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The Protonmotive Q Cycle in Mitochondria and Bacteria

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ABSTRACT: The cytochrome bc_1 complex is an oligomeric electron transfer enzyme located in the inner membrane of mitochondria and the plasma membrane of bacteria. The cytochrome bc_1 complex participates in respiration in eukaryotic cells and also participates in respiration, cyclic photosynthetic electron transfer, denitrification, and nitrogen fixation in a phylogenetically diverse collection of bacteria. In all of these organisms, the cytochrome bc_1 complex transfers electrons from ubiquinol to cytochrome c and links this electron transfer to translocation of protons across the membrane in which it resides, thus converting the available free energy of the oxidation-reduction reaction into an electrochemical proton gradient. The mechanism by which the cytochrome bc_1 complex achieves this energy transduction is the protonmotive Q cycle. The Q cycle mechanism has been documented by extensive experimentation, and recent investigations have focused on structural features of the three redox subunits of the bc_1 complex essential to the protonmotive and electrogenic activities of this membranous enzyme.

KEY WORDS: energy transduction, respiration, Q cycle, bc_1 complex, mitochondria.

I. INTRODUCTION

The cytochrome bc_1 complex transfers electrons from ubiquinol to cytochrome c and links this electron transfer to the establishment of a proton gradient across the inner mitochondrial or bacterial plasma membrane by a mechanism known as the protonmotive Q cycle.[23,48,89,125] The reaction catalyzed by the bc_1 complex is described by the following equation, in which the subscripts *n* and *p* designate negative and positive sides of the membrane and *ox* and *red* refer to oxidized and reduced cytochrome.

$$QH_2 + 2c_{ox} + 2\ H^+_n \rightarrow Q + 2c_{red} + 4\ H^+_p$$

Three subunits of the cytochrome bc_1 complex contain redox prosthetic groups, cytochrome b, cytochrome c_1, and the Rieske iron-sulfur protein.[124] As the protonmotive Q cycle pathway of electron transfer has been elucidated, questions have arisen as to how the structural arrangement of these three proteins allows electron transfer between them, how electrons are transferred to and from ubiquinone and ubiquinol, and how protons enter and exit the catalytic sites of ubiquinol oxidation and ubiquinone reduction.

In this review we explain the protonmotive Q cycle mechanism and describe how the experimental findings that substantiated the Q cycle are interpreted in terms of that mechanism. We also attempt to identify specific amino acids that form the catalytic sites of ubiquinol oxidation and ubiquinone reduction and that may participate in proton conduction. We then discuss some aspects of the Q cycle that are not yet agreed on, and attempt to reconcile some opposing views or suggest experiments that might lead to their resolution.

II. THE CYTOCHROME bc_1 COMPLEX

All cytochrome bc_1 complexes contain a diheme cytochrome b, cytochrome c_1, and an iron-sulfur protein known as the Rieske protein.[122,124] There is no bc_1 complex known that contains any additional redox prosthetic groups. Although the

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cytochrome bc_1 complexes of mitochondria contain multiple additional subunits that lack prosthetic groups,[110,124] these supernumerary subunits are not essential to the Q cycle mechanism, as evidenced by the fact that the Q cycle operates in the bc_1 complexes of bacteria in which only the three redox proteins are present.[146,147] Cytochrome b, cytochrome c_1, and the iron-sulfur protein are thus necessary and sufficient for electron transfer and proton translocation by the bc_1 complex.[66,106,146,147]

The cytochrome bc_1 complex is a member of the larger family of bc-type complexes, which includes the cytochrome bf complex found in chloroplasts,[140] algae,[83] and some Gram-positive bacteria.[72] The bc_1 and bf complexes differ in that the equivalent of the monomeric cytochrome b in the bc_1 complex is an α,β dimer in the bf complex, with the two hemes located in the larger α subunit.[141] Cytochromes c_1 and f differ in that the axial ligands to the covalently attached c-type heme are histidine and methionine in cytochrome c_1,[114] and two nitrogenous ligands in cytochrome f.[105]

The cytochrome bc_1 complex is an integral membrane protein that spans the inner mitochondrial membrane or bacterial plasma membrane as illustrated in Figure 1. Cytochrome b is a transmembrane protein[3,57] in which the two heme groups form an electrical circuit across the membrane,[139] with the low potential heme (b_L) near the positive side of the membrane and the high potential heme (b_H) more central in the membrane.[99] The iron-sulfur protein is anchored to the complex by a hydrophobic helix at the amino terminus of the protein, and the C-terminal domain that

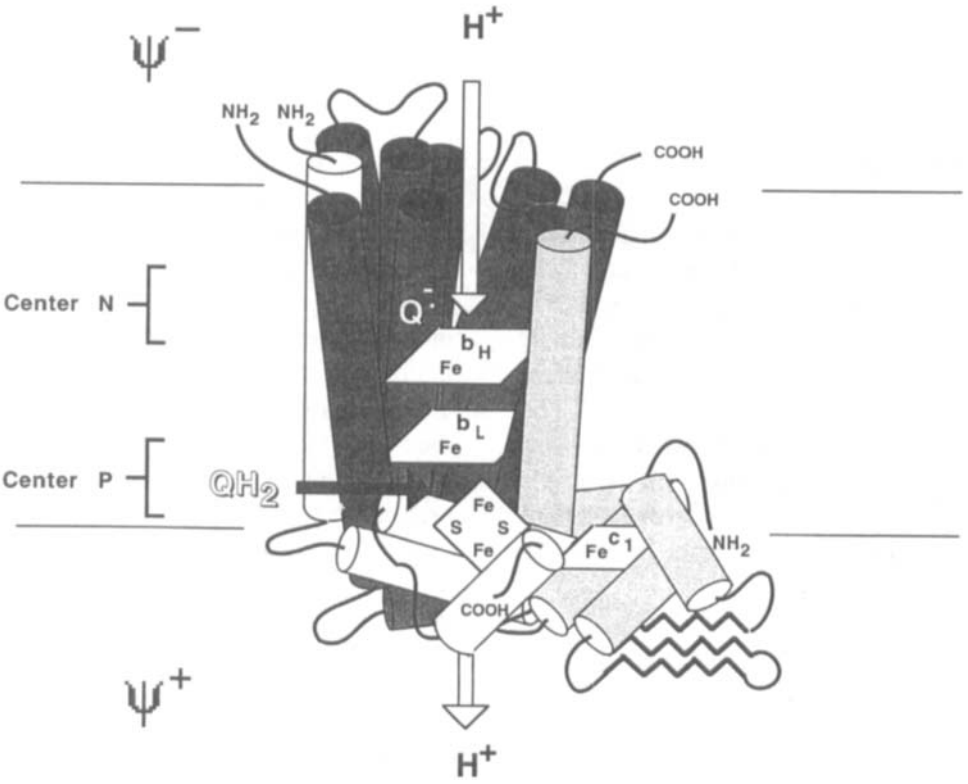

FIGURE 1. The cytochrome bc_1 complex. The figure shows the topographical arrangement of the three subunits that contain redox prosthetic groups in the inner mitochondrial membrane or bacterial cytoplasmic membrane. The figure also depicts ubiquinol (QH_2) entering the ubiquinol oxidase center P, and the stable ubisemiquinone anion ($Q^{\cdot-}$) near the b_H heme at the ubiquinone reductase center N. Protons are shown entering center N and exiting center P. The electronegative side corresponds to the matrix side of the mitochondrial membrane or the cytoplasmic side of the bacterial membrane.

contains the 2 Fe:2 S cluster is located near the electropositive surface of the membrane. Cytochrome c_1 is held to the complex by a hydrophobic helix at the C-terminus, and the domain of the protein that contains the heme is accessible at the electropositive surface of the membrane, where it reacts with cytochrome c.

Cytochrome bc_1 complexes have been purified from mitochondria and bacteria by extraction from the membranes with detergent followed by chromatography or fractionation with ammonium sulfate.[16,59,66,82,102,112,146,149] The purified cytochrome bc_1 complexes have ubiquinol-cytochrome c oxidoreductase activities comparable to those *in situ*, and when reconstituted into liposomes, the purified enzymes translocate protons with a H^+/e^- stoichiometry identical to that *in situ*.[75,147] All of the components necessary for energy transduction are thus present in the purified enzymes, which have been used to investigate the protonmotive Q cycle mechanism.

III. THE PROTONMOTIVE Q CYCLE

The protonmotive Q cycle is depicted in Figure 2 as numbered electron transfer reactions, with the redox groups of the bc_1 complex arranged topographically as illustrated in Figure 1. Ubiquinol is oxidized in two strictly coupled steps in which the first electron from ubiquinol is transferred to the 2 Fe:2 S cluster of the iron-sulfur protein to form a ubisemiquinone* ($Q\cdot_p$), which immediately reduces the low potential heme (b_L) of cytochrome b. This two-electron oxidation of ubiquinol (reaction 1 in Figure 2) releases two protons at the positive surface of the membrane. The ubiquinol oxidation site formed by the iron-sulfur protein and heme b_L is referred to as center P (Figure 1).

The two electrons removed from ubiquinol at center P diverge and follow separate pathways. The electron that was transferred to the iron-sulfur protein is then transferred to cytochrome c_1

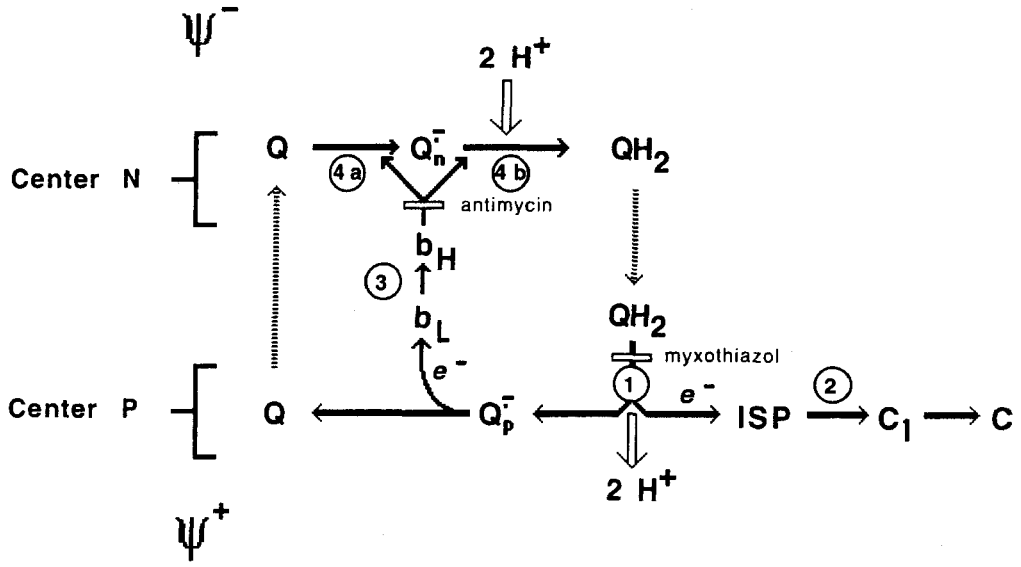

FIGURE 2. The protonmotive Q cycle. The path of electron transfer from ubiquinol to cytochrome c through the redox prosthetic groups of the cytochrome bc_1 complex is depicted as a series of numbered reactions shown by solid arrows. Dashed arrows represent movement of ubiquinol and ubiquinone between the site where ubiquinol is oxidized at the positive side of the membrane ("center P") and the site where ubiquinone and ubisemiquinone are reduced at the negative side of the membrane ("center N"). Open arrows show the reactions in which protons are released during oxidation of ubiquinol and taken up during reduction of ubiquinone. Open rectangles show the reactions that are blocked by myxothiazol and antimycin.

* Ubisemiquinone is ionized at physiological pH and thus depicted in the figures. The ionization state of the semiquinone does not affect the proton translocation stoichiometry of the Q cycle.

(reaction 2 in Figure 2), after which it is transferred to cytochrome c. The second electron from ubiquinol, transferred from $Q\cdot_p$ to heme b_L, recycles through the bc_1 complex from heme b_L to the high potential b heme (b_H) in reaction 3. The b_H heme then reduces ubiquinone to form a stable ubisemiquinone ($Q\cdot_n$) depicted as reaction 4a in Figure 2.

