Multiple Q-Cycle Bypass Reactions at the Q_o Site of the Cytochrome bc_1 Complex[†]

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Received January 23, 2002; Revised Manuscript Received March 29, 2002

ABSTRACT: The cytochrome (cyt) bc_1 complex is central to energy transduction in many species. Most investigators now accept a modified Q-cycle as the catalytic mechanism of this enzyme. Several thermodynamically favorable side reactions must be minimized for efficient functioning of the Q-cycle. Among these, reduction of oxygen by the Q_o site semiquinone to produce superoxide is of special pathobiological interest. These superoxide-producing bypass reactions are most notably observed as the antimycin A- or myxothiazol-resistant reduction of cyt *c*. In this work, we demonstrate that these inhibitor-resistant cyt *c* reductase activities are largely unaffected by removal of O_2 in the isolated yeast cyt bc_1 complex. Further, increasing O_2 tension 5-fold stimulated the antimycin A-resistant reduction by a small amount (~25%), while leaving the myxothiazol-resistant reduction unchanged. This most likely indicates that the rate-limiting step in superoxide production is the formation of a reactive species (probably a semiquinone), capable of rapid O_2 reduction, and that in the absence of O_2 this species can reduce cyt *c* by some other pathway. We suggest as one possibility that a semiquinone escapes from the Q_o site and reduces either O_2 or cyt *c* directly. The small increase in antimycin A-resistant cyt *c* reduction rate at high O_2 can be explained by the accumulation of a low concentration of a semiquinone inside the Q_o site. Under aerobic conditions, addition of saturating levels of superoxide dismutase (SOD) inhibited 50% of cyt *c* reduction in the presence of myxothiazol, implying that essentially all bypass reactions occur with the production of superoxide. However, SOD inhibited only 35% of antimycin A-resistant cyt *c* reduction, suggesting the presence of a second, slower bypass reaction that does not reduce O_2. Given that myxothiazol blocks cyt *b* reduction whereas antimycin A promotes it, we propose that this second bypass occurs by reduction of the Q_o site semiquinone by prereduced cyt b_L.

The cytochrome (cyt)[1] bc_1 complex, or complex III, plays a central role in mitochondrial electron transport-mediated energy conversion. It fulfills two key roles: (1) It transfers electrons between two mobile electron carriers, from the $n = 2$ chemistry of the ubiquinone/ubiquinol couple to the $n = 1$ chemistry of the cyt *c* (Fe^{3+}/Fe^{2+}) couple. (2) It uses the energy liberated by the electron-transfer reactions to transport protons across the energetic membrane, from the matrix to the inter membrane space.

Electron transfer through the cyt bc_1 complex occurs through the so-called "Q-cycle", first proposed by Mitchell and later modified by several groups (*1*−*6*). In the Q-cycle, ubiquinol (UQH_2) is oxidized at the quinol oxidase (Q_o) site, which is formed by the interaction of the cyt *b* protein with the "Rieske" iron−sulfur protein (ISP) (*3*, *7*). Electron transfer from UQH_2 is bifurcated, so that the first electron is transferred to the "high-potential chain", consisting of the Rieske 2Fe-2S cluster housed in the ISP and the cyt c_1 heme housed in the cyt c_1 subunit. The initial one-electron oxidation leaves an unstable semiquinone species at the Q_o site, which is oxidized by the "low-potential chain" consisting of cyt b_L and cyt b_H, which are embedded in the cyt *b* subunit. The two protons from the quinol oxidized at the Q_o site are released to the *p*-side of the membrane, i.e., the inter membrane space. After two turnovers of the Q_o site, the two electrons sent to the low-potential chain reduce a ubiquinone (UQ) molecule at the quinone reductase (Q_i) site to UQH_2, with an uptake of two protons from the *n*-side of the membrane (i.e., the matrix). As a result, for every two electrons transferred to the high-potential chain, two protons are shuttled to the *p*-side of the membrane, and two additional scalar protons are released on the *p*-side. The yield of the bifurcated reaction and proton pumping in the cyt bc_1/b_6f complexes is high over a wide range of conditions (e.g., refs *8* and *9*), implying that side reactions which bypass bifurcation are minimized.

Because bypassing the Q-cycle would be strongly favored by thermodynamics (*6*, *10*, *11*), it has been proposed that a catalytic switch gates electron transfer at the Q_o site, to prevent side reactions (*7*, *12*, *13*). There are at least four side reactions that could bypass proton pumping and must

[†] This work was supported by U.S. Department of Energy Grant DE-FG03-98ER20299 and by a Herman Frasch Foundation award.

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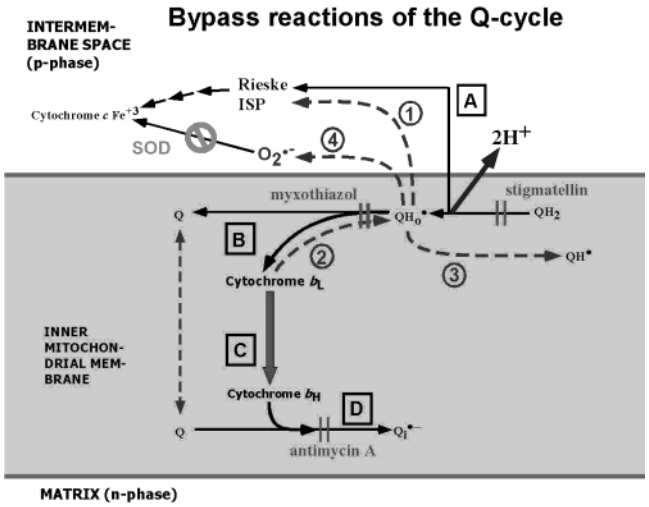
[1] Abbreviations: cyt, cytochrome; EPR, electron paramagnetic resonance; ISP, iron−sulfur protein; ISP-H, reduced, protonated Rieske iron−sulfur protein; SOD, superoxide dismutase; Mn-SOD, manganese-containing superoxide dismutase; TCA, trichloroacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MOA, E-β-methoxyacrylate; UQH_2, ubiquinol; UQ, ubiquinone; Q_o, ubiquinol oxidase site; Q_i, ubiquinone reduction site; E−S, enzyme−substrate complex.

