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Organization and Function of Cytochrome *b* and Ubiquinone in the Cristae Membrane of Beef Heart Mitochondria

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Abstract

The arrangement and function of the redox centers of the mammalian bc_1 complex is described on the basis of structural data derived from amino acid sequence studies and secondary structure predictions and on the basis of functional studies (i.e., EPR data, inhibitor studies, and kinetic experiments). Two ubiquinone reaction centers do exist—a QH_2 oxidation center situated at the outer, cytosolic surface of the cristae membrane (Q_0 center), and a Q reduction center (Q_i center) situated more to the inner surface of the cristae membrane. The Q_0 center is formed by the *b*-566 domain of cytochrome *b*, the FeS protein, and maybe an additional small subunit, whereas the Q_i center is formed by the *b*-562 domain of cytochrome *b* and presumably the 13.4 *k*Da protein ("QP-C"). The "Q binding proteins" are proposed to be protein subunits of the Q reaction centers of various multiprotein complexes. The path of electron flow branches at the Q_0 center, half of the electrons flowing via the high-potential cytochrome chain to oxygen and half of the electrons cycling back into the Q pool via the cytochrome *b* path connecting the two Q reaction centers. During oxidation of QH_2, $2H^+$ are released to the cytosolic space and during reduction of Q, $2H^+$ are taken up from the matrix side, resulting in a net transport across the membrane of $2H^+$ per e^- flown from QH_2 to cytochrome *c*, the H^+ being transported across the membrane as H ($H^+ + e^-$) by the mobile carrier Q. The authors correct their earlier view of cytochrome *b* functioning as a H^+ pump, proposing that the redox-linked *p*K changes of the acidic groups of cytochrome *b* are involved in the protonation/deprotonation processes taking place during the reduction and oxidation of Q. The reviewers stress that cytochrome *b* is in equilibrium with the Q pool via the Q_i center, but not via the Q_0 center. Their view of the mechanisms taking place at the reductase is a Q cycle linked to a Q-pool where cytochrome *b* is acting as an electron pump.

Key Words: Ubiquinone; bc_1 complex; cytochrome *c* reductase; cytochrome *b*; iron–sulfur protein; electrogenic e^- transport; redox-linked *p*K change.

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Introduction

This review concentrates on structural and functional interrelationships of ubiquinone and its two reaction centers within the mitochondrial bc_1 complex (ubiquinol : cytochrome *c* reductase). The discussion of the ubiquinol oxidizing center (Q_0 = outer center) and the ubiquinone reducing center (Q_i = inner center) includes a careful description of the topographical arrangement of the four redox centers and of the electron path connecting them, especially of the cytochrome *b* molecule, which contains two heme centers. The topographical view is based primarily on EPR and amino acid sequence data, the structure–function relationships on inhibitor binding studies, and on kinetic experiments.

The major part of the experiments discussed in this review was performed with a bc_1 complex solubilized in Triton X-100 (Engel *et al.*, 1983b). The multisubunit complex consists of 11 proteins (Schägger *et al.*, 1985), the primary structures of which have been established with the exception of the sequences of the two largest subunits, the two ill-defined "core proteins." The preparation is monodisperse, consisting of dimers (Von Jagow *et al.*, 1977), each dimer containing 2 times 11 subunits (Schägger *et al.*, 1986). The enzyme catalyzes electron transfer from ubiquinol-10 or its homologues to cytochrome *c* with high activity (turnover rate $800\,s^{-1}$). The complex can be incorporated into phospholipid vesicles with a homogeneous orientation in the membrane (over 90% of the complex oriented right-side out). The iron–sulfur protein and cytochrome c_1 are exposed to the outer phase, while the core proteins are exposed to the interior of the vesicles. The transmembrane electrogenic electron flow drives a transmembrane H^+ transport with a stoichiometry of $2H^+/e^-$.

When isolating our beef heart cytochrome *b* we were attracted by the idea of cytochrome *b* acting as a proton pump (Von Jagow and Engel, 1980). However, in the meantime an abundance of experimental data has failed to provide evidence in favor of this idea; therefore we have had to reconsider our hypothesis on the mechanism of proton translocation, as will be discussed below. A further topic of the review is the question, which step of electron transfer at the bc_1 complex is responsible for respiratory control.

For a more detailed description of the various *bc* complexes of bacteria, plants, and mammals the recent reviews of Hauska *et al.* (1983), Rich (1984), Berry and Trumpower (1985), and Crofts (1985) may be consulted. An excellent comparative review on cytochrome *b* has recently appeared (Mahler and Perlman, 1985). Analogies between complexes III and IV can be detected in the comprehensive review on cytochrome oxidase by Wikström *et al.* (1985).

Topographical Arrangement of the Redox Centers

The topographical arrangement of the redox centers of the bc_1 complex in the membrane was examined by utilizing a paramagnetic probe technique (Blum *et al.*, 1983; Ohnishi *et al.*, 1985). The effect of externally added dysprosium complexes on the spin relaxation of the $g_y = 1.90$ (Rieske Fe_2S_2 center), the $g_z = 3.8$ (b_L), and the $g_z = 3.4$ (b_H) signals was studied in proteoliposome vesicles, prepared by the cholate dialysis method from a Triton-X 100 bc_1 complex and egg yolk phospholipid micelles (Ohnishi and Von Jagow, 1985). Figure 1 gives a tentative model of the arrangement of the centers.

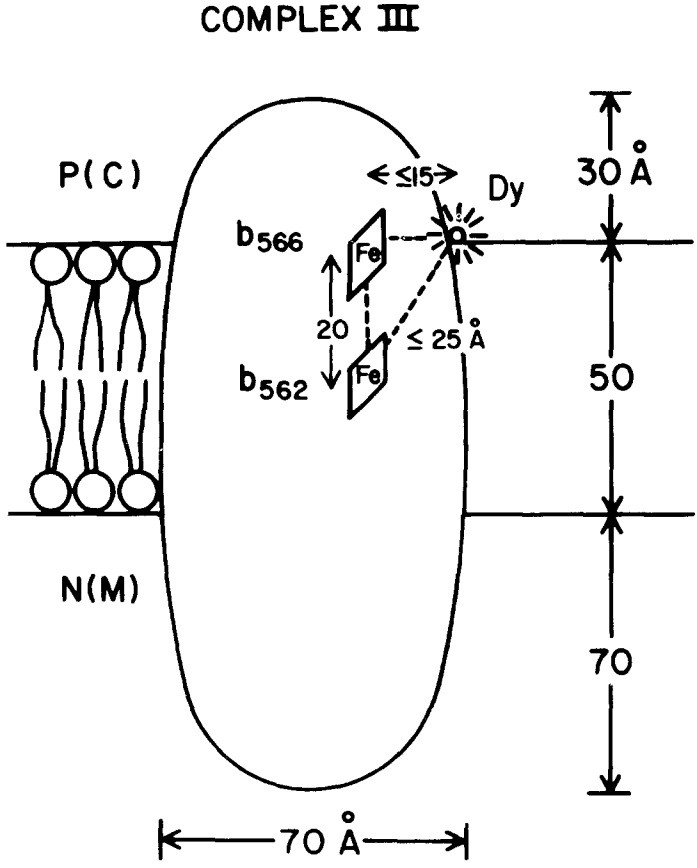


Fig. 1. Topographical arrangement of the redox centers of the bc_1 complex as deduced from EPR studies (Ohnishi and Von Jagow, 1985). P(C) = cytosolic side (positive); N(M) = matrix side (negative); Dy = dysprosium.

