

Evidence for a Concerted Mechanism of Ubiquinol Oxidation by the Cytochrome bc_1 Complex*

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To better understand the mechanism of divergent electron transfer from ubiquinol to the iron-sulfur protein and cytochrome b_L within the cytochrome bc_1 complex, we have examined the effects of antimycin on the presteady state reduction kinetics of the bc_1 complex in the presence or absence of endogenous ubiquinone. When ubiquinone is present, antimycin slows the rate of cytochrome c_1 reduction by ~ 10 -fold but had no effect upon the rate of cytochrome c_1 reduction in bc_1 complex lacking endogenous ubiquinone. In the absence of endogenous ubiquinone cytochrome c_1 , reduction was slower than when ubiquinone was present and was similar to that in the presence of ubiquinone plus antimycin. These results indicate that the low potential redox components, cytochrome b_H and b_L , exert negative control on the rate of reduction of cytochrome c_1 and the Rieske iron-sulfur protein at center P. If electrons cannot equilibrate from cytochrome b_H and b_L to ubiquinone, partial reduction of the low potential components slows reduction of the high potential components. We also examined the effects of decreasing the midpoint potential of the iron-sulfur protein on the rates of cytochrome b reduction. As the midpoint potential decreased, there was a parallel decrease in the rate of b reduction, demonstrating that the rate of b reduction is dependent upon the rate of ubiquinol oxidation by the iron-sulfur protein. Together these results indicate that ubiquinol oxidation is a concerted reaction in which both the low potential and high potential redox components control ubiquinol oxidation at center P, consistent with the protonmotive Q cycle mechanism.

Although the protonmotive Q^1 cycle mechanism of the cytochrome bc_1 complex is generally understood (1–3), the mechanism of ubiquinol oxidation at center P has not been fully elucidated. With the determination of the crystal structure of the cytochrome bc_1 complex (4–5), a more extensive examination of the structure-function relationships of the Q cycle mechanism is possible.

It is generally accepted that the mechanism of ubiquinol oxidation at center P involves a divergent oxidation in which the iron-sulfur protein oxidizes ubiquinol to semiquinone and the semiquinone reduces cytochrome b_L (1, 3). It is unclear,

however, whether the oxidation of ubiquinol occurs through semiquinone in a sequential mechanism or whether ubiquinol is oxidized by the iron-sulfur protein and cytochrome b_L in a concerted reaction. Earlier experiments suggested the presence of a transient semiquinone at center P (6), consistent with a sequential mechanism, although recent experiments suggest otherwise (7).

There have been two proposals for concerted reaction mechanisms at center P. Link (8) proposed a “proton-gated affinity change” mechanism in which stabilization of ubisemiquinone by anti-ferromagnetic coupling to the reduced iron-sulfur protein raises the potential of the iron-sulfur cluster such that the cluster cannot be oxidized by cytochrome c_1 until the semiquinone is oxidized. Jünemann *et al.* (7) suggested that the potential of the ubiquinol/ubisemiquinone couple at center P is more positive than that of the iron-sulfur cluster and that oxidation of the semiquinone is required to lower the potential of the couple. In both of these mechanisms ubisemiquinone must be oxidized to allow a thermodynamically linked reaction to occur. In one case this is required in order to allow reduction of cytochrome c_1 by the Rieske cluster (8), and in the other, to allow reduction of the Rieske cluster by the ubiquinol/ubisemiquinone couple (7).

The crystal structures of the cytochrome bc_1 complex suggest that movement of the iron-sulfur protein is necessary for divergent electron transfer to cytochrome c_1 and cytochrome b (5), and recent experiments have confirmed that mobility of the extra-membrane domain of the iron-sulfur protein is essential for function (9). Concerted electron transfer mechanisms can accommodate iron-sulfur protein movement either for thermodynamic reasons or if movement of the Rieske center makes the first electron transfer dependent upon the second. Similarly, mechanisms that propose occupancy of two ubiquinol molecules at center P (10, 11) can be concerted.

We have examined the effects of antimycin and endogenous ubiquinone on the presteady state rates of cytochrome b and c_1 reduction. We also examined the effects of decreasing the midpoint potential of the iron-sulfur protein on the presteady state reduction kinetics of the bc_1 complex. Our results indicate that oxidation of ubiquinol at center P occurs through a concerted mechanism in which both the iron-sulfur protein and cytochrome b_L must be oxidized.

EXPERIMENTAL PROCEDURES

Materials—Dodecyl maltoside was obtained from Roche Molecular Biochemicals. DEAE-Biogel A was obtained from Bio-Rad. Antimycin, diisopropyl fluorophosphate, phenylmethylsulfonyl fluoride, and menaquinone were purchased from Sigma. Yeast extract and peptone were from Difco. The yeast $\Delta coq2$ mutant was obtained from Dr. Catherine Clarke (UCLA). This mutant lacks ubiquinone due to deletion of the gene for the polyprenyl transferase that transfers the isoprenoid side chain to *p*-hydroxybenzoate at an early step in the ubiquinone biosynthetic pathway (12). Although the $\Delta coq2$ mutant is petite, it assembles a cytochrome bc_1 complex that is active with added quinol substrates and exhibits normal sensitivity to both center P and center N inhibitors (13).

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¹ The abbreviations used are: Q, ubiquinone; ISP, iron-sulfur protein.

