Characterization of Electron-Transfer and Proton-Translocation Activities in Bovine Heart Mitochondrial Cytochrome *c* Oxidase Deficient in Subunit III†

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ABSTRACT: The electron-transfer and proton-translocation activities of cytochrome *c* oxidase deficient in subunit III (M_r 29 884) prepared by native gel electrophoresis [Ludwig, B., Downer, N. W., & Capaldi, R. A. (1979) *Biochemistry 18*, 1401–1407] have been investigated. This preparation has been depleted of 82–87% of its subunit III content as quantitated by Coomassie Brilliant Blue staining intensity on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and [^{14}C]dicyclohexylcarbodiimide labeling. The maximum rate of electron transfer of the subunit III deficient enzyme at pH 6.5 is 383 s^{-1}, 78% of control enzyme. Neither the high-affinity site (K_m = 10^{-8} M) nor the low-affinity site (K_m = 10^{-6} M) of the cytochrome *c* kinetic interaction with cytochrome *c* oxidase is affected by the removal of subunit III. Subunit III deficient cytochrome *c* oxidase retains the ability to bind cytochrome *c* in both the high- and low-affinity sites as determined in direct thermodynamic binding experiments. Liposomes containing this preparation exhibit a respiratory control ratio [Hinkle, P. C., Kim, J. J., & Racker, E. (1972) *J. Biol. Chem. 247*, 1338–1341] of 3.9, while liposomes containing control enzyme exhibit a ratio of 4.3, suggesting that they have a similar proton permeability. Vectorial proton translocation initiated by the addition of ferrocytochrome *c* in liposomes containing subunit III deficient enzyme is decreased by 64% compared to those containing control enzyme. When the proton-translocated to electron-transferred ratio is measured in these phospholipid vesicles at constant enzyme turnover, removal of subunit III from the enzyme decreases the ratio from 0.52 to 0.21, a 60% decrease. When corrected for residual subunit III content in the preparation, the contribution of subunit III to vectorial proton translocation increased to 75%. These studies show that subunit III is not required for electron transfer from cytochrome *c* to molecular oxygen and that subunit III accounts for a majority of the observed transmembrane, vectorial movement of protons in the enzyme.

Bovine heart mitochondrial cytochrome *c* oxidase (EC 1.9.3.1) is the terminal electron carrier in the respiratory chain that reduces molecular oxygen to water (Azzi & Casey, 1979; Capaldi et al., 1983). The enzyme is an oxidation–reduction-linked proton pump both in isolated mitochondria (Wikstrom & Saari, 1977; Wikstrom, 1984) and in reconstituted artificial membrane systems (Krab & Wikstrom, 1978; Casey & Azzi, 1983; Proteau et al., 1983). The most convincing experiments delineating the ability of the enzyme to translocate protons across a membrane in a vectorial manner involve the reconstitution of the isolated enzyme into phospholipid vesicles. In this system, it has been shown that the proton vectorially translocated to electron-transferred ratio (H^+/e^-)[1] approaches 1 in the external aqueous phase (Krab & Wikstrom, 1978) and that two protons per electron transferred are abstracted from the intravesicular space (Thelen et al., 1985); one proton per electron is used in the catalytic reduction of molecular oxygen, and the other is pumped across the phospholipid bilayer. The external release of protons is dependent on the intravesicular buffering capacity (Krab & Wikstrom, 1978). The apparent stoichiometry is also dependent on the total number of electrons the enzyme catalytically transfers (Casey et al., 1979; Sigel & Carafoli, 1980; Proteau et al., 1983) and can only be observed in the presence of membrane potential dissipating agents such as valinomycin plus potassium (Krab & Wikstrom, 1978; Casey et al., 1984; Moroney et al., 1984).

One candidate for the subunit of the enzyme that mediates the translocation of protons is subunit III (M_r 29 884), a mitochondrially synthesized subunit (Anderson et al., 1982). Dicyclohexylcarbodiimide (DCCD) inhibits both proton translocation and, to a lesser extent, electron transfer in the enzyme (Casey et al., 1980; Prochaska et al., 1981). The major site of DCCD binding in the enzyme is in subunit III (Casey et al., 1980), which has a strong primary amino acid sequence analogy to the DCCD binding site in the putative proton channel of the Mg^{2+}-ATP synthetase (Prochaska et al., 1981). In addition, surface topographical studies of the enzyme in intact mitochondria and resolved submitochondrial particles (everted mitochondria) have shown that only subunits II (M_r 26 049) and III span the mitochondrial inner membrane (Ludwig et al., 1979).

A possible means of studying the role of subunit III in the mechanism of vectorial proton movement is to prepare cytochrome *c* oxidase deficient in subunit III and monitor the effects of that removal on the electron-transfer and proton-translocation activities of the enzyme. Different methods of preparation have been reported, all of which appear to retain significant electron-transfer activity (Saraste et al., 1981; Bill & Azzi, 1982; Thompson & Ferguson-Miller, 1983). Upon

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[1] Abbreviations: DCCD, *N*,*N*′-dicyclohexylcarbodiimide; TMPD, tetramethyl-*p*-phenylenediamine; Tris, tris(hydroxymethyl)aminomethane; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; $NaDodSO_4$–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; RCR, respiratory control ratio; H^+/e^-, ratio of vectorial protons translocated per electron transferred; K^+/e^-, ratio of potassium ions translocated per electron transferred.