This sequence of electron transfer reactions converts ubiquinol to ubisemiquinone and comprises the first half of the Q cycle. One electron is transferred to cytochrome c, and two protons are released to the outer P side of the membrane. However, only one electron from ubiquinol has been transferred to cytochrome c. The reaction catalyzed by this first half of the Q cycle is

$$QH_2 + c_{ox} \rightarrow Q\cdot_n + c_{red} + 2\ H_p^+$$

In the second half of the Q cycle reactions 1, 2, and 3 are repeated (Figure 2) as a second molecule of ubiquinol is oxidized by transferring one electron to iron-sulfur protein, which then reduces cytochrome c_1, and the resulting $Q\cdot_p$ again reduces heme b_L, which reduces heme b_H. Oxidation of the ubiquinol releases an additional two protons at the outer surface of the membrane. However, during the second half of the Q cycle the heme b_H reduces the ubisemiquinone ($Q\cdot_n$), which was formed in reaction 4a during the first half of the cycle, to ubiquinol in a reaction (4b in Figure 2) that consumes two protons from the inner side of the membrane. The site that catalyzes the two-step rereduction of ubiquinone to ubiquinol and that consumes protons from the negative side of the membrane is a domain of cytochrome b referred to as center N. The reaction catalyzed by the second half of the Q cycle is

$$QH_2 + c_{ox} + Q\cdot_n + 2\ H_n^+ \rightarrow c_{red} + QH_2 + Q + 2\ H_p^+$$

By summing the two reactions above, one can see that the net result of the Q cycle is that one molecule of ubiquinol is oxidized to ubiquinone, two molecules of cytochrome c are reduced, two protons are consumed on the negative (N) side of the membrane, and four protons are deposited on the positive (P) side of the membrane.

$$QH_2 + 2c_{ox} + 2\ H_n^+ \rightarrow Q + 2c_{red} + 4\ H_p^+$$

During one complete Q cycle two molecules of ubiquinol are oxidized to ubiquinones, but one molecule of ubiquinone is rereduced to ubiquinol. The iron-sulfur protein, cytochrome c_1, and the two hemes of cytochrome b are reduced and reoxidized twice. The reaction at center P is identical in the first and second half of the Q cycle. However, at center N heme b_H reduces ubiquinone to ubisemiquinone during the first half of the Q cycle (reaction 4a) and reduces ubisemiquinone to ubiquinol during the second half of the cycle (reaction 4b).

Oxidation of ubiquinol at center P is a second-order, diffusion-controlled reaction when the ratio of ubiquinol to bc_1 complex is 3 or less, with a half-time that varies from 300 μs to 7 ms, depending on the amount of quinol in the quinone pool.[25] As the quinone pool becomes more reduced and the ratio of ubiquinol to bc_1 complex increases, the rate of ubiquinol oxidation is limited by the rate at which iron-sulfur protein is reoxidized, which is determined by the rate at which cytochrome c_1 is oxidized by cytochrome c and cytochrome c oxidase. The electron transfer reactions that intervene between oxidation of ubiquinol and reduction of cytochrome c_1, and between ubisemiquinone oxidation and reduction of the b_H heme, are so rapid that they are not temporally resolved under normal experimental conditions. It has been deduced that electron transfer from the iron-sulfur protein to cytochrome c_1 has a half-time <200 μs, and that between the ubisemiquinone, $Q\cdot_p$, and the b_H heme has a half-time <300 μs.[25,129]

The two-step rereduction of ubiquinone to ubisemiquinone and ubiquinol at center N, which resembles the gated reduction of ubiquinone by the photosynthetic reaction center, occurs with a half-time of 1.7 to 7 ms.[25] Like the oxidation of ubiquinol at center P, the rate of this reaction is also controlled by the redox poise of the quinone pool, but in an opposite manner. Reduction of ubiquinone to ubiquinol by b_H at center N increases as the ratio of ubiquinone to ubiquinol increases. The net effect of the rates of reactions 1 to 4 (Figure 2) is that the amount of ubisemiquinone ($Q\cdot_n$) at the b_H reaction site is determined by the ratio of ubiquinol to ubiquinone

in the quinone pool, which paces ubiquinol oxidation at center P and ubiquinone reduction at center N.[25]

IV. EXPERIMENTAL EVIDENCE FOR THE Q CYCLE MECHANISM

A. Oxidant-Induced Reduction of Cytochrome b

Prior to the formulation of the protonmotive Q cycle, numerous attempts were made to construct linear pathways of electron transfer from ubiquinol to cytochrome c through the bc_1 complex (see Reference 142 and references therein). Any such pathway must account for the extensive observations regarding the effects of antimycin, an inhibitor that binds specifically to a single site in the bc_1 complex and that causes electrons to accumulate in ubiquinol and the b cytochromes when added to respiring mitochondria (see Reference 115 for an early review on the extensive literature regarding antimycin). Linear mechanisms of the type shown below account for the observation that addition of antimycin to respiring mitochondria causes electrons to accumulate in the b-cytochromes and ubiquinol while the iron-sulfur protein (ISP) and c-cytochromes become oxidized.

$$QH_2 \rightarrow [b_L, b_H] \xrightarrow{\text{antimycin}} ISP \rightarrow C_1 \rightarrow C$$

However, when a pulse of oxygen is added to mitochondria that are respiring slowly because of oxygen limitation, the oxygen burst causes increased oxidation of cytochromes c and c_1, as would be expected, but this oxidation is accompanied by an unexpected transient reduction of cytochrome b.[18] This oxidant-induced reduction of cytochrome b involves both b hemes and is increased by the addition of antimycin. Oxidant-induced reduction of cytochrome b was investigated extensively in early studies on the mechanism of energy transduction linked to respiration but could not be accounted for by any linear arrangement of the redox components of the cytochrome bc_1 complex of the type shown above.

The protonmotive Q cycle accounts for oxidant-induced reduction of cytochrome b. Wikström and Berden pointed out that divergent oxidation of ubiquinol could link formation of a cytochrome b reductant to oxidation of the c-cytochromes.[143] This observation was seminal to Mitchell's formulation of the Q cycle mechanism.[87] As can be seen from Figure 2, rapid oxidation of the c-type cytochromes and iron-sulfur protein accelerates ubiquinol oxidation, leading to a transient increase in $Q\cdot_p$, the reductant for cytochrome b. Antimycin blocks reoxidation of b_H, enhancing the oxidant-induced reduction.

B. Two Pathways for Presteady State Reduction of Cytochrome b

Evidence establishing the Q cycle mechanism came from examining the presteady state reduction of the cytochromes b and c_1 when iron-sulfur protein was reversibly resolved from the isolated bc_1 complex.[45,121,122,126,127] If iron-sulfur protein is extracted from the complex, cytochrome c_1 cannot be reduced by ubiquinol, but the b cytochromes can be reduced.[45,121] If iron-sulfur protein is extracted and antimycin is added, reduction of both cytochromes b and c_1 is blocked. These experiments showed that there are two pathways of cytochrome b reduction. One pathway, through center P, requires iron-sulfur protein. The second pathway, through center N, does not require iron-sulfur protein, but is inhibited by antimycin (Figure 2).

If antimycin blocks cytochrome b reduction through center N, as shown in Figure 2, one would predict that it would not inhibit reduction of cytochrome b through the alternative, center P pathway. Furthermore, one would also predict antimycin would not inhibit presteady state reduction of cytochrome c_1 during a single redox turnover of the bc_1 complex in a Q cycle-type of mechanism, but would inhibit c_1 reduction through any linear pathway in which the antimycin block is between the substrate (QH_2) and c_1. When these predictions were tested, it was found that antimycin does not block reduction of either cytochrome b or c_1 in a presteady state reduction of the complex, consistent with the Q cycle mechanism.[11]

An important tool for examining the two pathways of b reduction predicted by the Q cycle was

provided by the discovery of a second class of inhibitors specific for the bc_1 complex,[134] which act at a site different than antimycin, as evidenced by their differential effects on presteady state reduction of the cytochromes.[133–135] Myxothiazol, an example of these center P inhibitors, blocks reduction of cytochrome c_1, but does not block reduction of the b cytochromes through center N (Figure 2). When myxothiazol is added with antimycin, the two inhibitors block reduction of both cytochromes b and c_1. Myxothiazol thus mimics removal of iron-sulfur protein from the bc_1 complex, blocking oxidation of ubiquinol at center P (Figure 2). If myxothiazol blocks oxidation of ubiquinol at center P, and the iron-sulfur protein catalyzes this reaction, myxothiazol should inhibit, and iron-sulfur protein should be required for, oxidant-induced reduction of cytochrome b. These predictions were confirmed.[10,135]

C. Thermodynamic Properties of Ubiquinol/Ubiquinone Oxidation-Reduction

The protonmotive Q cycle also accounts for how the redox centers of the bc_1 complex, with oxidation-reduction potentials that span approximately 350 mV, participate in electron transfer through the complex. To appreciate the microscopic thermodynamic constraints of the Q cycle, it is useful to examine the relative midpoint potentials of the cytochromes and iron-sulfur protein and their relationship to that of the ubiquinone/ubiquinol couple, as shown in Figure 3. From this figure it would appear that, whereas electron transfer from ubiquinol to iron-sulfur protein is a thermodynamically favorable reaction, that from ubiquinol to cytochrome b_L is not. Recognition of this apparent thermodynamic barrier made it especially difficult to incorporate cytochrome b_L into any linear scheme of electron transfer. The Q cycle incorporates participation of both cytochrome b_H and b_L by mechanistically matching the redox potentials of the ubisemiquinone couples at center N and center P to the potentials of their electron acceptors and donors.

At center N, cytochrome b_H reduces ubiquinone to ubiquinol (Figure 3). In order for cytochrome b_H to reduce both ubiquinone to ubisemiquinone (step 4a in Figure 2) and ubisemiquinone to ubiquinol (step 4b), the potentials of the Q/Q· and Q·/QH_2 couples at center N must be similar. From the equilibrium constant for formation of ubisemiquinone and the Nernst equations for the ubiquinone/ubiquinol couple and the two ubisemiquinone couples, one can relate the oxidation-reduction potentials of the two ubisemiquinone couples and the concentration of ubisemiquinone.[88,123]

$$E_m\left(Q/Q^{\cdot-}\right) - E_m\left(Q^{\cdot-}/QH_2\right) = \frac{RT}{nF} \ln \frac{\left(Q^{\cdot-}\right)^2}{(Q)(QH_2)}$$

$$= 60 \log \frac{\left(Q^{\cdot-}\right)^2}{(Q)(QH_2)}$$

From this equation it can be seen that when the potentials of the two semiquinone couples are similar, the equilibrium constant for semiquinone formation approaches unity, and its concentration approaches those of ubiquinol and ubiquinone. This predicts that the ubisemiquinone at center N, $Q\cdot_n$, is relatively stable. A stable ubisemiquinone was detected in isolated mitochondrial bc_1 complex,[100] in mitochondrial[33] and bacterial membranes,[85] and the EPR signal from this semiquinone was eliminated on addition of antimycin.

At center P, the two electrons removed from ubiquinol follow divergent pathways in which the immediate electron acceptors, iron-sulfur protein and cytochrome b_L, differ by approximately 350 mV (Figure 3). Prior to the Q cycle mechanism, it was not obvious how ubiquinol could reduce heme b_L, because this appears to be a thermodynamically unfavorable reaction. This reaction is facilitated at center P by virtue of the fact that the oxidation-reduction potential of the ubiquinone/ubiquinol couple, E_m (Q/QH_2), is the arithmetic average of the oxidation-reduction potentials of the ubiquinone/ubisemiquinone couple, E_m (Q/Q·), and the ubisemiquinone/ubiquinol couple, E_m (Q·/QH_2).

$$2\ E_m\ (Q/QH_2) = E_m\ (Q/Q\cdot) + E_m\ (Q\cdot/QH_2)$$

Ubiquinol is oxidized at center P in a strictly coupled reaction by the iron-sulfur protein (E_m = +250 mV) and the heme of b_L (E_m =

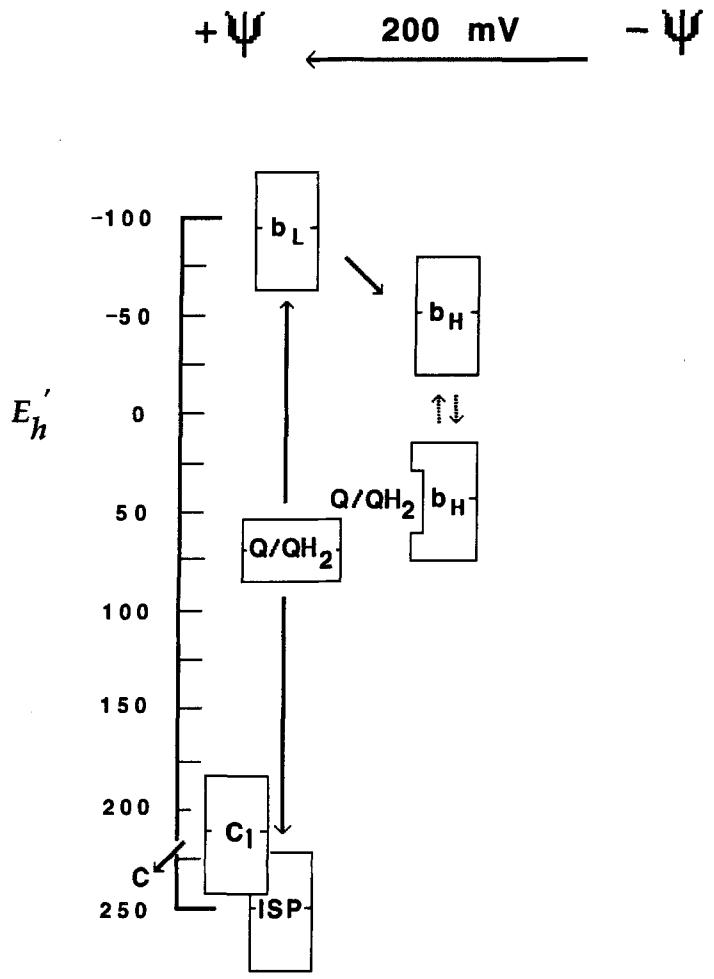

FIGURE 3. Thermodynamic profile of the Q cycle. In this depiction of the Q cycle the redox prosthetic groups are arranged vertically according to their oxidation-reduction potentials and displayed horizontally according to their electrochemical disposition across the inner mitochondrial membrane or bacterial plasma membrane. The open boxes delineate the approximate range along the vertical axis of oxidation-reduction potentials spanned by the redox components as their oxidation-reduction status varies in response to changes in rates of electron transfer through the cytochrome bc_1 complex. Cytochrome b_H is a mixture of two potentiometric species in which reversible binding to ubiquinol raises the potential of a variable portion of the b_H from approximately –50 to +40 mV.[108] The potentials of the stable $Q\cdot_n$ and unstable $Q\cdot_p$ ubisemiquinone couples are not shown.

