# Movement of the Iron−Sulfur Head Domain of Cytochrome $bc_1$ Transiently Opens the Catalytic $Q_o$ Site for Reaction with Oxygen[†]

Arkadiusz Borek, Marcin Sarewicz, and Artur Osyczka*

*Department of Biophysics, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Kraków, Poland*

*Received June 27, 2008; Revised Manuscript Received September 4, 2008*

ABSTRACT: Cytochrome $bc_1$, a key enzyme of biological energy conversion, generates or uses a proton motive force through the Q cycle that operates within the two chains of cofactors that embed two catalytic quinone oxidation/reduction sites, the $Q_o$ site and the $Q_i$ site. The $Q_o$ site relies on the joint action of two cofactors, the iron−sulfur (FeS) cluster and heme $b_L$. Side reactions of the Q cycle involve a generation of superoxide which is commonly thought to be a product of an oxidation of a highly unstable semiquinone formed in the $Q_o$ site ($SQ_o$), but the overall mechanism of superoxide generation remains poorly understood. Here, we use selectively modified chains of cytochrome $bc_1$ to clearly isolate states linked with superoxide production. We show that this reaction takes place under severely impeded electron flow that traps heme $b_L$ in the reduced state and reflects a probability with which a single electron on $SQ_o$ is capable of reducing oxygen. $SQ_o$ gains this capability only when the FeS head domain, as a part of a catalytic cycle, transiently leaves the $Q_o$ site to communicate with the outermost cofactor, cytochrome $c_1$. This increases the distance between the FeS cluster and the remaining portion of the $Q_o$ site, reducing the likelihood that the FeS cluster participates in an immediate removal of $SQ_o$. In other states, the presence of both the FeS cluster and heme $b_L$ in the $Q_o$ site increases the probability of completion of short-circuit reactions which retain single electrons within the enzyme instead of releasing them on oxygen. We propose that in this way, cytochrome $bc_1$ under conditions of impeded electron flow employs the leak-proof short-circuits to minimize the unwanted single-electron reduction of oxygen.

In respiratory and photosynthetic systems that couple electron transfer with a transmembrane proton gradient driving ATP production (*1*), cytochrome $bc_1$ (mitochondrial complex III) uses the Q cycle (*2, 3*) to catalyze electron transfer between quinone and cytochrome *c*. During the Q cycle, a reversible oxidation of quinol in the catalytic $Q_o$ site delivers one electron into the high-potential c-chain and the other into the low-potential b-chain. This reaction which is unique in biology relies on the energetic coupling of the two reduction/oxidation reactions, one involving the FeS[1] center of the c-chain and the other heme $b_L$ of the b-chain. The electrons are then exchanged between the FeS center and heme $c_1$ in the c-chain and among heme $b_L$, heme $b_H$, and the other quinone catalytic $Q_i$ site in the b-chain (Figure 1a) (*3, 4*). It appears that the two chains of cytochrome $bc_1$ have evolved to favor those productive electron transfers over the energy-wasting short-circuits of direct exchange of electrons between the chains or the uncontrolled leaks of electrons that produce damaging superoxide (*5*−*9*). Indeed, the enzyme working unperturbedly under driving force provided by substrates, quinol and cytochrome *c*, does not produce superoxide at detectable levels. This, however, may change when the impeded electron flow outbalances reducing equivalents in the two chains, which has long been known from the classic experiments with an inhibitor antimycin which demonstrated that blocking specifically the electron flow through the $Q_i$ site is sufficient to make the enzyme vulnerable to superoxide production (*10, 11*). These kinds of observations have raised an ongoing debate about whether cytochrome $bc_1$ does produce superoxide in living cells (*12*−*16*) and if so under what conditions and with what mechanism (*5, 17*−*24*). Answering those questions not only would bring clarity to many physiological and medical studies but also should improve our understanding of the molecular mechanisms of energy conservation supported by cytochrome $bc_1$.

Herein, by specifically manipulating the electron flow through the two chains of cytochrome $bc_1$, we were able to clearly isolate the states linked with superoxide production. This revealed that under the conditions of impeded electron flow, the leak of electrons from the $Q_o$ catalytic site to form superoxide competes with the short-circuit reactions and that the oscillatory movement of the FeS head domain causes transient suppression of certain short-circuits making the $Q_o$ site vulnerable to superoxide production.

## EXPERIMENTAL PROCEDURES

Wild-type and mutated cytochrome $bc_1$ were isolated from the appropriate strains (*25*−*27*) of *Rhodobacter capsulatus* as described previously (*28*). Mutated cytochrome $bc_1$ included the following cofactor knockout forms: the $c_1$ knockout with the M183L mutation in cytochrome $c_1$ (*26*) and the FeS motion knockout with the +2Ala insertion in the neck region of the FeS subunit (*27*). Steady-state

[†] This work was supported by The Wellcome Trust International Senior Research Fellowship to A.O.

