Journal of Bioenergetics and Biomembranes, Vol. 19, No. 2, 1987

MINI-REVIEW

On the Role of Subunit III in Proton Translocation in Cytochrome *c* Oxidase

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Received July 15, 1986

Abstract

Mammalian mitochondrial cytochrome *c* oxidase catalyzes the transfer of electrons from ferrocytochrome *c* to molecular oxygen in the respiratory chain, while conserving the energy released during its electron transfer reactions by the vectorial movement of protons across the inner membrane of the mitochondrion. The protein domain that translocates the protons across the membrane is currently unknown. Recent research efforts have investigated the role of one of the transmembrane subunits of the enzyme (III, M_r 29,884) in the vectorial proton translocation reaction. The data that favor subunit III as integral in vectorial proton translocation as well as the data that support a more peripheral role for subunit III in proton translocation are reviewed. Possible experimental approaches to clarify this issue are presented and a general model discussed.

Key Words: Mitochondria; respiratory chain; cytochrome *c* oxidase; electron transfer and energy transduction; vectorial proton translocation; membrane protein reconstitution; multi-subunit enzyme structure.

Introduction

Cytochrome *c* oxidase (EC 1.9.3.1) is an integral membrane enzyme complex that mediates the reduction of molecular oxygen into water (Azzi and Casey, 1979; Wikstrom *et al.*, 1981; Capaldi *et al.*, 1983). In prokaryotes, the enzyme is intercalated into the plasma membrane, whereas in eukaryotes, the enzyme is localized in the inner membrane of the mitochondrion. The complex can be easily isolated and purified from bacteria (Ludwig, 1980; Poole, 1983)

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0145-479X/87/0400-0143$05.00/0

and mammalian mitochondria (Hatefi, 1979; Thompson and Ferguson-Miller, 1983) by detergent extraction and ammonium sulfate fractionation. Recent research efforts have focused on understanding the structure and function of the enzyme by examining its isolated, purified form.

The Mitchell hypothesis (Mitchell, 1979) predicts that the electron transfer reactions in the respiratory chain drive the vectorial movement of protons across the mitochondrial inner membrane and the resulting pH gradient is then used to synthesize ATP. Thus, the energy generated by the oxidation-reduction reactions in actively respiring cells is transduced into chemical energy by the generation of a protonmotive force across the inner membrane of the mitochondrion. Cytochrome *c* oxidase is one site where the mitochondrion conserves the electrochemical energy released during electron transfer by the vectorial movement of protons across the inner membrane (Wikstrom and Krab, 1979; Casey and Azzi, 1983; Mitchell *et al.*, 1985). When the resulting pH gradient across the mitochondrial inner membrane is relaxed, ATP synthesis or translocation of ions occurs (Mitchell, 1979; Sordahl, 1979). Isolated mitochondrial cytochrome *c* oxidase has been used extensively to study the vectorial proton translocation process at the molecular level due to the large amount of information available about the structure of the enzyme (Wikstrom and Krab, 1979; Casey and Azzi, 1983). In addition, some work has focused on the bacterial enzymes due to their less complex structure, but less information is currently known (Poole, 1983). This review will limit its scope to work performed on the mammalian mitochondrial enzyme but will include salient information about other species when necessary.

Cytochrome *c* oxidase catalyzes the following reaction in the mitochondrial respiratory chain:

$$4 \text{ cytochrome } c^{2+} + 8H^+ + O_2 \rightarrow 4 \text{ cytochrome } c^{3+} + 2H_2O + 4H^+$$

The isolated enzyme complex contains four oxidation–reduction centers, two hemes (*a* and a_3), and two copper atoms (Cu_A and Cu_B) that mediate the transfer of electrons from ferrocytochrome *c* to molecular oxygen (Fig. 1) (Erecinska and Wilson, 1978; Wikstrom *et al.*, 1981; Malstrom, 1979). Heme a_3 can be distinguished from heme *a* by its ability to bind ligands such as oxygen, cyanide, azide, and carbon monoxide (Erecinska and Wilson, 1978; Wikstrom *et al.*, 1981).

The subunit structure of the mammalian heart mitochondrial enzyme is well defined (Buse *et al.*, 1983). Nine to thirteen distinct polypeptides (Buse *et al.*, 1983; Kadenbach and Merle, 1981) comprise the enzyme in a one-to-one stoichiometry. The subunits range in molecular weight from 56,965 to 4962 and their primary amino acid sequences are known from direct protein sequencing (Buse *et al.*, 1983) or from the mitochondrial DNA

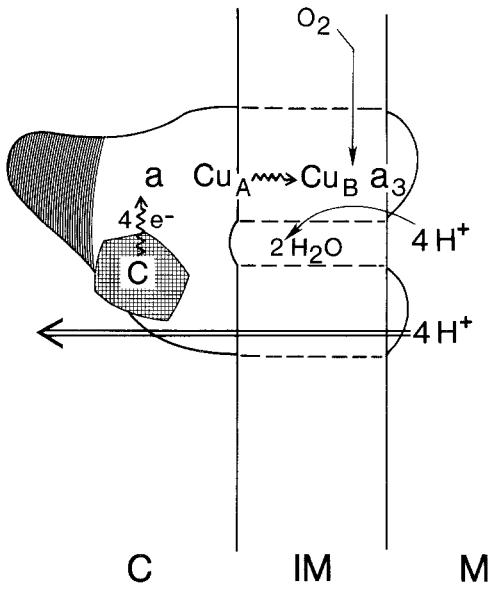

Fig. 1. Schematic drawing of the structure and function of mammalian mitochondrial cytochrome *c* oxidase. This schematic of bovine heart cytochrome *c* oxidase is based on the three-dimensional reconstruction of two-dimensional crystalline arrays as described by Fuller *et al.* (1979) and Deatherage *et al.* (1982). The locations of the oxidation–reduction centers are not drawn to scale.

sequence (Anderson *et al.*, 1982). The three largest subunits are encoded on mitochondrial DNA (Anderson *et al.*, 1982), whereas the remaining smaller subunits are encoded by nuclear DNA.

Two of the mitochondrially encoded subunits of the bovine heart enzyme [I and II, nomenclature of Capaldi *et al.*, (1983)] appear to contain the oxidation-reduction centers of the enzyme. Subunits I (M_r 56,965) and II (M_r 26,049) appear to contain heme *a* and Cu_A (Winter *et al.*, 1980). Subunit II has a primary amino acid sequence homologous to other blue copper proteins such as plastocyanin and azurin (Buse *et al.*, 1982). Subunit I has a primary amino acid sequence analogous to other oxygen-binding proteins and may contain the oxygen-binding site (heme a_3 and Cu_B) (Welinder and Mikkelsen, 1983). The substrate for the enzyme, cytochrome *c*, occupies a high-affinity binding site on subunit II, as demonstrated by chemical cross-linking (Briggs and Capaldi, 1978; Bisson *et al.*, 1978, 1980; Fuller *et al.*, 1981) and competitive binding experiments (Millet *et al.*, 1982, 1983).

