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MINI REVIEW

Is the Cytochrome b–c_1 Complex a Proton Pump? Probably Yes.

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Introduction

Complex III of the mitochondrial respiratory chain catalyzes electron transfer from ubiquinol to cytochrome *c* coupled to ATP synthesis and ion transport. In agreement with the principles of the chemiosmotic theory of energy coupling, this segment of the respiratory chain, when analyzed either in intact mitochondria or in a purified complex reconstituted into proteoliposomes, catalyzes the electrogenic translocation of protons across the mitochondrial membrane during the transfer of electrons down the respiratory chain. As a consequence of its role in oxidative phosphorylation, this complex has been the focus of intense research efforts to understand the mechanism of energy transduction.

Complex III, first isolated in 1961 from beef heart mitochondria (Hatefi *et al.*, 1962), has subsequently been isolated from mitochondria of many different sources including *Neurospora crassa* (Weiss and Kolb, 1979), yeast (Siedow *et al.*, 1978; Sidhu and Beattie, 1982), and rat liver (Gellerfors and Nelson, 1981). Furthermore, analogous enzyme complexes, which catalyze electron transfer from a reduced quinone to an acceptor coupled to proton translocation, have been isolated from the photosynthetic bacteria *Rhodopseudomonas spheroides* (Gabellini *et al.*, 1982) and *Anabaena variabelis* (Krinner *et al.*, 1982) as well as from chloroplasts (Hurt and Hauska, 1981). All such cytochrome *c* reductase complexes contain three chemically different electron-transfer centers, including two *b*-types and one *c*-type cytochrome as

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well as an iron–sulfur protein. In addition to these catalytically active centers, each complex has additional proteins which copurify with the complex to give a total subunit number ranging from 10 in the beef heart and *Neurospora* complex to 9 in that isolated from yeast mitochondria. The two largest polypeptides observed in the mitochondrial complex are usually referred to as the "core" proteins, while other polypeptides have been assigned functions such as the Q-binding or antimycin binding proteins. Interestingly, the same electron transfer and energy transduction reactions associated with proton pumping are also observed in the four subunit complexes isolated from *Rhodopseudomonas* and chloroplasts. Hence, it appears that the additional subunits observed in the complexes isolated from mitochondria are not strictly necessary for energy transduction, although recent studies have indicated that these subunits are required for catalytic activity in the mitochondrial complexes (Link *et al.*, 1985).

Considerable research efforts in the past decade have been focused on the mechanism of both electron transfer and energy transduction at this site of the respiratory chain. This span of the chain can be conveniently studied in intact mitochondria by measuring the rate of exogenous cytochrome *c* or ferricyanide reduction by the substrate succinate provided that the activity of cytochrome oxidase is blocked by cyanide. The number of protons ejected per two electrons transferred across this span of the chain ($H^+/2e^-$) is generally agreed to be four, when alternate proton-conducting pathways of the inner mitochondrial membrane are selectively inhibited. A net positive charge is also translocated outward during electron transfer. Moreover, identical proton stoichiometries have been observed, when complex III isolated from either beef heart (Guerrieri and Nelson, 1975; Leung and Hinkle, 1975) or yeast (Beattie and Villalobo, 1982) mitochondria was reconstituted into artificial proteoliposomes. Under these conditions, reduced analogues of coenzyme Q were used as electron donors, and exogenous cytochrome *c* (sometimes plus ferricyanide) was used as the electron acceptor. It is apparent from a close examination of the two reactions

$$\text{ubiquinol} \rightarrow \text{ubiquinone} + 2e^- + 2H^+$$

$$\text{succinate} \rightarrow \text{fumurate} + 2e^- + 2H^+$$

that two of the protons measured in the external medium during the reduction of cytochrome *c* arise directly from substrate oxidation. These are called "scalar" protons. The source of the other two electrogenic protons translocated across the membrane during electron transfer is currently an active area of research and is the subject of this short review.

Proposed Mechanisms for Electron Transfer and Proton Translocation in Complex III

The original formulation of the chemiosmotic hypothesis proposed by Mitchell (1961) required that electrons and protons traverse the membrane via redox "loops" in which electrons move across the membrane in one direction and protons in the other direction essentially coupled to the transport of electrons. The lack of an identifiable proton carrier in the cytochrome b–c_1 region of the respiratory chain led to the formulation of alternative explanations for proton pumping. Furthermore, it was imperative that any proposal to explain electron transfer in this region of the respiratory chain must also take into account the anomalous oxidation/reduction behavior of cytochrome b. The observation that cytochrome b is not as fully reduced as cytochrome c_1 under steady-state conditions and that it becomes more reduced, when cytochrome c_1 is oxidized especially in the presence of antimycin, the inhibitor of cytochrome b oxidation, cannot be satisfactorily explained by a linear scheme of electron transfer.

Currently, two mechanisms have been advanced to explain the pathway of electron transfer and proton translocation in complex III (Fig. 1). The Q-cycle, originally proposed by Mitchell (1976), suggests a central role for ubiquinone in the transfer of electrons and also proton translocation in the span between cytochromes b and c_1 in addition to its role as an electron acceptor for the primary dehydrogenases. In this ligand-conduction mechanism, the various cytochromes play a role solely in the transport of electrons. The other mechanism, the b-cycle proposed in several versions, mainly by Wikstrom *et al.* (1981) and Papa (1982), suggests that the cytochrome b–c_1 complex might undergo a protonation/deprotonation cycle coupled to the oxidation/reduction reaction such that a translocation of protons across the membrane occurs. In other words, complex III would act as a proton pump.