10.1021/bi025581e CCC: $22.00
Published on Web 00/00/0000 PAGE EST: 8.4

Scheme 1



be accounted for in Q_o site catalysis models (see Scheme 1). (1) UQH_2 at the Q_o site could be oxidized by two sequential electron-transfer reactions from the Q_o site quinol to the Rieske ISP, with the release of two protons to the *p*-side. (2) The unstable Q_o site semiquinone could oxidize ferrocytochrome (Fe^{2+}) b_L, re-forming a quinol. (3) Given the predicted instability of the Q_o site semiquinone, it is thought to be only loosely bound (*2*, *3*, *14*−*17*) and thus could escape from the site and reduce cyt *c* or disproportionate in the membrane. (4) An unstable semiquinone could also directly reduce O_2, forming superoxide (reviewed in refs *18* and *19*).

Recent advances in the mechanism of the cyt bc_1 complex offer a good explanation for how the complex can prevent some of these bypass reactions. Different crystal forms and crystals made in the absence and presence of Q_o site inhibitors showed the hydrophilic "head domain" of the ISP in distinct positions (*7*, *14*, *20*−*23*). One position, termed ISP_B, places the 2Fe-2S cluster close to the Q_o site and cyt b_L, and another, termed ISP_C, places the ISP close to cyt c_1, while intermediate positions have also been found (*7*, *14*, *20*−*23*). Similar conformational changes have been shown to occur in the chloroplast cyt b_6f complex and *bc*-type complexes (*24*, *25*).

The crystal structures of the cyt bc_1 complexes show that, in the ISP_B position, the 2Fe-2S cluster is too distant from the cyt c_1 heme to account for the observed rates of electron transfer (*7*) between these prosthetic groups. Likewise, when in the ISP_C position, the 2Fe-2S cluster is too distant from the Q_o site to effectively interact with quinol. Motion of the water-soluble ISP head domain would allow the 2Fe-2S cluster to interact with both ubiquinol at the Q_o site and cyt c_1 (*22*). The ISP head is constrained to the ISP_B position by the properties of the enzyme−substrate complex or intermediate states, and the Q_o site semiquinone is thereby prevented from reducing the high-potential chain components. Oxidation of the intermediate semiquinone releases this constraint, so that the second electron is forced to reduce the relatively low-potential cyt b_L, effectively preventing bypass reaction 1 (*2*, *14*, *17*, *26*). Recent independent evidence also supports the involvement of ISP pivoting in both cyt bc_1 and cyt b_6f catalysis (*24*, *25*, *27*−*38*).

In addition to the pivoting ISP mechanism, it has been previously suggested that two quinone/quinol species may bind at the Q_o site during turnover of the enzyme, forming an "electron shuttle" which could act to rapidly dissipate the reactive semiquinone and potentially prevent bypass reactions 2 and 3 (*6*, *15*, *39*). Data supporting this proposal have mostly come from EPR studies of the cyt bc_1 Rieske 2Fe-2S cluster, which is purportedly sensitive to the occupancy of the Q_o site (*15*, *39*, *40*). Independent spectroscopic data (*41*, *42*), as well as the recent X-ray structures (*7*), demonstrated that, in the cyt bc_1/b_6f complex, different classes of Q_o site inhibitors bind, albeit exclusively, to two distinct but overlapping binding niches within the Q_o site. As examples, the distal Q_o site binds stigmatellin (*43*) and 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole (*44*) and the proximal Q_o site binds E-β-methoxyacrylate (MOA) inhibitors (myxothiazol, MOA-stilbene) (*7*, *42*, *45*). More recently, evidence has emerged that two molecules of the inhibitor 2,5-dibromo-3-methyl-6-isopropylbenzoquinone (DBMIB) can bind simultaneously to each Q_o site in the cyt b_6f complex (*24*).

Other mechanistic models of Q_o site electron transfer suggest that only one quinoid species (i.e., quinone, semiquinone, or quinol) occupies the Q_o site during catalysis and can move between the proximal and distal niches of the Q_o site (*17*, *31*) or act in a concerted fashion between these niches (*46*). In either case, such models might effectively prevent side reaction 2.

Recent models from Link (*47*) and Brandt and co-workers (*11*) suggest that, during catalysis, the Q_o site semiquinone tightly associates with, and is thus stabilized by, the reduced 2Fe-2S cluster. This would effectively prevent oxidation of the 2Fe-2S cluster until electron transfer from the semiquinone to cyt b_L is complete. Such a scheme should also prevent the escape of the semiquinone into the bulk lipid phase, i.e., to prevent bypass reaction 3 (see Scheme 1). The predicted stable semiquinone intermediate has not yet been observed by EPR, though it is possible that spin coupling with the paramagnetic reduced 2Fe-2S cluster might render it EPR invisible (see refs *25* and *47*).

It has been known for some time that antimycin A, which blocks reoxidation of cyt *b* at the Q_i site, only incompletely inhibits steady-state cyt *c* reduction by the cyt bc_1 complex (*48*, *49*), even though its binding to the complex is extremely tight (*49*). More recently, it has emerged that while the Q_o site distal niche inhibitor stigmatellin completely abolishes electron transfer (superoxide production), the Q_o site proximal niche inhibitor myxothiazol does not (*50*, *51*). Since the simplest formulation of the Q-cycle would predict that both inhibitors should completely block cyt *c* reduction (*42*), it is likely that the residual activities represent Q-cycle bypass reactions. Furthermore, the antimycin A bypass reaction has been correlated with superoxide production (*48*, *49*) and thus may have relevance to a wide variety of human disease, including aging (reviewed in refs *19* and *52*).

In this work, we further characterize these side reactions by examining the special cases of turnover in the presence of antimycin A or myxothiazol in the isolated yeast cyt bc_1 complex under aerobic and anaerobic conditions.

MATERIALS AND METHODS

Purification of the Cyt bc_1 Complex. The yeast cyt bc_1 complex was isolated using the protocol of Ljungdahl et al. (*53*) with minor modifications. One kilogram of Fleishman's

baker's yeast was washed once with distilled water and once with isolation buffer I (100 mM Tris, 250 mM sorbitol, 5 mM $MgCl_2$, 150 mM potassium acetate, 1 mM dithiothreitol, pH 8.0). The washed yeast cells were resuspended with 240 mL of buffer I and frozen by pouring the suspension into liquid nitrogen. The frozen yeast were blended in liquid nitrogen in a 4 L stainless steel Waring blender for a total of ~5 min.

