environment about both these tyrosines was attained much sooner than the native state itself, as judged by other probes. The results indicate that different regions of the molecule attain their native environments at different rates. This observation shows that the folding pathway must involve partially folded intermediate states.

The process whereby a polypeptide chain folds into its native conformation is of major biological importance. Current interest in the mechanisms and pathway of protein folding centers on the nature of intermediate states. Difficulties exist in detecting intermediates during protein folding due to their low concentrations and short lifetimes. Preliminary results have indicated that the use of low temperatures and aqueous–organic cryosolvents may be very useful in permitting the stabilization of partially folded intermediates (Biringer & Fink,

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