* To whom correspondence should be addressed. Phone: +48-12-664-6348. Fax: +48-12-664-6902. E-mail: osyczkaa@biotka.mol.uj.edu.pl.

[1] Abbreviations: FeS, two-iron−two-sulfur cluster; SQ, semiquinone; $SQ_o$, semiquinone at the $Q_o$ site; SOD, superoxide dismutase.

10.1021/bi801207f CCC: $40.75 
Published on Web 10/29/2008

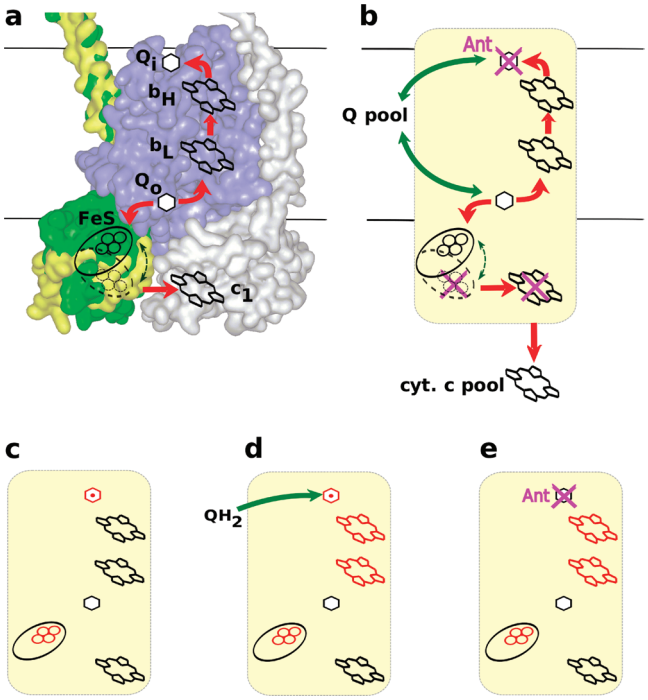


FIGURE 1: Manipulations with electron flow in cytochrome $bc_1$. (a) Three catalytic subunits [cytochrome *b* (blue), cytochrome $c_1$ (gray), and the FeS subunit (green and yellow for the $Q_o$ and $c_1$ positions, respectively)] embed two chains of cofactors that support electron transfers (red arrow) coupled to proton translocation across the membrane (not shown). (b) The cofactor knockouts or antimycin (crosses) affect the communication of the catalytic sites with the substrate (Q and cyt *c*) redox pools. (c−e) In the knockouts, oxidation of FeS is suppressed to seconds. Single electrons delivered from the $Q_o$ site to the b-chain travel freely to the $Q_i$ site (c), unless the $Q_i$ to heme $b_H$ reverse reaction saturates this portion of the b-chain with electrons (d) or inhibition with antimycin eliminates the action of the $Q_i$ site (e). This, in turn, may trap heme $b_L$ in the reduced state. Red and black colors in panels c−e represent reduced and oxidized states, respectively. Hexagons are quinones in the catalytic sites. A hexagon with a dot represents semiquinone. For the sake of simplicity, the cycling of sequential reactions at the $Q_i$ site is not shown. A different origin of suppression of FeS oxidation in the $c_1$ knockout and the FeS motion knockout is not shown either.

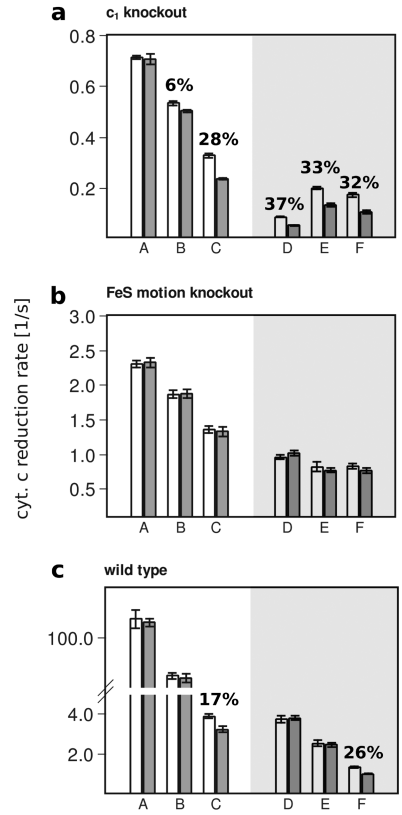


FIGURE 2: Superoxide production by isolated cytochrome $bc_1$ under various redox conditions. The activity of the $c_1$ knockout (a), the FeS motion knockout (b), and the wild type (c) was measured in the absence (white bars) and presence (gray bars) of SOD. Conditions: 50 mM Tris-HCl (pH 8) and 100 mM NaCl (A−C on a white background) or 50 mM Tris-HCl (pH 8) and 400 mM NaCl (D−F on a gray background); 20 $\mu$M quinol (A, C, D, and F) or 100 $\mu$M quinol (B and E); no inhibitor (A, B, D, and E) or 20 $\mu$M antimycin (C and F). Only a statistically significant amount of superoxide production is shown as a percentage above the bars.

enzymatic activity of cytochrome $bc_1$ was assayed by measuring the $DBH_2$ (2,3-dimethoxy-5-decyl-6-methyl-1,4-benzohydroquinone)-dependent reduction of mitochondrial horse cytochrome *c* as described previously (*25*). The measurements of superoxide production, based on superoxide dismutase-sensitive reduction of cytochrome *c*, were performed as described previously (*5*). The concentration of cytochrome *c* was 20 $\mu$M, and the concentration of quinol was as described in the figure legends.