The lysed cell powder was thawed with the addition of 720 mL of buffer I, with 1 mM diisopropyl fluorophosphate and 1 mM phenylmethanesulfonyl fluoride (PMSF) as protease inhibitors. The cell debris was sedimented at 3000*g* for 10 min, and the pellet was washed once in disruption buffer and sedimented at 3000*g* for 10 min. The supernatants were combined, and the mitochondrial membranes were sedimented at 20000*g* for 30 min. The mitochondrial membranes were washed twice in 50 mM Tris−acetate, 0.4 M mannitol, and 2 mM EDTA, pH 8.0, containing 1 mM diisopropyl fluorophosphate (buffer II) and once in 150 mM potassium acetate, 50 mM Tris−acetate, and 2 mM EDTA, pH 8.0 (buffer III). Membrane protein concentrations were determined by a modified Lowry method (Sigma DOC kit, Sigma Chemical Co., St. Louis, MO).

To purify the cyt bc_1 complex, mitochondrial membranes were suspended at 10 mg/mL in 50 mM Tris-HCl, 1 mM $MgSO_4$, and 1 mM PMSF, pH 8.0 (buffer IV), and dodecyl maltoside (Roche Chemicals) at a detergent/membrane protein ratio of 0.8 w/w was added and stirred for 45 min at 4 °C. The membrane extract was centrifuged at 100000*g* for 90 min (using a Sorval Ti 70 rotor at 37000 rpm). After the addition of 100 mM NaCl and stirring for 60 min, the extract was loaded onto a 2 × 20 cm (using 38 mL of Bio-Gel A, Bio-Rad) DEAE-Bio-Gel A chromatography column equilibrated with 50 mM Tris-HCl, 1 mM $MgSO_4$, 1 mM PMSF, 100 mM NaCl, and 0.1 mg/mL dodecyl maltoside, pH 8.0. After being loaded, the column was washed with two column volumes of the same buffer and eluted with a step gradient of 100−400 mM NaCl in 50 mM Tris-HCl, 1 mM $MgSO_4$, 1 mM PMSF, and 0.1 mg/mL dodecyl maltoside, pH 8.0. The cyt bc_1 complex eluted at approximately 180 mM NaCl. The combined cyt bc_1 fractions were concentrated to ~60 μM cytochrome bc_1 complex using Amicon Centriprep 30 tubes spinning at 750*g* (4 °C) for ~24 h. The concentrated cyt bc_1 complex was then diluted 1:1 with 100% glycerol and frozen. The concentration of the cyt bc_1 complex was estimated by the ferricyanide−ascorbate−dithionite absorbance difference spectra, using published values of the extinction coefficients (*53*).

Assay for Steady-State Turnover of the Cyt bc_1 Complex. Assays were conducted in 50 mM MOPS [3-(*N*-morpholino)-propanesulfonic acid] and 100 mM KCl, pH 6.90; 50 μM bovine heart cyt *c* [purified with TCA (trichloroacetic acid)], 50 μM decylubiquinol (for isolated cyt bc_1), and 5 mM succinate (for mitochondrial membranes) were used as substrates; 1 mM KCN was added to eliminate activity from trace contaminants of cyt *c* oxidase (*53*). The reaction was measured by the absorbance changes associated with cyt *c* reduction at 550 nm, using the α-band extinction coefficient of 17 mM^{-1} cm^{-1} (*54*). Rates were estimated after establishment of steady-state conditions and after subtraction of background rates determined in the presence of all substrates but in the absence of the cyt bc_1 complex. All activity measurements were expressed in moles of cyt *c* per second per mole of cyt bc_1 (turnovers per second per bc_1 complex) by dividing the initial rate of cyt *c* reduction by the concentration of the bc_1 complex.

Superoxide Production. The rate of superoxide production was estimated in two ways. The first was as the observed superoxide dismutase- (SOD-) sensitive reduction of cyt *c* (*48*). Superoxide reduces cyt *c* with a rate constant of around 10^6 M^{-1} s^{-1} (*55*, *56*), but this reaction can be outcompeted by SOD, which reacts with superoxide at a rate of ~10^9 M^{-1} s^{-1} (*55*, *56*). The experiments were carried out in 50 mM MOPS, 100 mM KCl, and 1 mM KCN, pH 6.90; 50 μM bovine heart cyt *c* (TCA purified) and 50 μM decylubiquinol (or 5 mM succinate for mitochondrial membranes) were also used as substrates. TCA-purified cyt *c* was used because this preparation is free of contaminating SOD. The rate of superoxide production was taken as the rate of cyt *c* reduction (measured spectrophotometricaly as described for the cyt bc_1 complex activity assay) in the absence of manganese-containing SOD (Mn-SOD) minus the rate of cyt *c* reduction in the presence of 300 units/mL Mn-SOD. Mn-SOD (Sigma) was used because it is resistant to KCN. One unit of SOD is defined as inhibiting the reduction of cyt *c* by xanthine oxidase by 50%, when the A_{550} increases from 0.025 to 0.05 in 1 min (*57*). The amount added was determined to be in large excess since increasing concentrations 4-fold had no further effects on results (see below). Inhibitors (antimycin A, myxothiazol, stigmatellin) were added to the buffer at the indicated concentrations (usually 10−20 μM). The rate of cyt *c* reduction in the presence of stigmatellin was not significantly different from background, i.e., stigmatellin, decyl-UQH_2 and cyt *c* without added cyt bc_1 complex.

Superoxide production was also measured via H_2O_2 formation using the Amplex Red horseradish peroxidase method (Molecular Probes, OR, product no. A-12212). Excess superoxide dismutase (400 units/mL) was added to the reaction buffer to convert superoxide into H_2O_2, which in the presence of horseradish peroxidase oxidizes Amplex Red to fluorescent resorufin. The increase in resorufin red fluorescence was followed using excitation at around 530 nm and emission between 590 and 670 nm, using an in-house-built kinetic fluorometer. The buffer was the same as for the cyt bc_1 complex activity measurements (above) and additionally contained 400 μM Amplex Red and 10 units/mL horseradish peroxidase. H_2O_2 production was calibrated using the standards provided in the Amplex Red kit.