The Fe_2S_2 cluster and the heme b_L center were found to have about the same effective distance from the aqueous bulk phase (16 and 15 Å, respectively), while the effective distance between the b_H center and dysprosium is significantly greater (25 Å). The data indicate that the Fe_2S_2 and the b_L center are located close to the cytosolic surface of the membrane, whereas the heme b_H center is embedded more deeply in the membrane.

These data agree fairly well with structural predictions derived from the amino acid sequences. The folding pattern of mitochondrial cytochrome *b* suggests nine transmembrane helices with short intervening hydrophilic stretches (Fig. 2A) (Saraste, 1984; Widger *et al.*, 1984). The comparison of the sequences of cytochrome *b* from mitochondria of six different species and from spinach chloroplast revealed four invariant histidines in the first five membrane-spanning segments. Both segments II and V contain a pair of invariant histidines, separated by 13 residues in each case. Since the transmembrane segments are assumed to form α-helices, the histidines of each segment are assumed to be situated on one side of the same helix with a distance of 21 Å between them. The two hemes are sandwiched between the membrane-spanning helices II and V (Fig. 2B). The Fe–Fe distance of 21 Å between the two heme centers is consistent with the EPR data. Since the structure predictions do not indicate a strong asymmetry in the positions of the hemes within the helices, it cannot be discerned whether the positions of the hemes are asymmetric with respect to the membrane, i.e., whether one heme is located near the phospholipid bilayer while the other heme is situated near the middle of the membrane, as indicated by the EPR data, or whether one heme is located near the M-side and the other near the C-side. However, the transmembrane arrangement of the two heme centers of cytochrome *b* is well established.

Helix V contains an invariant proline, four residues away from the N-terminal heme-binding histidine. This proline residue induces a twist of helix V between the two heme-binding histidines. The heme-binding histidines are not located on the same side of helix V but their α-carbon atoms form an angle of approximately 140° with the helix. Therefore the helices II and V cannot be arranged parallel to each other but at an angle of approximately 30° (Fig. 2B). Since the properties of the heme centers are determined by their protein environment, the tension caused by the proline residue could be the reason for the functional differences between the two heme centers (i.e., midpoint potentials, light absorption maxima, EPR *g* values).

Structural predictions of beef heart cytochrome c_1 and of the iron–sulfur protein from *Neurospora crassa* suggest that the redox centers of these proteins are located near the membrane surface (unpublished data). Both proteins have a hydrophobic membrane anchor near the C-terminal end while the rest of the molecule is moderately hydrophilic, consisting of various

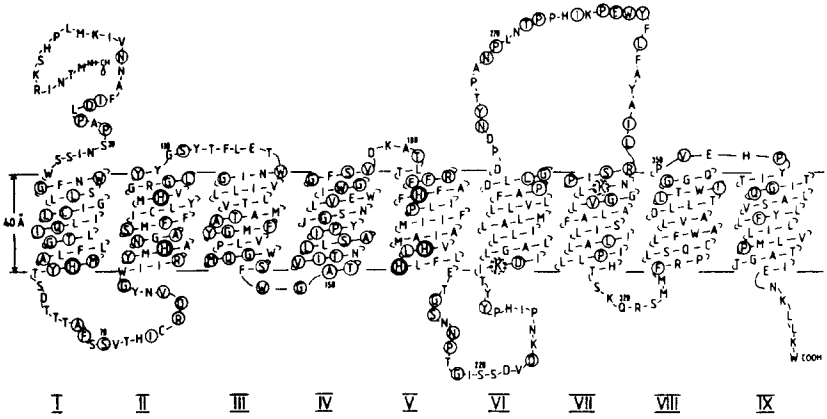


Fig. 2A. Folding pattern of cytochrome *b* from beef heart [reproduced from Saraste (1984) with kind permission of the author].

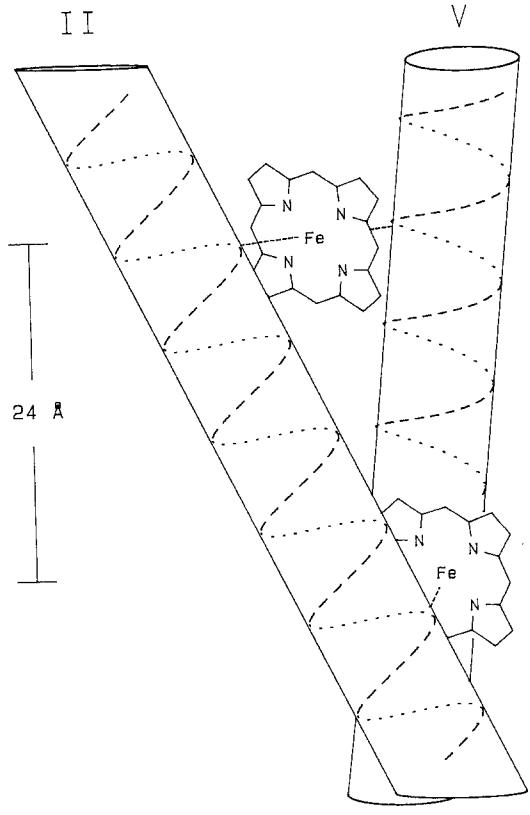


Fig. 2B. Presumptive position of the two hemes of cytochrome *b* between helices II and V (modified from Saraste, 1984).

amphiphilic helices and perhaps β-sheets. The redox centers (heme and Fe_2S_2 cluster, respectively) are probably bound between the amphiphilic helices and embedded in the phospholipid headgroup region.

The depicted arrangement of the redox centers is consistent with the function of the individual redox centers. The heme b_L center and the iron–sulfur cluster, both located near the cytosolic membrane surface, form one reaction center, as will be described in the next section. The heme b_H center is situated in a hydrophobic environment and therefore appropriate for the stabilization of a semiquinone radical. Cytochrome c_1 reacts with the largest reaction partner of the bc_1 complex, the water-soluble protein cytochrome *c*, and is therefore located at the surface of the complex.