The surface topography of the subunits of the bovine heart enzyme has been mapped with radioactive chemical modification reagents, including

diazonium benzenesulfonate (DABS)[2] (Ludwig *et al.*, 1979), *N*-(4-azido-2-nitrophenyl)-2-aminoethylsulfonate (NAP-taurine) (Prochaska *et al.*, 1980), and also with antibodies to each purified subunit (Chan and Tracy, 1978). In addition, an azido derivative of phosphatidylcholine has been used to examine the sites of phospholipid interaction with the bovine heart enzyme (Prochaska *et al.*, 1980). These studies have shown that subunits II and III (M_r 29,884) are membrane-spanning subunits, while subunits IV (M_r 17,153), V (M_r 12,436), and VII's (M_r 5441, 4962, and 6244) are localized on the matrix face of the enzyme. Subunits I–IV and VII's interact with the phospholipid bilayer, whereas subunit VI (M_r 8481) is buried or shielded from both the hydrophilic and hydrophobic milieu.

Mammalian cytochrome *c* oxidase is an oxidation–reduction-linked vectorial proton pump (Fig. 1) as demonstrated in both intact mitochondria (Wikstrom and Saari, 1977; Wikstrom, 1984) and in reconstituted systems using phospholipid vesicles that contain the isolated enzyme (COV) (Krab and Wikstrom, 1978; Casey and Azzi, 1983; Proteau *et al.*, 1983). The COV have been shown to translocate one proton per electron transferred across the membrane, and the external release of protons is dependent on the intravesicular buffering capacity (Krab and Wikstrom, 1978). This observed stoichiometry is dependent on enzyme turnover (defined as 4 electrons transferred/heme aa_3) (Sigel and Carafoli, 1980; Proteau *et al.*, 1983) and can only be observed in the presence of membrane potential-dissipating agents such as valinomycin and potassium (Krab and Wikstrom, 1978; Casey *et al.*, 1984; Moroney *et al*,1984; Singh and Nicholls, 1986). The proton translocation stoichiometry is unaffected by the phospholipid composition of the COV, and proton release is not observed upon the addition of ferricytochrome *c* or in the presence of azide (Casey and Azzi, 1983). In addition, the intravesicular pH has been observed to increase at a stoichiometry of two protons per electron transferred; one proton is used in the catalytic reduction of oxygen, and the second proton is translocated across the phospholipid bilayer (Thelen *et al.*, 1985). Therefore, mammalian cytochrome *c* oxidase is an oxidation–reduction-linked vectorial proton pump. A more detailed and rigorous treatment of this topic is available in Wikstrom and Krab (1979) and Casey and Azzi (1983).

[2]Abbreviations: DABS, diazonium benzenesulfonate; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; NCCD, *N*-(2,2,6,6-tetramethylpiperidyl-1-oxyl)-*N*′-cyclohexylcarbodiimide; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; COV, asolectin phospholipid vesicles containing control cytochrome *c* oxidase; COV-III, asolectin phospholipid vesicles containing subunit III-deficient cytochrome *c* oxidase; COV-T, asolectin phospholipid vesicles containing trypsin-treated cytochrome *c* oxidase; H^+/e^-, ratio of vectorial protons translocated per electron transferred; K^+/e^-, ratio of vectorial potassium ions translocated per electron transferred; RCR, respiratory control ratio; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine.

A focus of current research on the enzyme is an attempt to understand the molecular mechanism of proton translocation and how the subunit structure of the enzyme dictates its dual catalytic activities of electron transfer and vectorial proton translocation. The protein structural domain that mediates the transmembrane movement of protons in the mammalian enzyme is currently unknown. One potential candidate for this domain is subunit III (M_r 29,884), a mitochondrially synthesized subunit. Our focus in this review will be on the role of subunit III in the vectorial proton translocation reaction, and we will examine the data that support or dismiss the involvement of subunit III in the proton translocation reaction. We will limit the majority of our discussion to the role of subunit III from the mammalian enzyme, but will include results from other pertinent species when necessary.

Evidence Supporting the Involvement of Subunit III of Mammalian Cytochrome *c* Oxidase in Proton Translocation

Subunit III Is a Membrane-Spanning Polypeptide

One criterion that a putative proton-translocating domain in cytochrome *c* oxidase (Fig. 1) must satisfy is that it should span the inner mitochondrial membrane. Subunit III of bovine heart cytochrome *c* oxidase has been shown to be a transmembrane subunit by using two different techniques. One technique involved the use of water-soluble, lipid-insoluble, radioactive chemical modification reagents to map the surface topography of the subunits in the enzyme (Eytan *et al.*, 1975; Ludwig *et al.*, 1979). Mitochondrial membranes and submitochondrial particles (everted mitochondria) resolved with anti-ATP synthetase antibody were reacted with [^{35}S]-DABS, immunoprecipitated with anti-cytochrome *c* oxidase antibody, and run on SDS-PAGE to monitor the incorporation of [^{35}S]-DABS into each subunit (Ludwig *et al.*, 1979). These experiments showed that subunits II and III were membrane-spanning polypeptides.

Limited proteolysis of the rat liver (Jarausch and Kadenbach, 1985) and bovine heart enzyme (Zhang *et al.*, 1984) was used to determine the surface topography of the subunits. α-Chymotrypsin treatment of COV that had 90% of the bovine heart cytochrome *c* oxidase oriented with its cytochrome *c* binding face exposed to the extravesicular medium resulted in the cleavage of subunit III into at least three fragments with different molecular weights (Zhang *et al.*, 1984). Also, rat liver mitoplasts and submitochondrial particles were subjected to limited proteolysis using trypsin, pronase, and subtilisin, and polyclonal antibodies against each subunit were used to monitor the

orientation of subunits of the enzyme (Jarausch and Kadenbach, 1985). Subunit III was digested by pronase and subtilisin in mitoplasts and was uncleaved in the sonicated submitochondrial particles, suggesting that subunit III is exposed on the cytoplasmic side of the inner mitochondrial membrane.

The results from all of these experiments taken together suggest that subunit III of the mammalian enzyme is a membrane-spanning polypeptide. While this observation is *not* an absolute requirement for a polypeptide to be involved in proton translocation, it clearly emphasizes the important role of a transmembrane subunit in maintaining the three-dimensional structure of the enzyme.

Reagents That Bind to Subunit III Inhibit Proton Translocation in COV

Proton translocation through cytochrome *c* oxidase is likely to be facilitated by amino acid side chains in one or more subunits of the enzyme. One potential candidate for the functional group that could mediate the translocation of protons across a membrane is the free carboxyl group of glutamic and aspartic acid residues in proteins. An approach to studying the amino acid functional groups that are involved in a catalytic process in an enzyme is chemical modification of amino acid residues by group-specific chemical reagents (Means and Feeney, 1971).