–90 mV). If the first electron is transferred to iron-sulfur protein at a potential of +250 mV, and E_m (Q/QH_2) = +60 mV, it follows that the ubisemiquinone formed at center P must attain a potential as low as –130 mV, which is strongly reducing, even in comparison to heme b_L. If the potentials of the $Q/Q\cdot$ and $Q\cdot/QH_2$ couples at center P differ by 350 mV, the above equation indicates that the intermediate ubisemiquinone ($Q\cdot_p$) should be unstable. De Vries and co-workers detected a transient, unstable ubisemiquinone during oxidant-induced reduction of cytochrome b, and the EPR signal from this semiquinone was not eliminated by antimycin.[31]

D. Triphasic Reduction of Cytochrome b

If a rate limiting amount of ubiquinol is added to the bc_1 complex, the resulting presteady state reduction of cytochrome b is a triphasic reaction, while reduction of cytochrome c_1 is monophasic.[62,116,117] Within the first 50 ms there is a rapid partial reduction of cytochrome b, and c_1 reduction parallels this rapid phase of b reduction. Cytochrome b then undergoes reoxidation, while c_1 reduction continues. After approximately 100 ms c_1 reduction is complete, reoxidation of cytochrome b stops, and cytochrome b is slowly rereduced. Formation of $Q\cdot_n$ is delayed until reduction of c_1 nears completion.[32,150]

These redox responses are consistent with the Q cycle mechanism (Figure 2). When ubiquinol is added to fully oxidized bc_1 complex, it is oxidized at center P, due to the high potential of the iron-sulfur protein (+250 mV) in comparison to the b_H heme (+40 mV), and this reduction is linked to c_1 reduction. As $Q\cdot_n$ is formed, it catalyzes reoxidation of cytochrome b through center N and does not accumulate to detectable levels until c_1 and iron-sulfur protein are reduced, at which point further oxidation of ubiquinol through center P is blocked by lack of an electron acceptor. The b reoxidation phase then ends as ubiquinol oxidation "switches" to center N, and the b hemes are rereduced by reversal of reactions 4b and 4a in Figure 2. Myxothiazol inhibits the initial, rapid phase of b reduction and reduction of c_1 but allows the slower reduction of b to proceed. Antimycin does not inhibit the initial phase of b reduction but eliminates the subsequent reoxidation and slow rereduction.[117]

V. STRUCTURAL BASIS OF THE Q CYCLE

As the Q cycle gained acceptance, attention focused on elucidating the structure of the cytochrome bc_1 complex as it relates to the mechanism. Three-dimensional crystals of the mitochondrial bc_1 complex have been obtained, and a high-resolution structure of this oligomeric protein will eventually be available.[7,151,153] However, present knowledge of the structure of the complex is based on electron microscopy of membrane crystals,[7] chemical modifications that localize proteins at the membrane surface or interior,[3,50,57] spectroscopic probes that measure distances of the redox prosthetic groups from the membrane surface,[99] proteolytic digestions that release water-soluble domains of the iron-sulfur protein[76] and cytochrome c_1,[77] genetic methods that identify domains of cytochrome b involved in quinol oxidation and quinone reduction[20,27,37–43,48,61,73,119,120] and predictions of secondary structure by empirical algorithms.[15,26] The models that have resulted from a combination of these approaches reconcile the topography of the subunits within the membrane and the location of the redox prosthetic groups with the Q cycle mechanism.[20,48,124,125]

A. Cytochrome b

Sequences of cytochrome b's from approximately 900 species show a high degree of conservation of secondary structure, and identify the four conserved histidine heme ligands.[35] Cytochrome b is folded into an octet of transmembrane helices, and a ninth, extramembranous amphipathic helix, as in Figure 4. Genetic methods have been especially powerful in elucidating structure-function relationships in the cytochrome b of *S. cerevisiae* (for a review see Reference 20). Similar studies have been initiated with the cytochrome b of photosynthetic bacteria, which is amenable to site-directed mutagenesis (for a review see Reference 48). The mutations thus far characterized in *S. cerevisiae* and *Rh. capsulatus* are compiled in Tables 1 and 2. In order to relate the mutations in these two extensively studied systems, we have aligned the sequences of the two cytochrome b's in Figure 5, and in Tables 1 and 2 we have provided the numerical position of the amino acids for each species corresponding to a mutant allele in the other.

The two b heme groups are ligated through four conserved histidines,[35] and bridge the B-D helixes (Figure 4). The predicted folding of the protein (Figure 4) and oxidation-reduction of the two hemes in response to a membrane potential[139] infer that His82* and 183 ligate b_L

* Throughout the text, we have used the numbering of amino acids from the yeast sequences. The reader can cross-reference to the *Rh. capsulatus* sequences by referring to the sequence alignments in Figures 5, 8, and 10.

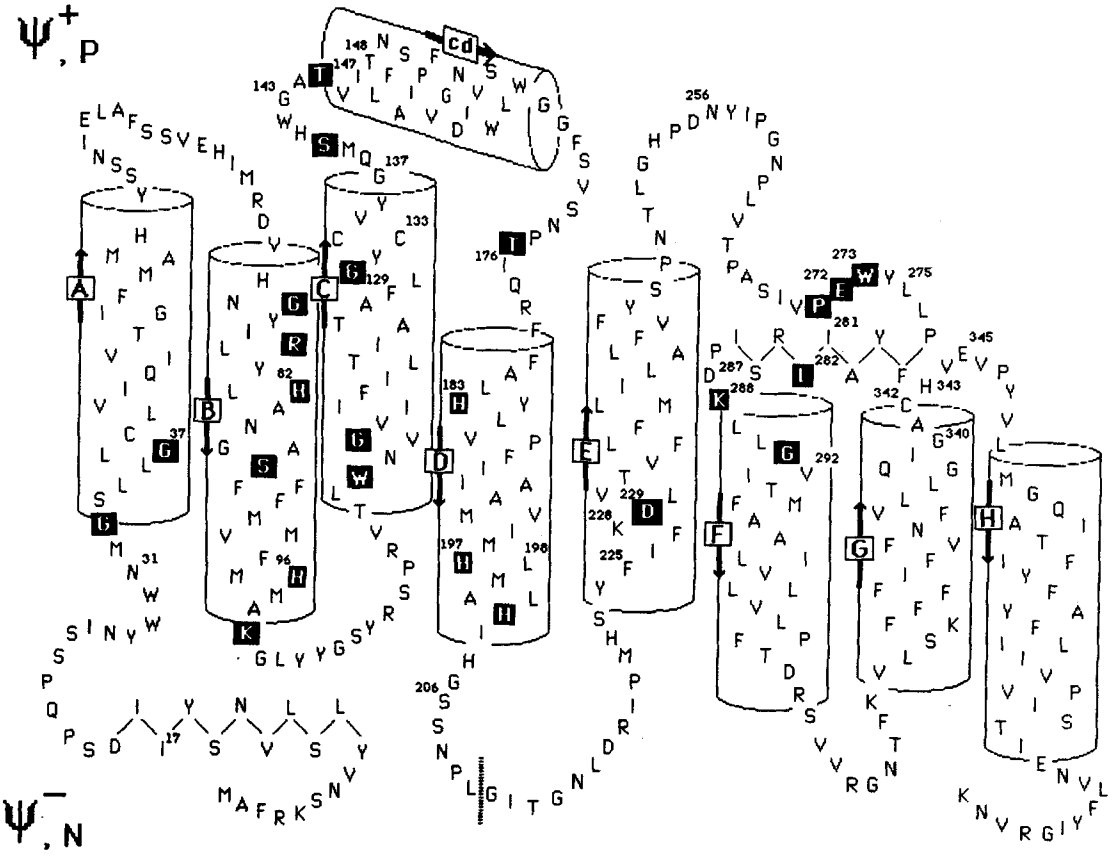

FIGURE 4. Predicted structure of cytochrome b from *S. cerevisiae*. The eight transmembrane helices are lettered **A** through **H**, the extramembranous helix at center P is lettered **cd**, and the orientation of the helices is indicated with a bold arrow pointing from the N-terminal to C-terminal direction. The dashed line between helices **D** and **E** depicts the approximate position at which cytochrome b is split into an α/β dimer in the cytochrome bf complexes. Numbers identify amino acids where mutations have been identified. The four histidine ligands to the hemes and the amino acids that are most highly conserved across the cytochrome b's of approximately 900 species are enclosed in bold boxes.

near the P side of the membrane, and His96 and 197 ligate b_H in a more electronegative location, near the center of the membrane. These assignments are supported by a point mutation, Cys133-Tyr, which alters the spectrum of b_L, and a second mutation, Ser206-Leu, which affects heme b_H.[73]

The centers N and P domains in cytochrome b have been identified by point mutations that confer resistance to inhibitors which block Q cycle reactions at these sites. Two additional inhibitors, strobilurin and stigmatellin, act at center P similar to myxothiazol, as indicated by their effects on presteady state reactions of the Q cycle[133,134] and cross-resistance in some of the inhibitor resistant cytochrome b mutants (Table 1). Point mutations conferring resistance to these center P inhibitors have been identified at Phe129, Cys133, Gly137, Ile147, Thr148, Asn256, Leu275, and Val292.[20,27,40,120] A mutation at Gly143 blocked oxidation of ubiquinol at center P, while leaving reactions through center N intact.[27] From these mutations it can be inferred that center P is formed from the amphipathic, extramembranous cd helix, the loop between helices E and F, and the distal regions of helices C and F, and that the b_L heme is near center P.

TABLE 1
Mutations in Cytochrome b Conferring Resistance to Inhibitors

Mutation			
Saccharomyces	*Rhodobacter*	Inhibitor resistance	Ref.
I17F	I33	Diu^r	42
N31K	I46	Diu^r, Ant^r	42
G37V	A52V	Ant^r, Fun^r	24, 37
M91	L106P	Myx^r, Str^r	27
I125	M140I	Myx^r, Stig^r	27
F129L	F144L	Myx^r, Stig^r	27, 38
F129	F144S	Myx^r	27
G137R	G152	Myx^r, Str^r	38
G137E	G152	Myx^r	120
G137V	G152S	Myx^r	27, 120
G143	G158P	Myx^r	1
I147F	I162	Stig^r	38
T148	T163A	Stig^r	27
L198F	I213	Fun^r	43
F225L,S	F248	Diu^4	37, 42
K228I	K251M	Ant^r	24, 37
D229	D252E,N,A	Ant^r	24, 54
N256Y	N279Y	Myx^r, Str^r, Stig^r	24, 38
E272	E295Q	Stig^r	24
W273	W296L	Myx^r, Stig^r	24
L275S,F	F298	Myx^r, Str^r	38
L275T	F298	Myx^r	38
V292	V333A	Stig^r	27

Note: The column on the left shows the numerical positions of the amino acids in the yeast sequence; the second column shows the numerical positions of the equivalent or homologous amino acid in the *Rh. capsulatus* or *Rh. sphaeroides* sequences. The abbreviations used are Ant, antimycin; Diu, diuron; Fun, funiculosin; Myx, myxothiazol, Sti, stigmatellin; Str, strobilurin A.

Mutations conferring resistance to antimycin, funiculosin, and diuron have been identified at Ile17, Asn31, Gly37, Leu198, Phe225, and Lys228.[37,42,43,73] In addition, a mutation at Ser206 specifically affects heme b_H.[73] These place the amino terminus of the protein, the connecting loop between helices D and E, the end of helix E, and the end of helix D, which includes one of the b_H histidine ligands, at the site that catalyzes oxidation-reduction of ubiquinol and ubiquinone at center N (reactions 4a and 4b in Figure 2). Because antimycin destabilizes $Q\cdot_n$,[33,85,100] and some of the antimycin-resistant mutants exhibit low rates of electron transfer, it would be interesting to determine whether the amino acid changes in these mutants affect the stability of the EPR-detectable semiquinone (see below).

