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MINI REVIEW

Is There Sufficient Experimental Evidence to Consider the Mitochondrial Cytochrome bc_1 Complex a Proton Pump? Probably No.[1]

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Abstract

The electron flow through the cytochrome bc_1 complex of the mitochondrial respiratory chain is accompanied by vectorial proton translocation, though the mechanism of the latter phenomenon has not yet been clarified. Several proposed hypotheses are briefly presented and discussed here. Recently, a number of papers have appeared claiming the existence of a proton pump in the enzyme mainly on the basis of the interaction of the complex with N,*N'*-dicyclohexylcarbodiimide. These data are reviewed here with the aim of showing their ability to fit multiple interpretations. This together with some other arguments leads to the conclusion that a proton pump in the mitochondrial bc_1 complex has not yet been demonstrated.

Key Words: Mitochondria; respiratory chain; bc_1 complex; proton translocation; proton pump.

Introduction

The cytochrome bc_1 complex of the mitochondrial respiratory chain is an oligomeric lipoprotein complex containing as electron carriers two spectroscopically distinguished species of cytochrome *b* (*b*-562 and *b*-566, the latter having a shoulder in the alpha-band absorption peak at 558 nm) (Slater, 1981), a high-potential iron–sulfur protein with a binuclear iron–sulfur cluster (Trumpower, 1981a) and cytochrome c_1 (Yu *et al.*, 1972). It is

[1] This article is dedicated to the memory of my friend and long-time close collaborator Dr. Robert P. Casey who has passed away after a short, tragic illness at the age of 34.

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also believed that the functional enzyme requires ubisemiquinone for electron transfer through the complex (Mitchell, 1975). The electron transport activity of the bc_1 complex is coupled to vectorial proton translocation though the mechanism of the latter remains unclear. Several models have been proposed for the proton translocation by the enzyme: the protonmotive Q-cycle (Mitchell, 1975, 1976), its derivation in a form of the so-called "double Q-cycle" (De Vries *et al.*, 1983; Slater, 1983), the "*b*-cycle" (Wikström and Krab, 1980), the "vectorial Bohr shift" where proton translocation results from protein conformational changes (Papa, 1976; Von Jagow and Engel, 1980), and a variation of the latter mechanism where protons are translocated through a specific channel in the enzyme (Papa, 1981). The present article will give a short look into the experimental evidence for and against these hypotheses.

Proton Translocation by the bc_1 Complex

In spite of the different mechanisms proposed, it is generally agreed that for each two electrons passing through the bc_1 complex, four protons appear on the electropositive, cytoplasmic side of the inner mitochondrial membrane with a charge/$2e^-$ ratio of 2 (for reviews see Rieske, 1976; Wikström *et al.*, 1981; Papa, 1982; Rich, 1984). There is no difference in the measured H^+/e^- ratio regardless of whether quinol or succinate is used as an electron donor to the system (see, e.g., Krab *et al.*, 1984). Two of the four protons are uncoupler-insensitive and result from the overall oxidation of QH_2 by cytochrome *c*. The quinol oxidation site is located close to the cytoplasmic side of the inner mitochondrial membrane (see, e.g., Trumpower, 1981a, b) and thus ubiquinone acts as a classical protonmotive redox component (Mitchell, 1961). The other two protons (and 2 charges per $2e^-$) are uncoupler-sensitive and are believed to be vectorially translocated from the electronegative, matrix side of the inner mitochondrial membrane. Different hypotheses have been used to explain the appearance of these two extra protons.

The same proton/electron stoichiometries are also obtained in a reconstituted system when using purified bc_1 complex (Leung and Hinkle, 1975 and many followers since then). The importance of this fact is clear: it enables the use of the purified protein complex to study its proton translocating function without any interference from other membrane components. Though proteoliposomes are not free from disadvantages (e.g., low internal buffering capacity that may limit the amount of proton transport, heterogeneity of vesicles, variable amount and sidedness of the reconstituted protein), they still serve as the most convenient model system. Most of the studies discussed below were performed on the isolated and reconstituted bc_1 complex.