## RESULTS AND DISCUSSION

*Steady-State Properties of Cofactor Knockouts.* To investigate the production of superoxide by cytochrome $bc_1$, we used the isolated forms of the bacterial enzyme which transfer electrons extremely slowly due to the drastically changed properties of one of the cofactors in the c-chain (Figure 1b). Those properties knock out the physiological competence of the enzyme by introducing either a thermodynamic or a steric barrier for one of the steps that follows the initial oxidation of quinol at the $Q_o$ site. In the FeS motion knockout, the FeS head domain is sterically arrested close to the $Q_o$ site, which severely interferes with its normal oscillation between the $Q_o$ site and heme $c_1$ required for efficient electron transfer (*27*). In the $c_1$ knockout, the midpoint potential of heme $c_1$ is lowered by more than 300 mV, making the electron transfer through this heme extremely uphill (*26*).

The overall effect of these changes is to slow the electron transfer through the c-chain to the time scale of seconds which severely inhibits the communication of the $Q_o$ site with the cytochrome *c* pool. Those effects can be combined with the use of antimycin which cuts off the communication between the b-chain and the quinone pool at the level of the $Q_i$ site. All those effects decrease the steady-state enzymatic activity to low but readily measurable levels. This, in combination with the biochemical assay for superoxide production and the experimental means of modulating the concentration of substrates and changing the pH, establishes the flexible system for analyzing conditions of impeded electron transfer under functional $Q_o$ and $Q_i$ sites or, with antimycin present, under the functional $Q_o$ site alone. Those conditions mimic some of the redox states encountered by cytochrome $bc_1$ in the cells, especially those with the cytochrome *c* and/or quinone pool over-reduced. In such cases, the enzyme is saturated with quinol but the flow of electrons out of the c-chain and/or the b-chain is greatly suppressed (Figure 1c−e).

*Isolation of States Linked with Superoxide Production.* Figure 2 compares the steady-state enzymatic activities

measured in the wild type and the knockouts at two different ionic strengths and at two different concentrations of quinol. In these assays, the difference in the activity measured in the absence and presence of superoxide dismutase reflects the level of superoxide production.

At 100 mM NaCl and 20 $\mu$M quinol, the $c_1$ knockout does not produce a measurable amount of superoxide (Figure 2aA); however, it is enough to increase the quinol concentration to 100 $\mu$M, and the level of superoxide production reaches 6% (Figure 2aB). This percentage is much higher at an ionic strength of 400 mM NaCl (37%) (Figure 2aD) or when antimycin is added at either of the ionic strengths (approximately 30%) (Figure 2aC,F). The same set of measurements conducted with the FeS motion knockout shows that this form does not produce superoxide at statistically significant levels under any condition, with or without antimycin (Figure 2bA−F). The native enzyme produces superoxide at levels of 17 and 26% at 100 and 400 mM NaCl, respectively, only when antimycin is present (Figure 2cC,F).

In the experiments depicted in Figure 2, an increase in the ionic strength and in the concentration of quinol introduces two distinct effects. The ionic strength acts primarily to inhibit the interaction between cytochrome $c$ and cytochrome $c_1$ and thereby slows the electron transfer in the c-chain and suppresses the overall enzymatic activity. On the other hand, quinol, which is the substrate for the $Q_o$ site, can also enter the $Q_i$ site to reduce heme $b_H$ in the reverse direction (*29, 30*), thereby competing with the forward reaction of oxidation of quinol at the $Q_o$ site. This may lead to a saturation of the b-chain with electrons (Figure 1d). Thus, increasing the concentration of quinol increases the extent to which the reverse reaction interferes with the forward reaction, which may generally lead to a decrease in the measured activity. This is evident in the wild type and in the FeS motion knockout at both 100 and 400 mM NaCl (B vs A and E vs D in panels b and c of Figure 2) and in the $c_1$ knockout at 100 mM NaCl (B vs A in Figure 2a). On the other hand, a high ionic strength in this form inhibits the electron flow through the c-chain to such extremely low rates that increasing the concentration of quinol appears actually to slightly increase the measured activity (E vs D in Figure 2a).