The locations of the center N- and P-resistant mutants provide strong support for the topographical disposition of transmembrane helices A through F and the extramembranous helix cd as shown in Figure 4. Analysis of second site, intragenic pseudorevertants has provided additional evidence for the topology of the transmembrane helices and has identified interacting amino acids within adjacent helices.[40,41] The locations of these mutations and the corresponding second site revertants are listed in Table 2. A mutation at Gly340 that leads to a nonfunctional cytochrome b[73] is complemented by an intragenic reversion mutant at Lys288, indicating that the C-terminus of helix

TABLE 2
Mutations in Cytochrome b Affecting Activity or Assembly of the bc_1 Complex

Mutation				Reversion	
Saccharomyces	*Rhodobacter*	Phenotype	Ref.	*Saccharomyces*	Ref.
G33	G48V	Q_N^-	24		
G33	G48D	nass$^-$	24		
G37	A52V	Q_N^-	24		
G37	A52D	nass$^-$	24		
Q43	Q58L	b_L	154		
F129	F144S	Q_P^-	24		
G131S	G146	act$^-$,CP$^-$	73		
C133Y	V148	b_L	40, 73	A126T	40
				I176S	40
				H343T	39
G137E	G152	act$^-$	120	C133SN	41
				H141Y	40
				256K	40
W142R	W157	act$^-$	73		
G143	G158D	Q_P^-	27		
G143	G158C,D,T,N,E, V,H,L,I,R,Y,W	act$^-$	1		
G167	G182S	act$^-$	54		
P187	P202L	b_L — b_H slow	154		
H202	H217A	Q_N^-	24		
S206L	N221	b_H	73		
M221K	F244	act$^-$	73		
M221	F244L	nass$^-$	24		
K228	K251M,I	Q_N^-	24		
D229	D251N,A	Q_N^-	24		
N256Y	N279Y	Q_P^-, act$^-$	24, 46		
E272	E295G,Q	Q_P^-	24		
W273	W296L	Q_P^-	24		
Y274	Y297S	Q_P^-	24		
L282F	L305	act$^-$, CP$^-$	73	N256I	39
				I281F	39
				D287H	39
G340E	G381	act$^-$, CP$^-$	73	K288N	39
				C342G	39
				E345G	39

Note: Mutations in yeast or *Rhodobacter* are listed in the first and second columns, and equivalent or homologous amino acids are identified in the two species as in Table 1. The abbreviations used for the phenotypes are act$^-$, catalytic activity decreased; nass$^-$, not assembled; b_H, heme b_H altered; b_L, heme b_L altered; CP$^-$, core protein I missing; Q_N^-, center Q_N activity decreased; Q_P^-, center Q_P activity decreased. Second site revertants in yeast are listed in the fifth column.

G and the N-terminus of helix F interact and are on the same side of the membrane.[39] A mutation of Cys133 is complemented by reversion mutations at His343 or Ile176, indicating that helix C interacts with helices G and D (Figure 4).

Based on these mutational studies, it can be concluded that on the positive side of the membrane domains from helices B, C, F and cd together with the ef loop form center P, and that on the negative side of the membrane domains from helices A, D, and E form center N. The results from random mutagenesis studies are complemented by naturally occurring resistance against inhibitors of the mitochondrial bc_1 complex. Based on the alignment of the approximately 900 known cytochrome b sequences, Degli Esposti and co-

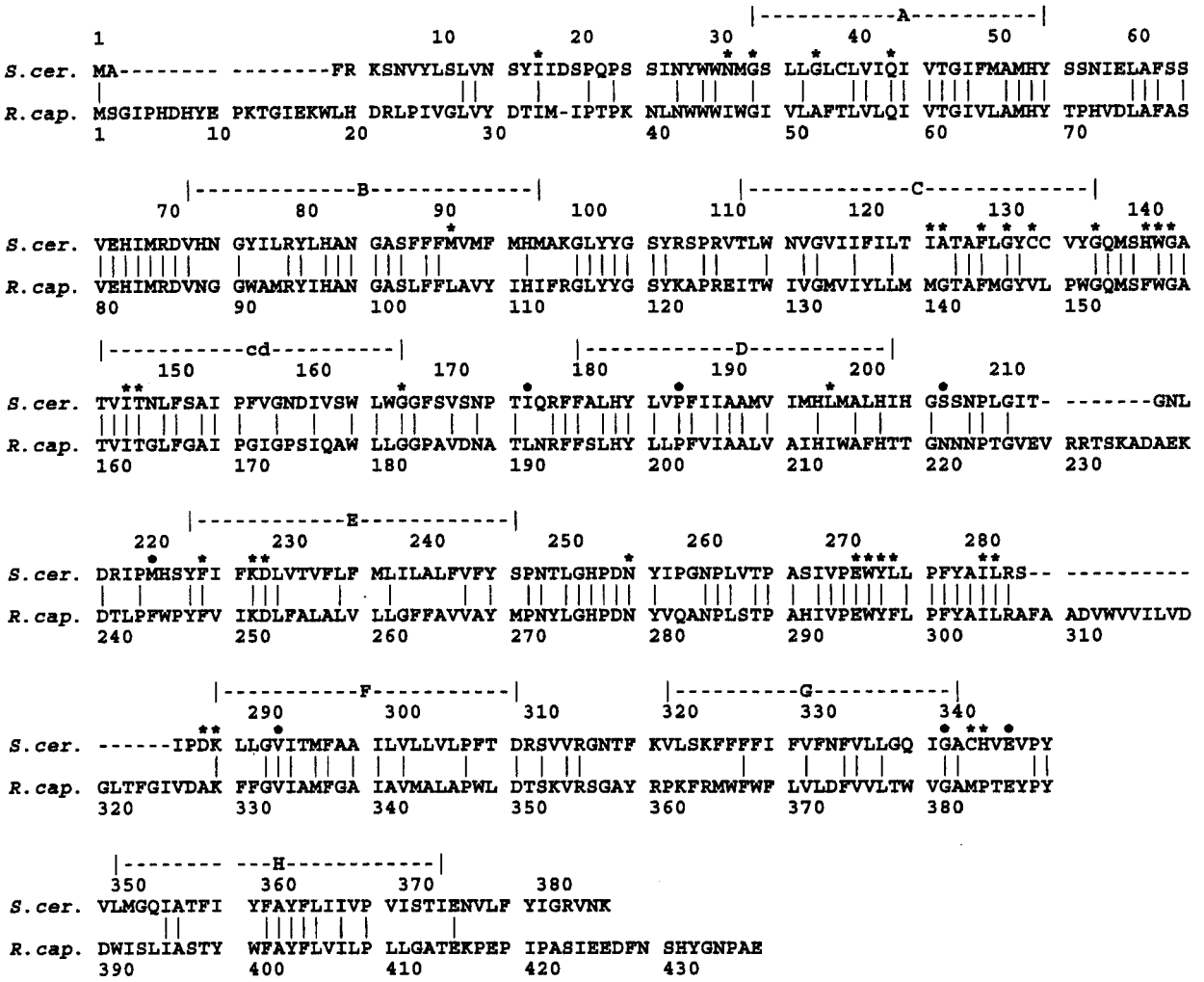

FIGURE 5. Alignment of cytochrome b sequences from *S. cerevisiae* and *Rh. capsulatus*. The sequences were aligned based on the multiple sequence alignment of Degli Esposti et al.,[35] except that the number of gaps was minimized. The asterisks (*) designate amino acids where mutations have been identified. The brackets lettered **A-H** delineate the eight transmembrane helices, and the bracket lettered **cd** delineates the extramembranous helix connecting helixes C and D.

workers have identified amino acid changes in various species that can account for natural inhibitor resistance of one species relative to the other.[35] For example, Val194 of the rabbit protein appears to be responsible for a 60-fold reduced sensitivity toward funiculosin when compared with bovine cytochrome b, which has a much smaller alanine in this position. Plant cytochrome b, which shows HQNO hypersensitivity, has a Gly substitution for Asp31, a position that confers resistance to center N inhibitors if it is mutated to Lys (Table 1).

This information was combined to deduce a hypothetical arrangement for helices A to F as shown at the bottom of Figure 6.[35] While this arrangement works well to predict center N (Figure 6, bottom), it poses some problems at center P. Although the ef loop, which carries the highly conserved "PEWY" sequence and the mutated position Asn256, is predicted to be approximately 40 amino acids long, it seems unlikely that the two helices it connects are as distant as predicted by the arrangement shown for center N (Figure 6, bottom). Complementing mutations between Gly137 and Asn256 infer that the conserved "PEWY" loop connecting helices E and F interacts with the C-terminus of helix C.[40] Therefore, it seems likely that the nearest neighbor relation-

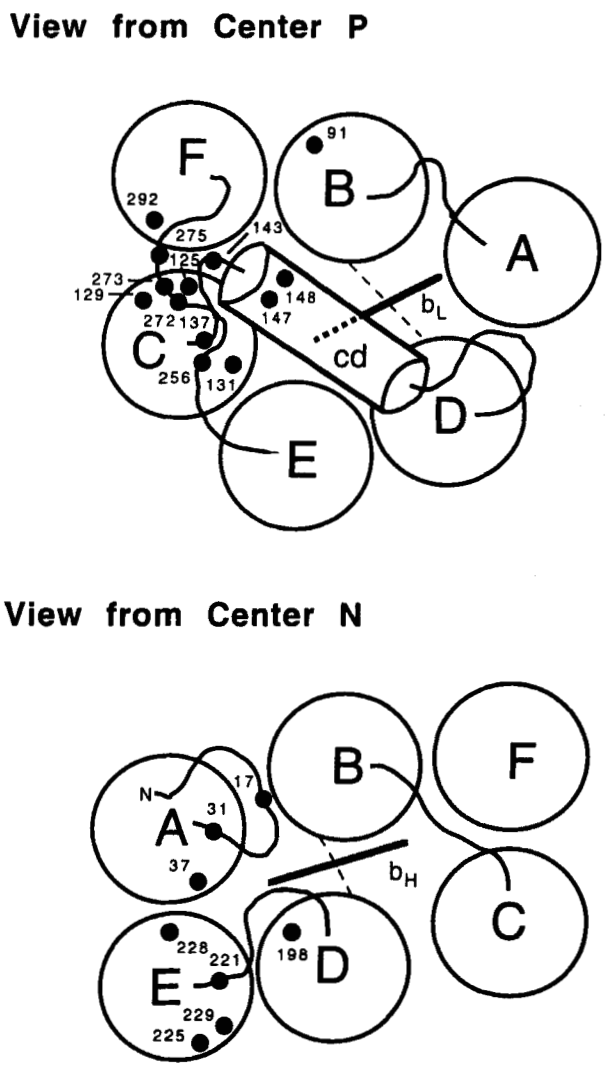

FIGURE 6. Arrangement of helices A-F of cytochrome b at center P and center N. The hypothetical views are derived from schemes discussed previously.[35,48] It was assumed, however, that the proposed bend introduced by the proline in helix D^{79} allows helix E to traverse from a location closer to helix A on the N-side to a location closer to helix C on the P-side. This also moves helix E closer to helix F at center P, making the ef-loop long enough to connect the two transmembrane spans. *Filled circles* indicate the approximate positions of mutations conferring resistance to center P (top) or center N (bottom) inhibitors. *Curved lines* indicate the interhelical loops. The heme b_L (top) and b_H (bottom) groups are indicated as bars and their coordination by the conserved histidine residues in helices B and D is indicated by *dashed lines*.

ship for the helices of cytochrome b might be different at centers P and N, placing helix E between helices D and C at center P (Figure 6, top), rather than between helices D and A as at center N (Figure 6, bottom). The bend in helix D induced by the highly though not completely

conserved Pro187[35] may allow helix E to traverse the membrane in a way that deviates significantly from being perpendicular to the membrane surface.