A similar proton translocating activity is observed when succinate-cytochrome *c* oxidoreductase (complexes II + III) is reconstituted instead of the pure bc_1 complex (Nałęcz and Azzi, unpublished data). In addition, coupling and H^+/e^- stoichiometries of such a system depend on the fluidity (cholesterol content) of the liposomal membrane with an optimum at about 5 mol % cholesterol. These data suggest that a relatively high fluidity accompanied by a decreased leakiness of the membrane is ideal for the functional enzyme (Nałęcz and Azzi, unpublished). All of this supports a possible involvement of a mobile proton carrier in the reaction mechanism and confirms the lack of a proton translocating step at the level of succinate dehydrogenase.

Different Hypotheses

Q-Cycle

The Q-cycle (Mitchell, 1975, 1976) assumes that mobile ubiquinone molecules transport both electrons and protons through the membrane. The model suggests a sequence of chemical reactions vectorially organized in the membrane in such a way that protons are taken from the matrix and released into the external phase. This idea may be currently treated as "classical," making use of mobile redox groups as the agents of proton movement. The original requirements of the Q-cycle, i.e., quinone and quinol mobility, the existence of a stable protein-bound ubisemiquinone species, and lack of a direct redox contact between *b* and *c* cytochromes, have all been experimentally proven (for reviews see Slater, 1981; Trumpower, 1981a, b; Slater, 1983; Rich, 1984). Further evidence in favor of the Q cycle came also from studies with different inhibitors distinguishing between two pathways of the reduction of cytochrome *b* (e.g., Bowyer *et al.*, 1982; Zhu *et al.*, 1982a, b; Von Jagow *et al.*, 1984) and from characteristics of the isolated iron–sulfur protein (Trumpower, 1981a). However, the Q-cycle in its recent formulation does not fully account for all experimental observations concerning the electron flow (see De Vries *et al.*, 1983 and Matsuura *et al.*, 1983). In order to explain these findings, modifications of the original Q-cycle have been proposed (see De Vries *et al.*, 1983 and Slater, 1983 for the "double Q-cycle" and Rich, 1984, for a review), but not all of them were found to be necessary later. For instance, assumptions made in the "double Q-cycle" were criticized on the basis of recent EPR studies of the bc_1 complex (Salerno, 1984). Some findings were alternatively interpreted (Rich, 1983), and the existence of such a mechanism has been questioned on the basis of isolation of an active bc_1 monomer (Nałęcz *et al.*, 1985b; Nałęcz and Azzi, 1985).

As to the proton translocating activity, the Q-cycle predicts both proton and charge stoichiometries observed experimentally for the bc_1 complex, although it is not clear whether the mobility of a Q/QH_2 couple is high enough to transfer protons across the membrane.

b-Cycle

The mechanism of electron transfer in the "*b*-cycle" (Wikström and Krab, 1980; Wikström *et al.*, 1981) is similar to that of the Q-cycle and also assumes no direct redox contact between *b* and *c* cytochromes: cytochrome c_1 is reduced via iron–sulfur protein (by the QH_2/QH couple) whereas cytochrome *b* is reduced directly by the QH/Q couple. Both schemes also have an antimycin-sensitive site at which cytochrome *b* is reoxidized by quinone and/or semiquinone, but the "*b*-cycle" assumes only one site of interaction with quinones whereas the Q-cycle assumes two. Recent findings that myxothiazol and antimycin are able to act independently (Von Jagow and Engel, 1981; Rich, 1983; Von Jagow *et al.*, 1984) suggest the existence of the two quinone-interacting sites. Moreover, it has been reported that the fully oxidized quinol may easily oxidize cytochrome *b* (Rich, 1983), which is not predicted by the "*b*-cycle" mechanism. All of this favors the Q-cycle as far as the electron flow is concerned. Transmembrane proton and charge translocation activity, however, may be a different case. In the "*b*-cycle" mechanism it is not mobile CoQ which transfers protons, but a redox-linked proton binding species in contact with different sides of the membrane. Such species, preferentially cytochromes *b*, would undergo conformational changes during the redox cycle and hence change pK values of the proton-binding group(s), as envisaged by the "vectorial Bohr shift" hypothesis.