Both the Q-cycle and b-cycle mechanisms involve a branched chain electron transfer pathway in which ubiquinol is oxidized donating one electron to the high-potential iron–sulfur protein/cytochrome c_1 couple, while the second electron is transferred to the low-potential cytochrome b. This concept has gained acceptance by the recent observation that the initial oxidation of ubiquinol and the concomitant reduction of the iron–sulfur protein are inhibited by myxothiazol (von Jagow and Ohnishi, 1985) as well as by the quinone analogues UHDBT[2] (Bowyer *et al.*, 1982) and HNQ (Matsuura *et al.*, 1983).

[2]Abbreviations: BAL, British antilewisite; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone;DCCD, dicyclohexylcarbodiimide; DCIP, 2,6-dichlorophenolindophenol; HMHQQ 7-(*n*-heptadecyl)mercapto-6-hydroxy-5,8-quinolinequinone; HNQ, 2-hydroxy-3-undecyl-1,4-naphthoquinone; UHDBT, 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole.

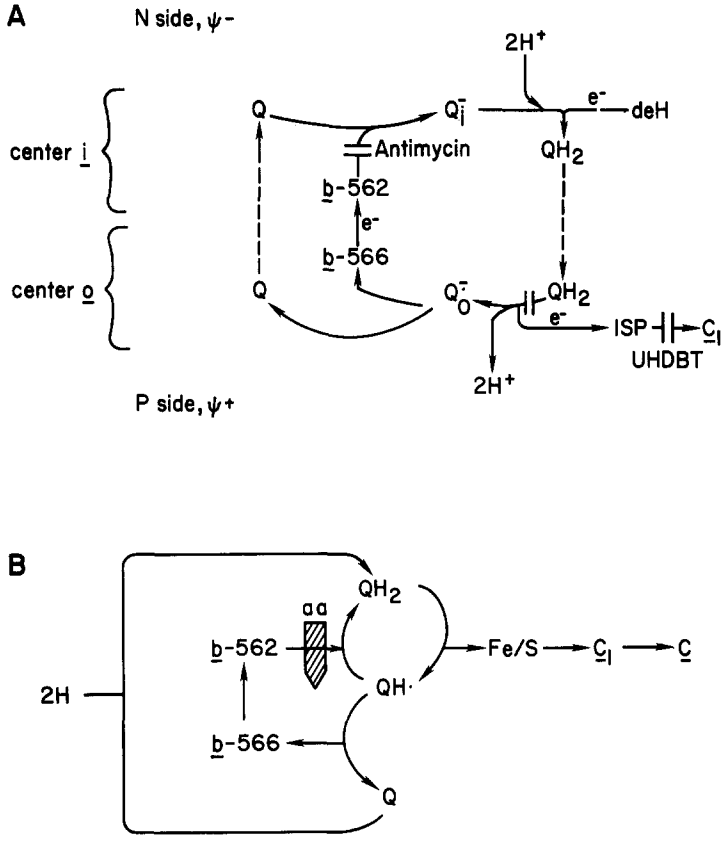

Fig. 1. (A) Proposed Q-cycle for electron transport in complex III. The dashed lines represent the movement of uniquinone and ubiquinol. The subscripts *i* and *o* refer to specific ubisemiquinone anions at the cytoplasmic and matrix side of the inner mitochondrial membrane respectively. ISP, iron–sulfur protein. deH, dehydrogenase. (From von Jagow *et al.*, 1984.) (B) Proposed *b*-cycle of complex III. 2H indicates reducing equivalents from the ubiquinone pool. Q, QH ·, and QH_2 indicate ubiquinone, ubisemiquinone, and ubiquinol bound to protein. aa, antimycin A. (From Wikstrom and Krab, 1980.)

The Q-cycle proposes that concomitant with the oxidation of ubiquinol at the cytoplasmic side of the inner membrane (center *o*), a release of two protons occurs at this side of the membrane forming the semiquinone anion. The low-potential $Q\dot{-}/Q$ couple thus produced would then act to reduce the low-potential *b* cytochromes. Cytochrome *b*-562 is subsequently oxidized in an antimycin-sensitive reaction by a second distinct semiubiquinone localized on the M side of the membrane with uptake of two protons from the matrix.

By contrast, the *b*-cycle while proposing a similar branched pathway of electron transport makes two very different assumptions. First, and most

important, is that the cytochromes are assumed to be carriers of the protons during the oxidation/reduction reactions of the b–c_1 region in a mechanism analogous to the hemoglobin Bohr effect (Wikstrom *et al.*, 1981). In many such proposals, cytochrome b is suggested to act as the proton pump, since its oxidation/reduction potential is strongly pH dependent (von Jagow and Engel, 1980). Second, only one tightly bound quinone is postulated to serve as both electron donor and acceptor connected through the cytochrome b system. In some formulations of the b-cycle, a protonated species of ubiquinone ($QH \cdot$) is the electron acceptor for cytochrome b-562.

As indicated by this brief introduction, many similarities exist in these two schemes, especially in the proposed pathways for electron transfer. Often, the differences between the Q-cycle and b-cycle have been the subject of intense debate thus obscuring the similarities in the two proposals. For example, both mechanisms explain the oxidant-induced reduction of cytochrome b in which the oxidation of cytochrome c_1 (and presumably the iron–sulfur protein which has a similar redox potential) causes an increased reduction of the low-potential cytochromes b especially in the presence of inhibitors such as antimycin. Furthermore, both schemes explain equally well the observation that cytochrome b can be reduced by two different pathways: one pathway inhibited by myxothiazol or UHDBT which also block the transfer of electrons by the iron–sulfur protein, while the second pathway involves reduction of cytochrome b by the antimycin-sensitive pathway.