An example of a chemical modification reagent that is specific for carboxyl groups in proteins is dicyclohexyl carbodiimide (DCCD) (Khorana, 1954; Azzi *et al.*, 1984). DCCD is a hydrophobic reagent that has been used extensively as an energy-transfer inhibitor of ATP synthesis in mitochondria (Beechey *et al.*, 1967), chloroplasts (McCarty and Racker, 1967), and bacteria (Evans, 1970). DCCD binds covalently to a hydrophobic membrane sector polypeptide (M_r 7500) of the ATP synthetase (Fillingame, 1980; Sebald and Hoppe, 1981), called the DCCD-binding protein, subunit 9, or subunit c (in *E. coli*) (Hoppe and Sebald, 1984). The locus of DCCD binding in the DCCD-binding protein of the ATP synthetase is on a single glutamic acid in mitochondria and chloroplasts and on an aspartic acid in bacteria (Hoppe and Sebald, 1982). The primary amino acid sequence of DCCD-binding sites from all ATP synthetases contains a buried carboxyl group in a sequence of conserved hydrophobic amino acids (Hoppe and Sebald, 1984).

Bovine heart mitochondrial cytochrome *c* oxidase incorporated into COV has been treated with DCCD and the effects of the modification on electron transfer and proton translocation activities monitored (Casey *et al.*, 1979; Azzi *et al.*, 1984). DCCD inhibited proton translocation activity, but induced much less inhibition of electron transfer activity (Casey *et al.*, 1979). Previous work suggested that a significant inhibition of electron transfer

activity could be induced by reacting the enzyme with DCCD in detergent solution (Carrol and Racker, 1978). Further investigations showed that under all conditions, including the uncoupled state of electron transfer (in the presence of a proton ionophore), the inhibition of electron transfer activity in COV induced by DCCD was much less than the inhibition of proton translocation activity (Casey *et al.*, 1980; Prochaska *et al.*, 1981; Casey *et al.*, 1981). The DCCD-promoted inhibition of proton translocation activity in COV was not a result of changes in the proton permeability of the COV (Casey *et al.*, 1980). In addition, the time course of covalent incorporation of [^{14}C]-DCCD into the enzyme was similar to that observed for the inhibition of proton translocation (Casey *et al.*, 1980; Prochaska *et al.*, 1981). The stoichiometry of [^{14}C]-DCCD that was covalently bound to the enzyme in COV was proportional to the degree of inhibition of proton translocation activity (0.6 mole reagent bound per mole aa_3, 60% inhibition of activity), suggesting that the covalently bound DCCD was responsible for the observed inhibition (Casey *et al.*, 1980; Prochaska *et al.*, 1981). SDS-PAGE of [^{14}C]-DCCD-labeled enzyme from COV showed that the primary site of DCCD binding was in subunit III (Casey *et al.*, 1980), although the absolute stoichiometry of DCCD-binding in subunit III was not sufficient to account for all of the inhibition of proton translocation activity (Prochaska *et al.*, 1981). A spin-labeled analogue (NCCD) of DCCD was also used to inhibit proton translocation activity in COV, and the NCCD competed with DCCD for reactive sites on the enzyme in COV (Casey *et al.*, 1981). The ESR signal from the NCCD was annihilated more quickly in the presence of lipid-soluble reductants (TMPD) than in the presence of water-soluble reductants, emphasizing the hydrophobicity of the NCCD (DCCD) binding site in subunit III (Casey *et al.*, 1981).

[^{14}C]-DCCD-labeled subunit III was isolated and the primary amino acid sequence of the DCCD-containing peptide was determined (Prochaska *et al.*, 1981). [^{14}C]-DCCD was found to be covalently bound to glutamic acid 90 in the primary amino acid sequence of subunit III (Fig. 2). When the primary amino acid sequence of the DCCD-binding site in subunit III from bovine heart is compared to a similar sequence from subunit III from rat and yeast enzyme, a strong amino acid homology near the DCCD-binding site is observed (Fig. 2). When these primary amino acid sequences are compared to those of the DCCD-binding protein from the bovine or yeast ATP synthetase, a striking amino acid sequence homology can be seen, suggesting that the DCCD-binding sites from two widely different polypeptides are similar.

In addition to the chemical modification experiments, the effect of polyclonal antibodies raised against purified subunit III on proton translocation activity in COV has also been determined (Chan and Freedman, 1983).

I L F I I S E* V	SUBUNIT III BOVINE CO
I L F I V S E V	SUBUNIT III RAT CO
L M F V L S E V	SUBUNIT III YEAST CO
L G F A L S E* A	DCCD BINDING PROTEIN - BOVINE ATP SYNTHETASE
L G F A L S E A	DCCD BINDING PROTEIN - YEAST ATP SYNTHETASE

*[^{14}C]-DCCD

Fig. 2. A comparison of the primary amino acid sequence of the DCCD-binding sites in subunit III of cytochrome *c* oxidase with that in the proteolipid of the mitochondrial ATP synthetase. References for the sequences are the following: bovine subunit III, Prochaska *et al.* (1981); yeast subunit III, Thalenfeld and Tzagoloff (1980); bovine ATP synthetase, Hoppe and Sebald (1982); yeast ATP synthetase, Sebald and Hoppe (1981).

Anti-subunit III serum blocked proton translocation in COV, although some uncoupling or stimulation of the basal rate of electron transfer was observed (Chan and Freedman, 1983).

These results indicate that electron transfer in cytochrome *c* oxidase is loosely coupled to proton translocation. The results obtained with DCCD suggest that blocking a carboxyl group on glutamic acid 90 in subunit III inhibits proton translocation. This result can be interpreted in two ways: (1) subunit III is involved in the proton translocation process, perhaps as a protein domain that translocates protons across the membrane (Casey *et al.*, 1980; Thompson *et al.*, 1985). The physical blocking of the proton translocation route by DCCD results in a decrease in proton translocation efficiency. The DCCD-reactive subunit c in the *E. coli* ATP synthetase has been recently proposed to interact with another membrane sector polypeptide (subunit a) to facilitate proton translocation (Cox *et al.*, 1986), and subunit III may interact with other subunits where the protons are released in cytochrome *c* oxidase in a similar manner; (2) DCCD may inhibit the oxidation–reduction linked regulation of proton translocation by binding covalently to subunit III or may perturb the control of the aggregation state of the enzyme leading to inhibition of proton translocation (Puettner *et al.*, 1985; Sarti *et al.*, 1985). (See later discussion.) The inhibition of proton translocation by the polyclonal antibodies to subunit III may be due to some of the antibodies physically blocking the release of the protons. Alternatively,

the antibody may bind to subunit III and induce a conformational change in the enzyme, resulting in a loss of proton translocation activity by an indirect mechanism. These results taken alone cannot conclusively delineate the role of subunit III in the vectorial proton translocation reaction in cytochrome *c* oxidase.