Redox Bohr Effects, a Proton Pump

This proposal involves a mechanism in which the protonmotive process is restricted to a single polypeptide or even a single group changing its pK value on different sides of the membrane upon redox-linked conformational changes of the electron carrier. The first formulation of the redox Bohr effects for metal centers in the respiratory chain (Papa, 1976) specified general features of the mechanism. For the bc_1 complex the idea soon became associated with the *b* cytochromes since they have pH-dependent midpoint potentials at physiological pH values and could therefore serve as proton translocators. They are also integral transmembrane proteins in contact with both sides of the membrane (Smith and Capaldi, 1977; Gellerfors and Nelson, 1977). The most elaborated proposal of the cytochrome *b* involvement in the proton translocation activity came from Von Jagow and Engel (1980). The

model assumes that the dimer of cytochromes *b*, *b*-562, and *b*-566 is composed of the same monomers in two opposite functional states having different midpoint potentials. When the redox state of the heme *b* center is one of the monomers changes, a hypothetical amino acid residue consequently changes its pK value (protonation or deprotonation). During this process the amino acid is moved from the "inner to the outer position" due to accompanying change of protein conformation. Such a movement, though, is unlikely to occur across the membrane and therefore the existence of some proton channels providing connection to the matrix and to the cytoplasm has been postulated. The hypothetical group would be in this case moved from a "matrix channel" (where it accepts proton) to a "cytoplasmic channel" (where proton is released).

This hypothesis was based on several thermodynamic measurements performed on the mitochondrial enzyme (Von Jagow and Engel, 1980, 1981) and especially on the fact that the pH-dependent midpoint potentials of -60 mV per pH unit were reported for cytochromes *b* (e.g., Dutton and Wilson, 1974; Erecińska and Wilson, 1976; Nelson and Gellerfors, 1976). This would allow one redox-linked proton translocation associated with each of the two cytochromes *b* thus fitting the $4H^+/2e^-$ and 2 charges/$2e^-$ stoichiometry (with two scalar protons being released from the oxidation of quinol). In addition, it is known that the potentiometric behavior of the cytochrome *b* is very sensitive to even small structural changes of the enzyme (see, e.g., Leigh and Erecińska, 1975; Yu *et al.*, 1979), and this makes the opposite (redox-induced conformational changes) more likely. However, there are also difficulties with this hypothesis. First, it is now clear that cytochrome *b* is a monomer containing two protoheme groups and not a dimer (see, e.g., Slater, 1981; T'sai and Palmer, 1983), i.e., the "vectorial Bohr shift" would occur within a single polypeptide between the two redox centers, yet this is more difficult to envisage. Second, the small pH-dependence of the cytochromes *b* of the chloroplast *bf* complex (Rich and Bendall, 1980; Hurt and Hauska, 1982) limits the applicability of the hypothesis and argues against such a mechanism for the chloroplast system. Third, recent studies on the yeast enzyme revealed that the pH-dependence of the midpoint potentials of both hemes *b* is -30 mV/pH unit, i.e., two electrons passing through the *b*-couple would be necessary for the translocation of each proton (T'sai and Palmer, 1983). In such a case the hypothesis does not account for the measured H^+/e^- stoichiometry.

Another candidate to serve as a redox-driven proton translocating agent has been proposed to be the Rieske iron–sulfur protein (Papa, 1976, 1981). The localization of a proton pump in the iron–sulfur protein was based on the fact that the midpoint potential of this electron carrier also depends on pH (Papa, 1976; Prince and Dutton, 1976) and that EPR investigations have

indicated two distinct forms of the mitochondrial Rieske center (De Vries *et al.*, 1979). This would allow formulation of a similar hypothesis to the one described for *b* cytochromes. Two main criticisms were raised against this hypothesis: first, the pH dependence of Fe–S midpoint potential occurs at nonphysiological pH values; second, the EPR spectra of the iron–sulfur center(s) are not fully understood and may meet different explanations (see Trumpower, 1981a for a review).