The similarities in the two proposals should also be considered when experiments are planned to elucidate the molecular mechanisms of proton translocation. As the subject of this review is the examination of the proposal that cytochrome b acts as a proton pump, several different experimental approaches and data will be discussed in an attempt to point out inconsistencies with the current formulations of the Q-cycle. Potentially useful experimental approaches will be suggested to help in the resolution of this controversy.

Pathways of electron transfer

A major experimental finding in support of the Q-cycle was the identification of two different species of ubisemiquinone in the cytochrome b–c_1 complex. Initially, the presence of an antimycin-sensitive semiquinone anion was reported and its properties studied in purified succinate : cytochrome c reductase preparations (Ohnishi and Trumpower, 1980). This ubisemiquinone, found in amounts of 0.5 mole per mole of cytochrome c_1 or less, would correspond to the quinone at center i in the Q-cycle (Fig. 1). A second antimycin-insensitive species of semiquinone anion was subsequently detected in ubiquinol : cytochrome c oxidoreductase in submitochondrial particles

under conditions of the oxidant-induced extrareduction of cytochrome *b* (deVries *et al.*, 1981). This ubisemiquinone was sensitive to treatment with British antilewisite (BAL), which destroys the iron–sulfur center and would correspond to the quinone at center *o* in the diagram of the Q-cycle (Fig. 1).

Subsequent studies with the Q analogue, 7-(*n*-heptadecyl)mercapto-6-hydroxy-5,8-quinolinequinone (HMHQQ), also revealed differences in the two ubiquinone-binding sites in complex III (Zhu *et al.*, 1982). Two binding sites with very different affinities for the inhibitor were observed. The inhibitory site with the highest affinity was shown to be responsible for electron transfer near the iron–sulfur cluster, while the second site of inhibition, the low-affinity site, was apparently similar to the antimycin-binding site. The affinity of HMHQQ for this site was not affected by the redox state of the iron–sulfur cluster, as was observed for the high-affinity site.

While these data appear convincing, it should be noted, however, that these two species of ubisemiquinone have never been detected simultaneously. Hence, it is possible that the two distinct EPR spectra observed under the different experimental conditions may actually represent an identical semiquinone molecule bound in slightly different environments thus leading to different spectra. Indeed, Rich (1984) has suggested that the detection of functionally different types of ubiquinone in different sites in the complex does not mean that these molecules are necessarily permanently fixed in these positions but may result from the properties of a pool quinone distorted by its occupation of a reaction site. Experiments to determine the actual location of the ubisemiquinone molecule in the lipid bilayer of the inner mitochondrial membrane relative to both the heme centers of cytochrome *b* and the iron–sulfur protein may clarify this situation.

Furthermore, in a detailed analysis of the E_m of the Q/QH · couple Hendler *et al.* (1985) have questioned the belief that the semiquinone is a spontaneous reducing agent for the *b*-cytochromes. In addition, these workers concluded that neither binding sites nor ionization of the semiquinone *per se* would alter the E_m of this couple sufficiently for it to act as a reductant. Hence, in their view, an alternate mechanism must be envisioned to drive the unfavorable reduction of cytochrome b_t by the semiquinone or for the simultaneous transfer of both electrons to cytochrome b_t and the iron–sulfur protein.

Some discrepancies in the observed pathways of electron transfer have also been reported that are not in agreement with either proposed mechanism for electron flow through the cytochrome b–c_1 complex. For example, both mechanisms predict that the reduction of cytochrome c_1 should not be antimycin sensitive in the first turnover of the complex. Bowyer and Trumpower (1981) have reported evidence in support of this prediction suggesting that cytochrome c_1 is reduced in the presence of antimycin in a

single turnover. These conclusions were questioned by Esposti and Lenaz (1982) who studied the rapid reduction of cytochrome c_1 by ubiquinol-1 or succinate in a mitochondrial fraction enriched in the b–c_1 complex. Their data indicated that cytochrome c_1 reduction was antimycin sensitive in the first enzymatic turnover and hence would contradict current concepts for the passage of electrons through the complex.

Further studies in which the pathway of electrons through the cytochrome b–c_1 complex were studied by pre-steady-state kinetics also provided data inconsistent with the Q-cycle (deVries *et al.*, 1982). In the absence of antimycin, cytochrome b-562 was reduced with biphasic kinetics in which the initial rapid reduction phase coincided with the formation of the antimycin-sensitive ubisemiquinone at center i. Both the iron–sulfur cluster and cytochrome c_1 were reduced monophasically but at a rate lower than the initial rapid reduction of cytochrome b-562. These results are clearly inconsistent with the Q-cycle which predicts that cytochrome b should be reduced more slowly and that the reduction of the iron–sulfur protein and cytochrome c_1 should occur simultaneously with the formation of the ubisemiquinone anion. Numerous other kinetic studies have been performed on the cytochrome b–c_1 complex; however, the data are often difficult to interpret and are subject to different explanations.