Phospholipid Vesicles Containing Subunit III-Deficient Cytochrome c Oxidase Lose the Ability to Translocate Protons

The role of subunit III in vectorial proton translocation in cytochrome *c* oxidase has also been investigated by the removal of subunit III from the enzyme and reconstituting this preparation into phospholipid vesicles. Subunit III-deficient bovine heart cytochrome *c* oxidase has been prepared by ion-exchange chromatography (Saraste *et al.*, 1981; Penttila, 1983), affinity chromatography using yeast cytochrome *c* (Bill and Azzi, 1982; Thelen *et al.*, 1985), native gel electrophoresis (Ludwig, *et al.*, 1979; Prochaska and Reynolds, 1986), and limited proteolysis using α-chymotrypsin (Puettner *et al.*, 1985; Sarti *et al.*, 1985). In addition, rat liver enzyme deficient in subunit III has been prepared using dodecyl β-D-maltoside and cytochrome *c* affinity chromatography (Thompson and Ferguson-Miller 1983; Thompson *et al.*, 1985). Initially, we will discuss results obtained with each preparation of subunit III-deficient enzyme and summarize this information in Table I.

Subunit III-Deficient Cytochrome c Oxidase Purified by Ion-Exchange Chromatography. Bovine heart enzyme deficient in subunit III has been prepared by incubation of the enzyme in high Triton X-100 at alkaline pH and subjected to ion-exchange chromatography (Saraste *et al.*, 1981). The preparation lost greater than 90% of its normal subunit III content and also lost components b (M_r 9419), c (M_r 10,068), and subunit VII-Phe (M_r 6244) as assayed by SDS-PAGE (Penttila, 1983). The nmoles heme *a*/mg protein ratio (an indicator of enzyme purity) increased in the preparation, emphasizing that the redox centers are not associated with subunit III (Saraste *et al.*, 1981). The reduced visible absorbance spectrum of heme aa_3 was unperturbed by the removal of subunit III, except that the reduced α-band observed in control enzyme at 604.5 nm was shifted to 603 nm upon the removal of subunit III (Penttila, 1983). Also, the midpoint potential of heme *a* was no longer pH dependent in this preparation of subunit III-deficient enzyme (Penttila, 1983). The subunit III-deficient enzyme appeared monodisperse in analytical ultracentrifugation (M_r 210,000; Saraste *et al.*, 1981) and was assigned a dimeric structure (4 heme *a*'s, 4 copper atoms) based on its high nmole heme *a*/mg protein ratio. The subunit III-deficient enzyme retained 90% of its electron transfer activity and the ability to interact with cytochrome *c* as assayed by enzyme kinetic experiments (Penttila, 1983).

Table I. Preparations of Mammalian Mitochondrial Cytochrome *c* Oxidase Deficient in Subunit III and their Proton Translocation Activity when Reconstituted into Phospholipid Vesicles

Preparation	References[a]	Subunit III content (%) (method of assay)	Percent inhibition[b] of proton translocation activity	Method of proton translocation assay
I. Ion exchange chromatography	1, 2	0 (SDS-PAGE)	100%	Potentiometric
II. Yeast cytochrome *c* affinity chromatography	3	0	100%	Potentiometric
	4	(SDS-PAGE)	100%[c]	Absorbance Spectrophotometric
III. Native gel electrophoresis	5, 6	5–15% (SDS-PAGE and [^{14}C]-DCCD Labeling)	80–100%	Potentiometric
IV. α-Chymotrypsin treatment	7	0	40%	Potentiometric
	8	(SDS-PAGE and [^{14}C]-DCCD Labeling)	47%[d]	Absorbance Spectrophotometric
V. Rat liver enzyme	9, 10	2–5% (SDS-PAGE)	30% & 82%[e]	Potentiometric

[a] References: (1) Saraste *et al.*, 1981; (2) Penttila, 1983; (3) Bill and Azzi, 1982; (4) Thelen *et al.*, 1985; (5) Prochaska and Reynolds, 1986; (6) Prochaska *et al.*, unpublished; (7) Puettner *et al.*, 1985; (8) Sarti *et al.*, 1985; (9) Thompson and Ferguson-Miller, 1983; (10) Thompson *et al.*, 1985.

[b] This value was calculated from data presented in each publication using the following equation:

$$\text{Percent inhibition} = \left(1.00 - \frac{H^+/e^- \text{ COV-III}}{H^+/e^- \text{ COV}}\right) \times 100.$$

[c] Calculated from changes in intravesicular pH value.

[d] Rapid flow kinetic measurement.

[e] The lower inhibition of proton translocation activity was obtained from multiple turnover assays, whereas the higher inhibition of activity was observed in ferrocytochrome *c* addition experiments. Bovine heart enzyme in COV was used as the control.

When reconstituted into phospholipid vesicles by cholate dialysis, phospholipid vesicles containing subunit III-deficient enzyme (COV-III) exhibited a decrease in the observed respiratory control ratio (RCR) (Hinkle *et al.*, 1972). RCR is defined as the rate of electron transfer catalyzed by COV in the presence of the ionophores, valinomycin and CCCP, corrected for the auto-oxidation rate of cytochrome *c* divided by the rate of COV-catalyzed electron transfer in the absence of ionophores corrected for the auto-oxidation rate of cytochrome *c* (Hinkle *et al.*, 1972). The RCR value is thought to be an indicator of endogenous proton permeability of the COV (Penttila, 1983; Puettner *et al.*, 1985; Sarti *et al.*, 1985). COV-III exhibited a complete loss of proton translocation activity (Saraste *et al.*, 1981) and also exhibited a 50% decrease in the observed K^+/e^- ratio compared to that of COV as determined by potassium uptake experiments (Penttila 1983) (See Table I). The loss of proton translocation activity in COV-III was shown to be a direct effect of subunit III removal by incorporating the enzyme treated with trypsin (to remove components b and c) into phospholipid vesicles (COV-T) and measuring K^+/e^-. COV-T exhibited a 25% decrease in the observed K^+/e^- as compared to COV (Penttila, 1983). This result suggested that the removal of subunit III in enzyme, not the removal of other polypeptides (components b and c), was responsible for the loss of proton translocation activity. (For an alternative explanation, see below and Puettner *et al.*, 1985, and Sarti *et al.*, 1985).