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reconstitution into liposomes, these preparations appear to lose from 40% to 100% of the ability to translocate protons in a vectorial manner (Saraste et al., 1981; Penttila, 1983; Thompson et al., 1984; Puettner et al., 1985; Thelen et al., 1985).

In this work, we characterize bovine heart mitochondrial cytochrome *c* oxidase deficient in subunit III isolated by native gel electrophoresis. This work is unique because we have quantitatively characterized the content of subunit III in our preparation. Also, we have characterized the ability of the subunit III depleted enzyme to interact with its substrate, cytochrome *c*, and have found that the interaction of cytochrome *c* with the enzyme is unaffected by the removal of subunit III. We have incorporated cytochrome *c* oxidase deficient in subunit III into liposomes and have shown that they have similar proton permeability as liposome containing control enzyme. These liposomes containing cytochrome *c* oxidase deficient in subunit III lose 75% of their ability to translocate protons in a vectorial manner.

Experimental Procedures

Enzyme Preparation. Mitochondria were isolated from bovine heart as described by Azzone et al. (1979). Cytochrome *c* oxidase was prepared as described by Yonetani (1967). Enzyme concentration was determined by using extinction coefficients of either 24 mM^{-1} cm^{-1} for heme aa_3 at 605 nm for reduced minus oxidized spectra (Van Gelder, 1966) or 16.5 mM^{-1} cm^{-1} for reduced heme *a* at $\Delta A_{605-630}$ (Briggs & Capaldi, 1977). Both extinction coefficients were found to give similar concentrations of heme *a* on any preparation, but 16.5 mM^{-1} cm^{-1} was used routinely. Protein was estimated as described by Lowry et al. (1951) with modifications in the presence of Triton X-100 (Wang & Smith, 1975). Phospholipid analysis was performed according to the procedure of Organisciak and Noell (1976).

Isolation of Cytochrome c Oxidase Deficient in Subunit III. Cytochrome *c* oxidase (10 mg/mL) was incubated with 7.5 mg of Triton X-100/mg of enzyme in 0.06 M Tris–phosphate, pH 6.9, and dialyzed against 2% Triton X-100 (w/v) and 0.06 M Tris–phosphate, pH 6.9, for 3 h at 4 °C with three changes. After centrifugation at 20000*g* for 10 min to remove a small amount of precipitated enzyme, the supernatant was applied to a slab gel containing 4% polyacrylamide, 0.16% bis(acrylamide), 0.2% Triton X-100, and 0.38 M Tris-HCl, pH 8.9. The gel (10 × 15 × 0.5 cm) was run at 4 °C for 8–10 h at 150 V. Two green protein bands were observed in the native gel. Each enzyme fraction was eluted from the gel by homogenization in 0.1% Triton X-100 and 0.01 mM KH_2PO_4, pH 7.4, and concentrated by ultrafiltration over PM-30 or XM-100A membranes (Amicon). Each enzyme fraction was then chromatographed over Sepharose 2-B (1.5 × 20 cm) at 4 °C in 0.1% Triton X-100 and 50 mM KH_2PO_4, pH 8.0, and the fractions containing absorbance at 422 nm were pooled.

Electron-Transfer Assays. Cytochrome *c* oxidase was activated for electron-transfer activity measurements as described by Vik et al. (1981), and all subsequent assays were performed polarographically at 25 °C as presented in Thompson and Ferguson-Miller (1983). The assay buffer for maximum velocity measurements contained 10 mM ascorbate, 0.6 mM *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD), 25 μM horse heart cytochrome *c* (type III, Sigma Chemical Co.), and 0.25 mM dodecyl β-D-maltoside (Calbiochem) in 50 mM KH_2PO_4, pH 6.5.

Enzyme kinetic assays were performed as described by Ferguson-Miller et al. (1978) with modifications of Vik et al. (1981). The activated enzyme was assayed in 6 mM ascorbate, 0.6 mM TMPD, 0.02% 1-oleoyllysophosphatidylcholine (Sigma Chemical Co.), 25 mM Tris–acetate, pH 7.8, and various cytochrome *c* concentrations from 20 to 0.02 μM.

Phospholipid vesicles inlaid with cytochrome *c* oxidase were assayed for electron-transfer activity in 15 mM ascorbate, 35 μM cytochrome *c*, and 50 mM KH_2PO_4, pH 7.4. The respiratory control ratio of the liposmes was determined as described by Hinkle et al. (1972), using 3 μM valinomycin and 5 μM CCCP to uncouple the rate of electron transfer.

Cytochrome c Binding Measurements. Cytochrome *c* binding to cytochrome *c* oxidase was performed as described by Ferguson-Miller et al. (1978) with modifications by Fuller et al. (1981) by chromatography on Sephadex G-100 (1 × 20 cm) columns equilibrated with 0.25% Tween 20 and 25 mM Tris–acetate, pH 7.8, containing either 0.6 μM or 20 μM cytochrome *c*. Cytochrome *c* bound to cytochrome *c* oxidase was calculated by using the dithionite-reduced spectrum as described in Ferguson-Miller et al. (1978).