Organization and Function of the Q_0 Center

At the Q_0 center the two-hydrogen (two-electron two-proton) carrier ubiquinol transfers its electrons onto one-electron redox centers (for a detailed scheme, see Fig. 3). Theoretically the problem could be solved either by means of an electron path where ubiquinol and the following redox centers are connected in series, the two electrons of ubiquinol being transferred to the same redox center one after another, or by means of an electron path where ubiquinol and two redox centers are connected in parallel, the electrons being transferred to the two different redox centers by a concerted type of reaction. The latter mechanism has been found at the ubiquinol oxidizing center, and the former mechanism at the ubiquinone reducing center, as will be discussed in the following section. The semiquinone occurring as an intermediate during the oxidation of quinol is extremely unstable in the free form. Therefore a concerted oxidation process can only take place in a reaction cleft where the ubiquinol molecule is bound and where the semiquinone species is stabilized. Moreover, a reaction center is required in which the two redox centers are arranged in close proximity to each other.

The two redox proteins involved in the formation of the Q_0 center are the b_L (*b*-566) domain of cytochrome *b* and the Fe_2S_2 cluster of the iron–sulfur protein; an additional involvement of the 8-kDa protein (Borchart *et al.*, 1985) is conceivable. This structure of the Q_0 center has been inferred from data obtained in three different types of experiments:

(i) preparative protein chemistry,
(ii) studies on the action of various highly specific inhibitors, and
(iii) kinetic experiments.

The branching of the electron flow and the sequence of the electron-transferring steps was established by Trumpower and Katki (1975) by means

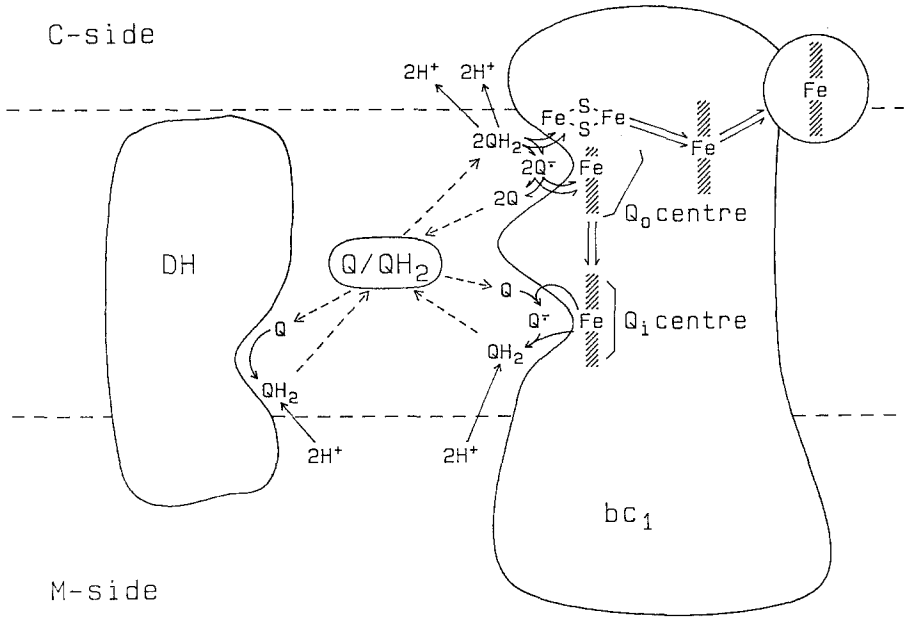


Fig. 3A. Scheme of the route of electron and proton flow of the mitochondrial bc_1 complex (modified from Crofts *et al.*, 1983). C-side = cytosolic side (positive); M-side = matrix side (negative); Q_0 center = ubiquinol-oxidizing center; Q_i center = ubiquinone-reducing center; DH centers = ubiquinone reducing centers of the dehydrogenases; b_L = low-potential heme *b*-566 center; b_H = high-potential heme *b*-562 center; c_1 = cytochrome c_1, *c* = cytochrome *c*. The double arrows indicate that two electrons flow via these routes during one catalytic cycle.

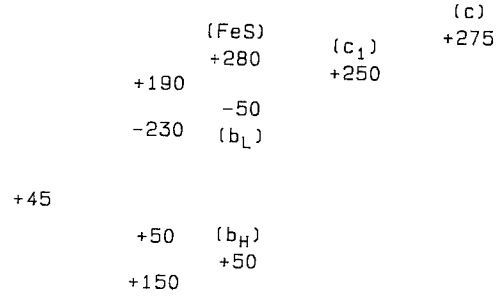


Fig. 3B. Midpoint potentials (mV) of the redox centers (Rich, 1984; Von Jagow *et al.*, 1978; Von Jagow and Ohnishi, 1985).

of a kinetic experiment, belonging to the type of experiments repeatedly described in the literature as "oxidant-induced reduction." In this experiment the Q_i center was blocked by antimycin and the high-potential Fe_2S_2-cytochrome chain reduced by ascorbate. A reduction of cytochrome *b* by succinate was not possible until the high-potential chain had been reoxidized

by ferricyanide, which proved that the first electron during the transition from QH^- to $QH\cdot$ goes to the Fe_2S_2 center, whereas the second electron during the transition from $Q^{\cdot-}$ to Q goes to the heme b_L center (cf. Fig. 3).

Due to the branching of the electron flow, observation of the single-electron transfer to cytochrome c_1 and cytochrome *c* is possible (Bowyer and Trumpower, 1981; Bowyer, 1982). During the first catalytic cycle, when the Q_i center is blocked, three electrons can still enter the high-potential cytochrome chain, since two electrons can enter the cytochrome *b* chain. The kinetics of single-electron transfers, as for instance from the iron–sulfur center to cytochrome c_1 (Meinhardt and Crofts, 1982) and from b_L to b_H (Meinhardt and Crofts, 1983), have been analyzed in detail in the *bc* complexes of chromatophores. In this system, the kinetics and thermodynamics could be resolved at each step by redox-poising the system and observing single-electron transfers triggered by light flashes.

Working with mitochondrial succinate-cytochrome *c* reductase, Trumpower and Edwards (1979) found that dissociation of the iron–sulfur protein leads to an inactivation of the quinol-oxidizing center while the quinone-reducing center retains full activity (cf. Fig. 3). After partial dissociation of the preparation, a reassociation of the iron–sulfur protein with the depleted preparation was possible as indicated by a reactivation of the catalytic activity. Later an improved method was established, using the reductase isolated in Triton X-100 (Engel *et al.*, 1983a). This system is governed by a dissociation equilibrium of the iron–sulfur protein. As in the case of succinate-cytochrome *c* reductase, the iron–sulfur protein was found to act only at the Q_0 center.

Up to a few years ago, the quinone analogues were the only inhibitors available for blocking the ubiquinol-oxidizing center. They were not specific, i.e., higher concentrations also affected the ubiquinone-reducing center (Zhu *et al.*, 1982). A clear picture of the Q_0 center did not emerge until a number of new inhibitors were discovered partly accidentally in the course of screening programs for new antibiotics (Von Jagow and Becker, 1982). Among these inhibitors, stigmatellin is the latest discovery. It binds in stoichiometric amounts, blocking all reactions at the oxidizing site, i.e., electron transfer both from QH^- to the iron–sulfur protein and from $Q^{\cdot-}$ to cytochrome b_L (Von Jagow and Ohnishi, 1985). Like the other antibiotics [the strobilurins, oudemansins, and myxothiazol; see Von Jagow and Link, 1986], stigmatellin displaces the ubiquinone molecule and quinone-analogous inhibitors like UHDBT from the reaction cleft (Fig. 4C). It seems to bind like a "cross-linker," forming ligand interactions to amino acid residues of the b_L domain of cytochrome *b* and to amino acid residues of the iron–sulfur protein. The strong interaction with the b_L center is indicated by a red shift of the absorbance maximum of the ferrous heme b_L center from 566 to 568 nm.