Subunit III-Deficient Enzyme Purified by Yeast Cytochrome c Affinity Chromatography. Subunit III-deficient bovine heart cytochrome *c* oxidase has also been isolated by affinity chromatography using yeast cytochrome *c* covalently attached to Sepharose 4-B (Bill and Azzi, 1982; Nalecz *et al.*, 1985; Thelen *et al.*, 1985). This method involved activating a free sulfhydryl group in the covalently attached yeast cytochrome *c* with 2,2′-dipyridyl disulfide and attaching the isolated, solubilized enzyme covalently to the column through a free sulfhydryl group in subunit III (Bill and Azzi, 1982). After subsequent elutions to wash unbound enzyme from the column, the column was incubated with high concentrations of Triton X-100 at high pH to cause the elution of subunit III-deficient enzyme. Purified subunit III was then eluted by washing the column with β-mercaptoethanol to break the disulfide bond between subunit III and yeast cytochrome *c*. The polypeptide composition of the subunit III-deficient enzyme was determined by SDS-PAGE and was found to be similar to the ion-exchange purified preparation (loss of subunit III, VII-Phe, and components b and c) (Bill and Azzi, 1982; Nalecz *et al.*, 1985).

The reduced absorbance spectrum of subunit III-deficient enzyme exhibited two absorbance maxima at 444.5 and 604.5 nm as compared to 445 and 605 nm for control enzyme. The reduced carbon monoxide minus reduced difference spectrum for the subunit III-deficient enzyme exhibited a blue shift of 4 nm for

the Soret band (424.5 nm) as compared to control enzyme (428.5 nm), a new spectral component at 410 nm, and a red shift in the α-peak (594 nm) as compared to control enzyme (590 nm) (Nalecz *et al.*, 1985). Analytical sucrose density gradient centrifugation showed that the subunit III-deficient enzyme had a molecular weight of 272,000 and was in the dimeric state of aggregation. In addition, the monomeric form of the subunit III-deficient enzyme was observed only during gel permeation chromatography at low concentrations of enzyme ($< 1\ \mu$M heme aa_3) (Nalecz *et al.*, 1985).

This preparation of subunit III-deficient enzyme retained 70% of its electron transfer activity when assayed in dilute detergent solution (Bill and Azzi, 1982) and approximately 50% of electron transfer activity when assayed in COV-III (Thelen *et al.*, 1985). This preparation exhibited monophasic kinetics with cytochrome *c* when assayed spectrophotometrically in the presence of dodecyl β-D-maltoside, but after the addition of asolectin, biphasic kinetics were observed. When the subunit III-deficient enzyme was assayed for electron transfer activity using TMPD and an oxygen electrode, monophasic kinetics were observed (Nalecz *et al.*, 1985). Comparison of the steady-state kinetics of the control enzyme and subunit III-deficient enzyme suggested that the interaction of cytochrome *c* with the enzyme was slightly perturbed by the removal of subunit III as shown by an increase in K_m and a decrease in V_{max}.

The affinity chromatography-purified subunit III-deficient enzyme was also incorporated into phospholipid vesicles by cholate dialysis and the effects on proton translocation monitored (Thelen *et al.*, 1985). COV-III exhibited RCR values of 3–4, as compared to 4–5 for COV, suggesting that COV-III had a somewhat higher endogenous permeability to protons. COV-III completely lost the ability to translocate protons in a vectorial manner as determined by measuring the extravesicular pH. In addition, the intravesicular pH of COV and COV-III was monitored by the use of fluorescein-labeled phosphatidylethanolamine incorporated into the phospholipid vesicles. COV were observed to extract 2.0 protons per electron transferred (H^+/e^-) from the intravesicular milieu, whereas COV-III abstracted 1.0 H^+/e^-, a result consistent with the data obtained in COV-III from the extravesicular pH measurements (Thelen *et al.*, 1985). The orientation of the subunit III-deficient enzyme in COV-III and the control enzyme in COV was determined to monitor the amount of the enzyme in each preparation with its cytochrome *c* binding face exposed to the extravesicular milieu, and it was observed that 77% of the subunit III-deficient enzyme in COV-III and 90% of the enzyme in COV was in the proper orientation for substrate binding (Thelen *et al.*, 1985). In addition, the physical size distribution of COV and COV-III was monitored by electron microscopy to ascertain the efficiency of incorporation of the enzyme into

COV and COV-III (Muller and Azzi, 1985). Both the size distribution and the incorporation of the enzyme into COV and COV-III were similar. These results suggested that the intravesicular volume or source of the protons for the vectorial proton translocation reaction was similar in COV and COV-III. If the intravesicular volume of COV and COV-III was similar, then the intravesicular buffering capacity for COV and COV-III was equivalent, emphasizing the net proton pool for utilization in vectorial proton translocation reaction was the same in both COV and COV-III. These experiments also showed that subunit III-deficient enzyme in COV-III lost the ability to translocate protons across the phospholipid bilayer and proved that the source of protons in cytochrome *c* oxidase vectorial proton translocation was the intravesicular space. Finally, the correlation of the loss of extravesicular proton translocation in COV-III with a decrease in efficiency of alkalinization in the intravesicular space provided strong evidence that subunit III of cytochrome *c* oxidase is involved in vectorial proton translocation.

Subunit III-Deficient Enzyme Purified by Native Gel Electrophoresis. Subunit III-deficient bovine heart enzyme has been prepared by native gel electrophoresis (Ludwig *et al.*, 1979; Prochaska and Reynolds, 1986). This method involved the incubation of enzyme in high Triton X-100 followed by gel electrophoresis on a 4% polyacrylamide gel in high salt at pH 8.9. SDS-PAGE showed that subunit III and components b and c were removed from the enzyme by the native gel treatment. The content of subunit III in the native gel preparation was determined to vary from 5 to 15% of control enzyme as monitored by [^{14}C]-DCCD labeling and quantitative Coomassie blue staining on SDS-PAGE (Prochaska and Reynolds, 1986). The reduced visible absorbance spectrum of the subunit III-deficient enzyme exhibited absorbance maxima at 443 and 603 nm as compared to 443 and 604 nm for the control enzyme. Subunit III-deficient enzyme retained 64% of control electron transfer activity when assayed in detergent solution and 50% of control electron transfer activity when in COV-III (Prochaska and Reynolds, 1986; Prochaska *et al.*, unpublished results). The subunit III-deficient enzyme exhibited biphasic enzyme kinetics with K_m values similar to control enzyme and bound similar amounts of cytochrome *c* as control enzyme in direct thermodynamic binding experiments, suggesting that the removal of subunit III did not perturb the interaction of cytochrome *c* with the enzyme (Prochaska and Reynolds, 1986).