The effect of saturating the b-chain with electrons is most enhanced when addition of antimycin completely blocks the outflow of electron from heme $b_H$ to the $Q_i$ site in the forward direction (Figure 1e), which generally leads to the inhibition of the measured activity (C and F in Figure 2). With those considerations, the experiments depicted in Figure 2 establish a broad range of conditions under which the sustained influx of electrons into heme $b_L$ cannot be balanced with the outflow of electrons from this heme to heme $b_H$ (Figure 1d,e). This traps heme $b_L$ in the reduced state which is expected to increase the probability of uncoupling of the FeS- and heme $b_L$-mediated two-electron oxidation/reduction of quinol/quinone at the $Q_o$ site and result in the formation of semiquinone ($SQ_o$) (*22, 31*). The highly unstable $SQ_o$ is then expected to be able to reduce oxygen generating superoxide. We see that indeed the conditions trapping heme $b_L$ in the reduced state make the wild-type cytochrome $bc_1$ and the $c_1$ knockout vulnerable to the production of superoxide. In the wild type, it is just when antimycin blocks the $Q_i$ site (Figure 2cC,F); in the $c_1$ knockout, it is also under the "pressure" from the $Q_i$ site to heme $b_H$ reverse reaction (Figure 2aB−F).

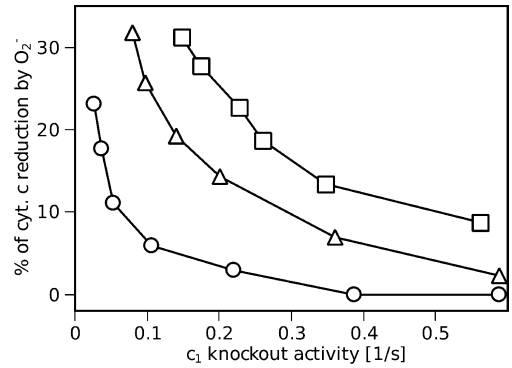


FIGURE 3: Influence of the $Q_i$ site to heme $b_H$ reverse reaction on the superoxide production in the $c_1$ knockout. The percent of superoxide production is plotted vs the SOD-insensitive enzymatic activity. The activity was manipulated by changing the ionic strength. The measurements were performed with 20 $\mu$M quinol and 50 mM MOPS (pH 7) (○), 20 $\mu$M quinol and 50 mM Tris-HCl (pH 8) (△), or 100 $\mu$M quinol and Tris-HCl (pH 8) (□).

On the other hand, the FeS knockout remarkably differs from the wild type and the $c_1$ knockout in that it does not produce a statistically significant amount of superoxide under any conditions, even when heme $b_L$ is trapped in the reduced form (Figure 2b). This appears as the first known example of the variant of cytochrome $bc_1$ for which the inhibition by antimycin is not able to stimulate superoxide production.

Since the reverse reaction is more prominent at alkaline pH (*29, 32*) or, as discussed earlier, with higher concentrations of quinol, we expect those factors to modulate the saturation of the b-chain with electrons and thereby influence the percentage of superoxide production. Indeed, the similarly active $c_1$ knockout produces more superoxide at pH 8 than at pH 7,[2] or with 100 $\mu$M quinol than with 20 $\mu$M quinol (Figure 3). On the other hand, the FeS motion knockout shows a consistent lack of observable superoxide production under the conditions described in the legend of Figure 3 (data not shown).

*The FeS Motion-Driven Unblocking of the $Q_o$ Site for Reaction with Oxygen.* The unprecedented resistance of the FeS motion knockout to production of superoxide under any conditions of our experiments implies that the structural changes that occur in the $Q_o$ site upon the movement of FeS are somehow related to the vulnerability of the enzyme to superoxide production.

In the FeS motion knockout, the FeS head domain is locked for seconds in the $Q_o$ position (*27*) and, thus, only occasionally leaves the site to reduce cytochrome $c_1$ and come back in the oxidized form to initiate the next reaction in the $Q_o$ site. If we assume that electrons from $SQ_o$ can leak on oxygen only during the time interval that FeS spends out of the $Q_o$ site, this would explain why no superoxide accumulates in this knockout on the time scale of minutes of our experiment. This is simply because FeS does not leave

[2] Given that the reaction rate constant of cytochrome $c$ with superoxide is $3 \times 10^5$ $M^{-1}$ $s^{-1}$ and the rates constants of spontaneous dismutation of superoxide in water at pH 7 and 8 are $6 \times 10^5$ and $4 \times 10^4$ $M^{-1}$ $s^{-1}$, respectively (*34*), we numerically solved the differential equations to estimate a contribution of superoxide dismutation on the level of experimentally measured superoxide production in our system at those two pH values. The increase in the rate of reduction of cytochrome $c$ resulting from the change in the dismutation rate is less than 0.1% of the reaction of superoxide with 20 $\mu$M cytochrome $c$ when the pH is changed from 7 to 8.