Modulation of Oxygen Tension. To create anaerobic conditions, a sealed redox cuvette containing reaction buffer was continuously stirred as ultrapure argon (Air Liquide) was flushed through the system. This process was carried out for 45 min at which point cyt *c* was added and stirring was slowed so as to minimize cavitation. After 15 min, 50 μM decyl-UQH_2 (or 5 mM succinate) was added. The experiment was started with the addition of the cyt bc_1 complex. The reduction of cyt *c* was then followed as described above.

The same redox cuvette was used to perform experiments under 100% oxygen. In this case, 100% O_2 was flushed through the system for ~45 min while stirring (no bubbling). Stirring was then slowed to prevent bubbling, cyt *c* was added, and the oxygen flow was continued for another 15 min. The experiment was started by adding the cyt bc_1

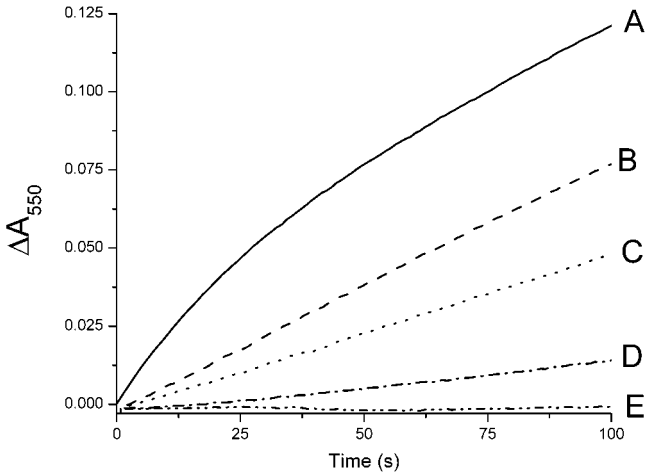


FIGURE 1: Effects of antimycin A and myxothiazol on cyt *c* reduction catalyzed by the cyt bc_1 complex. Under all conditions, 50 μM decyl-UQH_2 and 50 μM cyt *c* were used as substrates, and the background rate measured in the absence of the cyt bc_1 complex was subtracted from the traces. The buffer was 50 mM MOPS and 100 mM KCl, pH 6.90, and the experiment was carried out at room temperature. The reaction was initiated by the addition of the cyt bc_1 complex. Under uninhibited conditions (trace A), a lower concentration of the cyt bc_1 complex (0.85 nM) was added to allow resolution of kinetic data. 17 nM cyt bc_1 complex was added in inhibitory treatments, i.e., 20 μM antimycin A (trace B), 20 μM antimycin A + 300 units of Mn-SOD (trace C), 20 μM antimycin A + 20 μM myxothiazol (trace D), and 20 μM antimycin A + 10 μM stigmatellin (trace E). The experiments were repeated at least five times with similar results; representative traces are shown.

complex and following the reduction of cyt *c* as described above.

British Anti-Lewisite Treatment. Treatment with British anti-lewisite (BAL), which destroys the Rieske 2Fe-2S cluster, was carried out as described previously (*58*). Mitochondrial membranes were washed once in 100 mM NaCl, 50 mM Tris, and 1 mM $MgSO_4$, pH 8.0, and resuspended in the same buffer to a concentration of 10 mg/mL. The samples were then warmed to 40 °C, 10 mM British anti-lewisite (Sigma) was added, and samples were shaken (open to air) at 40 °C for 1 h. The mitochondrial membranes were then washed twice with buffer and resuspended in 100 mM NaCl, 50 mM Tris, 1 mM $MgSO_4$, and 1 mM PMSF, pH 8.0. For each experiment, a control was subjected to the same procedure except that 10 mM British anti-lewisite was omitted.

RESULTS

Effects of Antimycin A and Myxothiazol on Cyt c Reduction by the Cyt bc_1 Complex. The room temperature rate of decyl-UQH_2 to cyt *c* electron transfer catalyzed by the cyt bc_1 complex was about 160 mol of cyt *c* s^{-1} (mol of cyt bc_1)$^{-1}$ at pH 6.90 (Figure 1), similar to the rates previously reported for this system (*53*). Addition of saturating concentrations (20 μM) of antimycin A reduced the rate to about 2% ($\sim$3.25 s^{-1}), confirming that, under aerobic conditions, antimycin A can only partially block electron transfer (*49*, *59*). Addition of Mn-SOD inhibited the antimycin A-resistant reduction of cyt *c* by about 35% (see Figures 1 and 2). Increasing the concentration of cyt *c* to 200 μM or increasing the pH to 8.75, which should slow the spontaneous dismutation of superoxide (*55*), did not significantly increase the fraction of antimycin A-resistant reduction of cyt *c* that was inhibited by SOD (data not shown). Mn-SOD had no observable effect on the uninhibited (normal) turnover of the cyt bc_1 complex, indicating that the decrease in cyt *c* reduction was due to superoxide elimination rather than an effect on the turnover of the cyt bc_1 complex (data not shown).

These data are qualitatively consistent with previous results showing a 20% decrease in antimycin A-resistant cyt *c* reduction upon SOD addition (*49*). We note that SOD activity was found in some of our cyt *c* preparations, and similar contamination may have lowered the effect of addition of exogenous SOD in previous studies. In the present experiments, TCA-purified cyt *c* with no detectable SOD activity was used (see Materials and Methods).

The apparent rate of superoxide production estimated by this method was 1.13 mol of $O_2^{\bullet-}$ s^{-1} (mol of cyt bc_1 complex)$^{-1}$ at pH 6.90. The capacity of isolated cyt bc_1 complex to produce superoxide was verified (Figure 3) using the Amplex Red assay for the rate of H_2O_2 production, the stoichiometric product of superoxide dismutation (*18*, *55*). The rate of H_2O_2 production was 0.53 mol of H_2O_2 s^{-1} (mol of cyt bc_1 complex)$^{-1}$, i.e., considering the 2:1 stoichiometry for $O_2^{\bullet-}$:H_2O_2 (*60*), equal to 1.06 mol of $O_2^{\bullet-}$ s^{-1} (mol of cyt bc_1)$^{-1}$. Stigmatellin completely inhibited the antimycin A-resistant reduction of cyt *c* (Figure 1) and the H_2O_2 induced fluorescence on oxidation of Amplex Red (Figure 3). British anti-lewisite (BAL; see Materials and Methods) treatment decreased both normal steady-state turnover and antimycin A-resistant reduction of cyt *c* in membranes by about 99% (data not shown), consistent with previous work in submitochondrial particles (*61*). Furthermore, when (50 μM) reduced cyt *c* was added as a substrate or cyt *c* was omitted completely, preventing the 2Fe-2S cluster from becoming oxidized, no H_2O_2 production was observed (data not shown). These results indicate that the O_2-reactive intermediate was a product of quinol oxidation via the oxidized 2Fe-2S cluster.