Purification of Cytochrome bc_1 Complex—The $\Delta coq2$ yeast mutant was grown in 80 liters of yeast extract/peptone/dextrose medium, and the mutants with iron-sulfur mutations Y185F, S183T, S183A, and S183A/Y185F (14) were grown in 80 liters of minimal medium plus dextrose and harvested by centrifugation. Cytochrome bc_1 complex was isolated as described previously (13).

Kinetic Measurements—Kinetic measurements were performed at room temperature by stopped-flow rapid-scanning spectroscopy using the OLIS Rapid-Scanning Monochromator (On-Line Instrument Systems, Inc., Bogart, GA) equipped with a 1200 lines/mm of grating blazed at 500 nm. This produced a spectrum of 75-nm width, centered at 555 nm, with a resolution of 0.4 nm. The dead time of the instrument was ~ 2 ms, and the end of this period was chosen as time zero. Data was collected at 1000 scans/s.

Reactions were started by mixing $2 \mu\text{M}$ bc_1 complex in 50 mM potassium phosphate, pH 6.0, containing 250 mM sucrose, 0.2 mM EDTA, and 1.0 mg/ml bovine serum albumin against an equal volume of buffer containing menaquinol. A fresh solution of menaquinol substrate was prepared from menaquinone before each kinetic experiment, as described previously (13). An oxidized spectrum was obtained by mixing the oxidized bc_1 complex against buffer and averaging the data set to a single scan. For each experiment three data sets were averaged, and the oxidized spectrum was subtracted from each scan. From the three-dimensional data set comprised of wavelength, absorbance, and time, we extracted the time course of cytochrome b and c_1 reduction at 563.3 and 554.6 nm, respectively, using software from OLIS.

The kinetic plots were analyzed by a “successive integration” method (15) incorporated into the OLIS software. This software establishes whether the reaction consists of one or more kinetic phases and assigns first order rate constants to the exponentials. Second order rate constants were then calculated from the slopes of the curves obtained by plotting the first order rate constants against menaquinol concentration using Fig. P, v 2.2a software from Biosoft, Ferguson, MO.

RESULTS

Effect of Endogenous Ubiquinone and Antimycin on the Rate of Cytochrome c_1 Reduction through Center P—In the protonmotive Q cycle mechanism, oxidation of ubiquinol at center P in the bc_1 complex delivers the two electrons divergently to two separate redox chains within the enzyme: a high potential chain that includes the Rieske iron-sulfur protein and cytochrome c_1 and a low potential chain that includes the di-heme cytochrome b_H and b_L (Fig. 1). Whether and, if so, how these two thermodynamically separate redox chains influence the activity of each other has not been extensively examined.

We recently showed that endogenous ubiquinone residing at center N rapidly reoxidizes cytochrome b_H (13), and it is generally accepted that this reaction is inhibited by antimycin (1–3). To test whether the low potential chain affects reduction of the high potential chain, we examined the effects of antimycin or ubiquinone residing at center N on reduction of cytochrome c_1 by menaquinol. In the absence of inhibitors and in the presence of endogenous ubiquinone, the reduction of cytochrome c_1 by $25 \mu\text{M}$ menaquinol is monophasic, with a rate of 7.9 s^{-1} (Fig. 2), whereas the reduction of cytochrome b is triphasic, as was previously observed (13). When antimycin was added, the rate of cytochrome c_1 reduction slowed 4-fold to 1.8 s^{-1} , and cytochrome b reduction became a biphasic reaction, with each phase comprising 50% of the total absorbance change and rates of 19 s^{-1} and 1.9 s^{-1} (Fig. 2). Thus, antimycin binding at center N slows the rate of cytochrome c_1 reduction through center P, and the rate matches the slow phase of the biphasic b reduction.

We also examined cytochrome b reduction in a $\Delta coq2$ mutant. This mutant is completely devoid of ubiquinone due to deletion of the gene for the enzyme that attaches the isoprenoid side chain (12). Reduction of cytochrome b in the bc_1 complex from the $\Delta coq2$ mutant is no longer triphasic (13). Rather, in the absence of endogenous ubiquinone b , reduction is rapid and monophasic (Fig. 2, lower left). With $25 \mu\text{M}$ menaquinol, cytochrome b reduction occurred at 47 s^{-1} . Cytochrome c_1 reduction