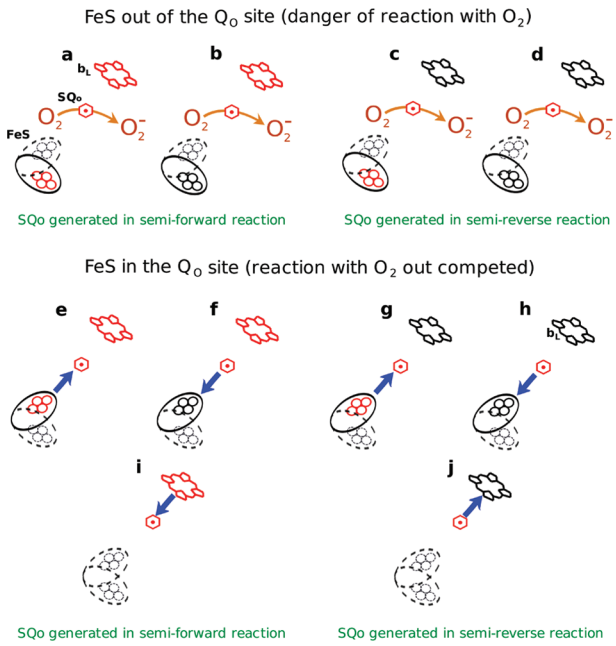


FIGURE 4: Short-circuits and leaks in the reversibly operating $Q_o$ site. Semiquinone ($SQ_o$) formed in semiforward (left panel) or semireverse (right panel) reaction undergoes rapid elimination by either oxygen (a−d), FeS in the $Q_o$ site (e−h), or heme $b_L$ (i and j). In panels a−j, the redox states after the initial formation of $SQ_o$ (hexagon with a dot) are colored red and black for the single-electron reduced and oxidized cofactor, respectively. Reaction of $SQ_o$ with oxygen in panels a−d is shown as a brown arrow; possible competitive reactions of $SQ_o$ with FeS or heme $b_L$ in panels e−j are shown as blue arrows.[3] For the sake of simplicity, FeS is shown at only two positions, in the $Q_o$ site (the $Q_o$ state) and out of the $Q_o$ site (the $c_1$ state). The "FeS out of the $Q_o$ site" should, however, be considered as a distribution of states (between the $Q_o$ site and the $c_1$ states) that lowers the density of FeS at the $Q_o$ site.

the $Q_o$ site a sufficient number of times to make such an accumulation possible.

Indeed, the motion of the FeS head domain may act to transiently "open" the $Q_o$ site for the reaction with oxygen. In this mechanism, the FeS center leaving the $Q_o$ site dangerously exposes single electrons of $SQ_o$ to an external oxidant by suppressing a completion of certain short-circuit reactions that otherwise effectively remove $SQ_o$ instead of oxygen. This should be regarded as a strictly kinetic effect of slowing the electron exchange within the $Q_o$ site cofactors caused by a transient increase in the distance between FeS and the remaining portion of the site (*33*). Figure 4 explains how, according to the mechanism described above, $SQ_o$ can be eliminated by either the reaction with oxygen or a completion of short-circuits. Those types of reactions inherently depend on the type of reaction that initially generates semiquinone in the $Q_o$ site.

*Competition of Short-Circuits with Leaks on Oxygen during the Forward and Reverse Electron Flow.* In the reversibly operating cytochrome $bc_1$, when electron flow through the c- and/or b-chain is impeded and the FeS center- and heme $b_L$-mediated two-electron oxidation/reduction of quinol/quinone at the $Q_o$ site is uncoupled, semiquinone in the $Q_o$ site can in principle be formed in two ways. The commonly evoked scenario assumes that the oxidized FeS center, as a part of a forward electron flow, withdraws a single electron from quinol bound to the $Q_o$ site even when heme $b_L$ is already reduced, and thus unable to support the thermodynamically coupled consumption of the second electron (*5, 17, 18, 21*) (we refer to it as the "semi-forward reaction"). The semiquinone so formed is highly unstable and can easily pass the electron to oxygen generating superoxide. Alternatively, the reduced heme $b_L$ can be a primary source of single electrons that reduce quinone to semiquinone as a part of the reverse electron flow (we refer to it as the "semi-reverse reaction"), and this semiquinone again can generate superoxide. Indeed, evidence of the involvement of heme $b_L$ in physiologically relevant production of superoxide has recently been presented from the observation that increasing the concentration of quinone increases the level of superoxide production by mitochondrial complex III (*24*).

Since the initial formation of $SQ_o$, under conditions of impeded electron flow, constitutes a first step of short-circuits, the subsequent reaction of $SQ_o$ with oxygen faces several competitive reactions that complete the short-circuit and effectively decrease the $SQ_o$ lifetime. Those reactions involve both the FeS center and heme $b_L$ (Figure 4).