Preparation of Phospholipid Vesicles. Control cytochrome *c* oxidase and subunit III deficient cytochrome *c* oxidase were reconstituted into phospholipid vesicles by the cholate dialysis procedure of Hinkle et al. (1972); 100 mg/mL asolectin (Sigma Chemical Co.) was dispersed in 100 mM HEPES–NaOH, pH 7.2, and 50 mM potassium cholate (Aldrich Chemical Co.) by sonication at 0 °C. Control or subunit III deficient cytochrome *c* oxidase (0.6–1.0 mg/mL) was added, and the sample was dialyzed at 4 °C according to a regime described in Prochaska et al. (1981).

Proton-Translocation Assays. Proton-translocation activity was monitored as described in Prochaska et al. (1981) at 25 °C in a water-jacketed cell using a Corning Model 12 pH meter equipped with a combination electrode and a Sargent–Welch XKR recorder. Liposomes containing control enzyme (0.28 nmol) or enzyme deficient in subunit III (0.17 or 0.14 mmol) were incubated in 2.5 mL of 100 mM KCl, 100 mM sucrose, 0.5 mM HEPES–NaOH, pH 7.2, and 1 μM valinomycin. Electron transport was initiated by the addition of various amounts of ferrocytochrome *c* (type VI, Sigma Chemical Co.). Ferrocytochrome *c* was prepared as described by Prochaska et al. (1981).

[^{14}C]DCCD Labeling of Cytochrome c Oxidase. [^{14}C]-DCCD (Research Products International, Inc., 50 mCi/mmol) labeling of cytochrome *c* oxidase and enzyme deficient in subunit III was performed in 0.2% Triton X-100 and 0.01 M NaH_2PO_4, pH 7.4, at a stoichiometry of 130 mol of DCCD/mol of enzyme for 1 h at 20 °C. All other conditions were as described in Prochaska et al. (1981).

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. $NaDodSO_4$–PAGE was performed on slab gels as described by Fuller et al. (1981) using 16% acrylamide and *N,N′*-methylenebis(acrylamide) (37.5/1 w/w) and on cylindrical gels by the procedure of Swank and Munkres (1971) using 12.5% acrylamide and *N,N′*-methylenebis(acrylamide) (10/1 w/w). Gels were stained, destained, and scanned as described by Downer et al. (1976). Radioactive gels were sliced into 1.2-mm slices by using a Bio-Rad gel slicer. The slices were incubated in 0.3 mL of 30% H_2O_2 at 70 °C overnight and, after addition of 0.7 mL of Bio-Hp (Fischer Scientific) scintillation fluid, were counted on a Packard liquid scintillation counter.

Results

Isolation of Subunit III Deficient Cytochrome c Oxidase. Enzyme was subjected to native gel electrophoresis (Maurer, 1971; Ludwig et al., 1979). Two green bands were observed in the native gel; the high-mobility band was cytochrome *c*

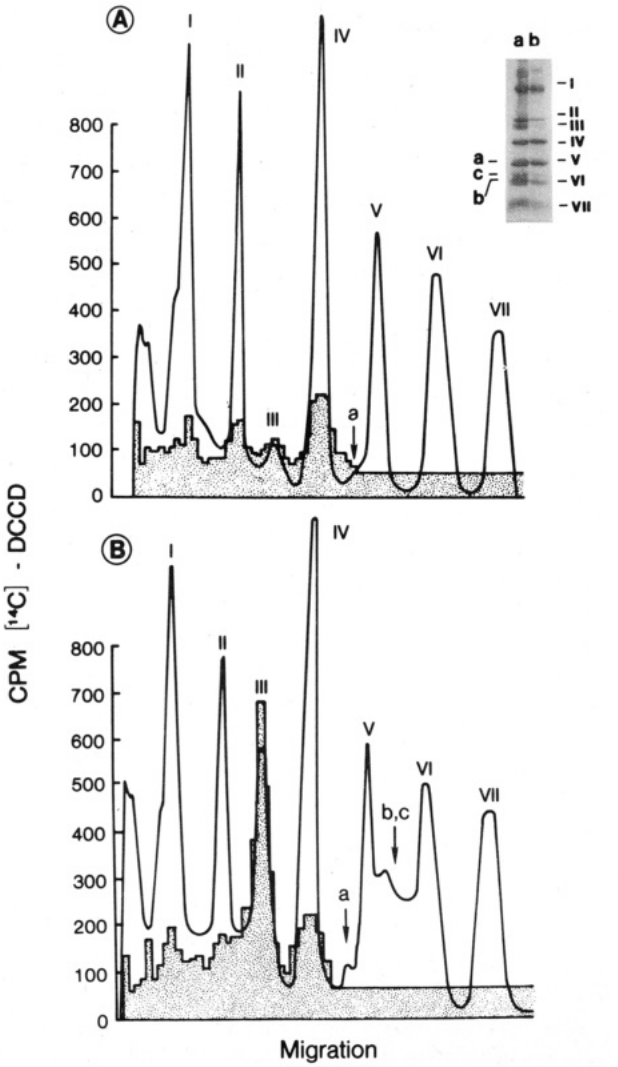

FIGURE 1: [^{14}C]DCCD labeling of control and subunit III deficient cytochrome *c* oxidase. [^{14}C]DCCD labeling was performed as described in Prochaska et al. (1981) and under Experimental Procedures. Control enzyme and subunit III deficient enzyme were prepared as described under Experimental Procedures. The stoichiometry of DCCD added to enzyme was 130 for subunit III deficient enzyme (A) and control enzyme (B). The insert shows control enzyme (lane a) and (lane b) subunit III deficient enzyme run on $NaDodSO_4$–urea slab–PAGE (Fuller et al., 1981).

oxidase deficient in subunit III, and the low-mobility band was control enzyme. Each fraction was assayed for heme *a* content, electron-transfer activity, and subunit content by $NaDodSO_4$–PAGE. Figure 1 shows that subunit III and components b (M_r 9419) and c (M_r 10068) (Buse et al., 1983) are almost completely removed from the enzyme by the native gel treatment; however, the polypeptide composition of control enzyme is unaffected by the native gel treatment.