Q-Gated Proton Pump

Recently, an attempt has been made to combine what was once the antagonistic point of view: the protonmotive ligand conduction (e.g., Q-cycle) and the cooperative proton transfer by apoproteins (redox Bohr effects). By taking elements from different proposals, the group of Papa (Papa, 1981; Papa *et al.*, 1983) formulated a hypothesis in which protonation of the ubisemiquinone/ubiquinol couple from the inner side of the membrane and deprotonation at the outer side results in transmembrane translocation of protons, provided, however, not by CoQ mobility but by a proton channel in one of the polypeptides. Access of protons into the channel and their release on the opposite side is favored by redox-linked pK shifts of ionizable groups in the channel, which, in turn, is a consequence of the redox state of the protein-bound quinone. The model incorporates a branched electron transfer mechanism for the bc_1 system (Wikström and Berden, 1972) and proposes either the Rieske Fe–S protein (Papa, 1981) or cytochrome *b* (Papa *et al.*, 1983) to serve as a proton pump. To confirm such a hypothesis, however, detailed studies on the $\Delta\psi$- and pH-dependencies of individual electron transfer steps are required as well as the identification and characterization of a still hypothetical proton channel.

Which Mechanism is True?

There is no straightforward answer to this question. The amount of data accumulated around the problem even exceeds the needs of separate hypothesis, e.g., the Q-cycle does not attribute any role to redox Bohr effects displayed by *b* cytochromes and the Rieske Fe–S protein whereas the proton pump mechanism does not utilize quinone mobility as its natural ability to transfer protons. The established sequence of electrogenic events within the complex and the effects of inhibitors and maximal measured stoichiometries are well implanted in both Q-cycle and proton-pump type mechanism. The Q-cycle, however, envisages the protonmotive activity of the complex as a direct consequence of transmembrane conduction of protons together with

electrons by quinol molecules, i.e., the tight coupling between these two transports. In such a scheme the H^+/e^- stoichiometry should be constant regardless of whether electron flow is activated in the oxidized or in the reduced enzyme, at different pH and at different rates of electron flow. The "vectorial Bohr shift" mechanism, instead, would expect the H^+/e^- stoichiometry of the pump to follow the pH profile of the midpoint potential of a redox-linked carrier. It would also envisage the possibility of rate-dependent changes in the coupling efficiency caused by interconversion of the enzyme between different conformational forms. In fact, the H^+/e^- stoichiometry appears stable under different experimental conditions and at very different electron flow rates (e.g., Lorusso *et al.*, 1983; Papa *et al.*, 1983; Nałęcz *et al.*, 1983a, b). A recent paper by the group of Beattie on rat liver mitochondria (Clejan *et al.*, 1984b) reported also a stable (approaching 4) H^+/e^- ratio in the 6.9–7.5 pH range, though at pH 6.7 the stoichiometry was found lower (3.3). Such a drop at relatively low pH value, however, is likely to reflect the formation of a proton barrier outside the membrane opposing the outward injection of protons, as originally suggested by the authors.

All this favors the Q-cycle over the direct "vectorial Bohr shift" mechanisms, though it does not exclude the "Q-gated proton pump" hypothesis. The latter also implies stable stoichiometries due to the involvement of the CoQ redox reactions as the source of protons.

Since it appeared difficult to elucidate the proton translocating mechanism on the basis of kinetic or thermodynamic data, a somewhat different experimental approach had to be utilized. Several laboratories have applied more structural studies, especially using amino acid modifiers, with the aim of identifying a hypothetical proton translocating polypeptide possibly involved in the proton-pump type mechanism. Most of these data concern the use of *N,N'*-dicyclohexylcarbodiimide (DCCD).[3]

Effects Induced by DCCD

General

The use of DCCD to study membrane-bound enzymes started from the observation (Beechey *et al.*, 1967) that DCCD strongly inhibits the proton translocating ATPase in mitochondria. It is now clear that this hydrophobic carbodiimide covalently binds to a single carboxylic group located in a hydrophobic domain in one of the subunits of the enzyme and thus blocks

[3] Abbreviations: BAL, British Anti-Lewisite (2,3-dimercaptopropanol); DCCD, *N,N'*-dicyclohexylcarbodiimide; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone; UHDBT, 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole.

the trans-membrane proton movements catalysed by the ATPase (see Fillingame, 1980, for a review). Similarly, cytochrome *c* oxidase has been shown to be inhibited in its proton pumping activity (Casey *et al.*, 1980) due to the interaction of DCCD with, again, a single carboxylic group (Prochaska *et al.*, 1981). In recent years many other membrane-bound enzymes have also been shown to covalently bind and to be inhibited by DCCD and, in several cases, such observations led to important conclusions about their reaction mechanisms (see Azzi *et al.*, 1984 for a review). It was therefore of great interest to study the effect of DCCD on the bc_1 complex. Before going into details of these studies, however, some additional comments are necessary.