The formation of $SQ_o$ in the semiforward reaction yields the state with reduced FeS and reduced heme $b_L$. The reduced FeS can return the electron back to SQ to re-form quinol (Figure 4e) or leave the $Q_o$ site toward heme $c_1$ (Figure 4a) and return in the reduced (Figure 4, back to reaction e) or oxidized form (Figure 4f), passing through the state in Figure 4b. The movement of FeS delays its possible participation in the removal of $SQ_o$, which means that in the states where FeS is not in the $Q_o$ site the probability of generation of superoxide by $SQ_o$ increases (Figure 4a,b). In the meantime, however, heme $b_L$ is ready to give away its electron reducing $SQ_o$ to quinol (Figure 4i); thus, if superoxide is to be formed in the states where FeS is not in the $Q_o$ site, reaction i (Figure 4) must be suppressed to the point that oxygen can effectively compete with it.

The distribution of all those states is greatly simplified in the knockouts. Suppression of the oxidation of FeS in the $c_1$ knockout shifts this distribution so that the contributions of reactions b and f (Figure 4) are much smaller than those of reactions a and e (Figure 4). Arresting the FeS in the $Q_o$ position further narrows the distribution of the two latter states, by increasing the probability of reaction e versus reaction a (Figure 4). With these considerations, we can explain the observed variations in the superoxide production in our system using the "FeS motion-driven unblocking" mechanism. The forms with frequently occurring "opened" states in panel a or b of Figure 4 (wild type and the $c_1$ knockout) produce detectable superoxide, while those with infrequently occurring states in panels a and b of Figure 4 (the FeS motion knockout) do not.

The formation of $SQ_o$ by heme $b_L$ in the semireverse reaction yields the state with oxidized heme $b_L$. $SQ_o$ can give an electron back to heme $b_L$ (Figure 4j). It can also reduce oxidized FeS (Figure 4h) or withdraw an electron from reduced FeS (Figure 4g) provided that the FeS center is in the $Q_o$ site at the time of $SQ_o$ formation. However, if this

[3] It is useful to refer the states described in this figure to the states described in Figure 6 of ref *6*. Reaction f is a third step of short-circuit 6c (*6*). Reaction h is a second step of short-curcuit 6f (*6*). Reaction i is a second step of short-circuit 6e (*6*). Reaction g is a part of reverse catalytic reaction 6b (*6*). Reactions e and j bring the $Q_o$ site to the state before the formation of $SQ_o$.

condition is not met (i.e., FeS is not in the $Q_o$ site at the time of $SQ_o$ formation), there is a chance that reaction with oxygen outcompetes the electron exchange within the $Q_o$ site (Figure 4c,d) simply because an increase in the distance between FeS and the remaining portion of the $Q_o$ site slows the rate of electron exchange between FeS and the occupant of the $Q_o$ site and thereby reduces the likelihood that FeS participates in the immediate removal of $SQ_o$.

In analogy to the previously described situation, the distribution of all those states is greatly simplified in the knockouts. The $c_1$ knockout shifts this distribution so that the contributions of reactions d and h (Figure 4) are much smaller than those of reactions c and g (Figure 4). The FeS motion knockout increases the probability of reaction g versus reaction c (Figure 4). Again, using arguments similar to those described above, we can understand the observed variations in superoxide production. Only the forms with the frequently occurring "opened" states of panel c or d of Figure 4 (wild type and the $c_1$ knockout) produce detectable superoxide.

We thus come to the conclusion that, irrespective of the origin of the $SQ_o$ (i.e., whether it is formed in a semiforward or semireverse reaction), the $Q_o$ site with the semiquinone faces the increased possibility of leaks of electrons to oxygen when the FeS head domain transiently leaves the site (Figure 4a−d). Apparently, even when the FeS domain moves unperturbedly (microseconds), the risk of an occurrence of the state with SQ at the $Q_o$ site and FeS away from the site does exist, as reflected by the various levels of superoxide production seen in the wild type and the $c_1$ knockout.[4]

Indeed, the variations in the production of superoxide by cytochrome $bc_1$ observed by us and others could reflect variations in the distribution of all states described in Figure 4 and, thus, result from reactions involving $SQ_o$ produced in both semiforward and semireverse reactions. It is, however, worth mentioning that there is one significant difference between the states encountered by the enzyme after formation of $SQ_o$ in semiforward and semireverse reactions. In the first case, when $SQ_o$ is formed due to the presence of oxidized FeS in the $Q_o$ site, reduced heme $b_L$ is already there close to $SQ_o$ and in readiness to give away its electron, while in the second case, $SQ_o$ can be formed when FeS is remote from the $Q_o$ site and, thus, unable to immediately participate in the reaction. This transient asymmetry in the geometrical arrangement of the $Q_o$ site cofactors at the time of $SQ_o$ formation could in principle shift the weight more toward the importance of the semireverse mode of $SQ_o$ formation in superoxide generation by cytochrome $bc_1$, in agreement with a recent proposal of a physiological significance of the heme $b_L$-driven mechanism of superoxide production in living cells (*24*).