Native gel treatment of cytochrome *c* oxidase results in a 7.5% yield of enzyme deficient in subunit III (Table I). The heme *a* to milligram of protein ratio increases in the enzyme depleted in subunit III, indicating a loss of protein in the preparation. The control enzyme heme to protein ratio is unchanged by the native gel electrophoresis and column chromatography purification steps. The enzyme is not significantly denatured during removal of subunit III as evidenced by a maximum electron-transfer turnover number of 383 s^{-1} (78% of control, Table I).

Quantitative Determination of Subunit III Content in Subunit III Deficient Cytochrome c Oxidase. The subunit III content of the preparation of cytochrome *c* oxidase deficient

Table I: Purification of Cytochrome *c* Oxidase Devoid of Subunit III

step	mg of protein[a]	nmol of heme *a*/mg of protein[b]	% yield	equiv of e^- (mol of CO)$^{-1}$ s^{-1}[c]
(I) starting material	60.7	8.5		490
(II) native gel + Sepharose 2B column	4.6	10.1	7.5	383
max heme *a* to protein ratio expected[d]		10.1–11.9		

[a] Protein was estimated by the Lowry method (1951) and by Coomassie Blue staining intensity on $NaDodSO_4$–urea gels (Prochaska et al., 1981). [b] Heme *a* concentration was monitored by its absorbance spectrum (Van Gelder, 1966). [c] Electron-transport assays were performed as described in Thompson and Ferguson-Miller (1983). Control and subunit III deficient enzymes were dispersed in Triton X-100 in a buffer containing 4 mg/mL asolectin, 0.2% cholate, and 50 mM KH_2PO_4, pH 7.4 (Vik et al., 1981). [d] Maximum heme *a*/protein ratio is based on the sums of molecular weights of the 12-subunit or 9-subunit enzyme (Buse et al., 1982).

Table II: Removal of Subunit III from Cytochrome *c* Oxidase As Detected by Different Methods

method	control enzyme	subunit III deficient enzyme	% decrease
(1) area of Coomassie stain (units/subunit III)	162	30	82
(2) [^{14}C]DCCD labeling (cpm/subunit III)[a]	1690	220	87

[a] [^{14}C]DCCD labeling was performed as described in Prochaska et al. (1981) and under Experimental Procedures. [^{14}C]DCCD labeling of subunit III was determined by integrating the area under the subunit III peak in control and subunit III deficient enzymes and correcting for differences in the amount of protein on the gel. The results presented are a typical preparation of subunit III depleted enzyme. The subunit III content of five preparations was determined. Four preparations were found to vary less than 5% from the presented data. One preparation was observed to have 94% of its subunit III removed.

in subunit III was monitored by Coomassie Blue staining intensity of $NaDodSO_4$–urea–PAGE. Standard curves of Coomassie Blue staining intensity for each of the subunits were generated with untreated enzyme. The subunit III band exhibited a linear increase in dye binding as a function of protein concentration (data not shown). Different amounts of subunit III deficient enzyme were loaded onto gels, and after similar staining and destaining regimes as control enzyme, areas under each subunit were integrated. This method suggests that 82% of subunit III was removed by the native gel electrophoresis method (Table II).

We also monitored subunit III content in our preparation by reacting both control and subunit III deficient enzyme with [^{14}C]DCCD under conditions where subunit III is the predominate reactive species (130 mol of DCCD/mol of enzyme) (Casey et al., 1980; Prochaska et al., 1981). Figure 1 shows $NaDodSO_4$–PAGE of control (B) and subunit III deficient cytochrome *c* oxidase (A) reacted with [^{14}C]DCCD. When corrected for differences in protein concentration on the gels, an 87% decrease of [^{14}C]DCCD incorporated into subunit III deficient enzyme was observed (Table II). It could be argued that subunit III, due to its relative hydrophobicity (Buse et al., 1983), could be aggregated on the top of the gel and thus lead to an underestimation of the subunit III content of the native gel preparation. Clearly, this is not occurring due to similar total radioactivity being observed on the top of both control and subunit III deficient enzyme gels. At least 82% but as much as 87% of subunit III is removed by the native