There is a certain tendency to treat DCCD as a king of probe for proton translocating enzymes (e.g., Solioz, 1984). This is not the case (see Azzi and Nałęcz, 1984). DCCD, in fact, is a highly reactive agent toward many organic functional groups (Kurzer and Duraghi-Zadeh, 1967) which may not only covalently interact with –COOH, –SH, –OH, and $-NH_2$ in proteins but also may induce intra- and intermolecular crosslinking of polypeptides (for reviews see Kurzer and Duraghi-Zadeh, 1967; Azzi *et al.*, 1984; Nałęcz *et al.*, 1985a). DCCD may also interact with lipids, being hydrophobic and thus easily soluble in the lipid bulk. A certain specificity, however, may be induced by the conditions of the reaction (as cited above).

It is not surprising that, on some occasions, with some enzymes under precise conditions, DCCD is able to act specifically. The protein structure plus the interactions provided by the lipid may create a special environment for some amino acid residues to express a high reactivity toward the carbodiimide. However, this should not be taken as a rule. The binding and the inhibition by DCCD successfully demonstrated for some cation transporting proteins cannot be used as a diagnostic tool for them.

Effects on the bc_1 Complex in Mitochondria and Submitochondrial Particles

The amount of DCCD necessary to inhibit the ATPase activity in mitochondria and submitochondria particles is about 2–20 nmol per mg protein, depending on the temperature and time of incubation (Beechey *et al.*, 1967). The amount necessary to influence the bc_1 complex, instead, was reported to be between 100 and 2000 nmol per mg protein (see Azzi *et al.*, 1984 for a review). Already this first observation puts a question mark on the specificity of such interaction. Indeed, it has been observed that the incubation of rat liver mitochondria with DCCD (200 nmol per mg protein) produces an increase in H^+ permeability of mitochondria (Beattie and Villalobo, 1982). Similar concentration of carbodiimide (150 nmol/mg protein) was observed to abolish the transmembrane electrical potential buildup during succinate oxidation in bovine heart submitochondrial particles

(Degli Esposti *et al.*, 1983). Inevitably, both effects were accompanied by a drop in H^+/e^- stoichiometry.

Another group (Price and Brand, 1982, 1983) did not observe similar signs of DCCD-induced alterations of the membrane but reported on a substantial increase of electron flow from succinate to oxygen, confirmed by the group of Lenaz (Degli Esposti *et al.*, 1982). This was also accompanied by a decrease of the H^+/e^- ratio. It should be noted here that whereas Beattie and Villalobo (1982) measured the enzyme activity spectrophotometrically as cytochrome *c* reduction, the other group of experiments used a polarographic technique. It is now well established that electron flow through the mitochondrial bc_1 complex may lead to superoxide generation which is also stimulated by agents destabilizing bound ubisemiquinone (e.g., Yu *et al.*, 1980; Ksenzenko *et al.*, 1983). It seems likely that DCCD may act as an agent allowing a rapid donation of electrons directly to oxygen and not reassociated with H^+ production. Verification of such a hypothesis could come from experiments using superoxide dismutase or other superoxide quenchers in the experimental system. However, the possibility of some other DCCD-induced electron flow bypasses cannot be excluded either. The high reactivity of this carbodiimide provides grounds for many speculations (e.g., omission of some electron carriers in the reaction chain, promotion of a direct reduction of cytochrome c_1 or cytochrome *c* by quinols, etc.) which cannot be excluded until the effects of different inhibitors of the bc_1 complex (BAL, UHDBT, myxothiazol) are presented for DCCD-treated samples. Such studies have not been done to date.