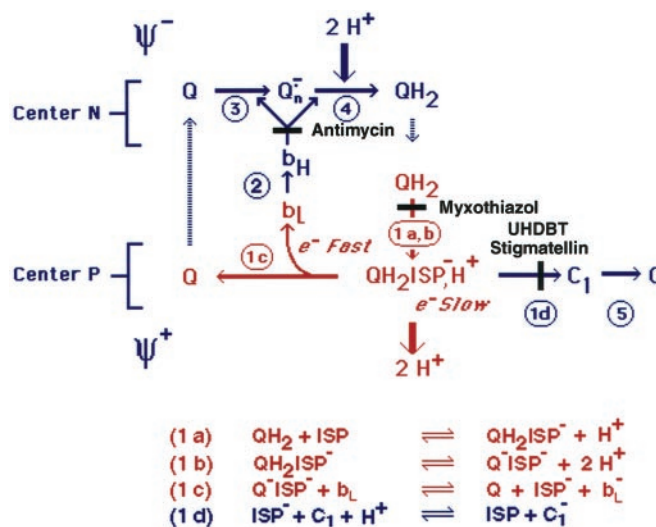


FIG. 1. Protonmotive Q cycle mechanism of electron transfer through the cytochrome bc_1 complex. The scheme shows the branched, cyclic pathway of electron transfer through the four redox centers of the cytochrome bc_1 complex. Dashed arrows represent movement of ubiquinol (QH_2) and ubiquinone (Q) between the site where ubiquinol is oxidized in a concerted reaction at center P on the positive side of the membrane and the site where ubiquinone and ubiquinol are sequentially reduced at center N on the negative side of the membrane. Movement of ubiquinol from center P of one monomer to center N of the second monomer through the two large cavities within the bc_1 dimer (4) would obviate exchange and diffusion through the bulk phase of the surrounding membrane lipid with each turnover of the enzyme but would not preclude such exchange on a somewhat slower time scale. The circled numbers designate electron transfer reactions. The divergent oxidation of ubiquinol at center P is represented as four steps, 1a–1d, described by the equations below the figure. Three of these, 1a–1c, occur in a concerted manner as discussed in the text and are shown in red in the figure. The solid rectangles show the sites at which antimycin, myxothiazol, UHDBT (5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole), and stigmatellin block binding of reactants or inhibit electron transfer reactions within the enzyme.

was considerably slower than the rate of cytochrome b reduction and occurred at 2.9 s^{-1} .

When antimycin was present, the reduction of cytochrome b in the absence of endogenous ubiquinone became biphasic, with each phase comprising 50% of the total absorbance change and rates of 18 s^{-1} , and 3.4 s^{-1} , respectively (Fig. 2, lower right). Antimycin changes the pattern of cytochrome b reduction in the bc_1 complex from the $\Delta coq2$ mutant from monophasic to biphasic with rates that are similar to those observed with wild-type complex in the presence of antimycin. This similarity in rates of b reduction is evident in the traces in the lower right and upper right panels of Fig. 2.

The biphasic reduction of cytochrome b in the bc_1 complex from the $\Delta coq2$ mutant in the presence of antimycin (Fig. 2, lower right) differs notably from that in the mutant in the absence of antimycin (Fig. 2, lower left), which is rapid and apparently monophasic. Also, the amount of b that is reduced in the bc_1 complex from the $\Delta coq2$ mutant is significantly greater than in the wild-type bc_1 complex (Fig. 2, upper left). The amount of b reduced in the wild-type enzyme in the presence of antimycin (Fig. 2, upper right) is similar to that reduced in the bc_1 complex from the $\Delta coq2$ mutant in the absence or presence of antimycin. The explanation for these differences in b reduction in the presence of antimycin and in the absence of endogenous ubiquinone is discussed below.

Cytochrome c_1 reduction in the bc_1 complex from the $\Delta coq2$ mutant was monophasic in the presence of antimycin and occurred at 3.1 s^{-1} (Fig. 2, lower right), compared with 2.9 s^{-1} in the bc_1 complex from the $\Delta coq2$ mutant in the absence of

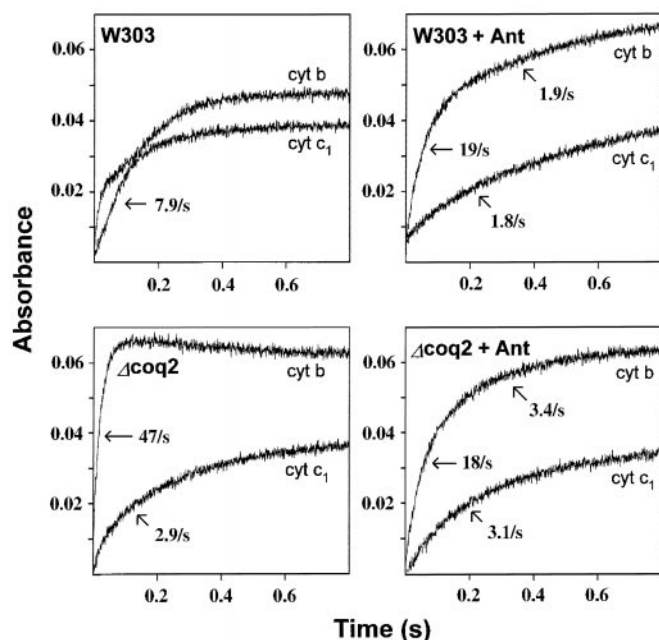


FIG. 2. Menaquinol reduction of wild-type and ubiquinol-deficient cytochrome bc_1 complex in the absence or presence of antimycin. The traces show the time course of reduction of cytochrome b and c_1 by $25 \mu\text{M}$ menaquinol in bc_1 complex from wild-type yeast (W303) or bc_1 complex from a yeast mutant lacking endogenous ubiquinone (Δcoq2) in the presence or absence of antimycin (Ant).

ubiquinone (Fig. 2, lower left). These results indicate that when there is no endogenous ubiquinone, antimycin does not slow the rate of cytochrome c_1 reduction. The rate of c_1 reduction in the bc_1 complex from the wild-type yeast in the presence of antimycin (1.8 s^{-1}) is comparable with the rates in the bc_1 complex from the Δcoq2 mutant in the absence (2.9 s^{-1}) or presence of antimycin (3.1 s^{-1}), allowing for some variation in activities of different enzyme preparations. Also, in the bc_1 complexes from both the wild-type yeast and the Δcoq2 mutant in the presence of antimycin, where the reduction of b is biphasic, the rate of c_1 reduction matches the slow phase of b reduction.