An interesting study was published from Lehninger's laboratory in which the location of the proton-extruding steps at this site of the respiratory chain was examined (Alexandre *et al.*, 1980). Measurements of $H^+/2e^-$ ratios in mitochondria were performed using either succinate or glycerol phosphate as substrate. It should be noted that the primary dehydrogenase for succinate faces the matrix, while that for glyerol phosphate faces the cytoplasmic side of the inner mitochondrial membrane. With both substrates the $H^+/2e^-$ ratio was four during the transfer of electrons to cytochrome c and ferricyanide, an observation not predicted by conventional Q-cycle modes which are all based on succinate dehydrogenase facing the matrix. Secondly, in the presence of antimycin ubiquinol could be oxidized from either substrate in the presence of a TMPD bypass which presumably accepts electrons from the ubiquinol pool resulting in the extrusion of $2H^+/2e^-$. From these observations, the authors concluded that the substrate hydrogens are first transferred to ubiquinone such that $2H^+/2e^-$ appears in the medium by protolytic dehydrogenation of a species of ubiquinol and that the other two protons are translocated from the matrix to the medium on passage of two electrons through the cytochrome b–c_1 complex (Alexandre and Lehninger, 1982). This proposal thus accommodates the Q-cycle mechanism for electron transfer with the b-cycle mechanism for proton translocation. In a recent study using TMPD and DCIP as bypasses of the antimycin-sensitive pathway of electron flow at this site of the respiratory chain, Alexandre and

Lehninger (1984) reported that these two bypasses are inhibited by myxothiazol and HNQ which both have been shown to act at center *o*. These results plus the observed subsequent reoxidation of cytochrome *b* were taken as further evidence for the transfer of electrons by the Q-cycle mechanism.

A very potent tool for the study of the role of ubiquinone in both electron transfer and proton translocation are mutants of yeast which lack any detectable ubiquinone (Sidhu and Beattie, 1985). The largest number of mutants isolated by Tzagoloff *et al.* (1975) lack NADH : cytochrome *c* reductase activity which could be restored by addition of exogenous ubiquinone analogues. Spectral analysis of petroleum ether–methanol extracts of some of these mutants showed an absence of ubiquinone in their mitochondria, while the wild type contained normal levels (Brown and Beattie, 1977). Both succinate and NADH : cytochrome *c* reductase activity as well as ATP synthesis can be restored in these mutant mitochondria by the addition of several analogues of ubiquinone (DeSantis *et al.*, 1982; Beattie and Clejan, 1986), suggesting that these mutants have not lost the capacity to assemble the complete respiratory chain and phosphorylating enzymes but simply cannot synthesize ubiquinone. The block in the ubiquinone biosynthetic pathway in the commonly used mutant strain, E3-24, has been shown to be

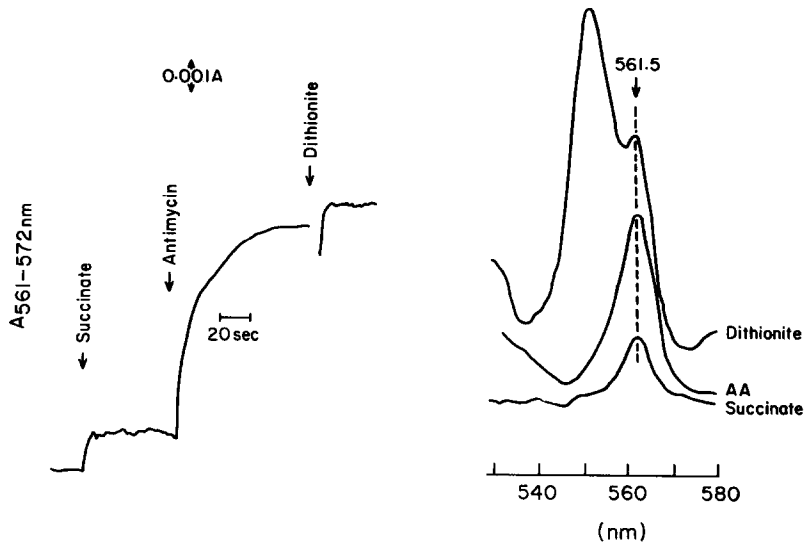

Fig. 2. Reduction of cytochrome *b* by succinate in the presence of antimycin in ubiquinone-deficient yeast. Mitochondria isolated from the mutant were resuspended at 2 mg/ml in the medium described by Meunier-Lemesle *et al.* (1980). After a baseline correction, succinate, 30 mM, antimycin, 22 μg, and a few crystals of dithionite were added. Left panel, kinetics of reduction in the dual-wavelength mode. Right panel, difference spectra recorded in the dual-beam mode.

at the level of an early intermediate, 3,4-dihydroxyhexoprenylbenzoate (Goewert *et al.*, 1981).

In recent studies in our laboratory (Clejan and Beattie, 1986), it was noted that in the presence of antimycin, succinate caused a slow biphasic reduction of cytochrome b in the mutants totally lacking ubiquinone to a level of 60–70% of the total dithionite-reducible cytochrome b (Fig. 2). This reductive pathway was sensitive to both myxothiazol and HNQ, suggesting that the pathway must involve cytochrome b-566 to which these inhibitors have been shown to bind (von Jagow *et al.*, 1984); however, no reduction of cytochrome c_1 was observed under these conditions. Similarly, DeSantis *et al.* (1985) have reported preliminary evidence that both NADH and succinate caused a reduction of cytochrome b in submitochondrial particles of the ubiquinone-deficient mutant and suggested that in the native membrane of mitochondria, a connection between the dehydrogenase and complex III may occur. Studies currently in progress using these mutants should be useful in further clarifying the pathway of electron transfer in this region of the respiratory chain.

Pathways for proton pumping

Proton translocation is an intrinsic property of the cytochrome b–c_1 complex as indicated by experiments in which an isolated complex III is reconstituted into proteoliposomes such that electrogenic proton movements are measured (Beattie and Villalobo, 1982). The substrate for electron transfer under these conditions is generally a reduced analogue of ubiquinone such as durohydroquinol or the decyl analogue. A careful consideration of the Q-cycle as depicted in Fig. 1 does not account for electrogenic proton pumping with exogenous quinols. It is possible that exogenous ubiquinols may reduce cytochrome c directly and not the ubiquinone "pool."