B. Rieske Iron-Sulfur Protein

Sequences of the Rieske iron-sulfur proteins have been deduced from 15 species, including mammals, plants, fungi, oxygenic and photosynthetic bacteria (for a review see Reference 52). The 2 Fe:2 S cluster is noncovalently held in the protein by two cysteines and two histidines as shown in Figure 7.[17,56,68] Because histidine is less strongly electron donating than sulfur, it is likely that the two histidine ligands account for the fact that the midpoint potential (+180 to +250 mV, see Reference 122) of this 2 Fe:2 S protein is 400 mV more positive than those of other ferredoxins,[9] which contain four sulfur ligands. The only conserved sequences in the Rieske proteins are two hexapeptides, corresponding to Cys159-THLGC and Cys178-PCHGS in the yeast sequence in Figure 8. These two sequences include the only conserved histidines, His161 and His181, which must be two of the ligands to the 2 Fe:2 S cluster, and

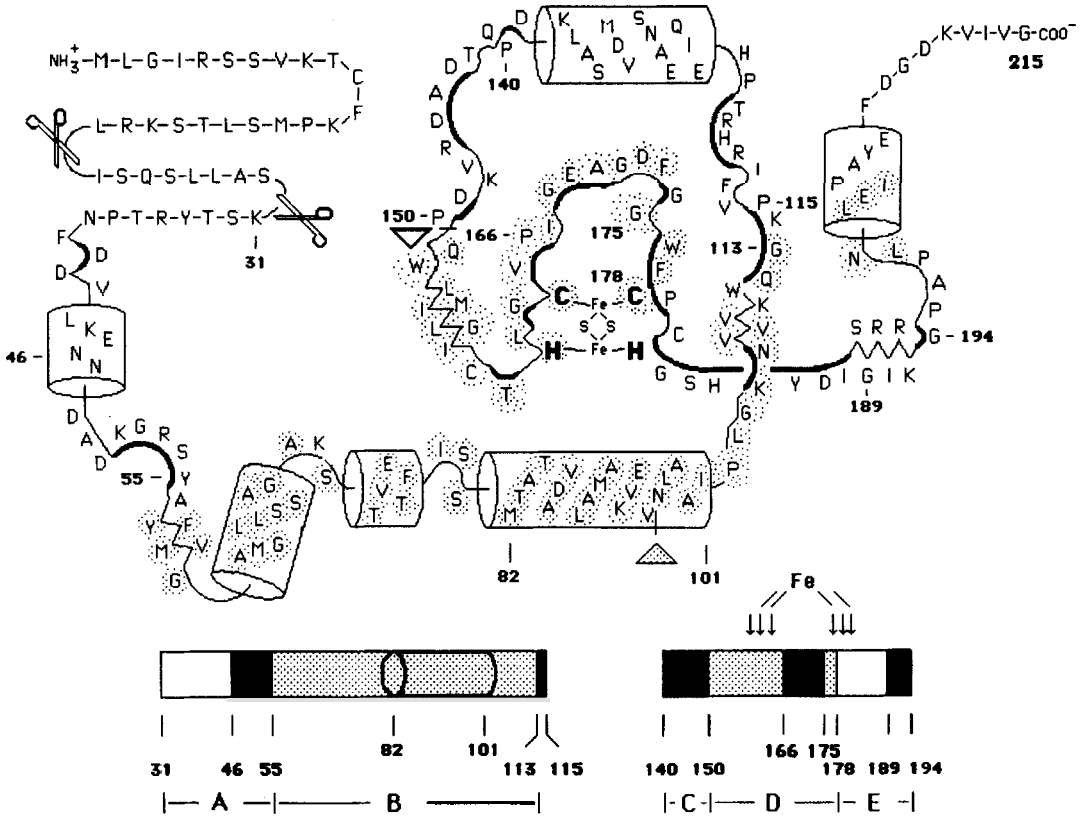

FIGURE 7. Predicted structure of the Rieske iron-sulfur protein from *S. cerevisiae*. The scissors indicate the two sites of posttranslational processing by mitochondrial proteases. Hydrophobic regions of the protein are indicated by stipling around the amino acids. The stipled triangle indicates the region in the yeast protein where there is a deletion of approximately 15 amino acids in the iron-sulfur proteins from spinach, *Nostoc,* and *Synechococcus.* The inverted open triangle indicates where there is an insertion of 10 to 15 amino acids in the iron-sulfur proteins from *P. denitrificans* and *Rh. capsulatus.*[52] Below the predicted structure is a map of the five structural domains **(A-E)** that are conserved in the Rieske iron-sulfur proteins characterized to date. Numbers below the map correspond to amino acids in the yeast protein. Open rectangular areas on the map indicate randomly coiled hydrophilic domains; solid black areas indicate flexible hydrophilic domains; stipled areas on a clear background indicate hydrophobic domains; and stipled black areas indicate flexible hydrophobic domains. Arrows point to locations of the possible ligands to the iron-sulfur cluster.

four conserved cysteines. Which two cysteines are ligands is still under investigation. It seems unlikely, for steric reasons, that an iron could be liganded to Cys180 if His181 is a ligand. However, site-directed mutagenesis of Cys159, Cys164, Cys178, and Cys180 have all resulted in nonfunctional iron-sulfur proteins, apparently lacking the Fe:S cluster.[28,52,53]

Although the amino acid sequence of the iron-sulfur protein is not highly conserved, the structure is. The diagram in Figure 7 depicts five structural domains in the yeast protein that are conserved across species. The N-terminus of the protein is distinguished by a bipartite structure in which a hydrophilic domain (A) is followed by a hydrophobic domain (B), which includes one or more extended α helices flanked by two highly flexible sequences.

The N-terminus of domain B appears to hydrophobically bind the iron-sulfur protein to the bc_1 complex. A synthetic peptide corresponding to Ser54-Lys72 in the yeast protein (Figure 7) prevented the full-length protein from rebinding to bc_1 complex from which it was extracted,[51] and trypsin releases a water-soluble form of the protein by cleavage at Lys72.[76] Whether this hydrophobic anchor spans the membrane or is adsorbed to cytochrome b is not clear. The iron-sulfur protein binds to the bacterial plasma membrane in the absence of cytochromes b or c_1, indicating that its anchorage is not dependent on other subunits of the complex.[132] However, extraction of the protein by guanidine[126] or sodium carbonate[58] is difficult to reconcile with a transmembrane anchor, and the protein was not labeled by hydrophobic probes that labeled phospholipids and cytochrome b[50,57] but was labeled by extrinsic, hydrophilic reagents.[47]

The Fe:S cluster is held in a flexible, hydrophobic pocket in the C-terminus of the protein, flanked by sequences enriched in glycines and prolines (Figure 7), which are sites of temperature-sensitive mutations.[52] Domain C is thought to be at the aqueous surface of the bc_1 complex. The hydrophilic, flexible character of this region is retained in the various Rieske proteins, and mutations that eliminate the acidic charges render the protein temperature sensitive. The hydrophobic C-terminus of the protein is thought to cap the Fe:S cluster, creating an environment in which one edge of the cluster, including His161 and one of the cysteines, is in a hydrophobic environment, while the other edge, including His181 and a second cysteine, is at an interface between hydrophobic domain D and hydrophilic domain E.[52]

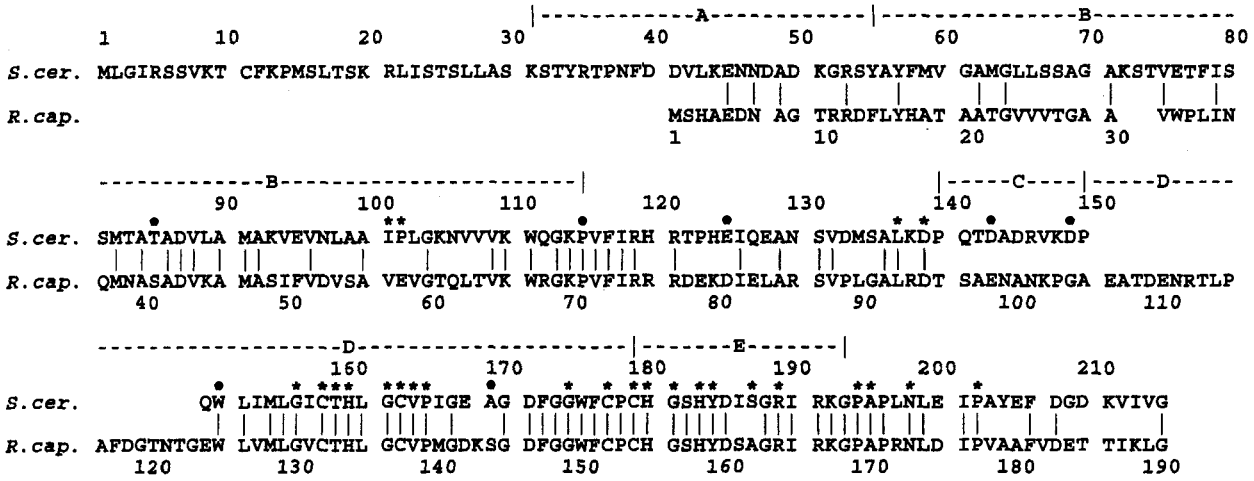

FIGURE 8. Alignment of Rieske iron-sulfur protein sequences from *S. cerevisiae* and *Rh. capsulatus*. The sequences were aligned with the GAP algorithm of the GCG software package and optimized manually. The asterisks (*) designate amino acids where mutations have been identified. The brackets lettered **A-E** delineate conserved structural domains shown in Figure 7.

The divergent oxidation of ubiquinol at center P requires that the Fe:S cluster and the b_L and c_1 hemes must have approximate relationships to each other and to the hydrophobic phase of the membrane as implied in Figure 1. The Fe:S cluster must be reducible by ubiquinol from within the membrane and must generate $Q\cdot_p$ within a few Ångströms of b_L. Additionally, the Fe:S cluster must transfer electrons to the heme of c_1, which must be inaccessible as a direct oxidant for either ubiquinol or ubisemiquinone, even when the iron-sulfur protein is removed.[45,121]

These considerations suggest that one edge of the Fe:S cluster is in a hydrophobic environment near the b_L heme, while the other must be electronically connected to the c_1 heme in a more hydrophilic region. Because electron transfer from ubiquinol to c_1 occurs on a microsecond time scale,[25,129] it is likely the electron is transferred from the quinol across the Fe:S cluster to the c_1 heme in an essentially solid state relay, in which one or both of the histidine ligands may also participate. As discussed below, double occupancy of the two histidines by two ubiquinol/ubiquinone ligands[44] may play a critical role in forming the ubisemiquinone $Q\cdot_p$ at a site sheltered from reactivity to the heme of c_1. To prevent spurious reactivities of the strongly reducing $Q\cdot_p$, water and oxygen must be excluded from center P, which must, however, conduct protons from ubiquinol to the surface of the membrane. Identifying amino acids that form this proton conduction pathway on the iron-sulfur protein or cytochrome b is currently under investigation.[2]

C. Cytochrome c_1

Cytochrome c_1 is anchored to the P surface of the bc_1 complex by a hydrophobic helix at its C-terminus[64] and released as a soluble heme-protein by proteolysis.[77,118] The c-type heme is usually covalently attached through thio-ether linkages to two conserved cysteines,[148] although protozoan c_1s have only a single thio-ether linkage.[93] A histidine adjacent to a cysteine ligand in the sequence CxyCH and a methionine corresponding to Met225 in yeast c_1 are thought to form the fifth and sixth axial ligands to the heme (Figures 9 and 10). These assignments are consistent with site-directed mutagenesis of these conserved residues[55,96] and optical[152] and magnetic circular dichroism[114] properties of the proteins.

Cytochrome c_1 is the exit point for electrons from the bc_1 complex and is more accessible to proteases at the surface of the membrane[118] than the iron-sulfur protein (Figure 1). Mitochondrial bc_1 complexes contain numerous subunits of unknown function, in addition to the three redox proteins.[110,124] One of these supernumerary subunits, an acidic protein, resides on the surface of the bc_1 complex in association with cytochrome c_1 and shuts down electron transfer from c_1 to c in half of the dimeric bc_1 complex in response to high protonmotive force.[111]

Cytochrome f, the equivalent of cytochrome c_1 in the cytochrome bf complex, has now been crystallized. The crystal structure of the 252-residue extrinsic domain of the 285-residue cytochrome f polypeptide has been solved to 2.3 Å, and the polypeptide chain has been completely traced.[22] Cytochrome f is an approximately 75 × 30 × 20 Å elongate structure, which is unique in several aspects. Unlike the other c-type cytochromes, which are single domain, predominantly α-helical proteins, cytochrome f has two domains, and the predominant secondary structure is β sheet. The structure previously predicted for cytochrome c_1,[124] which included a high content of β-pleated sheet structure as shown in Figure 9, thus agrees with the crystal structure of cytochrome f.

Cytochrome c_1 is a diffusionally constrained, membrane protein and conducts electrons from a hydrophobic environment to a hydrophilic, soluble protein, cytochrome c. Electron transfer to and from the c_1 heme probably follows different routes, requiring an electronic circuit through the protein. Identifying the electron pathway within this integral membrane cytochrome will be a focus of forthcoming investigations.