Addition of saturating concentrations of myxothiazol partially inhibited the reduction of cyt *c* by the cyt bc_1 complex, to a rate of about 0.9 mol of cyt *c* s^{-1} (mol of cyt bc_1 complex)$^{-1}$, or less than 1% of the uninhibited rate (Figures 1 and 2). Addition of Mn-SOD inhibited the myxothiazol-resistant reduction of cyt *c* by 50%, i.e., to 0.45 mol of cyt *c* s^{-1} (mol of cyt bc_1 complex)$^{-1}$ (Figure 2). Concomitant addition of stigmatellin and myxothiazol, or addition of stigmatellin alone, inhibited cyt *c* reduction completely (data shown). Addition of antimycin A to the myxothiazol-treated cyt bc_1 complex had virtually no effect on cyt *c* reduction or superoxide production, whereas addition of stigmatellin essentially completely blocked both reactions (data not shown), consistent with an essential role for the Q_o site in superoxide production.

Superoxide production in the presence of myxothiazol was confirmed by measuring H_2O_2 production in the presence of excess SOD using the Amplex Red method (Figure 3). In the presence of myxothiazol, the rate of H_2O_2 production was found to be 0.21 mol of H_2O_2 s^{-1} (mol of cyt bc_1)$^{-1}$, i.e., indicating the production of 0.42 mol of superoxide s^{-1} (mol of cyt bc_1)$^{-1}$, in agreement with the cyt *c*−superoxide measurement under these conditions. These data are consistent with recent reports showing that addition of myxothiazol induces superoxide production in mitochondria and submitochondrial particles (*50*, *51*). As with antimycin A,

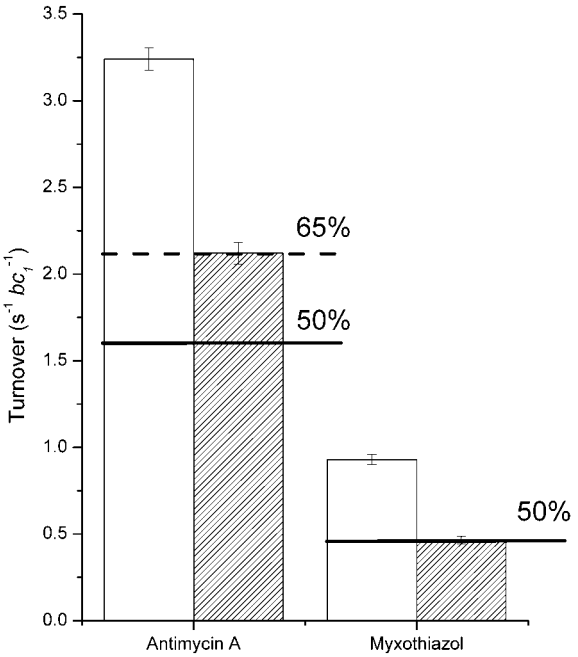


FIGURE 2: The antimycin A- and myxothiazol-resistant reduction of cyt *c* is partially inhibited by Mn-SOD. This figure shows the effect of the addition of excess Mn-SOD on the myxothiazol- and antimycin A-resistant reduction of cyt *c*. The results are expressed in moles of cyt *c* reduced per second per mole of cyt bc_1. Experimental conditions were the same as in Figure 1. The bar graph shown is an average of three repeats. Key: empty bars, no additions; hatched bars, +300 units of Mn-SOD.

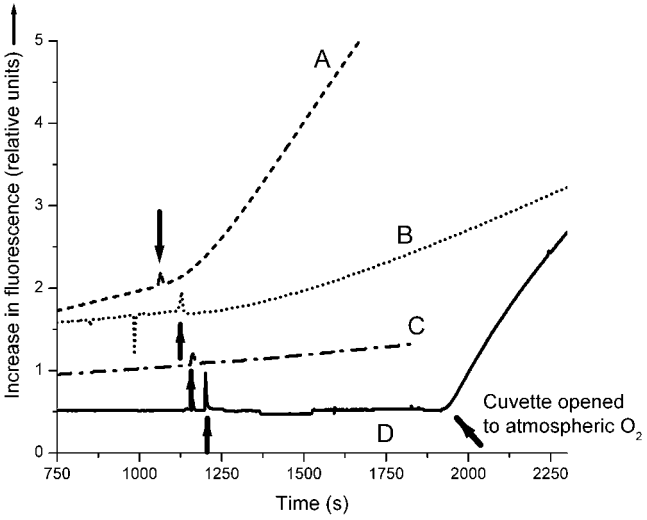


FIGURE 3: Myxothiazol and antimycin A but not stigmatellin induce $H_2O_2/O_2^{\bullet-}$ production in the isolated cyt bc_1 complex. The experiment was carried out in bc_1 reaction buffer (pH 6.90) at room temperature. The buffer also contained 400 μM Amplex Red, 10 units/mL horseradish peroxidase, and 300 units of Mn-SOD. Substrates (50 μM decylubiquinol, 50 μM cyt *c*) were added to start the experiment. A background trace was obtained, after which 42 nM cyt bc_1 complex was added to the reaction mixture (indicated by the dark gray arrow). Trace A contains substrates, Mn-SOD, and 20 μM antimycin A. Trace B contains the same as in (A) + 20 μM myxothiazol. Trace C contains the same as in (A) + 5 μM stigmatellin. Trace D contains the same as in (A) but conducted under anaerobic conditions. The light gray arrow at the end of the trace indicates where the argon flow was stopped, the anaerobic cuvette opened to the atmosphere, and air blown through the system.

when cyt *c* was omitted or replaced with reduced cyt *c*, no H_2O_2 production was seen (data not shown).