Recently, the group of Beattie published a new report on DCCD-induced effects in rat liver mitochondria (Clejan *et al.*, 1984b). Using 120 nmol DCCD per mg protein they observed no alteration of proton permeability of mitochondrial membranes, in disagreement with the previous report (Beattie and Villalobo, 1982) concerning the concentration of 200 nmol DCCD per mg protein. This stresses the variability of the effects upon even a slight modification of the experimental conditions. The paper also stated a drop of the H^+/e^- ratio due to addition of DCCD and suggested the existence of a proton pump in the bc_1 complex. In agreement with what was mentioned above, however, the study is missing an important argument showing that the measured reduction of cytochrome *c* or $K_3Fe(CN)_6$ from succinate is indeed enzymatic and catalyzed by a normally functioning bc_1 complex and not through a possible bypass. Since the binding and the inhibition by antimycin is influenced *per se* by DCCD (see below for a more detailed discussion), Clejan *et al.* presented only recorded rates of electron flow and proton appearance, not corrected for nonenzymatic activity. The same study also reported that DCCD decreases swelling of mitochondia in $CaCl_2$ and induces the loss of internal K^+. Both effects are in line with a more

complex alteration of the mitochondrial membrane than a single modification of one polypeptide, as postulated (Clejan *et al.*, 1984b).

For yeast mitochondria it has been observed that the antimycin sensitivity of cytochrome *c* reductase in samples treated with DCCD depends on whether succinate or quinol is used as substrate (Clejan and Beattie, 1983). Actually, only a small alteration of this sensitivity was found in the case of succinate : cytochrome *c* reductase whereas a profound decrease of the antimycin-induced inhibition was observed for quinol : cytochrome *c* reductase. Since both reactions should involve the same catalytic function of the bc_1 complex (and therefore the same inhibitor sensitivity), it seems likely that the externally added quinol preferentially participates in an alternative, antimycin-insensitive, electron pathway induced by DCCD.

At higher concentrations, up to 2000 nmol/mg mitochondrial protein, DCCD inhibits both electron transport and proton translocation by the bc_1 complex without affecting the H^+/e^- ratio (Degli Esposti *et al.*, 1981; Lenaz *et al.*, 1982b).

Effect on the Isolated and Reconstituted Enzyme

Table I summarizes presently available data on DCCD effects on the enzyme as well as basic information about the experimental conditions applied.

Beside listing the most important observations, short comments to some of them seem necessary.

Isolated Enzyme (Table IA)

a. Our report (Nałęcz *et al.*, 1983a, b) that 100 mol DCCD per mol heme *b* strongly inhibits the electron flow through the bc_1 complex has been recently reinterpreted (Clejan *et al.*, 1984b) as being due to the thermal inactivation of the enzyme. Though some loss of the activity was indeed observed during the incubation of the control sample, it was corrected for in the final plot of the DCCD-induced inhibition (Nałęcz *et al.*, 1983a).

b. Crosslinking between subunits of the bc_1 complex upon addition of DCCD (Nałęcz *et al.*, 1983a, b; Degli Esposti *et al.*, 1983; Lorusso *et al.*, 1983) has been ascribed to the presence of ammonium sulfate in the system (Clejan *et al.*, 1984a). However, no ammonium sulfate was present in our experiments and the extent of crosslinking was clearly time-dependent, correlating with the time course of the inhibition of electron flow (Nałęcz *et al.*, 1983a, b). Intermolecular crosslinking requires the subunits involved to be in close contact. The absence of such a crosslinking may point to the enzyme having slightly different conformation depending on the detergent, salt, and other variants of the medium. However, the fact that three separate groups

observed a similar phenomenon concerning the same subunits (Rieske Fe–S protein, subunits VII and VIII) suggests the vulnerability of these polypeptides to interaction with carbodiimide. It might therefore be that under conditions where the actual enzyme conformation does not allow intermolecular crosslinking to occur, an intramolecular phenomenon takes place within single polypeptides. Such alteration would most likely be undetectable by SDS-gel electrophoresis.

c. If this hypothesis is correct, it could explain the observations concerning DCCD alterations of antimycin binding (Clejan and Beattie, 1983; Clejan *et al.*, 1984a, b). Originally it has been postulated that antimycin binds to subunit VII (Das Gupta and Rieske, 1973; Rieske, 1976), the polypeptide which copurifies with cytochrome *b* (Marres and Slater, 1977). Later it was suggested that more than one antimycin binding site may exist in the enzyme (Slater, 1981). Whether or not the subunit VII is the only antimycin binding site, it is the polypeptide that is involved in intermolecular (and possibly intramolecular) crosslinking induced by DCCD. In such a case the structural alteration of this polypeptide would inevitably modify its interaction with antimycin and, due to a general conformational change of the enzyme, could as well lead to spectral modifications (Clejan and Beattie, 1983; Clejan *et al.*, 1984b). Modification of antimycin interaction with the enzyme would, in this case, come from the crosslinking event and not from binding of DCCD, as has been proposed (Clejan and Beattie, 1983). Indeed, it is interesting to note that incorporation of [^{14}C]DCCD into cytochrome *b* was studied at lower concentrations of carbidiimide and shorter incubation times than used to affect antimycin binding (see Clejan and Beattie, 1983; Clejan *et al.*, 1984a, b).