To more accurately determine the rates of cytochrome c_1 reduction in the presence of antimycin and in the absence of endogenous ubiquinone, the rates were measured at multiple menaquinol concentrations, and second order rate constants for cytochrome c_1 reduction were calculated from the plots shown in Fig. 3. With wild-type enzyme, in which endogenous ubiquinone is present, the second order rate constant for cytochrome c_1 reduction varied, declining ~ 4.5 fold from $6.9 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ to $1.55 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ as the concentration of menaquinol increased. The reason for the variable rate constant in the wild-type bc_1 complex in the absence of antimycin is discussed below. When antimycin was added, the rate constant decreased by 10-fold to $6.6 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$. With the bc_1 complex from the Δcoq2 mutant, the second order rate constant for c_1 reduction was $1.1 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, and when antimycin was added, the rate constant was unchanged. From these rate constants it is clear that in the presence of endogenous ubiquinone antimycin slows the rate of c_1 reduction, but in the absence of endogenous ubiquinone, antimycin has no effect. The absence of endogenous ubiquinone appears to mimic the effect of antimycin on presteady state reduction of c_1 by menaquinol when ubiquinone is present.

Effect of Iron-Sulfur Protein Midpoint Potential on the Rate of Cytochrome b Reduction through Center P —Using bc_1 complexes in which the midpoint potential of the Rieske iron-sulfur protein was altered by site-directed mutations, we previously

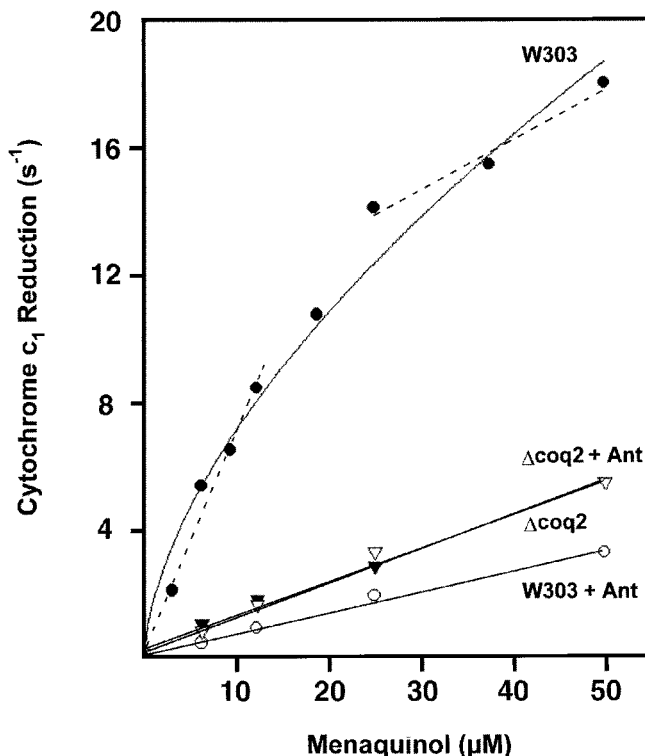


FIG. 3. Rates of cytochrome c_1 reduction in wild-type and ubiquinol-deficient cytochrome bc_1 complex in the absence or presence of antimycin. The rates of cytochrome c_1 reduction are plotted as a function of menaquinol concentration for cytochrome bc_1 complex from wild-type yeast (W303) or bc_1 complex from a yeast mutant lacking ubiquinone (Δcoq2) in the presence or absence of antimycin (Ant). Dashed lines indicate the slopes that were used to calculate the two second order rate constants for the bc_1 complex from the wild-type yeast (W303).

showed that there is a direct correlation between midpoint potential of the iron-sulfur protein and the catalytic activity of the enzyme (14). The mutations chosen for these studies did not change the stability of the protein or cause any structural change in the environment of the iron-sulfur cluster, as assessed by various spectroscopic methods (14).

We also previously established that menaquinol reacts directly with the bc_1 complex at center P and not through endogenous ubiquinone (13, 16). In the present study we have examined the effects of iron-sulfur protein mid-point potential on the presteady state reduction kinetics of cytochromes b and c_1 . In these experiments antimycin was included to isolate the reaction at center P , so that cytochrome b reduction is linked to iron-sulfur protein reduction.

With wild-type enzyme, in which iron-sulfur protein $E_{m,7} = +285 \text{ mV}$ (14), cytochrome b reduction was biphasic and occurred at 58 s^{-1} and 9.6 s^{-1} (Fig. 4, top left). Cytochrome c_1 reduction was monophasic and occurred at 8.4 s^{-1} . With the bc_1 complex containing iron-sulfur protein with a Y185F mutation ($E_{m,7} = +217 \text{ mV}$), b reduction was biphasic and occurred at 44 s^{-1} and 11.7 s^{-1} (Fig. 4, top right), whereas c_1 reduction was monophasic and occurred at 12.1 s^{-1} . The decrease in the fast phase of b reduction in the bc_1 complex with the Y185F iron-sulfur protein compared with the rate in the wild-type enzyme is consistent with the decrease in the potential difference between the iron-sulfur protein and the substrate menaquinol. The increase in the rate of c_1 reduction is also consistent with the decrease in iron-sulfur protein midpoint potential, which would be expected to increase the rate of electron transfer from the iron-sulfur protein to c_1 .