The native gel electrophoresis preparation of subunit III-deficient enzyme was also incorporated into phospholipid vesicles by cholate dialysis and the effects on proton translocation assayed. COV-III exhibited an RCR value of 5.2 $\pm$ 1.8, whereas COV had an RCR value of 6.2 $\pm$ 2.0, suggesting a similar endogenous proton permeability (Prochaska *et al.*, unpublished results). The endogeneous proton permeability of COV and COV-III was

also monitored by measuring the concentration dependence of uncoupler-stimulated electron transfer activity, and both types of liposomes exhibited similar concentration dependences. In addition, COV and COV-III containing a high concentration of intravesicular potassium ions exhibited similar extents and kinetics of valinomycin-induced proton-potassium exchange as liposomes without cytochrome *c* oxidase, emphasizing the similarities in endogenous proton permeabilities of COV and COV-III (Prochaska *et al.*, unpublished results). The proton translocation activity of COV-III was assayed by monitoring extravesicular pH with a sensitive pH meter equipped with a chart recorder, and electron transfer was initiated by the addition of ferrocytochrome *c*. Figure 3 shows proton translocation data from COV-III containing subunit III-deficient enzyme isolated by native gel electrophoresis (Prochaska and Reynolds, 1986). After addition of ferrocytochrome *c*, a suspension of COV exhibited the characteristic acidification followed by

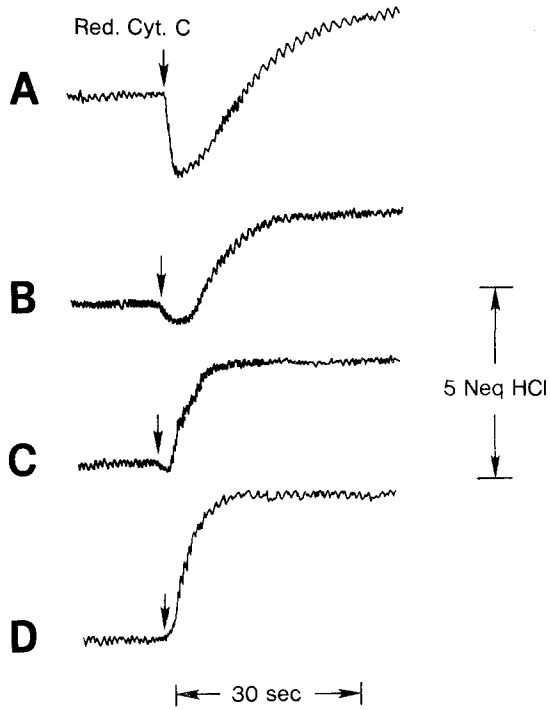

Fig. 3. Changes in extravesicular pH induced by electron transfer in phospholipid vesicles inlaid with control and subunit III-deficient cytochrome *c* oxidase. All conditions were as described in Prochaska and Reynolds (1986). The RCR value of the COV-III was 4.3 for trace B and 5.8 for trace C. Reprinted with permission from *Biochemistry*, copyright 1986 American Chemical Society.

alkalinization (Fig. 3A). The acid phase represents proton translocation, while the alkaline phase represents the consumption of protons in the catalytic reduction of oxygen (Krab and Wikstrom, 1978; Thelen *et al.*, 1985). Figure 3D shows that after the addition of CCCP, only the alkaline phase occurs. The extent of the acid phase is less in COV-III (Figs. 3B and 3D) that contain enzyme retaining 15 and 5% subunit III, respectively, than the COV. The apparent H^+/e^- for COV and COV-III was calculated by the extrapolation method of Krab and Wikstrom (1978) and it was observed that the COV-III lost at least 75% of the efficiency to translocate protons (Prochaska and Reynolds, 1986). Subsequent work performed at lower enzyme turnovers showed that COV-III containing 15 and 5% of its subunit III content lost 80 and 100% of their proton translocation activity, when the proton translocation activity was corrected for remaining subunit III content (Prochaska *et al.*, unpublished results). In addition, COV and COV-III were shown to have cytochrome *c* oxidase inserted into the phospholipid vesicles in a similar orientation (approximately 90% of the enzyme with the cytochrome *c* binding domain toward the extravesicular medium).

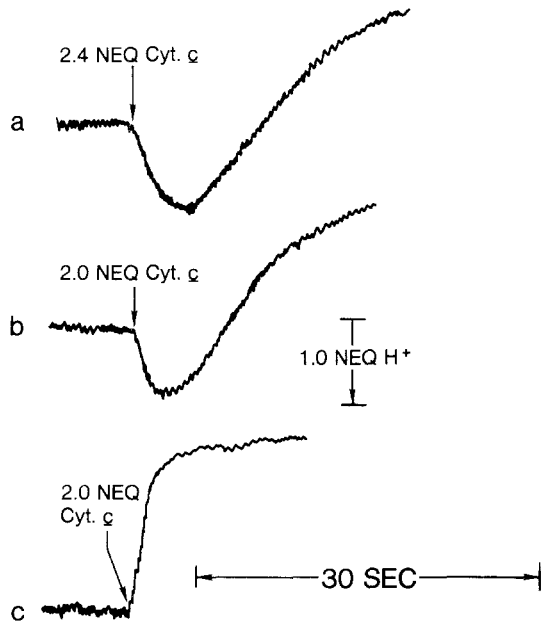

Fig. 4. Changes in extravesicular pH induced by electron transfer in phospholipid vesicles inlaid with control and trypsin-treated cytochrome *c* oxidase. All conditions were as described in DiBiase and Prochaska (1985). Reprinted with permission from *Archives of Biochemistry and Biophysics*, copyright 1985, Academic Press.

An important control experiment was performed to ascertain what effect the loss of components b and c and the Triton X-100 treatment had on proton translocation activity. The enzyme was first incubated in Triton X-100 and treated with trypsin to remove components b and c (Ludwig *et al.*, 1979; Malatesta *et al.*, 1983; DiBiase and Prochaska, 1985) and then incorporated into phospholipid vesicles by cholate dialysis. Proton translocations by COV-T (Fig. 4B) was equivalent to proton translocation activity in COV (Fig. 4A) (DiBiase and Prochaska, 1985). In addition, later work showed that in all conditions of enzyme turnover COV-T had the same H^+/e^- as COV. Control experiments showed that the trypsin-treated enzyme in COV-T and control enzyme had a similar orientation in the liposomes and both types of liposomes exhibited similar RCR values (6.0 ± 0.8 for COV-T). The proton translocation data presented in Fig. 4 suggest that the detergent treatment of the enzyme during trypsin treatment does not appreciably effect the H^+/e^- obtained for COV-T, and we conclude that our potentiometric measurements are kinetically competent for COV-T. Using this approach, the assay of proton translocation activity in COV-III presented in Fig. 3 appears also to be kinetically competent, and the results suggest that subunit III is important in the vectorial proton translocation process. (However, see Puettner *et al.*, 1985, and Sarti *et al.*, 1985, for an alternative.)