VI. QUESTIONS RELATED TO THE STRUCTURAL BASIS OF THE Q CYCLE

A. Functional Role of the Dimeric Cytochrome bc_1 Complex

Analytical ultracentrifugation,[137] gel filtration chromatography,[97] neutron scattering,[101] and high-resolution electron microscopy of membrane

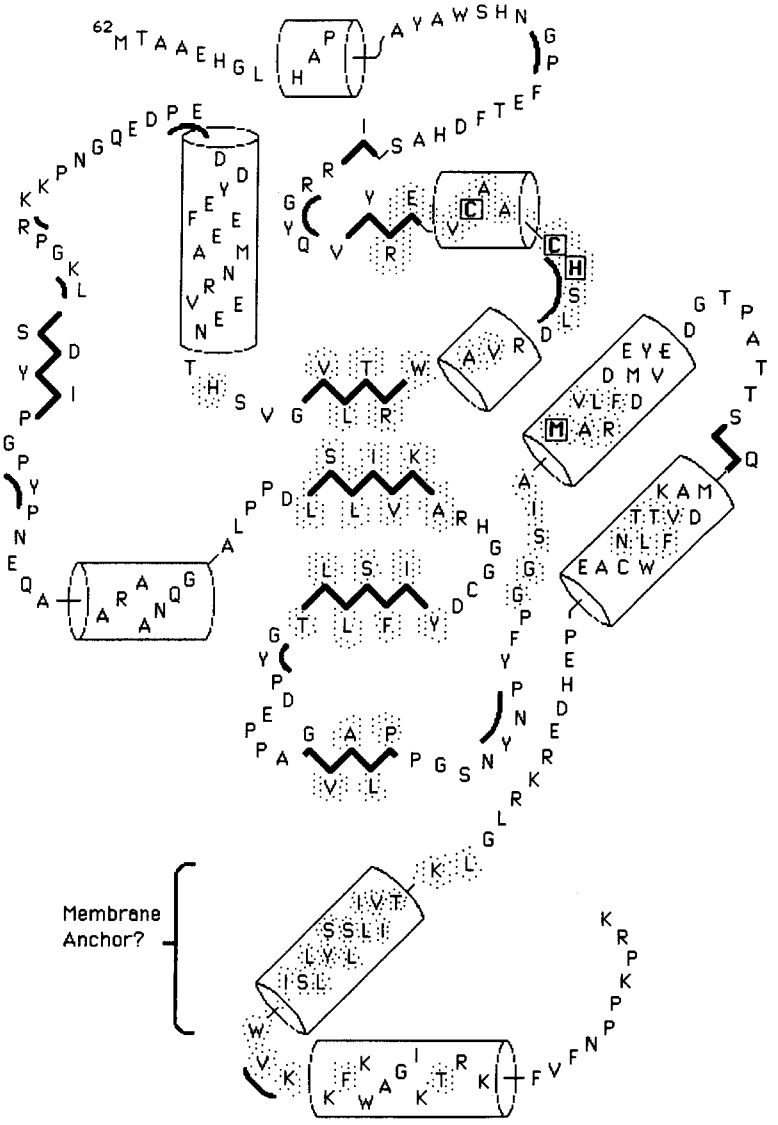

FIGURE 9. Predicted structure of cytochrome c_1 from *S. cerevisiae*. Methionine-62 is the N-terminus of the mature protein. The figure shows predicted regions of α-helix, ß-pleated sheet and ß-turns. Amino acids in regions predicted as hydrophobic are enclosed by a stipled area. The two cysteines to which the heme is covalently attached and the conserved histidine and methionine, which are the fifth and sixth axial ligands to the heme, are shown in bold type, enclosed in boxes. The postulated C-terminal hydrophobic membrane anchor is depicted as an α-helix, presumably capable of spanning the membrane, although the predicted secondary structure of this region includes a ß-pleated sheet.

crystals[74] indicate that the cytochrome bc_1 complex isolated from eukaryotic cells is a structural dimer with a twofold axis of symmetry. The existence of such a dimer has been offered as an explanation for some of the redox and spectroscopic properties of the mitochondrial complex.[30] In addition, as discussed below, the dimeric structure is essential for a regulatory mechanism that appears to operate in the mitochondrial cytochrome bc_1 complexes.[111]

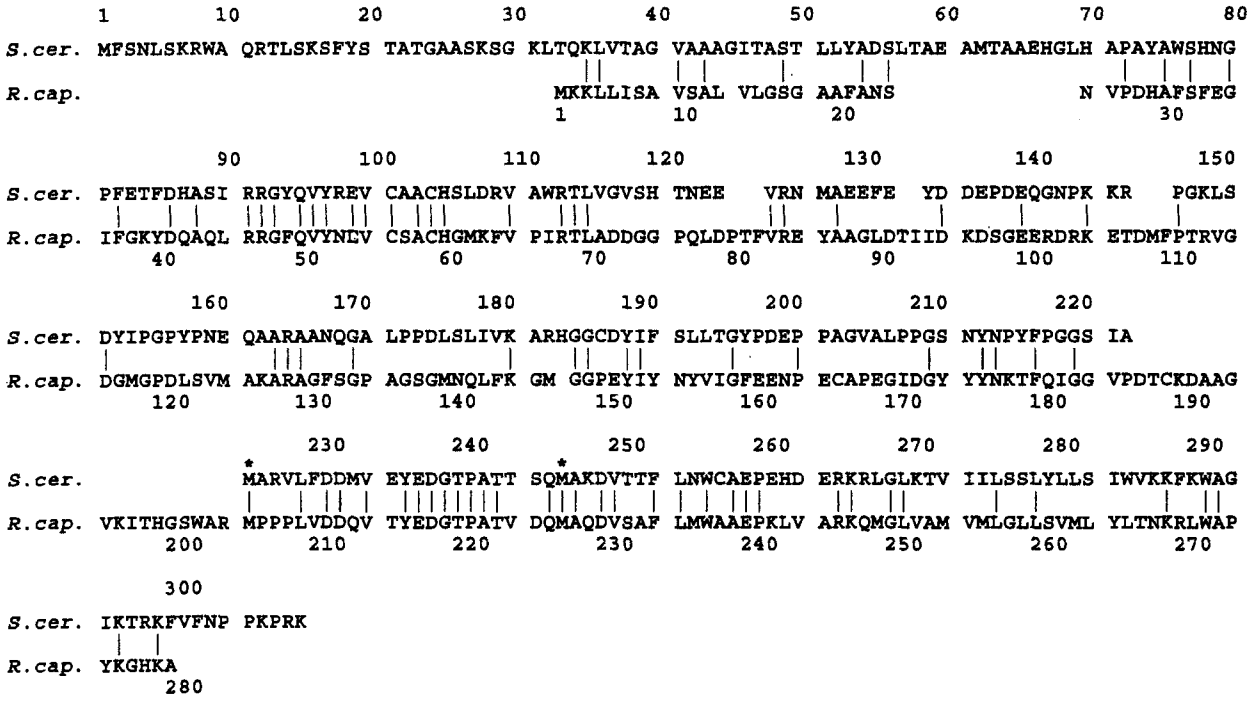

FIGURE 10. Alignment of cytochrome c_1 sequences from *S. cerevisiae* and *Rh. capsulatus*. The sequences were aligned with the GAP algorithm of the GCG software package and optimized manually. The asterisks (*) designate amino acids where mutations have been identified.

Although there is agreement that the cytochrome bc_1 complex of mitochondria is a structural dimer, the question of whether the two monomers cooperate catalytically via a "dimeric Q cycle" is still controversial. Early proposals for a dimeric Q cycle were deduced from the observation that one antimycin per bc_1 complex dimer seemingly was sufficient to inhibit both monomers.[80] However, binding studies[14] and kinetic studies[91] later showed that there is one inhibitor binding site per monomer, both for antimycin at center N and myxothiazol at center P, and that two inhibitor molecules per dimer are required for full inhibition.

The concept of a dimeric Q cycle was revived recently on the basis of triple inhibitor titrations.[98] These experiments were interpreted as indicating that limited treatment with DCCD leads to selective blockage of one antimycin binding site per dimer without affecting catalytic activity. An alternative to this interpretation was offered in a study of the steady state kinetics of the cytochrome bc_1 complex showing that the hyperbolic characteristics of inhibitor titration curves can be described quantitatively by assuming that even an inhibitor with a very low apparent K_d-like antimycin can exchange rapidly between the two halves of a dimer.[5]

The fact that the exchange of inhibitors between two monomers at center N is much faster than at center P fits a model in which the two centers N of a dimer are close to each other. Proximity of two centers N within a dimer is also consistent with the antimycin-sensitive quinol/quinone transhydrogenase activity of center N.[155] We conclude that the evidence supports a model of two monomers operating independently by a Q cycle mechanism as shown in Figure 2 within a structurally dimeric bc_1 complex in mitochondria, and that the centers N of the dimer are sufficiently close to each other to allow rapid exchange of inhibitor and substrate molecules between the two monomers at this center.

The dimeric structure of the mitochondrial bc_1 complex allows this enzyme to manifest half-

of-the-sites reactivity for cytochrome c reduction and for this activity to be regulated by subunit 6, one of the supernumerary subunits found in mitochondrial, but not in bacterial, cytochrome bc_1 complexes. The kinetic properties of yeast mitochondrial cytochrome bc_1 complex in which subunit 6 is deleted indicate that dissociation of subunit 6 reversibly lowers the activity of the dimeric bc_1 complex by half.[111] This finding suggests a mechanism for regulation of the linkage between ATP levels and the rate of respiration in mitochondria in which the negatively charged subunit 6 senses the positive charge at the P surface of the bc_1 complex and dissociates from the complex or undergoes a structural rearrangement at high $\Delta\mu H$, thus lowering the rate of respiration when the ATP level is high.[111]

Although there is extensive evidence that the mitochondrial bc_1 complexes are structurally dimeric[74,97,101,137] comparable data are not available for the bacterial complexes. In addition, bacterial bc_1 complexes lack an equivalent of the mitochondrial subunit 6. It is thus possible that the dimeric structure and attendant half-of-the-sites reactivity for cytochrome c reduction are properties of the mitochondrial and not of the bacterial bc_1 complexes. One exception to this generality may be the bc_1 complex of *Paracoccus denitrificans* in which a uniquely acidic amino terminal extension on cytochrome c_1 may serve the same function as the acidic subunit 6 of the mitochondrial complexes.[71]

B. Protonation and Deprotonation Pathways at Centers N and P

While the path of electrons through the cytochrome bc_1 complex by a Q cycle mechanism is understood,[2,5,14,25,44,87,125] little is understood on how protons move from the bulk phases to the sites at centers N and P, where protonation and deprotonation events occur. Protonable groups are expected to be an intrinsic part of the redox reactions, providing charge compensation and forming channels for the movement of protons. The nature and position of these groups determine how the generation of the chemiosmotic potential $\Delta\mu H$ is divided into electrogenic and protogenic events. In addition, fine tuning of the proton pathways could adapt the Q cycle to variations in bulk phase pH values and buffer capacities found in different species.

The distance between the two heme groups of cytochrome b is approximately 20 Å.[109,141] As this is only about 40% of the thickness of a phospholipid bilayer, and as the ubiquinone and ubiquinol binding sites have to be close to the hemes to ensure efficient electron transfer,[86] at least one of the two ubiquinol/ubiquinone redox centers has to be connected to the bulk phase by a proton channel of considerable length. Topology studies of both the mitochondrial[99] and the bacterial[84] cytochrome bc_1 complexes showed that heme b_H is located near the middle of the membrane and that heme b_L is closer to the positive surface of the membrane. This requires that protons have to travel 15 to 20 Å to center N through a channel (see Figure 1) that is sufficiently hydrophilic to allow membrane-impermeable redox agents to reach heme b_H.[70] Such a channel might consist of a hydrogen bond relay through one or more water molecules bound in a crevice between the cytochrome b helices, or may consist of one or more protonic amino acids that form a conductance path between heme b_H and the membrane surface. Based on the same evidence and the fact that inhibitor-resistant mutants put the end of helix C and the extramembranous helix cd into a common volume element (see above), center P is only a few Ångströms from the positive side of the membrane.

By examining the sequences of approximately 900 cytochrome b's,[35] it appears there are 14 candidate amino acids, including the heme-ligating histidines, for protonic residues that might be involved in forming proton channels. These residues are listed in Table 4. In 6 of these 14 positions exchanges with nonprotonic residues were reported. However, except for Ser140, the non-protonic residues were only reported for a single species (see Table 2 in Reference 35) and include two of the heme-ligating histidines, which raises the possibility that the nonconservation of protonic residues in these exceptions may be due to sequencing errors. Even if the exchanges are correct, another protonic residue located in the channel could assume the function of the mutated residue in these species.