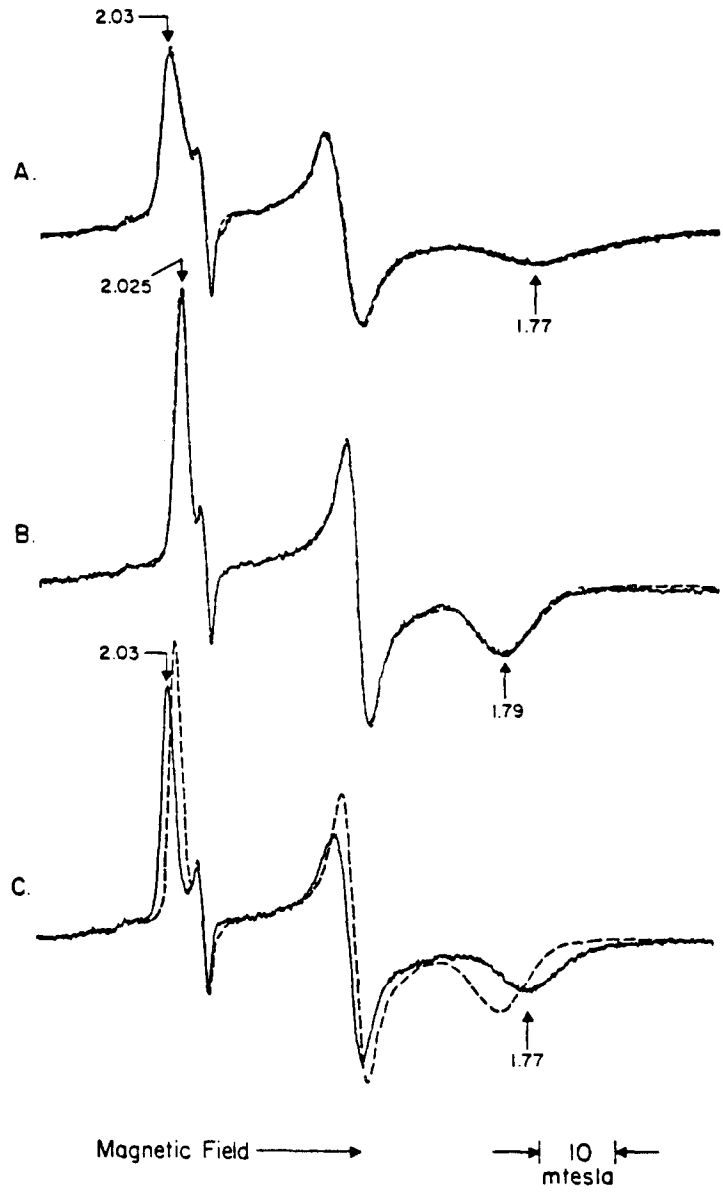


Fig. 4. EPR spectra of the Fe_2S_2 center of isolated bc_1 complex in the presence of the Q_0 site inhibitors stigmatellin and UHDBT (Von Jagow and Ohnishi, 1985). A: No inhibitor added; B: stigmatellin added; C: UHDBT plus stigmatellin added (dashed line).

Binding of stigmatellin does not seem to influence the ligand field of heme b_H, as became obvious from the absence of a change of the *b*-562 absorbance band (Von Jagow and Ohnishi, 1985).

Binding of stigmatellin to the iron–sulfur protein has striking effects on the EPR spectrum and on the midpoint redox potential of the Fe_2S_2 center (Fig. 4). The values of all three EPR signals are shifted and their linewidths are narrowed; the midpoint potential changes from +280 to +540 mV.

The question arises, which part of the stigmatellin molecule binds at the quinol-oxidizing center. The inhibiting quinol analogues UHDBT, UHNQ, and HMHQQ, which were studied in detail, all contain a bicyclic 1,4-quinone system with a hydroxy group at position 2 and an alkyl chain at position 3 which determines the hydrophobicity of the molecule. These common structural elements suggest that the bicyclic quinone systems, like the chromone ring system of stigmatellin, fit into the reaction center, whereas the alkyl side chains might be situated in the hydrocarbon core of the membrane (Von Jagow and Link, 1986). Therefore, the reaction cleft may have dimensions of about 12 × 10 × 6 Å.

It seems worth noting at this point that a structural similarity exists between the mitochondrial ubiquinol-oxidizing center and the Q_B centers of chloroplasts and of *Rps. sphaeroides*, since stigmatellin also binds with high affinity to these reaction centers (Oettmeier *et al.*, 1985; Robertson, personal communication).

Organization and Function of the Q_i Center

The Q_i center consists of a hydrophobic reaction cleft where Q is reduced to QH_2 and where the intermediate semiquinone radical has to be stabilized, i.e., its lifetime prolonged. The possible contribution of various protein subunits of the bc_1 complex to the ubiquinone-reducing center is a question that has not been completely settled. Presumably the main part of the reaction cleft is formed by the b_H domain of cytochrome *b*. This is indicated by a specific and pronounced red shift induced by binding of the classical inhibitor antimycin, namely a shift of the b_H absorbance maximum from 562 to 564 mm (Slater, 1973). Antimycin does not cause any absorbance change in the b_L center, which shows its high specificity, similar to that of the antibiotics which inhibit the Q_0 center (Becker *et al.*, 1981).

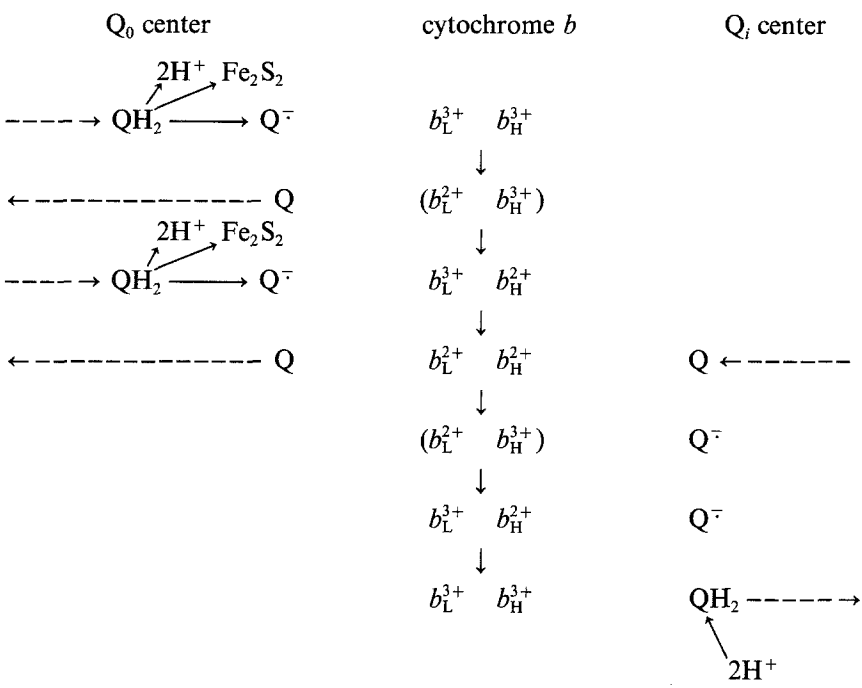
The high specificity of the inhibitors of either the Q_0 center or the Q_i center is an indication that the structures of the two reaction centers are