In the bc_1 complex containing the S183A iron-sulfur protein

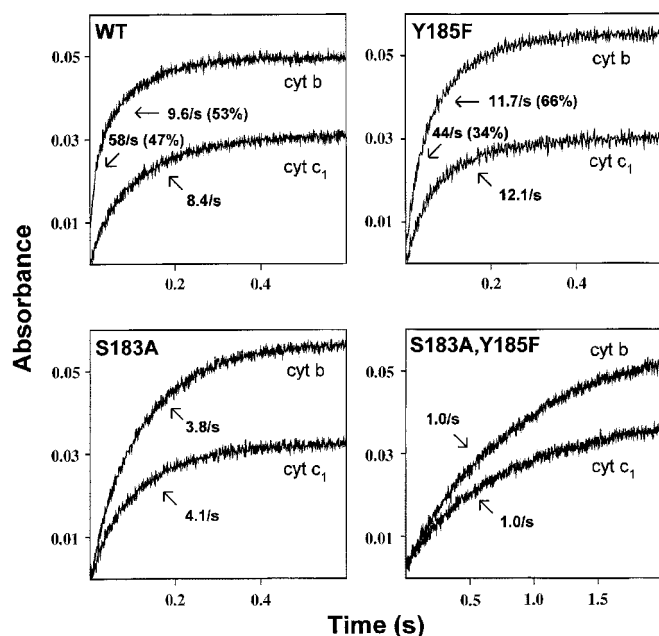


FIG. 4. Effect of Rieske iron-sulfur protein midpoint potential on the rates of cytochrome b and c_1 reduction by menaquinol. The traces show the time course of cytochrome b and c_1 reduction when cytochrome bc_1 complexes, isolated from either wild-type yeast (WT) or from the yeast mutants with Y185F, S183A, or S183A/Y185F double mutations in the Rieske iron-sulfur protein, are reduced by $100 \mu\text{M}$ menaquinol in the presence of antimycin. Rates of b and c_1 reduction are indicated under the traces. Numbers in parenthesis are the percentages of b reduced during the two phases of the biphasic reduction.

mutation ($E_{m,7} = 155 \text{ mV}$) and the bc_1 complex containing the S183A/Y185F double mutation ($E_{m,7} = 105 \text{ mV}$), reduction of both cytochrome b and c_1 was monophasic. With the S183A iron-sulfur protein mutation, the rate of b reduction was 15 times slower than the fast phase of b reduction in the wild-type enzyme, whereas the rate of c_1 reduction was 2 times slower (Fig. 4). With the bc_1 complex containing the S183A/Y185F iron-sulfur protein double mutation, the rates of both b and c_1 reduction were 1.0 s^{-1} , which are 58 times and 8 times slower than the corresponding rates in the wild-type enzyme.

To demonstrate the effects of iron-sulfur protein midpoint potential on the rate of b reduction, second order rate constants were calculated and plotted versus iron-sulfur protein midpoint potential (Fig. 5). For this plot the midpoint potentials of the iron-sulfur proteins at pH 7 were used (14). However, we also checked the mid-point potentials at pH 6.0 of the wild-type protein and the S183A and Y185F proteins and confirmed that the mutations do not eliminate the pH dependence of the midpoint potential.

For the wild-type bc_1 complex the rate constant for b reduction was $8.2 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$. There is a direct correlation between the rate of b reduction and the midpoint potential of the iron-sulfur protein. For the enzymes with the S183A and the S183A/Y185F double mutations the rate constants decreased 13.4- and 75-fold, respectively. These results demonstrate that cytochrome b reduction is linked to and limited by the oxidation of quinol by the iron-sulfur protein.

DISCUSSION

The oxidation of ubiquinol at center P has been thought to occur by a sequential mechanism in which the iron-sulfur protein oxidizes the quinol to semiquinone, which then reduces cytochrome b_L (1, 3). Our results suggest that ubiquinol oxidation occurs by a concerted mechanism that requires that both the iron-sulfur protein and cytochrome b_L be oxidized and that

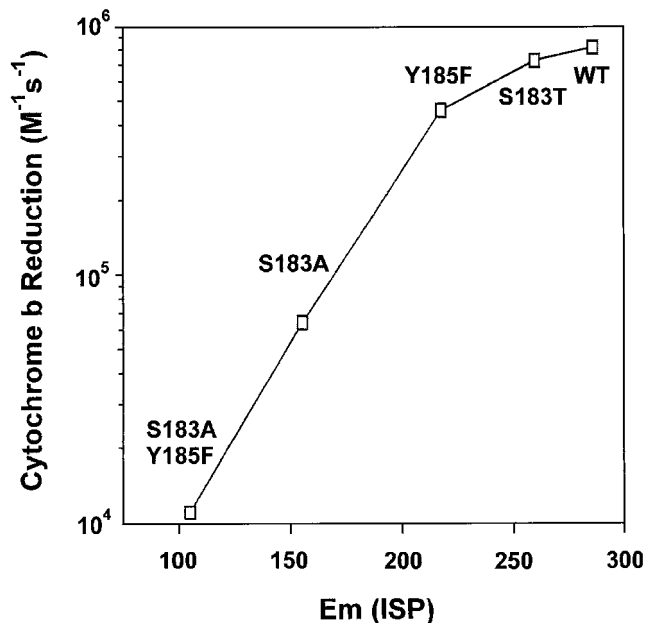


FIG. 5. Effect of Rieske iron-sulfur protein midpoint potential on the second order rate constants for cytochrome b reduction through center P. The second order rate constants for cytochrome b reduction by menaquinol through center P are plotted as a function of midpoint potential for the bc_1 complexes with the indicated iron-sulfur protein mutations. The plotted midpoint potentials are those at pH 7, although the presteady state rate constants were measured at pH 6 to prevent nonenzymatic reduction. The site-directed mutations used to alter the midpoint potential of the Rieske protein do not eliminate the pH dependence of the midpoint potential, and this is taken into account in the discussion of the results. WT, wild type; ISP, iron-sulfur protein.