The observation most often quoted as favoring the b-cycle for proton translocation in the cytochrome b–c_1 region of the chain is the pH dependence of the oxidation/reduction midpoint potential of cytochrome b. Such a linkage between protolytic events and oxidoreduction, called "redox Bohr" effects by analogy with hemoglobin (Papa, 1982), have been observed in several electron-carrying proteins. Several groups (Papa, 1982; Wikstrom *et al.*, 1981; von Jagow and Engel, 1980) have suggested that these changes may be linked to proton pumping. It should be noted that the Bohr effects in an isolated cytochrome b–c_1 complex were $0.84\,H^+$ at pH 7.0 for each electron transferred to the iron–sulfur protein (Guerreri *et al.*, 1981).

Recent observations with dicyclohexylcarbodiimide (DCCD), the well-known carboxyl-modifying reagent, have raised questions about the mechanisms of the Q-cycle for proton translocation at site 2 of the respiratory

chain. In recent years, DCCD has been widely used to study the mechanism of proton translocation in a variety of enzyme complexes of the respiratory chain. DCCD has been shown to inhibit the movement of protons in F_1–F_0 ATPases by covalent binding to a specific, single glutamyl or aspartyl residue (Fillingame, 1980). Similarly, DCCD has been reported to block electrogenic proton translocation in cytochrome *c* oxidase of the respiratory chain (Casey *et al.*, 1980) where it binds to a single glutamyl residue present in subunit III of the enzyme (Prochaska *et al.*, 1981). Interestingly, considerable sequence homology exists in the region of the protein adjacent to the glutamyl residue to which the DCCD binds, suggesting that a similar mechanism may exist for the translocation of protons in these different enzyme complexes (Senior, 1983). In addition, inhibition of proton translocation by DCCD has also been observed in the H^+-ATPases of the plasma membrane (Sussman and Slayman, 1983) and in the mitochondrial transhydrogenase (Pennington and Fisher, 1981; Phelps and Hatefi, 1981).

Recently, we reported that DCCD inhibited the proton-translocating device in the yeast complex III reconstituted into liposomes (Beattie and Villalobo, 1982) with only minimal effects on the cytochrome *c* reductase activity of either the isolated complex or that reconstituted into liposomes (Clejan and Beattie, 1983). Similarly, DCCD was reported to inhibit electrogenic proton movements driven by succinate or ubiquinol oxidation without significant effect on the rate of cytochrome *c* reductase activity in mitochondria isolated from beef heart (Esposti and Lenaz, 1982; Esposti *et al.*, 1983) or rat liver (Price and Brand, 1983). Esposti and Lenaz (1982) have also reported that DCCD inhibits proton translocation but not electron transport in complex III from beef heart mitochondria reconstituted into liposomes. By contrast, Nalecz *et al.* (1983) observed in studies on isolated and reconstituted complex III from beef heart mitochondria that DCCD inhibits equally electron flow and proton translocation. Subsequently, Lorusso *et al.* (1983) reported that treatment of complex III from beef heart mitochondria with DCCD caused a marked depression of proton translocation in vesicles under conditions where the rate of electron flow in the coupled state was actually enhanced.

Similar studies with rat liver mitochondria in our laboratory (Clejan *et al.*, 1984) indicated that the primary effect of DCCD at site 2 of the respiratory chain was an inhibition of proton translocation. The inhibitory effects of DCCD were time and concentration dependent and affected by the pH of the medium, suggesting that the processes of electron flow and proton pumping can be uncoupled. An uncoupling of these two processes would be contradictory to all proposed mechanisms of the Q-cycle in which an obligatory coupling of electron flow with the concomitant ejection of protons is depicted.

These specific inhibitory effects of DCCD on proton translocation in the cytochrome b–c_1 region of the respiratory chain have prompted investigations of the possibility that a covalent linking of DCCD with one or more subunits of complex III may occur, as has been demonstrated with other proton-translocating enzyme complexes (Senior, 1983). Initially, it was shown in this laboratory that radioactive DCCD binds selectively to cytochrome b in yeast complex III, suggesting that this protein may be involved in proton translocation at this site of the respiratory chain (Beattie and Clejan, 1982; Beattie *et al.*, 1984). Contrasting results were reported, however, with complex III isolated from beef heart mitochondria. In one laboratory, the binding of radioactive DCCD to all subunits of this complex was reported with a preferential binding to cytochrome b in the reconstituted complex (Nalecz *et al.*, 1983). Under certain conditions, cross-linking was observed between subunits V (the iron–sulfur protein) and VII after treatment with DCCD.

Subsequently, Lorusso *et al.* (1983) reported that DCCD was bound preferentially to the 8-kDa subunit of the complex concomitant with cross-linking of both the 8- and 12-kDa subunits of the iron–sulfur protein. By contrast, Esposti *et al.* (1983) reported that the preferential binding of DCCD to the 8-kDa peptide (subunit VIII) correlated with the selective inhibition of electrogenic proton ejection. Recently, we reported that DCCD binds to both cytochrome b and subunit VIII of the beef heart complex III in a time and concentration dependent manner (Clejan *et al.*, 1984). No cross-linking was observed after DCCD treatment unless the complex was reisolated by precipitation with ammonium sulfate (Beattie *et al.*, 1985).