Subunit III-Deficient Enzyme Prepared by Treatment with α-Chymotrypsin. Subunit III-deficient bovine heart mitochondrial cytochrome *c* oxidase has also been prepared by incubation of the enzyme in Triton X-100 at pH 8.5 with α-chymotrypsin (Malatesta *et al.*, 1983). SDS-PAGE showed that the α-chymotrypsin-treated preparation exhibited a loss of subunit III and components b and c (Puettner *et al.*, 1985). [^{14}C]-DCCD labeling showed that almost all of the subunit III was removed during the digestion. The reduced absorbance spectrum of this preparation exhibited a blue shift of the α-band to 602–603 nm (605 nm for control enzyme) and changes at 520 and 560 nm (Puettner *et al.*, 1985). No differences between control and subunit III-deficient enzyme were observed in the reduced carbon monoxide spectrum (Malatesta *et al.*, 1986). The enzyme retained up to 80 to 90% of its electron transfer activity (Puettner *et al.*, 1985). The transient kinetic properties of the α-chymotrypsin-treated enzyme with ferrocytochrome *c* were determined and the bimolecular rate constant decreased twofold after the α-chymotrypsin treatment (Malatesta *et al.*, 1986). Also, the reoxidation of reduced Heme *a* exhibited similar slow kinetics in both control and subunit III-deficient enzymes, emphasizing that no new oxygen binding site was created by the removal of subunit III. Overall, it was concluded that the subunit III-deficient enzyme exhibited a similar mechanism of electron transfer as control enzyme (Malatesta *et al.*, 1986).

The α-chymotrypsin-treated enzyme has been reconstituted into phospholipid vesicles by cholate dialysis and proton translocation studied by potentiometric (Puettner *et al.*, 1986) and rapid-flow kinetic methods (Brunori *et al.*, 1985a; Sarti *et al.*, 1985). The α-chymotrypsin-treated enzyme in COV-III exhibited an H^+/e^- of 0.48 by potentiometric measurement as compared to 0.8 for COV when assayed at similar enzyme turnovers, suggesting that COV-III retained a significant ability to translocate protons. The retention of proton translocation activity was shown to be dependent on the RCR of the COV-III (Puettner *et al.*, 1985). Similar H^+/e^- were observed using stopped-flow absorbance spectrophotometry with a pH indicator dye (phenol red); COV-III and COV exhibited H^+/e^- of 0.5 and 0.93, respectively (Sarti *et al.*, 1985). Both methods of assay suggested that removal of subunit III may affect proton translocation in the enzyme, but subunit III is not the primary constituent of the active proton pump and may provide a regulatory role in the proton translocation mechanism (Sarti *et al.*, 1985). A more complete discussion of the data from the α-chymotrypsin-treated subunit III-deficient enzyme will follow in the next section.

Rat Liver Cytochrome c Oxidase Isolated with Dodecyl β-D-Maltoside. Subunit III-deficient cytochrome *c* oxidase has been isolated from rat liver by solubilizing the mitochondrial inner membrane with dodecyl β-D-maltoside and purifying the enzyme with a horse heart cytochrome *c* affinity column (Thompson and Ferguson-Miller, 1983). SDS-PAGE showed that this preparation lacked 95–98% of subunit III as compared to the bovine heart enzyme. In contrast to the results obtained with bovine subunit III-deficient enzyme, the pH dependence of the midpoint potential of heme *a* in the rat liver enzyme deficient in subunit III was similar to that of control bovine heart enzyme (Thompson *et al.*, 1985). The enzyme (M_r 152,000) was determined to be a monomer as isolated, as determined by sedimentation equilibrium analysis (Suarez *et al.*, 1984). The maximum electron transfer activity of the isolated rat liver enzyme was 72% of its membrane-bound form and exhibited biphasic steady-state enzyme kinetics with kinetic parameters similar to the membrane-bound form (Thompson and Ferguson-Miller, 1983).

The isolated rat liver enzyme was incorporated into phospholipid vesicles and the proton translocation activity determined (Thompson *et al.*, 1985). COV-III exhibited RCR values of 6.3 ± 1.4 with some values as high as 9.0, whereas bovine heart enzyme in COV exhibited an RCR of 5.4 ± 0.8. Upon the addition of ferrocytochrome *c*, COV-III exhibited a DCCD-insensitive H^+/e^- of 0.12, whereas COV showed H^+/e^- values of 0.65 and 0.24 in the absence and presence of DCCD (Thompson *et al.*, 1985). Different results were obtained when a multiple turnover assay was used; COV-III showed an H^+/e^- of 0.34 (+DCCD it was 0.11), while COV exhibited an H^+/e^-

of 0.48 (+DCCD, it was 0.11). These results suggested that the rat liver enzyme retains from 15%–50% of its ability to translocate protons in COV-III and that the remaining activity was insensitive to DCCD treatment. Thompson *et al.* (1985) suggested that subunit III acts as a proton channel in the enzyme rather than an active proton pump.

Summary

The preparations of subunit-III-deficient enzyme and their ability to translocate protons in COV-III are summarized in Table I. The similarities of the preparations of subunit III-deficient enzyme are the following: (1) they all appear to lose at least 85% of their native subunit III content and also components b and c during their isolation; (2) the reduced absorbance spectrum of all preparations of subunit III-deficient enzyme is somewhat perturbed with the α-band exhibiting a blue shift; (3) all preparations of subunit III-deficient enzyme retain at least 60% of the maximum electron transfer activity of the native enzyme; (4) upon incorporation into phospholipid vesicles, all preparations of subunit III-deficient enzyme exhibit a decrease in the observed H^+/e^- as compared to COV, and this decrease is independent of the method of assay. Some inconsistencies arise with the different preparations of subunit III-deficient enzyme: (1) the aggregation state of the subunit III-deficient enzyme is either a monomer (ion-exchange chromatography and rat liver preparations) or a dimer (affinity column preparation); (2) the contribution of subunit III to the vectorial proton translocation in COV-III (80–100% for the ion-exchange chromatography, affinity chromatography, and native gel electrophoresis preparations, < 50% for the α-chymotrypsin treated preparations). These results cause a controversy: what is the absolute contribution of subunit III to vectorial proton translocation and what, if any, potential artifacts are involved in the measurement of H^+/e^- in COV-III?

Evidence against the Involvement of Subunit III of Mammalian Mitochondrial Cytochrome *c* Oxidase in Proton Translocation

Phospholipid Vesicles Containing Subunit III-Deficient Enzyme Prepared by α-Chymotrypsin Treatment Exhibit Significant Proton Translocation Activity

When reconstituted into COV-III, α-chymotrypsin-treated enzyme lost about 40% of its proton translocation activity as assayed by a potentiometric method (Puettner *et al.*, 1985). These COV-III exhibited RCR values of 4. When proton translocation activity of COV-III that had RCR values of 2.5 was measured, a complete loss of proton translocation activity was observed.

RCR values greater than 3 were required to observe proton translocation in COV-III, and an additional requirement was that the enzyme undergo less than five turnovers (see Figs. 3 and 4 for alternative data). In addition, the half-time for the decay of the acid phase of proton translocation (the proton decay rate) was stimulated by the addition of CCCP, suggesting that slight changes in proton permeability of COV can create an artificial lowering of the observed H^+/e^- (Puettner *et al.*, 1985).