One may speculate that the natural design of the $Q_o$ site has evolved to maintain the short-circuits at rates safely relegated from the catalytic time scale but, under conditions of impeded electron transfer, efficiently competing with the rate of the leak of electrons to oxygen. With such a defense mechanism, the living cells would compromise the energy-wasting formation of damaging reactive oxygen species with the energy-wasting but leak-proof short-circuits. The overall effects can be similar to those seen in the knockouts, which revealed that hypoxic-like (*16*) blocking of an oxidation of cytochrome *c* and the outflow of electrons from the c-chain while maintaining the influx of quinol presents a real danger of leaks of electrons to oxygen at the level of cytochrome $bc_1$. The mechanism developed here makes this understandable.

[4] With an unknown mechanism, the two- or three-step short-circuits of the $Q_o$ site are suppressed to seconds, most likely by the first electron transfer reaction (formation of $SQ_o$). The motion of FeS decreases the probability of its participation in the rapid removal of $SQ_o$ (by decreasing the density of FeS at the $Q_o$ site) to the point that oxygen can compete with it. If native FeS moves with a rate of approximately $3.3 \times 10^4$ $s^{-1}$, as suggested (*27*), the maximum rate of interaction of $SQ_o$ with oxygen should be approximately $1 \times 10^5$ $s^{-1}$ to match the maximum level of superoxide production of 36% observed in the $c_1$ knockout with antimycin.

## ACKNOWLEDGMENT

We thank Prof. Fevzi Daldal (University of Pennsylvania, Philadelphia, PA) for providing us with the mutated *Rhodobacter capsulatus* strains used in this work.

## REFERENCES

1. Nicholls, D. G., and Ferguson, S. J. (2002) *Bioenergetics*, Vol. 3, Academic Press, New York.
2. Mitchell, P. (1975) Protonmotive redox mechanism of the cytochrome b-$c_1$ complex in the respiratory chain: Protonmotive ubiquinone cycle. *FEBS Lett. 56*, 1–6.
3. Brandt, U., and Trumpower, B. L. (1994) The protonmotive Q cycle in mitochondria and bacteria. *Crit. Rev. Biochem. Mol. Biol. 29*, 165–197.
4. Darrouzet, E., Moser, C. C., Dutton, P. L., and Daldal, F. (2001) Large scale domain movement in cytochrome $bc_1$: A new device for electron transfer in proteins. *Trends Biochem. Sci. 26*, 445–451.
5. Muller, F., Crofts, A. R., and Kramer, D. M. (2002) Multiple Q-cycle bypass reactions at the $Q_o$ site of the cytochrome $bc_1$ complex. *Biochemistry 41*, 7866–7874.
6. Osyczka, A., Moser, C. C., Daldal, F., and Dutton, P. L. (2004) Reversible redox energy coupling in electron transfer chains. *Nature 427*, 607–612.
7. Osyczka, A., Moser, C. C., and Dutton, P. L. (2005) Fixing the Q cycle. *Trends Biochem. Sci. 30*, 176–182.
8. Rich, P. R. (2004) The quinone chemistry of *bc* complexes. *Biochim. Biophys. Acta 1658*, 165–171.
9. Crofts, A. R. (2006) Proton pumping in the $bc_1$ complex: A new gating mechanism that prevents short circuits. *Biochim. Biophys. Acta 1757*, 1019–1034.
10. Boveris, A., and Cadenas, E. (1975) Mitochondrial production of superoxide anions and its relationship to the antymycin insensitive respiration. *FEBS Lett. 54*, 311–314.
11. Ksenzenko, M., Konstantinov, A. A., Khomutov, G. B., Tikhonov, A. N., and Ruuge, E. K. (1983) Effect of electron transfer inhibitors on superoxide generation in the cytochrome $bc_1$ site of the mitochondrial respiratory chain. *FEBS Lett. 155*, 19–24.
12. Zhang, L., Yu, L., and Yu, C. A. (1998) Generation of superoxide anion by succinate-cytochrome *c* reductase from bovine heart mitochondria. *J. Biol. Chem. 273*, 33972–33976.
13. St-Pierre, J., Buckingham, J. A., Roebuck, S. J., and Brand, M. D. (2002) Topology of superoxide production from different sites in the mitochondrial electron transport chain. *J. Biol. Chem. 277*, 44784–44790.
14. Muller, F. L., Liu, Y., and Van Remmen, H. (2004) Complex III releases superoxide to both sides of the inner mitochondrial membrane. *J. Biol. Chem. 279*, 49064–49073.
15. Chen, Q., Vazquez, E. J., Moghaddas, S., Hoppel, C. L., and Lesnefsky, E. J. (2003) Production of reactive oxygen species by mitochondria. Central role of complex III. *J. Biol. Chem. 278*, 36027–36031.
16. Guzy, R. D., and Schumacker, P. T. (2006) Oxygen sensing by mitochondria at complex III: The paradox of increased reactive oxygen species during hypoxia. *Exp. Physiol. 91*, 807–819.
17. Snyder, C. H., Gutierrez-Cirlos, E. B., and Trumpower, B. L. (2000) Evidence for a concerted mechanism of ubiquinol oxidation by the cytochrome $bc_1$ complex. *J. Biol. Chem. 275*, 13535–13541.