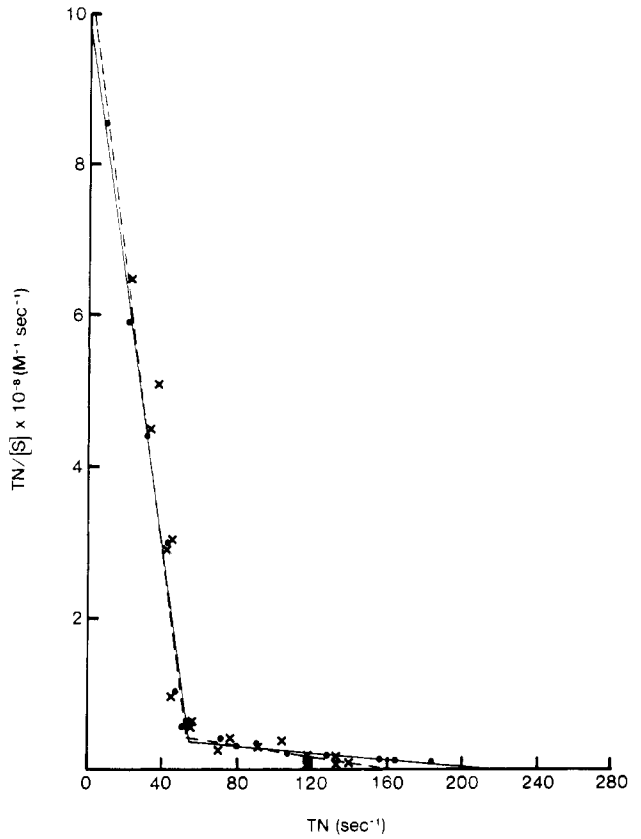

FIGURE 2: Eadie–Hofstee plot of the reaction of cytochrome *c* with control and subunit III deficient cytochrome *c* oxidase. Control (●) and subunit III deficient (×) cytochrome *c* oxidase was dispersed in Triton X-100 (3 mg/mg of enzyme) and activated in a buffer containing 4 mg/mL asolectin, 0.2% cholate, and 50 mM KH_2PO_4, pH 7.4, as described in Vik et al. (1981). Enzyme kinetic assays were performed as described in Ferguson-Miller et al. (1978) with modifications of Vik et al. (1981). All other conditions were as described under Experimental Procedures. The turnover number (TN) is the moles of cytochrome *c* oxidized per second per mole of cytochrome *c* oxidase (heme aa_3), and [S] is the molar concentration of cytochrome *c*.

gel electrophoresis treatment (Table II).

Interaction of Cytochrome c with Subunit III Deficient Cytochrome c Oxidase. We investigated the effects of the removal of subunit III on the ability of the enzyme to interact with its substrate, cytochrome *c*. An Eadie–Hofstee plot of the interaction of cytochrome *c* with control cytochrome *c* oxidase exhibits both the high-affinity (apparent $K_m = 5.7 \times 10^{-8}$ M) and low-affinity (apparent $K_m = 2.4 \times 10^{-6}$ M) phases of kinetic activity (Figure 2) (Ferguson-Miller et al., 1978). After removal of subunit III, both the high-affinity (apparent $K_m = 5.3 \times 10^{-8}$ M) and low-affinity (apparent $K_m = 2.4 \times 10^{-6}$ M) phases are unaffected. The V_{max} of the high-affinity site is unperturbed by removal of subunit III (57 s^{-1}) as compared to control enzyme (56 s^{-1}). However, the V_{max} for the high- and low-affinity sites for subunit III deficient enzyme (160 s^{-1}) is decreased by 25% as compared to control enzyme (212 s^{-1}). When corrected for the activity of the high-affinity site, the inhibition in the low-affinity site is 35%. The low-affinity site has been shown to be endogenous diphosphatidylglycerol associated with the enzyme (Vik et al., 1981). Enzyme deficient in subunit III contains 0.02–0.04 μg of phospholipid/μg of protein, whereas the phospholipid content of untreated enzyme ranges from 0.03 to 0.06 μg/μg of protein, enough to satisfy the minimum diphosphatidylglycerol requirement for enzymatic activity (Robinson et al., 1980; Vik et al., 1981).

The substrate binding capacity of the control anf subunit III deficient enzyme was also examined (Ferguson-Miller et al., 1978). Control enzyme bound 1.10 ± 0.19 mol of cytochrome *c*/mol of heme aa_3 at the high-affinity site ([cytochrome *c*] = 0.6 μM) and 2.20 ± 0.43 mol of cytochrome *c*/mol of aa_3 at the high- plus low-affinity site ([cytochrome *c*] = 20 μM). Enzyme deficient in subunit III bound 0.98 ± 0.37 and 2.39 ± 0.80 mol of cytochrome *c*/mol of heme aa_3 at the high-affinity and high- plus low-affinity sites. This suggests that there is little or no change in the interaction of cytochrome *c* with cytochrome *c* oxidase after the removal of subunit III and components b and c.

Proton Translocation in Phospholipid Vesicles Containing Control and Subunit III Deficient Cytochrome c Oxidase. Phospholipid vesicles containing the control fraction from the native gel exhibited respiratory control ratios (Hinkle et al., 1972) ranging from 2.7 to 7.2 with a mode of 4.3 and an electron-transfer turnover number of 122 s^{-1} at pH 7.4 (seven preparations). Subunit III deficient enzyme containing phospholipid vesicles showed respiratory control ratios ranging from 3.1 to 9.3 with a mode of 3.9 (six preparations) and a turnover number at pH 7.4 of 99 s^{-1}.

We monitored the ability of these phospholipid vesicles to retain [^{14}C]sucrose during dialysis (Uratani & Cramer, 1981). After the dialysis regime, both types of liposomes exhibited high respiratory control ratios and had an internal aqueous volume of 3.5–4.0% of the initial volume, suggesting liposomes of similar size. Upon chromatography on Sephadex G-75 to separate intravesicular from extravesicular [^{14}C]sucrose, all of the [^{14}C]sucrose in both control and enzyme deficient in subunit III containing liposomes eluted at the void volume. These results suggest that the liposomes containing enzyme deficient in subunit III are intact and not freely permeable to small molecules such as sucrose (M_r 342) or protons.