These results demonstrating the binding of DCCD to cytochrome b in complex III from yeast mitochondria and possibly to cytochrome b of the beef heart complex suggest a major role for this protein in proton translocation at this site. Interestingly, the pH profile for the DCCD inhibition of proton translocation in rat liver mitochondria parallels the profile of the extent to which both the b cytochromes and the iron–sulfur protein function as effective hydrogen carriers in line with earlier suggestions that Bohr effects in cytochrome b might account for the movement of protons across the membrane. In this context it should also be noted that cytochrome b has been shown to span the inner mitochondrial membrane (Chen and Beattie, 1981; Beattie *et al.*, 1981), a necessary prerequisite for a proton carrier in energy transductions.

It is possible that the effects of DCCD on proton ejection are a consequence of a change in the membrane leading to increased proton conductance, thus masking the measurement of electrogenic proton ejection. However, direct measurements of proton conductance by using Li^+-loaded

liposomes suspended in a K^+-medium, driven by a valinomycin-induced diffusion potential, suggested that the DCCD-treated cytochrome b–c_1 complex actually had a lower proton conductance than the untreated complex similarly embedded in liposomes (Beattie and Villalobo, 1982).

Other reagents have also been studied for their ability to dissociate proton translocation from electron transfer in complex III. The amine-reactive agent, fluorescamine, was reported to block electrogenic proton movements in rat liver mitochondria without significant effects on the rate of electron transfer in the succinate-cytochrome c region of the respiratory chain (Tu *et al.*, 1981), suggesting that an obligatory coupling between proton ejection and electron transfer may not exist.

In a similar study, Ting and Wang (1982) treated beef heart mitochondria with specific amino acid-modifying agents and subsequently studied proton extrusion and respiration. Reagents such as 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (a carboxyl reagent) and tetranitromethane (a tyrosyl reagent) blocked proton movements to a greater extent than oxygen uptake, leading to a decreased H^+/O ratio. Ting and Wang (1982) proposed an indirect mechanism for proton pumping based on protein conformational changes driven by electron transport. It should be noted that these studies involved oxygen uptake representing electron transport by the entire respiratory chain, and hence it is impossible to pinpoint which proton translocating event is modified. Certainly, further experiments with these reagents to clarify exactly where the modification occurs would appear reasonable.

By contrast, Lorusso *et al.* (1983) reported that tetranitromethane inhibited electron transport as well as proton translocation in complex III incorporated into proteoliposomes. More recently, this same group (Lorusso *et al.*, 1985) reported that papain treatment of the cytochrome b–c_1 complex from beef heart mitochondria caused the proteolysis of several subunits of the complex resulting in some inhibition of electron transfer but complete blocking of proton translocation. The identification of specific amino acid or protein-modifying reagents that under certain experimental conditions act to "uncouple" proton translocation and electron transfer will be invaluable in an eventual understanding of proton ejection at this site of the respiratory chain.

In some recent experiments in our laboratory, we have attempted to demonstrate directly the presence of a proton channel in the cytochrome b–c_1 complex. The method involves measurements of proton conductance in liposomes containing the complex compared to that of "simple" liposomes without protein using the procedure of Okamoto *et al.* (1977) who demonstrated proton conductance in the TF_0 portion of the proton-translocating ATPase complex of the thermophilic bacterium PS3. Liposomes were

prepared by sonication using Sigma Type V phosphatidylcholine which is actually a mixture of phospholipids in the presence of KCl to form K^+-loaded vesicles. Subsequently, complex III purified from yeast mitochondria by the procedure of Sidhu and Beattie (1982) was incorporated into the liposomes by the cholate dialysis method (Beattie and Villalobo, 1982) modified such that the final dialysis steps were against a medium containing LiCl. The fluorescence quenching of the pH-sensitive dye, 9-aminoacridine, was used to measure pH changes within the liposomes. As indicated in Fig. 3A, the vesicles were essentially impermeable to protons, since after the establishment of a diffusion potential upon addition of valinomycin, only a very slight quenching of fluorescence was observed. Addition of the uncoupler, CCCP, collapsed the potential immediately as indicated by the rapid quenching of fluorescence observed. Figure 3B suggests that the pH within the liposomes is identical to that outside, since the initial addition of CCCP had no effect upon the fluorescence.

In the liposomes containing the b–c_1 complex, the rate of 9-aminoacridine fluorescence quenching was almost three times that of the simple liposomes (Fig. 3C). These results suggest that when the b–c_1 complex is present in the liposomes, it may form a channel which facilitates the movement of protons across the membrane. It should be noted that the amount of complex added in comparison to the total amount of phospholipid present should not be sufficient to cause a nonspecific leakage of protons. Further studies are underway to investigate the proton conductance observed in the b–c_1 complex.

The Topographical Organization of Complex III in the Membrane

Before conclusions as to the data supporting either the b-cycle or the Q-cycle for electron transfer and proton translocation at this site of the respiratory chain can be firmly made, it is important that the subunit structure of the complex be compatible with the kinetic or enzymatic data. The topography and subunit interaction of the polypeptides of complex III in both the soluble and membrane-bound states have been studied by several different methods including labeling with diazobenzenesulfonate (Bell *et al.*, 1979; Beattie *et al.*, 1981), lactoperoxidase iodination (Gellefors and Nelson, 1977; D'Souza and Wilson, 1982), chemical cross-linking (Smith *et al.*, 1978), photoaffinity labeling with lipid analogs (Gutweniger *et al.*, 1981), electron microscopy (Leonard *et al.*, 1981), proteolytic digestion (Sidhu and Beattie, 1982; Sidhu *et al.*, 1983), and immunoinhibition studies with antibodies against specific subunits (Sidhu *et al.*, 1983).