cytochrome b_L reduction precedes iron-sulfur protein reduction of cytochrome c_1 . In the presteady state experiments, two molecules of menaquinol are sequentially oxidized at center P. During the first turnover, the iron-sulfur protein and cytochrome b_L are reduced (reactions 1a–1c in Fig. 1). The iron-sulfur protein will remain predominately reduced since its potential is higher than that of cytochrome c_1 . However, cytochrome b_L will immediately reduce cytochrome b_H , which then reduces ubiquinone to ubisemiquinone at center N (reactions 2 and 3 in Fig. 1). The second turnover at center P is dependent upon the redox state of cytochrome b_L and the iron-sulfur protein and will only occur when both are oxidized. After the second turnover, both the iron-sulfur protein and cytochrome c_1 are reduced, and the second electron in the low potential chain reduces semiquinone to ubiquinol at center N (reaction 4 in Fig. 1).

When ubiquinone is absent or when antimycin occupies center N, the single electron introduced during the first turnover will remain in the low potential chain. If the second turnover at center P is not dependent upon the redox state of the low potential chain and solely dependent upon the iron-sulfur protein, then the rate of cytochrome c_1 reduction should remain unchanged. However, we have shown that when ubiquinone is absent or when antimycin is present, the rate of cytochrome c_1 reduction is dramatically slowed. Furthermore, antimycin had no additional slowing effect when ubiquinone is absent. These results show that equilibration of an electron between cytochrome b_H and b_L in the low potential chain slows reduction of the high potential chain. This suggests that ubiquinol oxidation at center P occurs by a concerted mechanism in which the redox status of the low potential chain exerts reciprocal control on reduction of the high potential chain.

This same effect accounts for the decline in the second order rate constant for c_1 reduction in the wild-type bc_1 complex as

the concentration of menaquinol increases. We previously showed that menaquinol reduces the endogenous ubiquinone by a transhydrogenase reaction at center N, and this reaction is slower than the direct reduction of b and c_1 through center P (13). It would be expected that as the menaquinol concentration increases, the endogenous ubiquinone would become partly reduced, resulting in equilibration of an electron between ubiquinone and the low potential chain and a reciprocal slowing of reduction of the high potential chain. Consequently, as the concentration of menaquinol increases, the second order rate constant for c_1 reduction in the wild-type enzyme approaches the rate constants seen in the presence of antimycin or absence of ubiquinone.

The slowing effect of antimycin on presteady state reduction of cytochrome c_1 that we have described here was first reported by Degli-Esposti and Lenaz (17), although they interpreted this result as a block of electron transfer between cytochrome b and c_1 and inconsistent with the protonmotive Q cycle mechanism. We attribute this effect of antimycin to lack of equilibration of ferro-cytochrome b_H with ubiquinone through center N (reactions 4 and 5 in Fig. 1), as evidenced by duplication of the effect in bc_1 complex that lacks ubiquinone, and explain these results in terms of a concerted oxidation mechanism in the Q cycle. Elsewhere we have shown that the inhibitory effect of antimycin on catalytic activity of the bc_1 complex is distinct from the slowing effect manifested during presteady state reduction of the enzyme (18).

We have also shown that there is a direct correlation between the midpoint potential of the iron-sulfur protein and the rate of cytochrome b reduction, the other half of the reciprocal control implicit in a concerted mechanism whereby the high potential chain controls reduction of the low potential chain. These results agree with previous findings that the oxidation of ubiquinol by the iron-sulfur protein is the rate-limiting step under conditions of catalytic turnover and that the midpoint potential of the iron-sulfur protein is quantitatively the predominant determinant of that rate (14).

The effects of iron-sulfur protein midpoint potential on rates of cytochrome b reduction are incompatible with linear mechanisms of the type $QH_2 \rightarrow [b_H, b_L] \rightarrow ISP \rightarrow c_1 \rightarrow c$, in which ubiquinol reduces cytochrome b_H , which then reduces the iron-sulfur protein via cytochrome b_L (19). If ubiquinol reduces cytochrome b directly, the midpoint potential of the iron-sulfur protein should have no effect upon the rate of b reduction. However, our results clearly show that as the midpoint potential of the iron-sulfur protein decreases, the rate of b reduction decreases.

We observed that the amount of cytochrome b that is reduced in the bc_1 complex from the $\Delta coq2$ mutant is greater than in the wild-type bc_1 complex (Fig. 2). Furthermore, the addition of antimycin to the wild-type enzyme caused the extent of b reduction to be identical to that in the $\Delta coq2$ mutant. These differences are due to reoxidation of cytochrome b through center N, which is obviated by the absence of endogenous ubiquinone or inhibited by antimycin when ubiquinone is present (13).