Reconstituted System (Table IB)

a. The group of Azzi was the only one to report a parallel inhibition of electron flow and proton translocation by DCCD in the reconstituted bc_1 complex (Nałęcz *et al.*, 1983a, b). As stated in the methodological sections of these papers, such a result was obtained when subtracting antimycin-insensitive cytochrome *c* reduction from the measured rate of electron flow. Actually, when recalculating these data without this correction, the same result was obtained as reported by others, i.e., a substantial drop in the H^+/e^- ratio coming from a time-dependent decrease of antimycin sensitivity of the enzyme (Nałęcz, Casey, and Azzi, unpublished). The reverse is also true, i.e., the data of Clejan and Beattie (1983), when submitted to our way of calculation, show a stable H^+/e^- stoichiometry upon addition of DCCD. Therfore the difference between these two results does not concern experimental observations, but rather their interpretation. The latter appears vital

	100	15 min or 60 min	35°C 12°C	Binding to many subunits with some preference for cyt. *b* and phospholipids, no crosslinking in the absence of $(NH_4)SO_4$	Not reported	Clejan *et al.*, 1984a
	50 or 25	10–60 min 60 min	22°C 22°C	Preferential binding to cyt. *b* and phospholipids, some binding to subunit VIII, no crosslinking	Not reported	
Yeast mitochondria	100	180 min	12°C	Preferential binding to cyt. *b*	Not reported	Beattie and Clejan, 1982
	100–300	120 min	12°C	Displacement of antimycin from its binding site	Decreased sensitivity to antimycin, inhibition of electron flow (20%)	Clejan and Beattie, 1983
	50–100	30–60 min	12–35°C	Preferential binding to cyt. *b* and phospholipids, no crosslinking	Inhibition of electron flow (up to 60%), substantial termal inactivation at 35°C	Beattie *et al.*, 1984
	50	60 min	12°C	Preferential binding to cyt. *b* and phospholipids, lowered by a pretreatment with antimycin	Small inhibition of electron flow (up to 10%)	

[a]Electron flow is understood here as a reduction of cytochrome *c* by quinol.

Table IA. Isolated bc_1 Complex in Detergent

Source of enzyme	Amount of DCCD (mol/mol heme *b*)	Incubation conditions		Observed modifications		Reference
		Time	Temperature	Structural	Functional	
Beef heart mitochondria	Not specified	Not specified		Preferential binding to core proteins	Inhibition of electron flow[a] (up to 75%)	Lenaz *et al.* 1981a
	100	45 min	35°C	Binding to many subunits, crosslinking between subunits V and VII and V and VIII	Inhibition of electron transfer (85%), correlated with the crosslinking	Nałęcz *et al.*, 1983a, b
	30–50 or 1000	30–240 min 5–10 min	25°C 25°C	Binding to many subunits, crosslinking between subunits V, VII and VIII	Inhibition of electron flow (up to 75%)	Degli Esposti *et al.*, 1983
	30–50	5–10 min	25°C	Preferential binding to subunit VIII	No effect	
	25–30	30 min	25°C	Preferential binding to subunit VIII, labeling of cyt. *b* and core proteins being extractable with acetone, crosslinking of subunits V and VII and/or V and VIII	Not reported	Lorusso *et al.*, 1983

Yeast mitochondria	100–250	Free enzyme treated with DCCD prior to the reconstitution	60–180 min	12°C	Alterations as reported for the isolated enzyme (part A of this table; see Beattie and Clejan 1982)	Inhibition of proton translocation (30%), lower coupling of liposomes, total inhibition of reversed electron flow[b]	Beattie and Villalobo, 1982
	100–200	DCCD added directly to liposomes	180 min	20°C	Not studied on reconstituted enzyme	Inhibition of proton translocation (30–60%), inhibition of cyt. *c* reduction by quinol under coupled conditions (up to 25%), decreased sesitivity to antimycin (50%)	Clejan and Beattie, 1983
	or 100–300	Free enzyme treated with DCCD prior to the reconstitution	120 min	12° C	Alterations as reported for the isolated enzyme (part A of this table)		

[a] Electron flow is understood here as reduction of cytochrome *c* by quinol.
[b] Reversed electron flow is understood here as transfer of electrons from reduced cytochrome *b* to coenzyme Q (reoxidation of cytochrome *b* by quinone), driven by a K^+-diffusion potential.