After the addition of ferrocytochrome *c*, a solution of liposomes containing the control enzyme exhibited the characteristic initial acidification followed by alkalinization (Figure 3A). The acid phase represents vectorial proton translocation, whereas the alkaline phase represents consumption of protons in the oxygen reduction reaction (Krab & Wikstrom, 1978). Figure 3D shows that, in the presence of the uncoupling ionophore CCCP, only the alkaline phase of the reaction occurs. The extent of the acidification phase is less in phospholipid vesicles containing subunit III deficient enzyme retaining 15% (Figure 3B) and 5% (Figure 3C) of its subunit III content. Phospholipid vesicles containing control enzyme show a maximum ejection of 1.94 ± 0.49 nequiv of protons at 4.2–6.0 turnovers (17–24 nequiv of cytochrome *c* added/nmol of heme aa_3), whereas liposomes containing subunit III deficient enzyme show a 64% decrease in the measured extent, yielding 0.71 ± 0.23 nequiv of protons ejected (Table III) at a similar number of enzyme turnovers. Apparent H^+/e^- ratios were calculated by the extrapolation method of Krab and Wikstrom (1978). Phospholipid vesicles containing control enzyme had an apparent H^+/e^- of 0.52 ± 0.11, whereas liposomes containing enzyme deficient in subunit III had an H^+/e^- of 0.21 ± 0.04. When the apparent H^+/e^- ratio of the phospholipid vesicles containing subunit III deficient enzyme was corrected for the content of subunit III (Table III), H^+/e^- decreased to 0.13. The calculated value appears correct, since enzyme containing 5% of its native subunit III content undergoes an apparent H^+/e^- ratio of 0.13 (Figure 3D). This calculated 75% decrease in the H^+/e^- ratio of the subunit III depleted cytochrome *c* oxidase liposomes suggests that subunit III is responsible for most of the vectorial proton translocation in

Table III: H^+ Translocating Properties of Phospholipid Vesicles Inlaid with Control or Subunit III Depleted Cytochrome *c* Oxidase[a]

prepn	extent of acid phase (nequiv of H^+)[b]	% inhibn	app H^+/e^-[c]	% inhibn	corrected H^+/e^-[d]	% inhibn
control enzyme	1.94 ± 0.49		0.52 ± 0.11		0.52 ± 0.11	
subunit III deficient enzyme	0.71 ± 0.23	64	0.21 ± 0.04	60	0.13 ± 0.04	75

[a] Phospholipid vesicles inlaid with control or subunit III deficient cytochrome *c* oxidase were prepared as described in Figure 4, and proton translocation activity was monitored as described under Experimental Procedures. Control enzyme and subunit III deficient enzyme containing phospholipid vesicles underwent from 4.2 to 6.0 and from 3.8 to 6.4 turnovers, respectively. Five separate preparations of control enzyme containing liposomes and seven preparations of subunit III deficient enzyme containing phospholipid vesicles were used to obtain the data. [b] This column represents the extent of the acid phase observed upon addition of ferrocytochrome as presented in Figure 3. [c] The apparent H^+/e^- was measured as described by Krab and Wikstrom (1978). This apparent H^+/e^- was extrapolated from the intercept of the maximum rates of H^+ consumption (alkaline phase) and production (acid phase) from the base line and dividing this number of nanoequivalents of protons (H^+) released by the amount of cytochrome *c* added to the assay. [d] The corrected apparent H^+/e^- ratio was calculated by assuming the 15% of cytochrome *c* oxidase containing subunit III in the subunit III deficient enzyme preparation has a similar H^+/e^- ratio as the control.

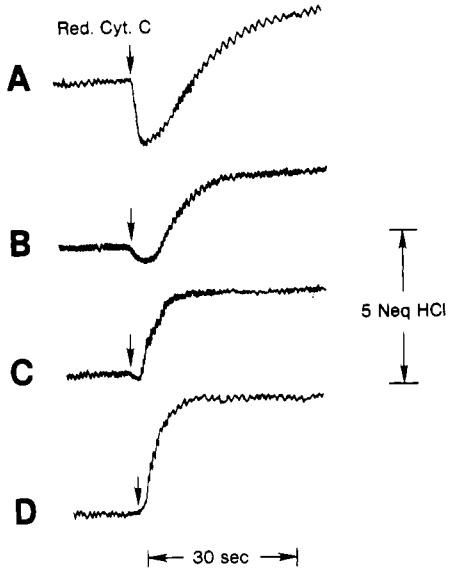

FIGURE 3: Changes in extravesicular pH induced by electron transfer in phospholipid vesicles inlaid with control and subunit III deficient cytochrome *c* oxidase. Phospholipid vesicles inlaid with control (traces A and D) or subunit III deficient (traces B and C) enzyme were prepared as described under Experimental Procedures. Assay conditions were as described under Experimental Procedures. Electron transfer and subsequent proton translocation were initiated by the addition of 5.5 (traces A and D), 3.0 (trace B), and 2.4 nequiv (trace C) of ferrocytochrome *c*. The assay mixture in trace D contained 5 μM CCCP. The content of subunit III in the preparations assayed in trace B was 15% and in trace C was 6% as estimated by methods detailed under Experimental Procedures. The buffering capacity of the suspension was estimated by the addition of 5 nequiv of standard HCl.

the enzyme. [For an alternative explanation, see Discussion and Puettner et al. (1985).]