Rapid-flow kinetics was also used to measure H^+/e^- of COV and α-chymotrypsin-treated enzyme in COV-III (Brunori *et al.*, 1985a; Sarti *et al.*, 1985). COV-III were observed to lose 47% of their proton translocation activity compared to COV (Sarti *et al.*, 1985). Greater extents of inhibition (> 80%) of proton translocation activity were correlated with lower RCR values. Lower H^+/e^- values were observed for both in COV-III and COV when the RCR value was less than 3.0. This suggested that the back leak of protons through the phospholipid vesicles strongly affects the extent of the extravesicular acidification. These data were interpreted as providing evidence that subunit III of the enzyme plays a regulatory role in the mechanism of proton translocation, not an obligatory role.

Other Related Experimental Evidence

Cytochrome *c* oxidase has been isolated from *Paracoccus denitrificans* (Ludwig and Schatz, 1980; Ludwig, 1980), and it has been shown to have two subunits that have a primary amino acid sequence homology to subunits I and II of the bovine enzyme (Steffens *et al.*, 1983). In addition, polyclonal antibodies against subunit II of the bacterial enzyme crossreact with subunit II of the bovine enzyme (Ludwig, 1980). *The enzyme does not contain a polypeptide that migrates at a molecular weight equivalent to the bovine subunit III.* The *P. denitrificans* enzyme has been reconstituted into phospholipid vesicles and it has been observed to translocate protons at an efficiency of 0.6 compared to approximately 1.1 for bovine enzyme in COV (Solioz *et al.*, 1982). Furthermore, the proton translocation activity in the reconstituted bacterial enzyme was found to be DCCD-insensitive (Puettner *et al.*, 1983). These results suggested that subunit III (or DCCD-homologous protein domain) was not required for proton translocation activity in *P. denitrificans* enzyme.

A final line of experimentation that suggests that subunit III might have a regulatory role in proton translocation rather than a participant role is the heat treatment of the bovine heart enzyme (Sone and Nicholls, 1984). These experiments showed that incubation of COV with heat (43°C, 1 h) inhibited 80% of the proton translocation activity without affecting electron transfer activity of the COV. The RCR value of COV was unchanged by the heat treatment, emphasizing that the endogeneous proton permeability of COV

was unaffected. In addition, the pH dependence of the midpoint potential of heme *a* was unperturbed by the heat treatment, suggesting tentatively that the site of action of the heat treatment was different from that of subunit III. However, Sone and Nicholls (1984) proposed that the results could not rule out that a functional dissociation of subunit III from the enzyme complex occurred during the heat treatment. In support of this mechanism of inhibition of proton translocation by heat treatment, DCCD was observed to stimulate the dissociation of subunit III from the enzyme complex (Azzi *et al.*, 1985).

Concluding Remarks

The experimental evidence for mammalian cytochrome *c* oxidase acting as an oxidation–reduction linked proton pump is voluminous and proposals describing the molecular mechanism of the proton release are beginning to be described (Malstrom, 1984; Mitchell *et al.*, 1985; Brunori *et al.*, 1985b). However, the protein domain that mediates this process is still uncharacterized. This review has attempted to provide a summary of the experimental evidence that has been obtained on the role of one of the subunits (III) of the enzyme in vectorial proton translocation. Although the descriptive characteristics of proton translocation of the mammalian enzyme reconstituted into phospholipid vesicles are well defined, experimental inconsistencies remain pertaining to the role of subunit III in the vectorial proton translocation reaction. The major area of disagreement on the role of subunit III in proton translocation arises when the results from proton translocation experiments from preparations of subunit III-deficient enzyme reconstituted into phospholipid vesicles are discussed. A number of laboratories observe a loss of proton translocation activity in different preparations of COV-III and interpret this result when taken together with other experimental evidence as an indication of an intimate role for subunit III in proton translocation. Alternatively, a number of other laboratories observe a significant retention of proton translocation activity in COV-III and suggest that subunit III plays a regulatory, not participant, role in proton translocation. Two experimental approaches may yield more explicit information on the role of subunit III in proton translocation. The first approach is to attempt reconstitution of isolated, purified mammalian subunit III with mammalian subunit III-deficient enzyme and study the proton translocation properties of the reassociated enzyme in COV. The second approach is to study proton translocation in structurally less complex cytochrome *c* oxidases such as those from lower eukaryotes (Bisson *et al.*, 1985) or bacterial species (Poole, 1983). The bacterial species will have a particular advantage, for they will allow recombinant DNA technology via site-specific mutagenesis to be

used in an investigation of the amino acid functional groups involved in vectorial proton translocation.

In conclusion, a model for proton translocation in mammalian cytochrome *c* oxidase that may reconcile the conflicting data described in this review merits discussion. Cox *et al.* (1986) have recently published a model for proton translocation in ATP synthetase where proton conduction is mediated by amphiphilic α-helices in two membrane sector subunits, a and c (DCCD binding proteolipid). In mammalian cytochrome *c* oxidase, one amphiphilic α-helix could be located on subunit III; subunit III could help facilitate the translocation of protons and not be the active constituent of proton release (Casey *et al.*, 1980; Thompson *et al.*, 1985). The primary amino acid sequence of mammalian subunit III contains at least six α-helices that are conserved across many different species (Bisson and Montecucco, 1985). In addition, these sequences appear to contain a number of conserved histidine residues that may be involved in proton translocation (Senior, 1984). If another subunit is involved in proton translocation, the removal of subunit III of the mammalian enzyme may not completely inhibit this process. The experiments described above may lead to a more focused insight into the molecular mechanism of proton translocation in cytochrome *c* oxidase.

Note Added in Proof

Recently, Finel and Wikstrom (1986) have shown that the ion-exchange purified preparation of subunit III-deficient enzyme incorporated into COV-III lost 60–80% of its proton translocation activity when compared to COV. Alkaline-treated enzyme that still retained subunit III when incorporated into liposomes also exhibited a similar decrease in proton translocation activity, emphasizing that removal of subunit III is not exclusively required for a decrease in proton translocation activity. Finally, the region of *Paracoccus denitrificans* DNA that encodes the genes for cytochrome *c* oxidase has been cloned and DNA sequencing revealed an open reading frame that codes for a protein homologous to subunit III of the eukaryotic mitochondrial enzyme (Saraste *et al.*, 1986). One interpretation of this finding is that the loss of proton translocation activity observed in liposomes containing the bacterial enzyme was due to the removal of subunit III during the isolation of the enzyme.

Acknowledgments

The authors wish to thank V. DiBiase and K. Reynolds for performing some of the experiments presented here and H. Liming for help with the

manuscript. This work was supported in part by grants from National Institutes of Health HL-29051 and the Miami Valley Chapter of the American Heart Association. P.S.F. was supported by a Wright State School of Medicine post-doctoral fellowship.

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