18. Muller, F. L., Roberts, A. G., Bowman, M. K., and Kramer, D. M. (2003) Architecture of the $Q_o$ site of the cytochrome $bc_1$ complex probed by superoxide production. *Biochemistry 42*, 6493–6499.
19. Forquer, I., Covian, R., Bowman, M. K., Trumpower, B. L., and Kramer, D. M. (2006) Similar transition states mediate the Q-cycle and superoxide production by the cytochrome $bc_1$ complex. *J. Biol. Chem. 281*, 38459–38465.
20. Sun, J., and Trumpower, B. L. (2003) Superoxide anion generation by the cytochrome $bc_1$ complex. *Arch. Biochem. Biophys. 419*, 198–206.
21. Cape, J. L., Bowman, M. K., and Kramer, D. M. (2006) Understanding the cytochrome *bc* complexes by what they don't do. The Q cycle at 30. *Trends Plant Sci. 11*, 46–55.
22. Cape, J., Bowman, M. K., and Kramer, D. M. (2007) A semiquinone intermediate generated at the $Q_o$ site of the cytochrome $bc_1$ complex: Importance for the Q-cycle and superoxide production. *Proc. Natl. Acad. Sci. U.S.A. 104*, 7887–7892.
23. Cape, J. L., Strahan, J. R., Lenaeus, M. J., Yuknis, B. A., Le, T. T., Shepherd, J. N., Bowman, M. K., and Kramer, D. M. (2005) The respiratory substrate rhodoquinol induces Q-cycle bypass reactions in the yeast cytochrome $bc_1$ complex. Mechanistic and physiological implications. *J. Biol. Chem. 280*, 34654–34660.
24. Drose, S., and Brandt, U. (2008) The mechanism of mitochondrial superoxide production by the cytochrome $bc_1$ complex. *J. Biol. Chem. 283*, 21649–21654.
25. Atta-Asafo-Adjei, E., and Daldal, F. (1991) Size of the amino acid side chain at position 158 of cytochrome *b* is critical for an active cytochrome $bc_1$ complex and for photosynthetic growth of *Rhodobacter capsulatus*. *Proc. Natl. Acad. Sci. U.S.A. 88*, 492–496.
26. Gray, K. A., Davidson, E., and Daldal, F. (1992) Mutagenesis of methionine-183 drastically affects the physicochemical properties of cytochrome $c_1$ of the $bc_1$ complex of *Rhodobacter capsulatus*. *Biochemistry 31*, 11864–11873.
27. Darrouzet, E., Valkova-Valchanova, M. B., Moser, C. C., Dutton, P. L., and Daldal, F. (2000) Uncovering the [2Fe2S] domain movement in cytochrome $bc_1$ and its implications for energy conversion. *Proc. Natl. Acad. Sci. U.S.A. 97*, 4567–4572.
28. Valkova-Valchanova, M. B., Saribas, A. S., Gibney, B. R., Dutton, P. L., and Daldal, F. (1998) Isolation and characterization of a two-subunit cytochrome $b$-$c_1$ subcomplex from *Rhodobacter capsulatus* and reconstitution of its ubihydroquionone oxidation ($Q_o$) site with purified Fe-S protein subunit. *Biochemistry 37*, 16242–16251.
29. Glaser, E. G., Meinhardt, S. W., and Crofts, A. R. (1984) Reduction of cytochrome *b*-561 through the antymycin-sensitive site of the ubiquinol-cytochrome $c_2$ oxidoreductase complex of *Rhodopseudomonas sphaeroides*. *FEBS Lett. 178*, 336–342.
30. Robertson, D. E., and Dutton, P. L. (1988) The nature and magnitude of the charge-separation reactions of ubiquinol cytochrome $c_2$ oxidoreductase. *Biochim. Biophys. Acta 935*, 273–291.
31. Zhang, H., Osyczka, A., Dutton, P. L., and Moser, C. C. (2007) Exposing the complex III Qo semiquinone radical. *Biochim. Biophys. Acta 1767*, 883–887.
32. Robertson, D. E., Giangiacomo, K. M., de Vries, S., Moser, C. C., and Dutton, P. L. (1984) Two distinct quinone-modulated modes of antymycin-sensitive cytochrome *b* reduction in the cytochrome $bc_1$ complex. *FEBS Lett. 178*, 343–350.
33. Page, C. C., Moser, C. C., Chen, X., and Dutton, P. L. (1999) Natural engineering principles of electron tunneling in biological oxidation-reduction. *Nature 402*, 47–52.
34. Bielski, B. H. J., Cabelli, D. E., Arudi, R. L., and Ross, A. B. (1985) Reactivity of $HO_2/O_2^-$ radicals in aqueous solution. *J. Phys. Chem. Ref. Data 14*, 1041–1100.

BI801207F