rather dissimilar. At the Q_0 center, the QH_2 molecule is activated by deprotonation and subsequently undergoes two oxidation steps, which proceed far from equilibrium and drive the reaction sequence, while at the Q_i center, the reduction of ubiquinone occurs near equilibrium in two steps which are assumed to have similar redox potential and require a strong binding of the semiquinone radical. Therefore the structure of the Q_i center must be designed for an "induced fit" of $Q^{\cdot-}$.

Since cytochrome *b* has only short extramembrane stretches between the membrane-spanning α-helices (Saraste, 1984; Widger *et al.*, 1984), it seems unlikely that cytochrome *b* on its own is able to constitute the whole reaction site. Looking for other subunits, we came to regard the 13.4-kDa protein as a conceivable partner of cytochrome *b*. It has been characterized by Yu *et al.* (1978) as a ubiquinone-binding protein, and was designated by them as "QP-C". It is a protein of extremely high polarity, containing a considerable number of tyrosine residues (Wakabayashi *et al.*, 1985). Labeling experiments performed by Yu and Yu (1982) have shown that ubiquinone analogues substituted with an arylazido label at the end of an alkyl side chain (NAPA-ubiquinone) tagged the 13.4-kDa protein and cytochrome *b*. The predicted folding pattern of the protein shows no membrane-spanning segments but a short membrane-anchoring α-helix at the N-terminus and several surface-seeking amphiphilic domains. This secondary structure prediction suggests that QP-C may serve as the lid of the Q_i reaction center (Von Jagow *et al.*, 1986).

Unfortunately it has not yet been shown whether dissociation of the protein affects specifically the Q_i center. We expect that a closer characterization of the Q-binding proteins will soon reveal their real function. It seems to be very likely that they may after all not "constitute a separate class of Q-bound proteins comparable to heme proteins and flavoproteins" (Wang and King, 1982), but may represent structural constituents of ubiquinone reaction centres.

The original version of the Q-cycle implied that for Q reduction at site Q_i one electron is supplied by the low-potential cytochrome *b* chain while the other one is supplied by a dehydrogenase complex (Mitchell, 1975). This original version was conceived for thermodynamic reasons, but was soon rejected by Garland *et al.* (1975), who stressed that full electron turnover could be reached with bc_1 complexes devoid of dehydrogenases. The alternative mechanism which he proposed is a "double-turnover mechanism." If we assume that the two heme centers of cytochrome *b*, which equilibrate with each other rapidly, and the quinone at the Q_i center behave like a capacitor, requiring a critical voltage for their discharge, the reactions taking place may be described as follows:



Therefore the mitochondrial Q reducing center functions as a two-electron gate, like the Q_B centers of photobacteria and chloroplasts.

Another mechanism has been proposed for the reduction of ubiquinone at the Q_i center (De Vries *et al.*, 1982). It implies that the two Q_i centers of the dimeric complex interact with each other. In a cycle of this kind, one ubiquinone molecule and two heme b_H centers would be connected in parallel (similar to the situation found in the Q_0 center) and ubiquinone would receive one electron from each of the two high-potential cytochrome *b* centers. We have so far no experimental data in favor of such a functional cooperativity of the dimeric complex ("double Q-cycle"), although the structural features of the dimer, including 2 two-heme cytochrome *b* molecules situated in the core of the complex (Karlsson *et al.*, 1983), seem to indicate such a possibility. On the contrary, a lot of data exists that speak against, rather than for, the existence of a dimer functional in electron transfer:

(i) The titers for full inhibition of electron flow of the inhibitors of the Q_0 site (i.e., myxothiazol and stigmatellin) as well as of the inhibitor of the Q_i site (antimycin) are 2 mol of inhibitor per dimer (Von Jagow *et al.*, 1982).

(ii) The isolated reductase complex has been resolved into monomeric and dimeric species, the monomeric complex showing high electron-transferring activity and both kinds of complexes showing titers for

full inhibition of 1 mol inhibitor per cytochrome c_1 (Nalecz and Azzi, 1985).

(iii) The analysis of kinetic data obtained in chromatophores gave no indication of a cooperativity of this kind (Crofts, 1985).

The Ubiquinone Pool as a Redox Buffer

In beef heart mitochondria, ubiquinone is present in about tenfold excess compared to the individual components of the cytochrome chain, e.g., cytochrome c_1, and is largely situated in the hydrophobic membrane core (Lenaz *et al.*, 1984). Fifteen years ago Kröger and Klingenberg (1973a, b) proved in careful studies that ubiquinone is fully involved in the reaction sequence from the dehydrogenases to the cytochromes. The rates of electron input from the dehydrogenases into the Q pool and the rates of electron output from the Q pool into the cytochromes were found to depend on the ratio of oxidized and reduced Q species. The reactions could be described applying apparent first-order kinetics. The authors concluded that the electron transfer between the dehydrogenases and the bc_1 complex was affected by two bimolecular collision processes, firstly a reaction between oxidized Q and the respective dehydrogenases, and secondly a reaction between reduced QH_2 and the bc_1 complex.

Presumably this view was too clear-sighted and premature for the time, since in the course of the following decade numerous efforts were made to prove both the existence of kinetically different Q species (Gutman, 1980), as well as the binding of part or even all of the ubiquinone to the individual redox components (King, 1982). It was envisaged that the rate of electron transfer might not be determined by the lateral and transverse diffusion rates of ubiquinone, but by the diffusion rates of the protein complexes. During the last few years this view has been revised, based on the following observations:

(i) "The mitochondrial inner membrane is structurally a fluid rather than a solid-state membrane" and "electron transport is coupled to the lateral diffusion of all redox components and does not require ordered chains, assemblies, or aggregates of redox components" (Gupte *et al.*, 1984).

(ii) Q acts kinetically homogeneously as a pool and controls the electron transfer rates (Ragan and Cottingham, 1985). Experiments to demonstrate this was performed in the mammalian system (Rich, 1983; Von Jagow and Link, 1984), in chromatophores (Crofts *et al.*, 1983, Robertson *et al.*, 1984), and in hybrid systems consisting of photoreaction centers and mitochondrial cytochrome bc_1 complexes (Robertson *et al.*, 1985; Moser *et al.*, 1986).