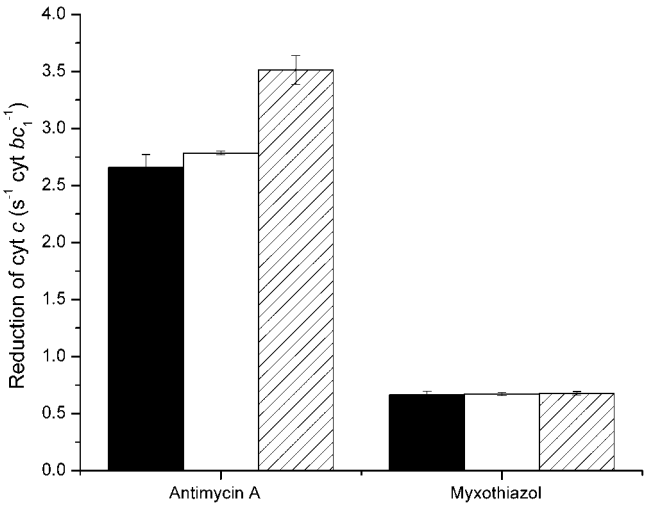


FIGURE 4: Effect of oxygen tension on the antimycin A- and myxothiazol-resistant reduction of cyt *c*. Conditions were as in Figure 1 except that the atmosphere above the samples contained 0% (filled bars), 21% (empty bars), and 100% O_2 (hatched bars). $n = 3$ for each experiment (see Materials and Methods). The rates of cyt *c* reduction were taken as the sustained maximal slopes of absorbance changes as in Figure 1. Each data point was the average of three separate experiments.

The effect of SOD was also determined for the antimycin A-resistant and myxothiazol-resistant succinate:cyt *c* oxidoreductase activity in mitochondrial membranes (obtained as intermediates in the isolation of the cyt bc_1 complex; see Materials and Methods). This reaction involves the use of the endogenous ubiquinone-6 as a substrate. The results were qualitatively similar to those reported here for the isolated complex (data not shown). We emphasize the results obtained with the isolated complex because this system avoids complications from other redox systems present in the membranes.

Effects of Oxygen Tension on the Inhibitor-Insensitive Reduction of Cyt c. The generation of superoxide, as measured by H_2O_2-induced fluorescence change on oxidation of Amplex Red in the presence of excess Mn-SOD (Figure 3), was completely inhibited by removal of O_2. However, as is shown in Figure 4, anaerobiosis had no significant effects on the rates of antimycin A- and myxothiazol-resistant reduction of cyt *c* when compared to results taken under air. These data are in contrast to those of Zhang et al. (*59*), which suggested a complete inhibition of the antimycin A-resistant reduction of cyt *c* by O_2 removal. In our work, complete O_2 removal was achieved through argon bubbling, and anaerobiosis was verified by inhibition of the H_2O_2 production (above) and the inhibition of KCN-sensitive cyt *c* oxidation via the cytochrome *c* oxidase (data not shown), which, as noted previously, is a trace contaminant in the cyt bc_1 complex preparation (*53*). Replacement of O_2 by briefly opening the cuvette seal resulted in rapid cyt *c* oxidation, indicating that that cyt *c* oxidase inhibition was achieved through removal of O_2 and not simply destruction of the enzyme. Similarly, superoxide production (measured via H_2O_2 production) resumed immediately after the anaerobic cuvette was opened to atmospheric oxygen (Figure 3). We conclude from these data that our system was completely anaerobic.

We repeated these experiments measuring antimycin A- and myxothiazol-resistant succinate:cyt *c* reductase in mi-

tochondrial membranes, such as to use endogenous ubiquinone-6 (as opposed to decyl-UQH_2) as a substrate for the cyt bc_1 complex. The results were the same as those obtained in the isolated cyt bc_1 complex with decylubiquinol as a substrate: complete removal of oxygen via argon purging failed to significantly decrease the rate of either the myxothiazol-resistant or the antimycin A-resistant reduction of cyt *c* (data not shown).

When samples were saturated with 100% O_2, i.e., a 5-fold increase in pO_2, the antimycin A-resistant reduction of cyt *c* (in the isolated cyt bc_1 complex) was found to be ca. 25% higher (Figure 4). On the other hand, elevated O_2 had no effect on the rate of myxothiazol-resistant cyt *c* reduction (Figure 4).

DISCUSSION

As previously discussed in the pioneering work of Chance (*18*, *48*), antimycin A-resistant cyt *c* reduction at the cyt bc_1 complex is closely associated with superoxide production. Previous research has suggested a likely model, wherein a reactive Q_o site semiquinone species reduces O_2 to form superoxide (*19*). Both electrons from quinol oxidation will end up on cyt *c*, but via disparate pathways (see Scheme 1). One electron is delivered to cyt *c* via the high-potential chain (Rieske ISP, cyt c_1) upon the formation of the Q_o site semiquinone. The other is passed to O_2, forming $O_2^{\bullet -}$, which in turn is rapidly oxidized by cyt *c*.

A similar mechanism can be proposed for myxothiazol-resistant cyt *c* reduction, but it is necessary to propose that the oxidized ISP in the ISP_B position can interact with quinol in the Q_o distal niche even in the presence of myxothiazol. Such a suggestion is surprising in view of the structure of the Q_o site in the presence of myxothiazol as determined by X-ray crystallography, which, despite the bifurcated volume of the site, shows both a relatively constricted tunnel for access from the lipid phase, fully occupied by the "tail" of myxothiazol, and a closure of the port through which ISP His-161 accesses the occupant (*22*). The generation of superoxide in the presence of myxothiazol must involve prior formation of an ES complex through occupancy by UQH_2 and, therefore, suggests a degree of flexibility of the structure not apparent from the crystallographic studies. This would also be required by so-called "double occupancy" models where two quinone/quinol species bind at the Q_o site (*15*).

In the context of this type of model, we would expect that the addition of SOD should prevent electrons in the superoxide pathway from reaching cyt *c*, lowering its rate of reduction by exactly half. Our results with myxothiazol (Figure 2) are consistent with this view, and we conclude that the vast majority of the bypass reaction in the presence of this inhibitor occurs via the above-described model.