TABLE 3
Mutations in The Rieske Iron-Sulfur Protein Affecting Respiration in Yeast or Growth of *Rhodobacter* Under Photosynthetic Conditions

Mutation			
Saccharomyces	***Rhodobacter***	**Phenotype**	**Ref.**
T851	S41	ts. UHDBT^{hs}	6, 81
I101T	V57	ts	52
P102L	E58	ts	6
P115L,S	P71	ts	52
E125G	D81	ts	52
L137S	L93	ts	52
D139G	D95	ts	52
D143N,G	E99	ts	52
D149G	G105	ts	52
W152R	W126	Petite	52
G157S	G131	ts	6
C159S,A	C133R,S,G	Petite/Ps–	28, 52, 53,131
T160A	T134	ts	52
H161R,C	H135P,L,C	Petite/Ps–	28, 52, 63,131
G163	G137D	Ps+	131
C164S,A	C138R,S,F	Petite/Ps–	28, 52, 53,131
V165A	V139	ts	52
P166S	P140	ts	52
A170T	S145	ts	52
G175S	G150	ts	6
G175D	G150	Petite	52
C178S,A,R	C153R,S,Y	Petite/Ps–	28, 52, 53,
131			
C180S,A,R	C155D,G,S	Petite/Ps–	28, 52, 53,
131			
H181C,R	H156L,P,F,T,Y	Petite/Ps–	28, 52, 53
G182D,S	G157	ts	52
H184R	H159A,S	Normal	28, 53
Y185C	Y160	Petite	52
S188F	A163	ts	52
S188P	A163	Petite	52
R190G	R165	Petite	52
P195L,S	P170	ts	52
A196T	A171	ts	52
A196V	A171	Petite	52
N199S	N174	ts	52
N199K	N174	Petite	52
P203S	P178	ts	6

Note: Equivalent or homologous amino acids are identified by number in the two species. Abbreviations used for the phenotypes are Ps$^-$, no photosynthetic growth *(Rh. capsulatus);* ts, temperature sensitive, UHDBT^{hs}, 2-undecyl-5-hydroxybenzoxythiazol hypersensitive.

For the same reasons, the number of residues potentially involved in conducting protons to and from the reaction centers of the cytochrome bc_1 complex is probably not limited to those listed in Table 4 (see also Section C).

C. Role of Proton Sinks and Proton Traps In the Electrogenic Reactions

The predicted locations of protein domains and of the b hemes relative to the membrane

TABLE 4
Conserved Protonic Amino Acids in *S. cerevisiae* Cytochrome b

Amino acid	Helix/loop location	Topographical location	Exchanges
Arg-79	B	Membrane/N side	His, **Phe**
His-82	B	Heme b_L ligand	**Gln**
Ser-87	B	Membrane center	Asp, Thr
His-96	B	Heme b_H ligand	—
Lys-99	B	N side	Arg
Ser-140	c d	P side	**Gly**
Thr-145	c d	P side	Lys, **Val**
Thr-175	c d	P side	**Met**
His-183	D	Heme b_L ligand	—
His-197	D	Heme b_H ligand	Asp
His-202	D	Membrane/N side	Arg
Asp-229	E	Membrane/N side	Glu
Glu-272	e f	P side	His
Lys-288	F	Membrane/P side	His, **Phe**

Note: The table lists protonic amino acids that are highly conserved among approximately 900 cytochrome b's that have been sequenced to date,[35] and indicate their location in one of the helices, or in the connecting span between two helices. Reported amino acid exchanges, which retain a protonic residue in most cases, are also listed. Except for Ser-140, which is Gly in chloroplast cytochrome b_6, all exchanges to nonprotonic residues (boldface) were found only in a single species.

surfaces allow chemiosmotic principles[86] to be applied to explain the electrogenic properties of the bc_1 complex in molecular terms. By this approach, it may also be possible to reconcile reported differences in the electrogenic steps of the Q cycle between mitochondrial and bacterial cytochrome bc_1 complexes and an apparent discrepancy between the predicted structural and electrical location of the cytochrome b hemes.

The protonmotive force ($\Delta\mu H$) across a biological membrane is composed of a difference in electrical ($\Delta\Psi$) and protonic (ΔpH) potential that are related by the equation

$$\Delta\mu H = \Delta\Psi - 59\Delta pH$$

The cytochrome bc_1 complex generates protonmotive force by translocating protons, and thus initially establishes solely a protonic potential. Depending on the buffering capacity of the two compartments that are separated by the energized membrane, protons are bound on the positive side of the membrane or regenerated on the negative side by protonable solutes, phospholipids, and amino acids on proteins. This buffering converts most of the protonic potential into an electrical potential.

While the interconversion between protonic and electrical potentials occurs readily, they represent two forms of chemiosmotic energy. The protonic potential is a function of the concentration difference for protons between the bulk phases of the two compartments, and it is independent of the thickness and properties of that part of the membrane/protein barrier separating the two phases that are not accessible to protons. The electrical potential, on the other hand, is purely a charge difference between the two phases and drops across the membrane, depending on the thickness and properties of the membrane dielectric, which behaves like a capacitor in this respect. It follows from this distinction that proton channels that extend the bulk phase into the membrane dielectric or fixed charges and protonable groups that are located on the membrane/protein surface can change the local distribution between protonic and electrical potential.

Figure 11a illustrates how a proton-conducting channel that crosses part of the membrane dielectric from the negative (N) side would form a proton sink, which is the complement to the initially described proton well[90] formed by a proton-conducting crevice into the membrane from the positive (P) side. The electrical potential increase ($\Delta\Psi$) along the proton sink is compensated by an equivalent decrease of the protonic potential $-59\Delta pH$ (arrows in Figure 11a), keeping the protonmotive force constant. In effect, the pH at the bottom of the proton sink is predicted to be higher than in the bulk phase on the negative side. As a result the uptake of protons along the proton channel is electrogenic rather than protogenic.

As shown in Figure 11b, the potential profile across the energized membrane can be modified by so-called proton traps, a concept also introduced by Peter Mitchell.[90] A proton trap is established by protonable groups with a pK_A near the pH of the respective bulk phase (X-H and Y-H in Figure 11b) and results in a localized increase of the buffering capacity. Because this means a decrease of the protonic potential, the electrical component of the protonmotive force shows a local compensatory increase. Consequently, the pH gradient into the proton sink becomes more shallow, rendering the uptake of protons along the proton channel less electrogenic and more protogenic. A proton trap on the P side would

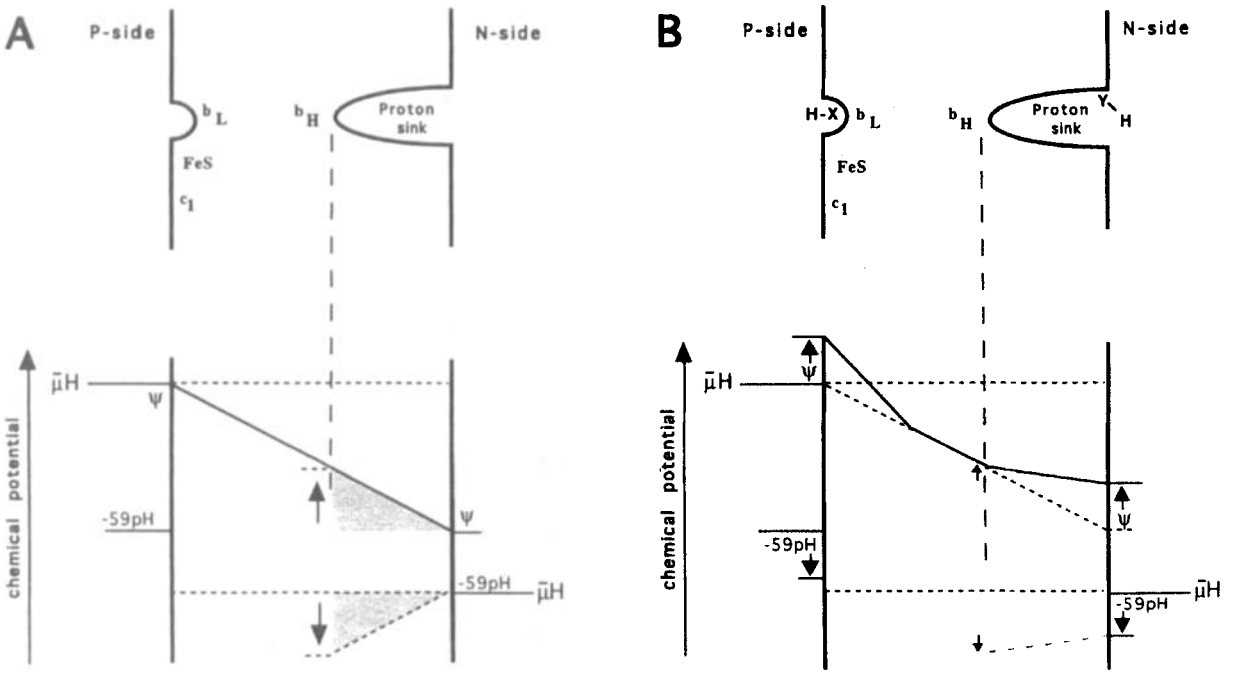

FIGURE 11. Proton sink and proton traps in the cytochrome bc_1 complex. Illustration of the effects of a proton sink **(A)** and a proton sink with proton traps on both sides of the membrane **(B)** on the energy profile across the cytochrome bc_1 complex. The top of the figure shows the arrangement of the redox centers of the cytochrome bc_1 complex and the proton channels necessary for proton release and uptake on the P and N sides, respectively. The bottom gives diagrams of the energy profiles across the membrane. The scale for the chemical potential is arbitrary, as is the relative contribution of the electric and protonic components, Ψ and –59 pH, respectively, to the total protonmotive force $\Delta\mu H$. b_L, heme b_L; b_H, heme b_H; FeS, iron sulfur center; c_1, heme c_1. **(A)** Proton sink. The arrows indicate the direction of change of the electrical and protonic components at the bottom of the proton sink marked by a vertical dashed line relative to the surface of the membrane. The part of the profile leading into the proton sink is highlighted by the shadowed area. **(B)** Proton sink and proton traps. In addition to the proton sink illustrated as **A**, changes of the electric and protonic portions of the protonmotive force by proton traps on the P-side (H-X) and N-side (Y-H) are indicated by arrows outside of the membrane. The profile for the electrical portion undistorted by the proton traps is indicated by a hatched diagonal.

have the opposite effect and result in a local increase of the electrogenic gradient at the expense of the protonic potential.

Figure 11 is a simplified view of how proton sinks and proton traps could influence the energetics of proton uptake and release in the cytochrome bc_1 complex. No attempts were made to take into account inhomogeneities of the membrane dielectric and surface potentials. Despite these simplifications, one can see how the application of these concepts, which are derived from the basic principles of chemiosmosis, can change the electrogenic profile of the Q cycle. This follows from a mere consideration of the location of the protonation and deprotonation sites and the buffering capacity of some residues at a given $\Delta\mu H$ and bulk pH; no detailed knowledge of the individual steps of proton conduction is necessary for, or can be deduced from, the concepts of a proton sink and a proton trap. However, interpretations of experimental results that address mechanisms of proton conduction require an exact analysis of the electrogenic profile.

A reported discrepancy between the structural[99] and electrical[65] topology of mitochondrial cytochrome bc_1 complexes might be explained by the effects of proton traps. Distance measurements with paramagnetic probes[8] indicate that heme b_L is structurally much closer to the positive surface of the membrane than heme b_H, the latter being near the middle of the bilayer.[99] On the other hand, heme b_L seems to be electrically localized near the middle and heme b_H near the negative surface of the membrane.[65]

From Figure 11b, it can be seen how proton traps at the entrances of the proton channels on both sides of the membrane could allow the electrical distance between the negative surface and center N to appear to be much shorter and make the electrical gap between the positive surface and center P appear to be much wider. Remarkably, in *Rhodobacter* the discrepancy between structural and electrical topology is not observed,[49,106] and a comparison to the mitochondrial cytochrome b sequences (Figure 5) reveals that several protonable groups found in mitochondria are not conserved in *Rhodobacter*. It can be speculated, for example, that Asp160 (yeast) or Glu163 (bovine) at center P and Arg218 (yeast) or Lys218 (bovine) at center N could function as proton traps, if their effective pK_A could be brought into the range of the bulk phase pH by the protein environment and membrane potential.

The effects of proton traps on the electrogenic profile not only can be applied to resolve the discrepancy between the structural and electrical topology of the mitochondrial cytochrome bc_1 complexes,[103] but can also offer an explanation for the differences ascribed to the bacterial system without transmembrane control of proton release as postulated by Konstantinov.[65] Moreover, the reported dependence of the electrogenic reactions on $\Delta\mu H$ and the bulk phase pH[94,95] is a direct implication of the proton trap concept.

D. Decoupling of the Protonmotive Q Cycle

One attractive feature of the protonmotive Q cycle is that the vectorial release of a second proton on the positive side of the membrane can be explained solely on the basis of the bifurcation of electron flow at center P, ensuring the return of the second electron generated on oxidation of ubiquinol to the negative side via the b hemes. While no experimental conditions have been found that decrease the H^+/e^- ratio at center P using native preparations of the cytochrome bc_1 complex, it has been shown that modification of Asp160 in the yeast cytochrome bc_1 complex with DCCD has just that effect, without drastically slowing the electron transfer activity of the complex.[4,19]

One way to mechanistically decouple the Q cycle is if both electrons from ubiquinol sequentially reduce the iron-sulfur protein. This has been proposed to be the cause of the DCCD induced decoupling.[2] However, this mechanism would require that the two electron oxidation of ubiquinol would not lead to a concerted reduction of heme b_L and the iron-sulfur cluster, which is still a matter of debate, or that the DCCD modification would specifically disable a switching mechanism proposed to guarantee the bifurcation of electron flow in the native enzyme,[14] or that transiently reduced heme b_L would reduce the iron-sulfur center at a high rate in the DCCD-modified enzyme.