When antimycin is present, reduction of cytochrome b is biphasic, both in the absence or presence of endogenous ubiquinone. This is because when antimycin occupies center N, the first electron to enter the low potential chain cannot equilibrate with ubiquinone but remains in the b cytochromes. Entry of a second electron into the low potential chain is then limited by the distribution of the first electron between the two b hemes if they cannot equilibrate with ubiquinone. Consequently, in these presteady state experiments antimycin inhibits entry of the second electron into the high potential chain but has no effect on the first turnover of the enzyme.

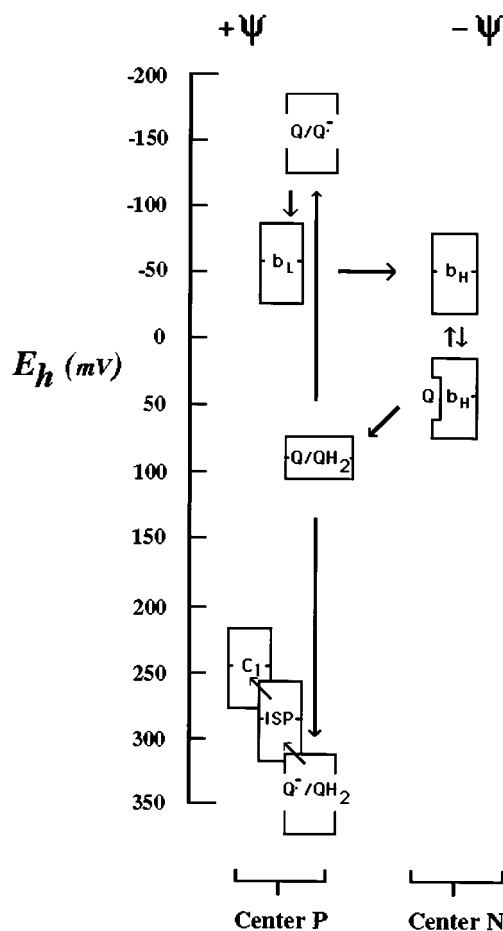


FIG. 6. Thermodynamic profile of the Q cycle. The figure depicts the thermodynamic relationship between the redox components of the cytochrome bc_1 complex in wild-type yeast at pH 7. The redox groups are arranged vertically according to their oxidation-reduction potentials and horizontally according to their disposition across the inner mitochondrial membrane. The open boxes delineate the approximate range of potentials spanned by the redox components as their oxidation-reduction status varies in response to changes in rates of electron transfer through the bc_1 complex. The centers of the boxes are positioned vertically at the midpoint potentials of the redox centers. Cytochrome b_H is a mixture of two potentiometric species in which ubiquinone is bound proximal to the b_H heme, and reduction of the quinone to quinol lowers the potential of a portion of the b_H heme from approximately +40 to -50 mV (20). The ubisemiquinone couples at center N are not shown. The potentials of the ubisemiquinone couples at center P are estimates that account for the concerted nature of ubiquinol oxidation and the predicted properties of this semiquinone.

The basis for the concerted mechanism of ubiquinol oxidation can be seen in a thermodynamic profile of the redox components of the bc_1 complex as shown in Fig. 6. Reciprocal control of ubiquinol oxidation by the low potential and high potential redox components of the bc_1 complex results from thermodynamic coupling of the electron transfer from ubiquinol to iron-sulfur protein to electron transfer from ubisemiquinone to cytochrome b_L . The essential feature of this coupling is that the potential of the Q^-/QH_2 couple is too high to reduce the Rieske iron-sulfur cluster unless the potential is lowered by removal of Q^- by reduction of cytochrome b_L as suggested by Jünemann *et al.* (7). If a conservatively estimated 10-fold lowering of the semiquinone:quinol ratio is required to bring the Q^-/QH_2 couple within the redox range of the Rieske center ($E_{m,7} = +285$ mV), this would require that $E_{m,7} (Q^-/QH_2)$ be at least $+340$ mV. The resulting low potential of the Q/Q^- couple (-160 mV) would provide a significant kinetic advantage to reduction of heme b_L ($E_{m,7} = -60$ mV) by ubisemiquinone. The exergonic

nature of this reaction and the proximity of the semiquinone to heme *b*_L (5) would ensure that this electron transfer is extremely rapid provided heme *b*_L is oxidized.

Several consequences follow from the potentials of the semiquinone couples associated with a concerted mechanism of this type. If $E_m(Q^{\cdot-}/QH_2) = +340$ mV, then the equilibrium constant for formation of ubisemiquinone from quinone and quinol would be $\sim 4.6 \times 10^{-9}$. In EPR experiments in which the concentration of *bc*₁ complex is typically 10–50 μ M, this equilibrium would have to be shifted 8 orders of magnitude to detect one equivalent of semiquinone. Clearly, this paramagnetic species, if it exists, is below the limits of detection by EPR spectroscopy.