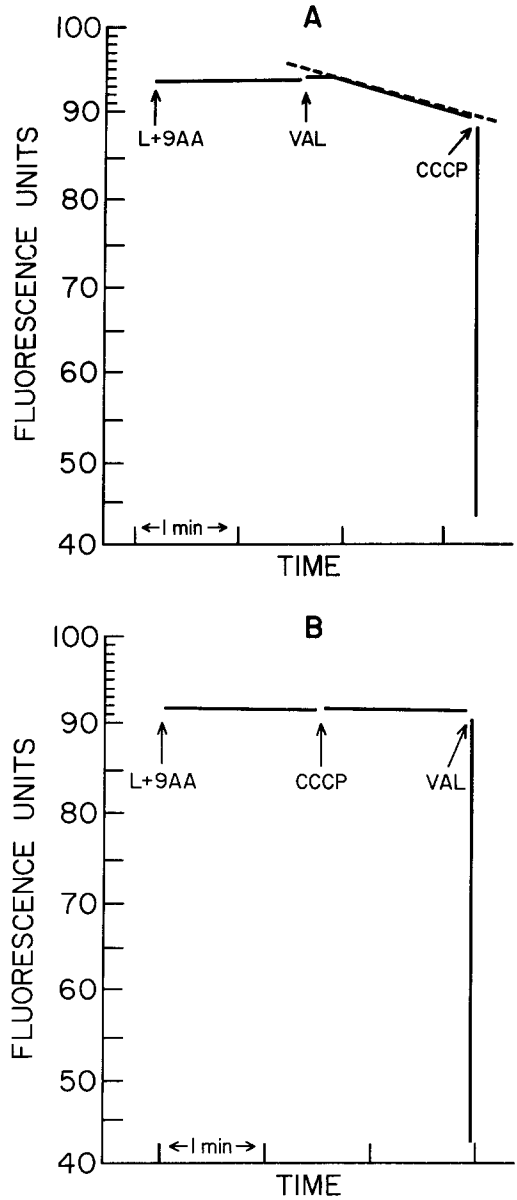

Fig. 3. Fluorescence quenching of 9-aminoacridine in "simple" liposomes and liposomes containing complex III. Liposomes were prepared by sonication of phospholipids in 100 mM KCl, 20 mM Hepes, pH 7.2, followed by cholate dialysis in 100 mM LiCl, 20 mM Hepes, pH 7.2 (Beattie and Villalobo, 1982). (A) Fifty μl of simple liposomes and 5 μM 9-aminoacridine were added to 950 μl of the LiCl buffer. The quenching of 9-aminoacridine was recorded upon addition of 30 ng of vilinomycin. CCP, 100 ng, was added to collapse the ΔpH. (B) Identical to panel A, with the addition of CCCP prior to valinomycin. (C) Liposomes (50 μl) containing the cytochrome b–c_1 complex were prepared by cholate dialysis and added to 950 μl of LiCl buffer containing 5 μM 9-aminoacridine.

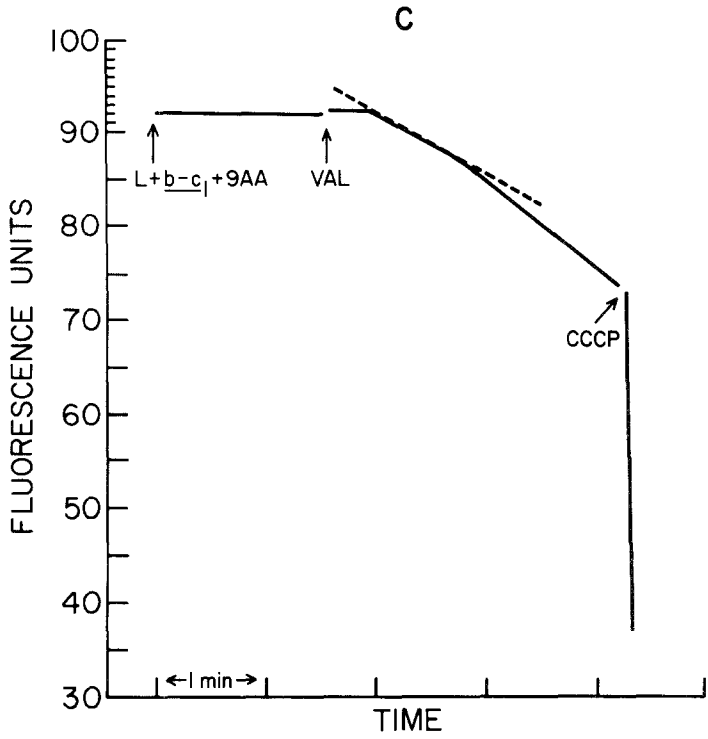

Fig. 3. Continued.

The results of the above studies are all in general agreement that cytochrome b and the two core proteins span the membrane, while cytochrome c_1 and the iron–sulfur protein are substantially exposed to the outer surface of the inner membrane. A transmembrane orientation of the complex was indicated by the immunoinhibition data obtained with IgG against complex III (Sidhu *et al.*, 1983). Similarly, labeling with diazobenzene sulfonate had indicated that cytochrome b and the two core proteins were accessible to this hydrophilic reagent from both sides of the membrane (Chen and Beattie, 1981).

The extent to which the polypeptide chains of cytochrome b and c_1 protrude from the cytoplasmic surface of the membrane was measured by digestion of mitoplasts, mitochondria from which the outer membrane has been removed, with chymotrypsin (Sidhu *et al.*, 1983). The results indicated that a considerable mass of both these hemoproteins are susceptible to chymotrypsin digestion. Furthermore, spectral analysis of the digested mitoplasts revealed a 60% decrease in the cytochrome c_1 content and an 80%

decrease in cytochrome *c*; however, in terms of heme content, cytochrome *b* was unaffected by digestion with chymotrypsin (Sidhu *et al.*, 1983). These results suggest that the heme of cytochrome *b* is buried within the membrane, in agreement with previous studies using electron paramagnetic measurements which had indicated that the hemes are far from the surface of the membrane (Ohnishi *et al.*, 1982), but closer to the cytoplasmic surface (Case and Leigh, 1976).