Another way to explain a DCCD-induced decrease in the observed H^+/e^- ratio is depicted in Figure 12. This model assumes that Asp160, the residue that is modified by DCCD in the yeast cytochrome bc_1 complex, is part of a proton channel that carries the second proton from ubiquinol

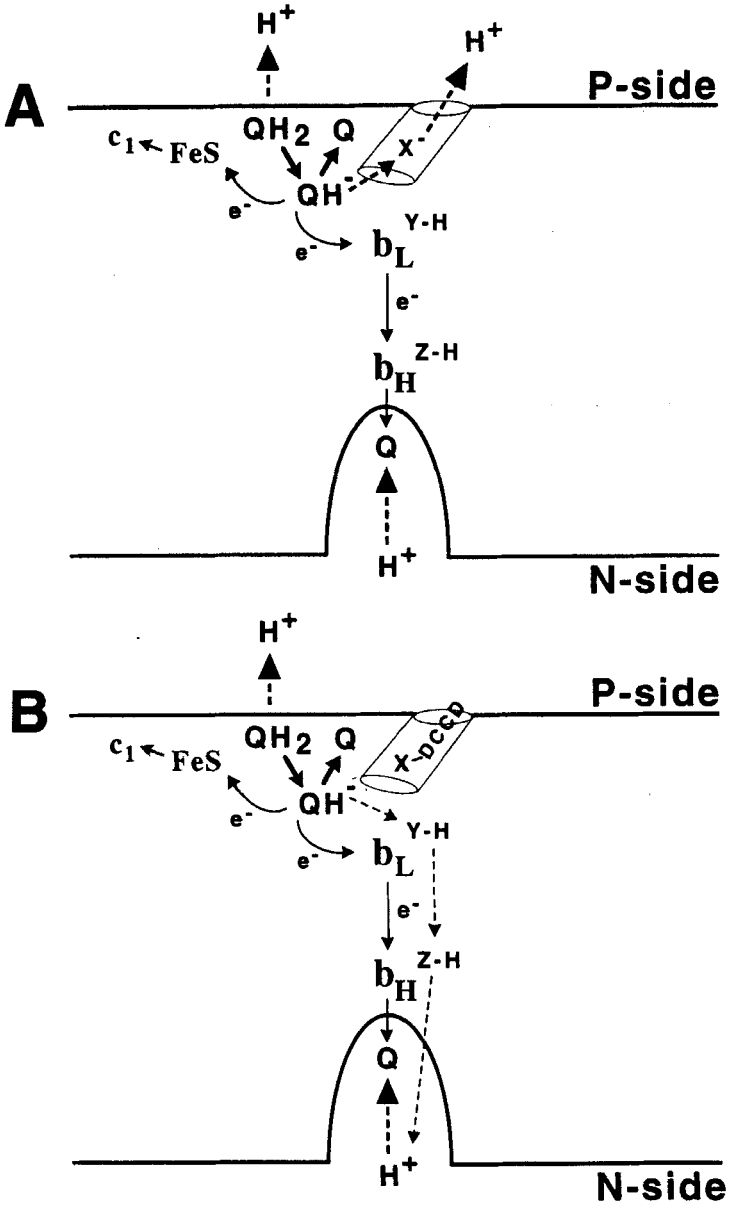

FIGURE 12. Decoupling of the protonmotive Q-cycle by DCCD. Blockage of a residue (X^-) in the proton channel conducting the second proton generated on oxidation of ubiquinol at center P by DCCD forces the proton along an artificial pathway leading to a proton channel at center N, which might involve protonable residues associated with heme b_L (Y-H) and heme b_H (Z-H). Solid arrows indicate electron transfers, hatched arrows indicate proton movements, and bold solid arrows indicate conversion of ubiquinol to ubiquinone. No details of the reactions at center N are given (see Figure 2). b_L, heme b_L; b_H, heme b_H; FeS, iron sulfur center; c_1, heme c_1; Q, ubiquinone. **(A)** Native enzyme. **(B)** DCCD-modified enzyme.

oxidation to the bulk phase (Figure 12a). If such a channel would be blocked by covalent modification, the proton might encounter an alternate path leading to the negative side of the membrane (Figure 12b), thus decoupling the protonmotive activity of the Q cycle from the electron transfer activity. The pH dependence of the midpoint potentials of heme b_L and b_H[70,78,103,130,145] suggests that protonable groups are associated directly with these redox centers. These groups (Y-H and Z-H in Figure 12) could provide an alternate proton path across the membrane that might be unmasked by a change in the cytochrome b structure.

Variations on the model in Figure 12 may also explain how certain point mutations in cytochrome b lower the efficiency of energy transduction in the cytochrome bc_1 complex while retaining relatively high rates of electron transfer.[73,120] This effect has now been observed with two mutations at center P, conversion of Gly137 to Val or Glu and conversion of Cys133 to Tyr, and a third mutation at center N, in which Ser206 is changed to Leu. It is conceivable that these mutants are decoupled due to unmasking of a low-resistance proton pathway into or out of center P or center N.

E. Properties of Bound Ubiquinol, Ubiquinone, and Ubisemiquinone

The number of ubiquinol and ubiquinone binding sites in the bc_1 complex cannot be measured directly because of the low affinity of the sites for the hydrophobic ligands, which partition between the membrane in which they are dissolved and the binding sites.[138] The Q cycle requires two catalytic sites and thus implies that there are two corresponding binding sites, one at center N and a second at center P. If the center N and center P sites arise from a single common binding domain accessible to the two sides of the complex,[104,144] the ubisemiquinone anions at center N and center P must be insulated from reacting at the opposite center, otherwise their transmembranous migration would decouple the vectorial proton transfer.

It is generally accepted that center N has a quinone binding site that is capable of stabilizing a semiquinone anion.[33,85,100] This site binds quinol and quinone with approximately equal affinity in the mitochondrial bc_1 complex and binds quinol 10- to 100-fold more tightly than quinone in the *Rh. sphaeroides* complex. The site also binds ubisemiquinone anion with an affinity comparable to that of the quinone and quinol, such that the semiquinone is present in amounts detectable by EPR spectroscopy at room temperature. The concentration of EPR-detectable semiquinone, $Q\cdot_n$, approaches 0.1 to 0.5 per complex, reflecting a stability constant between 2×10^{-3} and 2×10^{-1}, depending on what portion of the quinone pool is in equilibrium with the binding site.[85,100]

Evidence that the center N quinone binding site is near heme b_H comes from the observation that antimycin, which binds near heme b_H, eliminates the EPR signal arising from the EPR detectable, stable $Q\cdot_n$.[33,85,100] Further evidence that the semiquinone ring must be within a few Ångströms of the heme came from reductive titrations of purified bc_1 complex, in which it was observed that a subpopulation of the semiquinone and oxidized heme b_H are diamagnetically coupled such that both are EPR silent.[29,113] An additional implication of these findings is that there are two populations of stably bound semiquinone at center N, one that is paramagnetic and a second that is diamagnetic due to exchange coupling to oxidized heme b_H.

The EPR-detectable and EPR-silent semiquinones may represent a single bound semiquinone anion, equilibrating between a position proximal to heme b_H and a position electronically isolated from the heme. However, it is difficult to envision the structural basis for such an equilibrating mixture. An alternative explanation is that this behavior arises from a semiquinone pair, one of which is coupled to heme b_H. This seems unlikely because the EPR silent semiquinone amounts to only 20 not 50% of the total.[29,113] Furthermore, it would be difficult to explain why the two semiquinones in a pair would not spin couple to each other, as has been observed for the semiquinone pair in succinate-ubiquinone reductase,[100] but that has not been observed for semiquinone in the bc_1 complex.

A third possibility, based on a model suggested by Rich and co-workers,[103] is that a single semiquinone is bound proximal to heme b_H, and that the pK_a of a protonable group gives rise to a mixture of $[b_H][Q\cdot^-]$ and $[b_H][Q\cdot^-][H^+]$, in which

only the protonated form is diamagnetically coupled. Obviously, the semiquinone oxygen is the most likely candidate for the protonable group, and it is conceivable that protonation changes the conformation of the semiquinone ring such that the π orbitals are available for coupling to ferroheme b_H.

It is also generally agreed that center P has a second quinone binding site at which ubiquinol is oxidized and where the intermediate ubisemiquinone anion is not stabilized to the extent as at center N. By observing the quinone-induced changes of the EPR lineshape of the iron-sulfur cluster as a molar excess of ubiquinone is extracted in stepwise increments from the membranous bc_1 complex, it has been postulated that two quinones are bound at center P.[44] Each of the double-occupancy sites binds ubiquinone and ubiquinol with approximately equal affinity, but the two sites differ in that one has low affinity whereas the other has high affinity for both quinone and quinol. From this double-occupancy model, which has been compared to the situation in the bacterial reaction center,[36] a Q cycle accommodating two quinone molecules at center P has been proposed.

The double-occupancy model proposes that each of the two quinones are proximal to one of the two histidine ligands to the iron-sulfur cluster. If the two quinones are in electronic communication with the cluster, they would form an electron conduit into and across the redox center. Such an arrangement could have the effect of sequestering the strongly reducing ubisemiquinone anion from the quinone pool and protect against dismutation at center P. Structural segregation of the semiquinone anion may also prevent it from reducing cytochrome c_1, while allowing reduction of the c_1 heme via the opposite edge of the iron-sulfur cluster.

Another consequence of a double-occupancy model is that the two quinones may be sufficiently close to form a mixed redox status quinone pair to provide a chemical basis for the obligatory linkage of the divergent electron transfer at center P. For example, although a quinhydrone formed from ubiquinol plus ubiquinone would be expected to be stable and optically detectable, one can envision variations on such a structure, possibly involving ubiquinol and ubisemiquinone anion, in which removal of two electrons is obligatorily linked. It may be useful to search for possible inhibitors that mimic such structures.

A second quinone binding site could also provide an explanation for the noncompetitive inhibition of center P by the E-ß-methoxyacrylate inhibitors.[13] One can envision that these ligands may mimic one molecule of an obligate quinone pair, while the second quinone reacts with the quinone pool and is noncompetitive with the inhibitor. Such an explanation would not preclude the possibility that some center P inhibitors may block electron transfer from the iron-sulfur cluster to c_1 by virtue of their effects on the redox potential of the cluster.[136]

F. Obligatory Bifurcation of Electron Flow at Center P

The protonmotive stoichiometry of the Q cycle relies on the bifurcation of electron flow at center P. By reducing heme b_L, the second electron generated from oxidation of ubiquinol is transferred back to center N at the negative side of the membrane. It is remarkable that, except for the DCCD-treated enzyme, no experimental conditions could be found that override this "specific next" character of the ubiquinol oxidation at center P. It is not obvious why ubiquinol does not transfer both electrons to the iron-sulfur center when both b hemes are locked in the reduced form by antimycin. In other words, if semiquinone is generated on reduction of the iron-sulfur cluster by ubiquinol, the question arises why the semiquinone does not rereduce the iron-sulfur cluster after its rapid reoxidation by heme c_1, especially in the instance where heme b_L is locked in the reduced form by the antimycin block at center N.

One possible explanation is that ubiquinol oxidation at center P is an obligatorily concerted reaction because of the very negative midpoint potential of the semiquinone generated by the first oxidation step. However, no data are available that demonstrate the concerted nature of the reaction. On the other hand, a significant amount of ubisemiquinone is formed in the antimycin-inhibited complex,[67,128] leading to a slow leak

through the antimycin block. This indicates that formation of semiquinone at center P is not a thermodynamically prohibited reaction, even when it is not linked to a subsequent oxidation. Remarkably, the semiquinone still does not reduce the iron-sulfur protein but leaves center P to reduce molecular oxygen, forming superoxide.

While it is clear that the instability of the semiquinone demands that the reduction of cytochrome b has to occur immediately after reduction of the iron sulfur cluster, there is still the requirement for a mechanism that ensures the bifurcation of electron flow at center P. One such possible mechanism, based on a mixed redox quinone pair, was mentioned above. Another possibility is that there is a redox-dependent structural switch built into center P. Based on changes of the affinity of E-ß-methoxyacrylate inhibitors linked to redox changes of the prosthetic groups in the absence of ubiquinone, which indicate a redox-linked conformational change at center P, a catalytic switch mechanism was proposed that explains the obligatory "specific next" at center P and antimycin inhibition.[12,14] The essential feature of any such mechanism is that the oxidized iron-sulfur cluster must be structurally blocked from oxidizing ubiquinol when heme b_L is reduced.

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