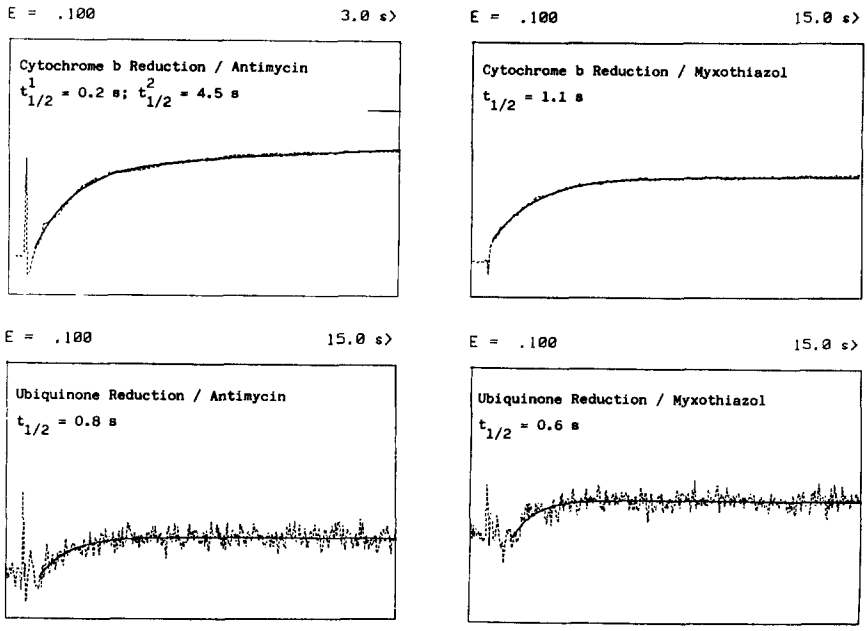


Fig. 5. Reduction kinetics of cytochrome *b* and the Q-pool by succinate in submitochondrial particles in the presence of antimycin and myxothiazol (cf. Von Jagow and Link, 1984). A: Cytochrome *b* reduction via Q_0 site (antimycin added); B: cytochrome *b* reduction via Q_i site (myxothiazol added); C: Q-pool reduction in the presence of antimycin; D: Q-pool reduction in the presence of myxothiazol.

We have analyzed the reduction kinetics of mitochondrial Q and of the two heme *b* centers in submitochondrial particles of beef heart. During the reversed mode of electron flow through the Q_i center, with the ubiquinone-oxidizing center blocked by myxothiazol, only cytochrome b_H was reduced. The *b* reduction (Fig. 5B) followed pseudo-first-order kinetics, showing approximately the same half-reduction time as the reduction of the Q pool in the presence of either antimycin or myxothiazol (Figs. 5C and 5D) or in the absence of inhibitors of the bc_1 complex. The reduction kinetics of cytochrome *b* via the ubiquinone-oxidizing center will be discussed in the next section.

The similarity of the ubiquinone reduction kinetics, independent of whether an inhibitor was present or not, indicates a reduction of the Q-pool via succinate dehydrogenase without the involvement of the bc_1 complex.

Furthermore, the synchronous reduction kinetics of the Q-pool and cytochrome b_H confirm that these are almost in equilibrium. The homogeneous pseudo-first-order reduction kinetics of ubiquinone gave no indication of a specifically bound ubiquinone which might behave kinetically differently.

Corroborating results were obtained in chromatophores of *Rps. sphaeroides* where a photosystem serves, in place of the dehydrogenases, to reduce Q (Robertson *et al.*, 1984). The reduction of b_H via the quinone-reducing center ($Q_c = Q_i$ site) was also mediated via the ubiquinone pool. An effective electron transfer from the photosystem to the bc_1 complex was found to depend on the redox potential of the Q pool, i.e., on the degree of reduction of the Q pool. An extreme depletion of the Q pool led to a change of the mode of electron transfer and to a direct interaction of the Q_B site of the photoreaction center with the quinone-reducing center of the bc_1 complex.

The Series Connection of the Two Heme *b* Centers

The scheme of the electron flow (Fig. 3) shows that cytochrome *b* transfers electrons from a semiquinone molecule at the Q_0 center to a quinone molecule at the Q_i center. The consequence of the recycling of half of the electrons fed into the bc_1 complex is obvious: it leads to a doubling of the number of protons transported across the membrane per electron flowing from QH_2 to cytochrome *c* (Mitchell, 1975).

A description of the stoichiometry of the bc_1 complex should also consider the dehydrogenases (Fig. 3). During the flux of $2e^-$ from ubiquinol to cytochrome *c*, i.e., during the oxidation of 2 molecules of QH_2 at the Q_0 center, $4H^+$ are released via the Q_0 center to the cytosolic phase. In the course of the completion of the catalytic cycle, $4H^+$ are taken up from the matrix phase, namely $2H^+$ via the Q_i center during the reduction of one molecule of Q, and $2H^+$ via the corresponding dehydrogenase during the reduction of the second molecule of Q. QH_2 produced at the Q_i site may react with the Q_0 site without exchanging freely with the Q pool (Rich, 1984); however, this may not be an essential point except when describing Q-depleted systems.

The electron flow to or from the cytochrome *b* chain can be blocked only when both ubiquinone reaction sites are obstructed by inhibitors. This can be effected by a simultaneous binding of antimycin and one of the inhibitors of the Q_0 site (Von Jagow and Engel, 1980). The experiment, called the "double kill," leads to a complete electric insulation of the two heme *b* centers. When they are in ferric form prior to inhibitor binding, they remain in an oxidized state, even after addition or production of a vast excess of QH_2; when they are in the ferrous form prior to inhibitor binding, they remain in a fully

reduced state even after complete oxidation of the Q pool. The "double kill" cannot be achieved when inhibitors of either the Q_0 center or the Q_i center are added alone.

Another reaction demonstrating the series connection of the two heme *b* centers, the "oxidant-induced reduction" of cytochrome *b*, has already been discussed when dealing with the reaction sequence during QH_2 oxidation.

With respect to the reversibility of electron flow, we have shown in the preceding section that cytochrome *b* can quickly equilibrate with the Q pool via the Q_i site. The equilibrium was demonstrated by the backward reaction of the Q_i site, the reduction of cytochrome *b* by QH_2 in the presence of myxothiazol. The equilibrium could also be demonstrated by the forward reaction, the reduction of Q by ferrous cytochrome *b*: The *b* centers of succinate : cytochrome *c* reductase were prereduced by succinate, and the Q_0 center inhibited by myxothiazol (Von Jagow *et al.*, 1984). An oxidant pulse by fumarate (in 1000-fold excess over succinate) led to a rapid oxidation of the Q-pool and caused a rapid reoxidation of cytochrome *b* (Fig. 6C). A similar result was obtained in the absence of an inhibitor (Fig. 6A).