Discussion

In an attempt to delineate the role of subunit III in vectorial proton translocation, subunit III depleted bovine heart mitochondrial cytochrome *c* oxidase has been prepared by native gel electrophoresis (Ludwig et al., 1979), ion-exchange chromatography (Saraste et al., 1981; Pentilla, 1983), limited proteolysis using α-chymotrypsin (Puettner et al., 1985), and affinity chromatography using yeast cytochrome *c* (Bill & Azzi, 1982). In addition, rat liver mitochondrial cytochrome *c* oxidase depleted in subunit III has been isolated by using dodecyl β-D-maltoside and cytochrome *c* affinity chromatography (Thompson & Ferguson-Miller, 1983).

The removal of subunit III from the bovine heart and rat liver enzyme appears to inhibit its electron-transfer activity, although most preparations retain at least 70% of the activity (Saraste et al., 1981; Bill & Azzi, 1982; Thompson & Ferguson-Miller, 1983). The effect of removal of subunit III on steady-state enzyme kinetic parameters of the interactions of cytochrome *c* with cytochrome *c* oxidase is more controversial. The alkaline-treated (Penttila, 1983) and rat liver (Thompson & Ferguson-Miller, 1983) preparations of subunit III deficient enzyme exhibit characteristic biphasic Eadie–Hofstee plots (Ferguson-Miller et al., 1978) with K_m and V_{max} values for each site similar to those for the control enzyme when assayed polarographically in the presence of TMPD. The cytochrome *c* affinity column purified preparation shows monophasic kinetics in both the spectrophotometric and polarographic methods of assay and biphasic kinetics in the spectrophotometric method when assayed in the presence of exogenous added asolectin (Nalecz et al., 1985). The differences observed may relate to changes in the aggregation state of the enzyme when subunit III is removed [see Nalecz et al. (1985) for a complete discussion].

Subunit III deficient enzyme has been incorporated into liposomes, and the effects on vectorial proton translocation have been investigated. The alkaline-treated (Penttila, 1983) and cytochrome *c* affinity column purified (Thelen et al., 1985) preparations completely lack the ability to translocate protons. The affinity column purified preparation can abstract 1.0 H^+/e^- in the intravesicular space (for oxygen reduction) as compared to 2.0 H^+/e^- for liposomes containing control enzyme (Thelen et al., 1985). The alkaline-treated preparation exhibits a decrease of 50% in the observed K^+/e^- uptake compared to control enzyme, consistent with an abolition of vectorial proton movement (Penttila, 1983). The rat liver preparation exhibits a 15–50% decrease in the observed H^+/e^- ratio when compared to control bovine heart enzyme, suggesting that subunit III is involved in proton translocation but not necessary for the mechanism of proton release (Thompson et al., 1984). In contrast, subunit III deficient enzyme isolated by α-chymotrypsin treatment (Puettner et al., 1985) exhibited an external H^+/e^- ratio of 0.5 compared to 0.8 for control enzyme. Puettner et al. (1985) only observed a complete loss of vectorial proton translocation in phospholipid vesicles containing subunit III depleted enzyme that exhibited a RCR of less than 3.0, suggesting that the proton permeability of the liposome not the removal of subunit III was responsible for the loss of vectorial proton translocation. They have shown that the proton decay rate in control enzyme containing phospholipid vesicles can be stimulated by different concentrations of CCCP, thus suggesting that slight changes in proton permeability can create a lowering of the observed H^+/e^- ratio. Also, they suggest that changes in subunit interactions in cytochrome *c* oxidase are induced during the removal of subunit III that could cause perturbations in the efficiency of proton pumping.

In this study, we observed biphasic steady-state enzyme

kinetics for the interaction of cytochrome *c* with subunit III deficient cytochrome *c* oxidase prepared by native gel electrophoresis and confirmed the results of Penttila (1983) and Thompson and Ferguson-Miller (1983) for other preparations of subunit III depleted enzyme. Furthermore, we have shown that cytochrome *c* binding to the enzyme is unaffected by the removal of subunit III by direct thermodynamic binding experiments. Upon incorporation into phospholipid vesicles, the native gel preparation lost 60% of its efficiency (H^+/e^- ratio) of proton translocation as compared to liposomes containing control enzyme. Also, we observed a loss of the apparent H^+/e^- ratio dependent on the subunit III content in the preparation, and when corrected for unremoved subunit III, the contribution of subunit III to vectorial proton translocation was calculated to be 75%. We think the criticisms raised by Puettner et al. (1985) merit consideration, but our preparation of liposomes containing subunit III deficient enzyme has an RCR of greater than 6 yet still exhibits a decrease in proton-pumping efficiency as compared to control enzyme containing liposomes. We observe very little change in the visible absorbance spectrum (reduced absorbance maxima at 443 and 605 nm) and in the electron-transfer activity of the enzyme induced by subunit III removal. However, positive evidence for the role of subunit III in the transmembrane movement of protons in cytochrome oxidase must be obtained by reconstitution experiments in which purified subunit III is added back to the subunit III depleted enzyme and recovery of the efficiency (H^+/e^- ratio) is observed in order to completely resolve this controversy.