Table IB. bc_1 Complex Reconstituted into proteoliposomes

Source of enzyme	Amount of DCCD (mol/mol heme *b*)	Incubation conditions			Observed modifications		Reference
		Way of treatment	Time	Temperature	Structural	Functional	
Beef heart mitochondria	400	DCCD added directly to liposomes	30–240 min	35°C	Preferential binding to cyt. *b*, crosslinking of subunits V and VII and V and VIII	Parallel inhibition of electron flow[a] and proton translocation (up to 75%), correlated with the crosslinking	Nałęcz *et al.*, 1983a, b
	30–300	DCCD added directly to liposomes	5 min	25°C	Not studied on reconstituted enzyme	Inhibition of proton translocation (up to 80%), stimulation of electron flow in the absence of valinomycin (up to 60%)	Degli Esposti *et al.*, 1983
	300–750	DCCD added directly to liposomes	15 min	25°C	Not studied on reconstituted enzyme	Inhibition of proton translocation (up to 50%), stimulation of coupled electron flow up to 5 times, no change or inhibition of electron flow in the presence of valinomycin or FCCP, respectively	Lorusso *et al.*, 1983
	or 25–30	Free enzyme treated with DCCD prior to the reconstitution	15 min	25°C	Alterations as reported for the isolated enzyme (part A of this table)		

for the understanding of the overall effect of DCCD. For example, if binding of antimycin is diminished by a crosslinking which in turn inhibits the normal electron pathway through the enzyme (apparently replaced by a bypass), the antimycin-insensitive reaction should be subtracted and the H^+/e^- ratio would stay the same. If, instead, antimycin binding is influenced by the covalent interaction of DCCD with cytochrome *b*, which *per se* is not inhibitory for the normal electron flow, the antimycin-insensitive reaction should not be subtracted and the H^+/e^- ratio would drop. As already stated for the mitochondrial system, the use of different bc_1 inhibitors seems necessary to clarify this point.

b. One should note here that the above discussion concerning antimycin does not apply to the results of Degli Esposti *et al.* (1982, 1983) and Lorusso *et al.* (1983) who claim no alteration of antimycin sensitivity of their system upon DCCD treatment. Instead, they reported on a substantial increase of electron flow under certain conditions (see Table IB), accompanied by a decrease of the H^+/e^- ratio. This controversy clearly examplifies the difficulties in interpreting DCCD-involved data. However, a different electron-flow bypass might have occurred in this system.

c. Even if a decrease in proton/electron stoichiometry is a true effect of DCCD, the observation may have different explanations. As recently proposed by Rich (1984), DCCD may be envisaged as binding to the region important for the mobility of a classical proton translocator such as CoQ. By changing local charges in this region, DCCD could profoundly alter proton stoichiometries.

d. Correlation between structural and functional effects induced by DCCD in the reconstituted system is essential for any conclusion. For the bc_1 complex, however, different conditions were often applied to study different effects, and structural modifications observed on the isolated enzyme were extrapolated to the reconstituted system (Table I; for a review, see Azzi *et al.*, 1984).

Conclusions

The large amount of literature on the proton translocating activity of the mitochondrial cytochrome bc_1 complex, of which only a small part was reviewed here, points to the importance of the subject. There is, however, no concensus as to the mechanism of this proton translocation. Kinetic and thermodynamic studies are still lacking the decisive experiment to distinguish between different hypotheses. DCCD-involved studies have supplied complicated and often conflicting data which are consistent with different interpretations. It is therefore too early to decide on the mechanism of proton

translocation in the bc_1 complex. Maybe reexamination of the present data together with some new experimental approach will, in the future, lead to a better understanding of the coupling between the H^+ translocation and the redox events in the bc_1 region of the mitochondrial respiratory chain.

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