A disequilibrium between the Q pool and cytochrome *b* existing at the Q_0 site was observed when testing the reaction in the forward and backward direction. The backward reaction of the Q_0 site was tested by the fumarate pulse technique when the Q_i site was blocked by antimycin. The reoxidation of cytochrome *b* occurred with very slow kinetics (Fig. 6B). The forward reaction of the Q_0 site was tested by a reduction experiment, as described in the previous section (Fig. 5A). In the presence of antimycin both *b* centers were reduced with biphasic reduction kinetics, 50% of cytochrome *b* being reduced when only 10% of the Q pool had been reduced. During the fast phase the b_H center was reduced, and during the slow phase the b_L center. The biphasic redox kinetics indicate a disequilibrium at the Q_0 site in the absence of a chemiosmotic membrane potential.

The electron input into the bc_1 complex is determined by the practically irreversible reaction

$$QH_2 + Fe_2S_2 + R\text{-}b_L^{3+} \rightarrow Q + Fe_2S_2^- + {}^+HR\text{-}b_L^{2+} + H^+ \qquad (1)$$

Under standard conditions the equilibrium constant of this reaction can be estimated to be 70 (Rich, 1984), assuming the midpoint potentials of the redox components as given in Fig. 3B. However, the electron flow of the respiratory chain in whole mitochondria and in submitochondrial particles is reversible, due to the influence of the membrane potential, which is the principle of respiratory control. Reaction (1) seems to represent one of the reaction steps inducing respiratory control.

The influence of the electrostatic potential on the midpoint potential of the redox components seems to relate to the topography of the redox centres

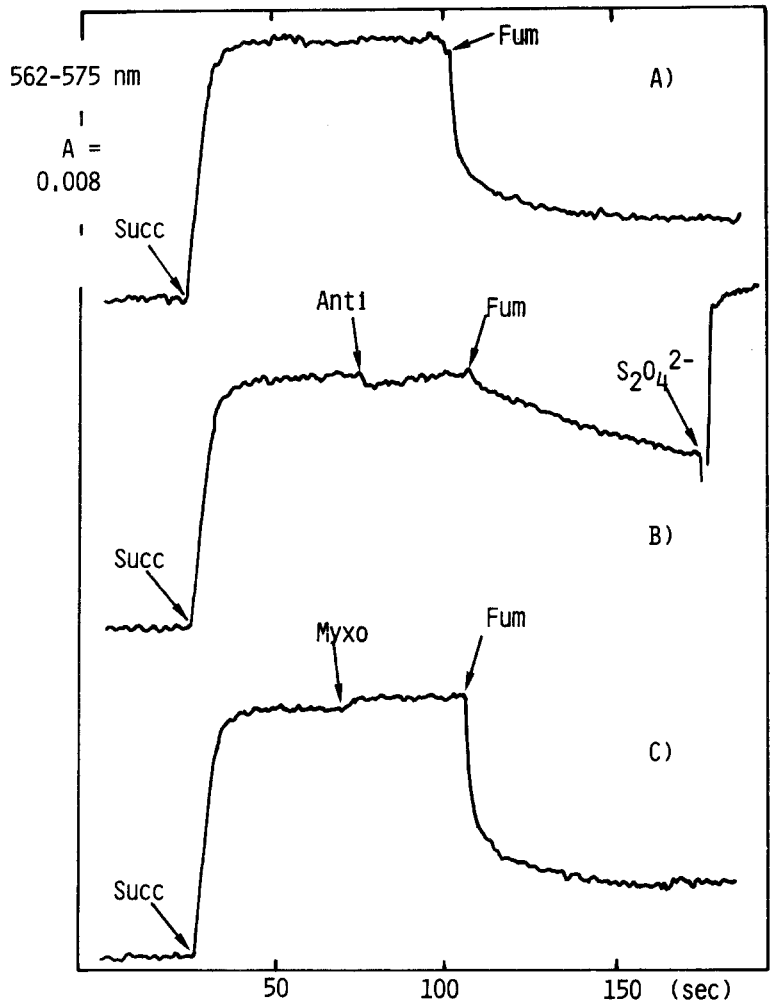


Fig. 6. Reoxidation response of the two ubiquinone reaction sites of cytochrome *b* in isolated succinate : cytochrome *c* reductase upon oxidation of the Q-pool by a fumarate pulse (Von Jagow and Link, 1984). A: No inhibitor added; B: Q_i site blocked by antimycin; C: Q_0 site blocked by myxothiazol.

in the membrane. On energization of intact mitochondria, Wilson and Dutton (1970) observed a change to a more positive midpoint potential of cytochrome b_L, while b_H showed no energy-dependent change of the midpoint potential. This agrees fairly well with the assumed location of the redox centers. As discussed in the second section, the b_L center seems to be arranged at the "positive" C-side of the membrane while the b_H center is situated near

the middle of the membrane. Therefore in energized mitochondria the b_L center has a higher potential than the b_H center and the equilibrium constant between the two heme *b* centers changes significantly, the b_L center becoming more reduced than the b_H center.

However, the midpoint potentials of cytochrome *b* given by Wilson and Dutton (1972) cannot explain the reversibility of the electron transfer, since the value of +245 mV for the b_L center does not agree with a straightforward reaction sequence between cytochrome b_L and ubiquinone. There are two possible explanations which might overcome this difficulty:

(i) The midpoint potential of the $Q_0/QH_{2,0}$ couple may change due to the influence of the membrane potential.
(ii) The data given by Wilson and Dutton (1970) may represent a correct evaluation of the changes of the midpoint potentials, but the actual values themselves may be incorrect due to experimental difficulties, e.g., the distribution of the membrane-permeable redox dyes between the internal and the external bulk phases under the influence of the electrostatic potential.

Discussion

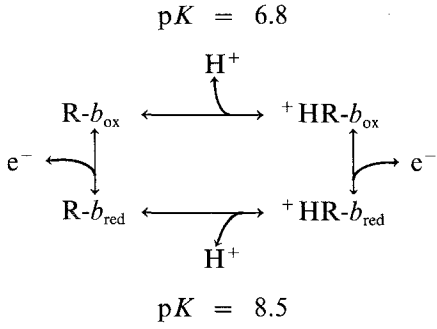
In the previous sections we have outlined the main aspects of the electron path through the mitochondrial bc_1 complex. Several basic features seem to be generally accepted, i.e. the existence of two distinct ubiquinone reaction centers, the bifurcation of the electron flow occurring at the QH_2-oxidizing center, and the backflow of half of the electrons from the bc_1 complex to ubiquinone (cf. Fig. 3). However, there is still a controversy about the exact route of electron flow, the mode of action of ubiquinone (bound or free), and the mechanism of proton translocation.

In our opinion, the simplest model explaining all the experimental data is that of a protonmotive Q cycle in which Q is reduced at the Q_i site by means of a double-electron turnover and which presupposes the existence of a Q pool. We assume that the two reaction sites of the bc_1 complex are not permanently occupied by a specific Q species; however, there may be a preferential diffusion of Q or QH_2 between the two reaction sites without complete equilibration with the Q pool.