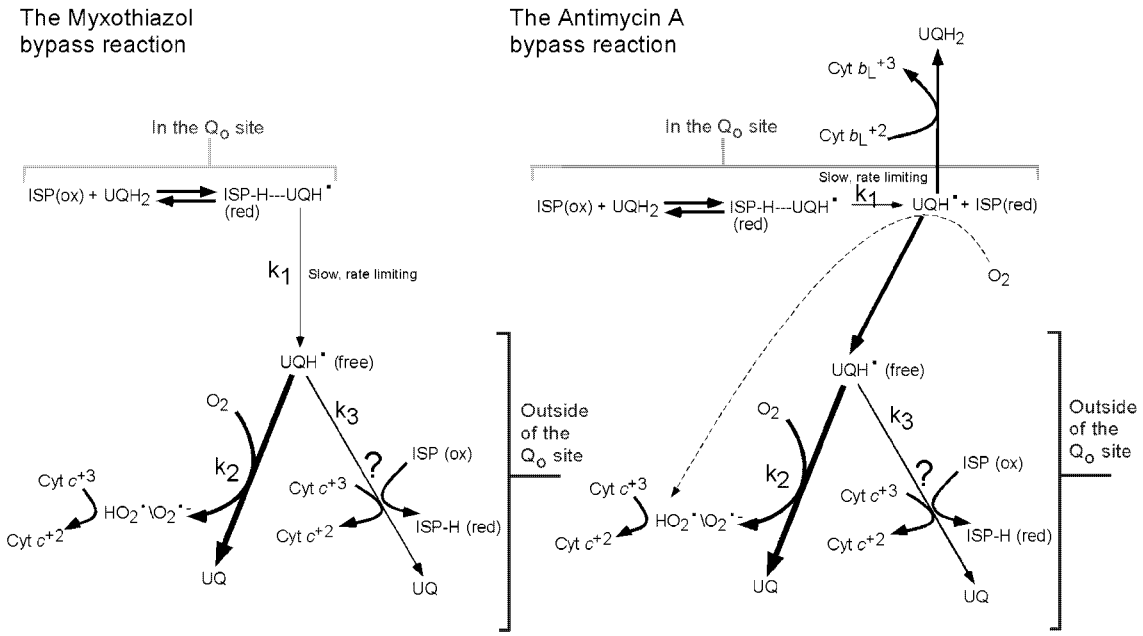
On the other hand, the antimycin-resistant rate of cyt *c* reduction was only lowered by 35% upon the addition of SOD (Figures 1 and 2). The difference could be attributed neither to inadequate SOD activity nor to incomplete reaction of superoxide with cyt *c*, since increasing cyt *c* and SOD concentrations 10-fold and 4-fold, respectively, had no further effects (data not shown). Superoxide production can thus account for no more than 70% of the bypass reaction, and we interpret these results as indicating the presence of an additional bypass reaction in the antimycin A-inhibited cyt bc_1 complex. We propose that this additional bypass reaction occurs via reaction 2 (see Scheme 1). Since myxothiazol is known to bind at the proximal niche of the Q_o site (*31*, *42*), it should block the flow of electrons between the cyt *b* hemes and the Q_o site semiquinone (*42*) and eliminate bypass reaction 2 (see the Introduction). We expect the maximal rates of reaction 2 in the presence of antimycin A, since this would maximize the concentrations of both reduced cyt b_L and the Q_o site semiquinone. On the basis of the fraction of cyt *c* reduction remaining in the presence of SOD, we estimate that about 30% of the total bypass occurs via reaction 2.

This by itself is insufficient to account for the 3−4-fold difference between myxothiazol- and antimycin A-insensitive cyt *c* reduction rates (Figures 1 and 2). We thus suggest that myxothiazol also interferes with the binding of quinol to the Q_o site distal niche as readily inferred from the structure of the site in the presence of the inhibitor (*17*). The extent of this interference will clearly depend on the reactivity of the semiquinone generated under the two conditions. Assuming equal reactivity, myxothiazol would be expected to reduce the occupancy about 3-fold (under our specific experimental conditions).

A striking finding of our work is that complete removal of O_2 had little effect on the rate of cyt *c* reduction, in the presence of both antimycin A and myxothiazol, while it completely eliminated superoxide production (Figures 3 and 4). Apparently, when the superoxide pathway was eliminated, electrons were transferred via an alternate route from the semiquinone to cyt *c*. To explain the absence of effect, we suggest the model shown in Scheme 2. The formation of a reactive intermediate (semiquinone), defined by the rate constant k_1, is followed by two competitive reactions for its destruction, one leading to superoxide production (k_2) and the other to an unidentified intermediate (k_3), both of which eventually reducing cyt *c*. Since k_1 is much slower than either k_2 or k_3, the overall flux will not be affected by elimination of the superoxide pathway, whereas the extent of partitioning of flux into the two pathways will (i.e., under aerobic conditions, $k_2 > k_3 \gg k_1$).

There are two clear possibilities for the alternative pathway (k_3), which reflect different models for the mechanism by which the bypass reaction is constrained. The first possibility, suggested by models positing a tightly bound Q_o semiquinone (*11*, *47*), is through oxidation of the ISP-H (reduced, protonated ISP) by the normal tethered diffusion to cyt c_1 and its return in the oxidized form to the ISP_B position where it could react with the semiquinone at the Q_o site. However, this could occur only if the ISP-H- - -$UQH^{\bullet}$ complex (see Scheme 2) was able to dissociate to give free products, the mobile ISP-H and $UQH^{\bullet}$ in the Q_o site. Such a dissociation would make this model formally equivalent to the alternative model (*62*) in which the first electron transfer leads to separation of products in a reaction with large positive ΔG. In either case, this opens a second possibility, suggested by models positing a highly unstable loosely bound semiquinone (*2*). Oxidation of the semiquinone could occur by diffusion of semiquinone from the site and its oxidation by an alternative pathway outside the Q_o site. Our data suggest that, if this second possibility represents a major pathway, the semiquinone does not disproportionate (since this would reduce the rate of cyt *c* reduction) but more likely reduces

Scheme 2



cyt *c* directly. The second-order rate constant for semiquinone reduction of cyt *c* is similar to that of semiquinone disproportionation (*63*), but the latter is dependent on the square of the semiquinone concentration, which is expected to be quite low under all of our conditions, whereas cyt *c* is present in excess. Although the aliphatic tail of the quinone is buried in the hydrophobic portion of the lipid bilayer, the headgroup is sufficiently polar to reside near the membrane−aqueous phase boundary, potentially allowing collision with soluble cyt *c* (*64*, *65*).