Our results support the findings of Penttila (1983), Thompson et al. (1984), and Thelen et al. (1985) and provide further evidence that electron transfer in cytochrome *c* oxidase is loosely coupled to vectorial proton translocation. Other experimental evidence that supports this concept is that DCCD blocks proton-translocation activity to a much greater extent than electron-transfer activity and that polyclonal antibodies raised against subunit III block vectorial proton translocation in phospholipid vesicles (Chan & Freedman, 1983). These results taken together suggest that the subunit(s) where protons are mechanistically released by electron transfer and the subunit domain (perhaps subunit III) that translocates the protons across the membrane is (are) different (Casey et al., 1980: Thompson et al., 1984).

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Variations in the Oxidation–Reduction Behavior of Liganded Species of *Pseudomonas* Cytochrome Oxidase†

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ABSTRACT: In an effort to determine the steady-state redox properties of *Pseudomonas aeruginosa* cytochrome cd_1, changes in absorption spectra after the addition of excess reductant (ascorbate, ferrous ethylenediaminetetraacetic acid) were monitored for degassed unliganded enzyme and samples in the presence of CO and CN^- at pH 6.0, 8.0, or 10.0. Plots of $[c^{2+}]/[c^{3+}]$ vs. $[d^{2+}]/[d^{3+}]$ indicate that a "pseudoequilibrium" was reached for all samples at pH 8.0. Calculated values of ΔE_{d-c}, the difference in reduction potential between the heme *c* and heme *d* moieties, at pH 8.0 were -25 ± 5 (unliganded), -10 ± 5 (enzyme–CO), and -25 ± 5 mV (enzyme–CN). Relative rates of heme *c* and heme *d* reduction were found to be dependent upon type of ligand, reductant, and pH. Evidence for a cooperative heme *c*–heme *d* interaction is discussed.

Pseudomonas aerguinosa cytochrome cd_1 (ferrocytochrome *c*-551:oxygen oxidoreductase, EC 1.9.3.2) is the terminal enzyme in the electron-transport chain of bacteria grown anaerobically in the presence of nitrate (Yamanaka et al., 1963). This water-soluble enzyme is a dimer of M_r 120 000; each subunit containing a heme *c* and a heme *d* (Kuronen & Ellfolk, 1972; Gudat et al., 1973; Kuronen et al., 1975; Parr et al., 1976; Saratse et al., 1977). Its physiological function is the one-electron reduction of NO_2^- to NO; however, the enzyme is also capable of catalyzing the four-electron reduction of O_2 to H_2O (Yamanaka et al., 1961), utilizing electrons donated either from *Pseudomonas* ferrocytochrome *c*-551 or reduced azurin. Electrons are donated to the enzyme at the heme *c* sites (Wharton et al., 1973; Parr et al., 1977), and substrate reduction occurs at heme *d* (Yamanaka & Okunuki, 1963a). While the former is covalently bound to the protein, the latter heme moiety is bound noncovalently and may be extracted and subsequently reinserted (Yamanaka & Okunuki, 1963b; Hill & Wharton, 1978). Therefore, those portions of the enzyme's absorption spectrum belonging to each heme type have been identified and found to be significantly separated, so that changes at the heme sites due to ligand binding and electron transfer may be easily monitored.

While extensive information is available on the rather complex kinetics of ligand binding and electron transfer within *Pseudomonas* cytochrome oxidase (Parr et al., 1975, 1977; Barber et al., 1978; Greenwood et al., 1978; Shickman & Gray, 1981), relatively little is known about the steady-state behavior of this enzyme. Previous investigators have carried out reductive titrations of the enzyme by using both excess (Shimada & Orii, 1976) and stoichiometric (Horio et al., 1961; Blatt & Pecht, 1979) amounts of reductant [ascorbate, ferrous ethylenediaminetetraacetic acid (FeEDTA), and/or durohydroquinone] and monitoring changes in the absorption spectrum with time. Using these two techniques has produced somewhat different results. Both Horio et al. (1961) and Blatt and Pecht (1979) present absorption spectra where the oxidized heme *d* peak at 640 nm shifts to ~625 nm upon reduction with no isosbestic points in the 600–700-nm region and calculate a positive difference in reduction potential between heme *c* and heme *d* (ΔE_{c-d}) of 60–70 mV. In the heme *d* absorption spectrum of Shimada and Orii (1976), the 640-nm peak shifts to 654 nm in the reduced spectrum, an isosbestic point appears at 650 nm, and ΔE_{c-d} is calculated to be 24 mV. Previously reported variations in the reduced heme *d* spectrum were only observed when dithionite was used as the reductant (Parr et al., 1974).

In an effort to resolve these discrepancies, we present the results of reductive titrations of *Pseudomonas* cytochrome oxidase under a variety of anaerobic conditions at room temperature: (1) Unliganded enzyme, pH 8.0, was examined with both excess and stoichiometric amounts of ascorbate and ferrous EDTA as reductants; (2) in the presence of excess ascorbate, the reduction process was examined at pH 6.0, 8.0, and 10.0 for the unliganded enzyme and samples to which either CO or CN^- had been added. Relative rates of heme *c* and heme *d* reduction have been found to vary under these conditions, differences in reduction potential of the two hemes occur as a function of ligand, and there is apparently cooperative interaction between heme *c* and heme *d*.

Experimental Procedures

Materials. Pseudomonas cytochrome oxidase was prepared according to the method of Gudat et al. (1973) and used without further purification. Enzyme concentration was 1–5 μM in titrated samples. Sodium ascorbate was prepared by neutralization of a saturated solution of ascorbic acid with 10

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