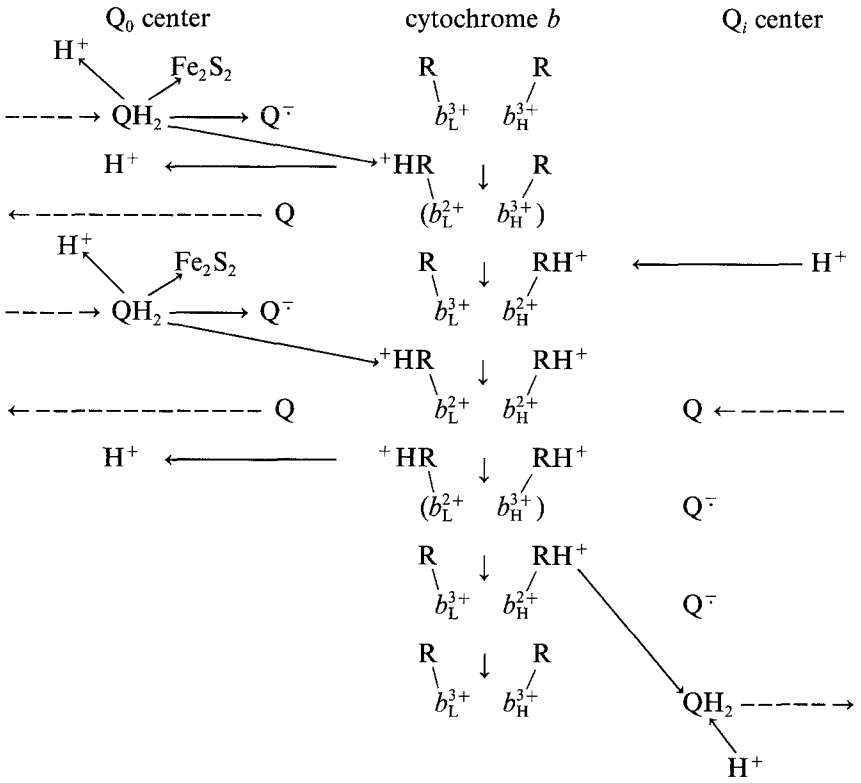
Alternative models of the path of electron flow and the mechanism of proton translocation have been considered in the past: A "*b* cycle" together with a proton pump has been suggested by Wikström (1980) and a "double Q cycle" by De Vries *et al.* (1982).

On the whole, our knowledge of the mechanism of H^+ transfer is more limited than that of the route of electron flow. The different models conceived for the electron flow impose, however, constraints on the putative pathways of H^+ transport. The assumption of the classical Q cycle implies transport of the H^+ by a Mitchellian "proton-pumping loop" (Mitchell, 1976). The interaction of the dehydrogenases with the bc_1 complex leads to an alternating sequence of hydrogen transport, electron transport, and again hydrogen transport (Fig. 3). The first transmembrane hydrogen transport occurs via diffusion of QH_2 from the dehydrogenase at the matrix side of the membrane to the Q_0 center; it is followed by an electronic charge separation across the membrane by means of the cytochrome *b* chain; the second transmembrane hydrogen transport occurs by diffusion of QH_2 from the Q_i center to the Q_0 center.

The question that arises is: What is the role of cytochrome *b* in the process of H^+ translocation? Urban and Klingenberg (1969) found a pH dependence of the midpoint potential of cytochrome b_H. Later, when it became possible to differentiate between the two heme *b* centres, it was found that both heme *b* centers show a pH dependence of their midpoint potentials (Wilson *et al.*, 1972; Von Jagow *et al.*, 1978). Assuming cytochrome *b* to act as a proton pump, Papa (1976) described the phenomenon as a "vectorial Bohr effect." As we have demonstrated, at least one acidic group per heme center is involved in this process (Von Jagow *et al.*, 1978), the sequence of the reactions being (Von Jagow and Engel, 1980)



However, the existence of redox-linked pK changes is not conclusive proof for a proton pumping mechanism. Another explanation for the significance of the redox-linked pK changes should be considered: the redox-linked acidic groups could be involved in the protonation–deprotonation reactions of ubiquinone during the catalytic cycle. We wish to postulate the following reaction sequence for the catalytic cycle:



At the Q_0 center, in a reaction preceding the oxidation of quinol and the formation of the semiquinone, QH_2 delivers one proton to the acidic group of either the heme b_L or of the iron–sulfur protein. After the release of the second proton from QH^- and the transfer of the electron from $Q^{\cdot-}$ to b_L, the pK of the acidic group of the heme b_L changes from 6.8 to a value higher than 8.5; thus one proton is tightly bound to the b_L site while the Q molecule can leave the reaction site. When b_L is reoxidized during the transfer of the electron from b_L to b_H, the proton is released, probably into a proton channel guiding the proton from the buried Q_0 reaction cleft to the surface of the complex at the C-side.

At the Q_i center, the acidic group of cytochrome b_H becomes protonated when heme b_H accepts an electron from heme b_L, and the pK of this acidic group changes from 6.8 to a value higher than 8.5. The proton is subsequently transferred to the intermediate semiquinone radical during the reduction of $Q^{\cdot-}$ to QH^- and the concomitant oxidation of heme b_H.

Therefore, we believe that cytochrome b does not function as a transmembrane proton pump. The pK changes of the acidic groups of this

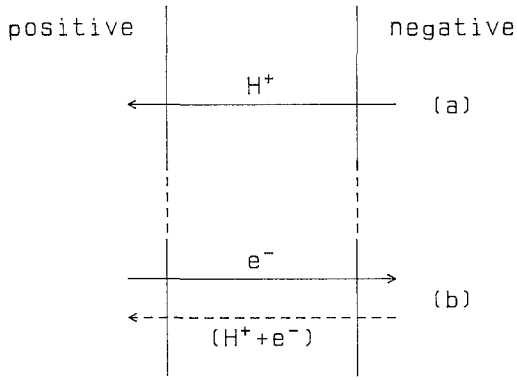


Fig. 7. Schematic representations of two possible mechanisms for proton translocation (cf. text). A: "Proton pumping" (e.g., bacteriorhodopsin); B: "electron pumping," proton transport by a Mitchellian loop (e.g., bc_1 complex).

transmembrane protein seem to have an auxiliary function in the protonation–deprotonation processes during the catalytic cycle. Proton pumping as a primary event occurs, for example, in bacteriorhodopsin and rhodopsin (cf. Ovchinikov *et al.*, 1985). Cytochrome *b*, however, acts as a charge separator, catalyzing an electrogenic electron transfer across the membrane. The proton translocation observed is the net result of two transmembrane transport processes—electron transport from the (positive) C-side to the (negative) M-side and an opposing hydrogen (proton plus electron) movement (Fig. 7). "Electron pumping" could be regarded as the primary event and proton translocation therefore as "secondary active transport."

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