The formation of superoxide anion by the *bc*₁ complex, a reaction that occurs at center P and is stimulated by antimycin (21), also depends on the potentials of the semiquinone couples and, by inference, the stability of the semiquinone. This reaction most likely results from aberrant reduction of oxygen instead of *b*_L by ubisemiquinone. The one-electron reduction of oxygen requires a relatively strong reductant, since $E_{m,7}(O_2/O_2^{\cdot-}) = -330$ mV in aqueous solvents (22). If one assumes $E_m(Q/Q^{\cdot-}) = -280$ mV is sufficiently low to allow the observed rates of superoxide anion formation, it would infer that $E_m(Q^{\cdot-}/QH_2) = +460$ mV and that the stability constant of the reacting ubisemiquinone is extremely low, $\sim 4.6 \times 10^{-13}$. These considerations suggest that the semiquinone that reacts with oxygen is highly unstable.

The thermodynamic profile in Fig. 6 also illustrates why cytochrome *b* reduction in the *bc*₁ complex from the wild-type yeast and in the enzyme with the Y185F iron-sulfur mutation is biphasic, whereas *b* reduction in the enzymes with the S183A and S183A/Y185F iron-sulfur mutations is monophasic. Oxidation of the second menaquinol molecule at center P is limited by the distribution of the first electron in the high potential chain between iron-sulfur protein and cytochrome *c*₁. Only when the iron-sulfur protein is oxidized can the second molecule of menaquinol reduce the iron-sulfur protein and cytochrome *b*_L. The midpoint potential of the Rieske iron-sulfur protein is pH-dependent, and this pH dependence is also manifested in the iron-sulfur proteins carrying site-directed mutations. At pH 6 the midpoint potential is +305 mV in the wild-type protein and +250 mV in the iron-sulfur protein with the Y185F mutation (23, 24). With both of these iron-sulfur proteins the equilibrium of the first electron in the high potential chain between the iron-sulfur protein and cytochrome *c*₁ lies more toward the iron-sulfur protein. This causes *b* reduction to be biphasic. The fast phase of *b* reduction is linked to the optically invisible reduction of the Rieske center, whereas the rate of the second, slow phase matches that of *c*₁ reduction (Fig. 4).

The midpoint potential of the iron-sulfur protein with the S183A mutation, $E_m = +185$ mV at pH 6 (24), is lower than that of *c*₁ (+240 mV), and the midpoint potential of the S183A/Y185F iron-sulfur protein is even lower (14). Thus, in the *bc*₁ complexes from these mutants, the first electron entering the high potential chain will be predominately located in cytochrome *c*₁, and cytochrome *b* reduction will proceed as fast as the menaquinol can be oxidized at center P. The latter reaction is slowed so much by the decrease in iron-sulfur protein midpoint potential that entry of a second electron into the low potential chain is not limited by the distribution of the first electron between the two *b* hemes. Consequently, in the *bc*₁ complexes with the S183A and the S183A/Y185F iron-sulfur protein mutations, reduction of *b* is slow and monophasic.

From these results, we propose a mechanism for the divergent oxidation of ubiquinol at center P that incorporates iron-sulfur protein mobility and concerted electron transfer. The

overall mechanism can be summarized by a minimum of four steps designated *Ia–Id*, the first three of which are concerted as described in Fig. 1. The concerted reaction begins when ubiquinol replaces the ionizable proton from the imidazole nitrogen of histidine-181, which is one of the ligands to the redox active iron of the Rieske cluster (25), to form a ubiquinol-imidazololate complex (*reaction Ia* in Fig. 1). Formation of a ubiquinol-imidazololate complex in this manner circumvents prerequisite ionization of ubiquinol, which has a $pK_a = 11.25$ (26). It is this reaction that is responsible for the $pK_a = 6.5–6.7$ component of the bell-shaped activity *versus* pH curves documented in both bovine and yeast *bc*₁ complexes by Brandt and Okun (27).

The ubiquinol-imidazololate complex is the electron donor for the redox active iron (*reaction Ib* in Fig. 1). This electron transfer does not occur unless the resulting semiquinone is simultaneously removed by reduction of cytochrome *b*_L (*reaction Ic* in Fig. 1), which only occurs when the iron-sulfur protein is in proximity to cytochrome *b*_L. This linkage is the basis of the concerted reaction. As noted above, the low potential of the *Q/Q*^{•-} couple and the proximity of the semiquinone to heme *b*_L cause this reaction to be extremely fast, provided that *b*_L is oxidized.

Binding of ubiquinol only occurs when the iron-sulfur protein is proximal to cytochrome *b*, since formation of the ubiquinol-imidazololate complex (*reaction Ia*) is dependent on reduction of *b*_L by the semiquinone (*reaction Ic*). If either cytochrome *b*_L or iron-sulfur protein is reduced, ubiquinol oxidation cannot occur, and ubiquinol cannot bind. As cytochrome *b* is reduced and ubiquinone dissociates, the reduced iron-sulfur protein moves to the cytochrome *c*₁ position, and electron transfer to *c*₁ ensues (*reaction Id* in Fig. 1). In this manner association of ubiquinol and dissociation of ubiquinone are linked to the movement of the iron-sulfur protein between the interfaces of cytochrome *b* and cytochrome *c*₁.

The overall rate of the concerted reaction can be influenced by several constituent reactions. The rate of electron transfer into the cluster is determined by the increment in redox potential between the ubiquinol-imidazololate complex and the iron-sulfur cluster, and this increment is the predominant determinant of the catalytic activity of the enzyme (14). In addition to the dependence on midpoint potential of the Rieske protein, the rate of ubiquinol oxidation is also partly determined by the rate of formation of the ubiquinol-imidazololate complex, reflected in the pH dependence of the ubiquinol-cytochrome *c* reductase reaction (27).

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