A comparison of the amino acid sequences of *b* cytochromes from a number of species have indicated a high degree of homology including two conserved histidine residue pairs which are considered to be the ligands for the hemes of cytochrome *b* (Widger *et al.*, 1984; Saraste, 1984). Hydropathy plots of the protein have suggested the possibility of eight to nine membrane spanning domains with the hemes located perpendicular to the plane of the membrane. None of these studies discussed above have indicated that the two hemes of cytochrome *b* are localized on different sides of the membrane as suggested by the Q-cycle. By contrast, the experimental evidence that cytochrome *b* spans the membrane is consistent with its proposed role as a proton pump.

The close relationship in the membrane between cytochrome *b* and the iron–sulfur protein should also be considered. Protein–protein interactions between these two subunits of complex III were studied in our laboratory by examining the immunoprecipitates obtained from Triton X-100 solubilized mitochondria and subunit specific antisera. The specific antiserum developed against the iron–sulfur protein was observed to immunoprecipitate cytochrome *b* along with the iron-sulfur protein, while the other subunits of the complex were not observed in the immunoprecipitate (Sidhu *et al.*, 1983). It should be noted that after solubilization with SDS, this same antibody only immunoprecipitated the iron–sulfur protein.

A similar close relationship of these two proteins was suggested by Capeillère-Blandin and Ohnishi (1982) who reported a deficiency of the Rieske iron–sulfur clusters in mutants of yeast lacking cytochrome *b*. They suggested that an intact cytochrome *b* containing a heme may be necessary to the synthesis of the iron–sulfur protein. Using these same cytochrome *b*-deficient mutants of yeast, we have recently demonstrated that in the absence of cytochrome *b*, there is a 50% decrease in the amount of the iron–sulfur protein in the mitochondrial membrane (Sen and Beattie, 1985). Furthermore, this protein appeared to be less tightly bound to the membrane in the mitochondria lacking cytochrome *b*, since mild sonication released significant amounts of this protein from the membrane. A close relationship between the polypeptide chains of cytochrome *b* and the iron–sulfur protein may reflect an interaction between their active sites. This concept is strengthened by recent observations that inhibitors such as stigmatellin bind

to and interact with the active site of both the heme b-566 domain of cytochrome b and the iron–sulfur clusters of the iron–sulfur protein (von Jagow and Ohnishi, 1985).

The localization of ubiquinone in the lipid bilayer of the membrane should also be considered in discussions of proton translocation in the b–c_1 complex. Measurements using a variety of techniques have indicated that ubiquinone is located relatively deep within the hydrophobic region of the bilayer near the midplane (Lenaz and Esposti, 1985). Measurement of the lateral diffusion coefficients of ubiquinones in lipid vesicles by a fluorescence quenching technique revealed diffusion coefficients higher than 10^{-6} $cm^2 \cdot s^{-1}$ at 27°C, suggesting the localization of the ubiquinones in the low-viscosity midplane region of the bilayer (Fato *et al.*, 1985). Similar conclusions were reached by Ulrich *et al.* (1985) using a different approach. Furthermore, studies of the transmembrane mobility of ubiquinone in which exogenous ubiquinols were used to reduce ferricyanide trapped within lipid vesicles indicated that the transmembrane diffusion rates were not rapid enough to explain the rates of cytochrome c reduction by ubiquinol in mitochondria, if the ubiquinols and ubiquinones must diffuse across the membrane as suggested in some versions of the Q cycle (Fig. 1A). More recently, however, the Q-cycle has been modified such that the mobile-hydrogen conducting elements of the QH_2/Q couple are suggested to be concentrated in a ubiquinone-rich zone near the center of the bilayer. This "Q zone" is envisioned to become oriented toward the inner or outer surface by the organization of the lipids and proteins in the membrane (Mitchell and Moyle, 1985). From this discussion, it is clear that more studies of ubiquinone chemistry are necessary to establish its localization in the membrane plus more information about its actual mobility in the membrane before definite statements can be made.

Conclusions

In this review, an attempt has been made to summarize the experimental data supporting the view that the cytochrome b–c_1 complex may function as a proton pump. While it appears clear that the pathway of electrons through this part of the respiratory chain must proceed via a branched-chain mechanism to explain the oxidant-induced reduction of cytochrome b, certain experimental results indicate that inconsistencies remain in the proposed flow of electrons in both the Q and b cycles as currently drawn. The major area of disagreement, however, in energy coupling at site 2 is currently centered on the mechanism of proton translocation during electron transport. It is clear that the availability of purified complexes from a number of organisms

will now permit detailed studies of the polypeptide composition and relationships among proteins in the complex. Such information as to the structural organization of the complex coupled with knowledge as to the localization of both the ubiquinone "pool" and the bound ubiquinones relative to the proteins will be invaluable in deciding between different hypotheses as well as in designing further experiments. The ability to reconstitute the complex into proteoliposomes and measure proton pumping directly will also permit experiments on the molecular level in a system considerably simpler than the intact mitochondrial membrane. Furthermore, the availability of mutants in complex III isolated from yeast or *Neurospora* plus the use of the recombinant DNA technology for site-directed mutagenesis may provide another approach to our understanding of energy transduction at site 2 of the respiratory chain.

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