We note striking similarities between this alternative pathway (direct reduction of cyt *c*) and the findings of Rich et al. regarding the nonenzymatic oxidation of various water-soluble quinols by cyt *c* (*63*). Just as in our work, the nonenzymatic reduction of cyt *c* was inhibited precisely 50% via the addition of SOD under aerobic conditions whereas O_2 removal had no effect on the overall rate. These authors concluded that oxidation of quinol was rate limiting to the overall reaction and that dissipation of the semiquinone was fastest via reduction of O_2 (forming superoxide), followed by reduction of cyt *c* and by dismutation. The comparison is important for our discussion because the work of Rich et al. clearly demonstrated that cyt *c* oxidation of the semiquinone can readily outcompete its disproportionation.

The model in Scheme 2 predicts that increasing the effective concentration of O_2 should not affect the overall rate of cyt *c* reduction (since k_1 would remain rate limiting). This behavior was observed for the myxothiazol-inhibited system (Figure 4). On the other hand, it was previously reported that raising oxygen concentration did increase the rate of cyt *c* reduction in the presence of antimycin (*18*), and we confirm this result here (Figure 4). The effect is rather small (about 25% increase with a 5-fold increase in pO_2) but suggestive of an additional, albeit inefficient, pathway for O_2 reduction. The fact that it is inhibited by myxothiazol suggests that it involves a Q_o site-bound species, most likely a transient semiquinone species resident at the site.

Taking all of these data together, we propose a model for bypass reactions at the Q_o site, as illustrated in Scheme 2. All of the proposed bypass reactions begin with the oxidation of quinol by the 2Fe-2S cluster, leading to the formation of a semiquinone at the Q_o site, as proposed for the normal Q-cycle (e.g., refs *15*, *16*, and *66*). This suggestion is supported by the fact that destroying the Rieske cluster with British anti-lewisite or maintaining it in the reduced state inhibits superoxide formation (see Results). Moreover, there are strong arguments that the Q_o site semiquinone species is the only one in the cyt bc_1 complex cycle capable of reducing O_2 at appreciable rates (see reviews in refs *19*, *67*, and *68*).

In the presence of myxothiazol, which presumably lowers the occupancy of UQH_2 at the site, the semiquinone is rapidly oxidized. Under aerobic conditions, our data indicate that the semiquinone reacts essentially exclusively with O_2. Under anaerobic conditions, the semiquinone is either lost from the site and reacts rapidly with cyt *c* or remains in the site and is oxidized by a returning ISP, thus reducing cyt *c* indirectly.

An important conclusion from our data is that production of superoxide, and the alternative bypass reactions under anaerobic conditions, must require dissociation of the intermediate complex after the first electron transfer to products. Whether the semiquinone escapes from the site and diffuses to find a reaction interface suitable for direct reduction of cyt *c* or stays in place long enough for the Rieske ISP to rotate to cyt c_1, become oxidized, and rotate back to oxidize the semiquinone will depend on relative rate constants and occupancies. Distortion of the Q_o site necessary for occupancy by quinol in the presence of myxothiazol would enhance the leaving tendency of unbound semiquinone and increase the probability that the semiquinone could escape from the site. Our data do not allow us to estimate whether this leaving time would be greater or less than the time needed for the reoxidized Rieske ISP to rotate to back to oxidize the semiquinone. This latter value lies between the rate of electron transfer between ISP-H and cyt c_1 (~10 μs) and the reaction lag time (~100 μs). These constraints are in the same range as the estimated leaving time for product quinone (<200 μs) (*4*).

The situation appears more complex in the presence of antimycin, both because superoxide production can only account for part (~70%) of the bypass reaction and also because the Q_o semiquinone can interact with additional redox carriers. All of the possibilities discussed in the context of the myxothiazol occupied site are available, but various additional possibilities are opened up by the availability of an open proximal domain of the Q_o site. These obviously depend on the role of occupancy of this domain and, hence, on single or double occupancy mechanistic scenarios. Since our data do not allow discrimination between these scenarios, we will limit our discussion to more general considerations. The semiquinone could be thermodynamically stabilized by its preferential binding to the site, as suggested by several models where it is proposed to H-bond to a 2Fe-2S cluster His ligand (e.g., refs *17*, *47*, and *69*), but this complex could dissociate to products with low (but sufficient to account for the bypass rate) probability. Alternatively, a free semiquinone could be effectively caged by diffusion to the volume of the Q_o site proximal to heme b_L and thus stabilized kinetically rather than thermodynamically, or it could be distributed between two quinoid species (*15*, *30*). In either case, a semiquinone could still either react with oxidized ISP or escape from the Q_o site and react with cyt *c*. In this latter case, the rate would be determined by the rate of escape. However, this would reflect the more physiological state (undistorted by myxothiazol occupancy), allowing for the accumulation of a small but significant concentration of semiquinone at the Q_o site. We propose that the differences between the bypass reactions in the presence of antimycin alone, or with myxothiazol, can best be explained by the existence of a longer lived Q_o site semiquinone species in the former case that allows two additional side reactions to occur. One of these is the oxidation of previously reduced cyt *b* (reaction 2 in Scheme 1), as suggested by the SOD-insensitive fraction of cyt *c* reduction (Figures 1 and 2). The other is the direct interaction of O_2 with the Q_o site bound semiquinone, as suggested by the increase in cyt *c* reduction rate under elevated O_2 (Figure 4).

Overall, the data indicate that multiple side reactions can occur in the cyt bc_1 complex. Because the rates of these reactions are low—a few percent under the extreme conditions of antimycin A poisoning—we do not anticipate effects in the energetic budget of the mitochondrion. However, the production of superoxide is likely to be of physiological importance, while understanding the general mechanism of these reactions should give us greater insights into the key intermediates of the Q-cycle.

ACKNOWLEDGMENT

We thank Dr. Bernard Trumpower for helpful discussions. We also thank Eiji Yamamoto for technical assistance.

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BI025581E