Oxidation Process of Bovine Heart Ubiquinol-Cytochrome c Reductase as Studied by Stopped-flow Rapid-scan Spectrophotometry and Simulations Based on the Mechanistic Q Cycle Model*

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Received for publication, December 3, 1996, and in revised form, April 14, 1997

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Stopped-flow rapid-scan spectrophotometry was employed to study complicated oxidation processes of ubiquinol-cytochrome c reductase (QCR) that was purified from bovine heart mitochondria and maximally contained 0.36 mol of ubiquinone-10/mol of heme c1. When fully reduced QCR was allowed to react with dioxygen in the presence of cytochrome c plus cytochrome c oxidase, the oxidation of b-type hemes accompanied an initial lag, apparently low potential heme b1, oxidized first, followed by high potential heme b1H. Antimycin A inhibited the oxidation of both b-type hemes. The oxidation of heme c1 was triphasic and became biphasic in the presence of antimycin A. On the other hand, starting from partially reduced QCR that was poised at a higher redox potential with succinate and succinate-cytochrome c reductase, the b-type hemes were oxidized immediately without a lag. When the ubiquinone content in QCR was as low as 0.1 mol/mol heme c1, the oxidation of the b-type hemes was almost suppressed. As the Q-deficient QCR was supplemented with ubiquinol-2, the rapid oxidation of b-type hemes was restored to some extent. These results indicate that a limited amount of ubiquinone-10 found in purified preparations of QCR is obligatory for electron transfer from the b-type hemes to iron-sulfur protein (ISP) and heme c1.

The characteristic oxidation profiles of heme b1, heme b1H, and heme c1 were simulated successfully based on a mechanistic Q cycle model. According to the simulations the two-electron oxidation of ubiquinol-10 via the ISP and heme c1 pathway, which is more favorable thermodynamically than the bifurcation of electron flow into both ISP and heme b1, does really occur as long as heme b1L is in the reduced state and provides ubiquinone-10 at center i. Mechanistically this process takes time, thus explaining the initial lag in the oxidation of the b-type hemes. With the partially reduced QCR, inherent ubisemiquinone at center i immediately oxidizes reduced heme b1H thus eliminating the lag.

The mechanistic Q cycle model consists of 56 reaction species, which are interconnected by the reaction paths specified with microscopic rate constants. The simulations further indicate that the rate constants for electron transfer between the redox centers can be from 106 to 107 s–1 and are rarely rate-limiting. On the other hand, a shuttle of ubiquinone or ubiquinol between center o and center i and the oxidation of heme c1 can be rate-limiting. The interplay of the microscopic rate constants determines the actual reaction pathway that is shown schematically by the “reaction map.” Most significantly, the simulations support the consecutive oxidation of ubiquinol in center o as long as both heme b1 and heme b1H are in the reduced state. Only when heme b1 is oxidized and ISP is reduced can SQO donate an electron to heme b1. Thus, we propose that a kinetic control mechanism, or “a kinetic switch,” is significant for the bifurcation of electron flow.

Ubiquinol-cytochrome c oxidoreductase (QCR1; bc1 complex or complex III) is one of the three sites for energy conservation in the mitochondrial respiratory chain. This mitochondrial enzyme consisting of 11 subunits (1) translocates protons from the matrix side of mitochondrial inner membranes toward the cytoplasmic side coupled with electron transfer from ubiquinol to cytochrome c (2, 3). QCR contains four redox centers, two b-type hemes (heme b2sg2/β1h and heme b2sg1/β1h), a c-type heme (heme c1), and a 2Fe–2S cluster (ISP) (3). In mitochondria, low potential heme b1L is located near the outside surface of the inner membrane, whereas high potential heme b1H is situated inside the membrane about 20 Å away from heme b1L. ISP and heme c1 are largely extramembranous on the cytoplasmic side of the inner membrane (4). In addition to these four prosthetic groups, ubiquinone-10 is usually found in purified QCR prepared from mammalian mitochondria (5, 6).

Previous work on the mechanism of electron transfer and proton translocation by QCR revealed characteristic features that lead to various hypothetical mechanisms (7, 8). To date, the protonmotive Q cycle, originally proposed by Mitchell (2), has become the generally accepted hypothesis. The central idea of the cyclic model is “branching” of electron flow from ubiquinol that delivers an initial electron to ISP at center o on the cytoplasmic side, one of the assumed Q-reactive centers. This electron is transferred from ISP to heme c1, and then to cytochrome c. Ubisemiquinone (SQ) thus generated is an intermediate and subsequently donates an electron to low potential heme b1L, at center o yielding quinone in turn. Then, quinone is...
translocated to center i, another Q-reactive site on the matrix side, and reduced to SQ, by accepting an electron from high potential heme b$_{1/2}$ to which the electron has been transferred from heme b$_{1}$ (3, 9, 10). Ubiquinone at center o or i is usually referred to as Q, or Q$_{c}$, respectively. These reactions constitute the first half of the Q cycle. In the second half, the oxidation of another molecule of quinol at center o proceeds following the same processes as before and provides a second electron at center i to reduce SQ, to quinol. The quinol then goes back to center o completing the promotomotive Q cycle. Thus bifurcation of electron flow from ubiquinol into ISP and heme b$_{1}$ at center o as well as electrogenic electron transfer from heme b$_{1}$ to heme b$_{1/2}$ is most pivotal in the energy conservation mechanism because this supports proton translocation across the membrane.

Despite a considerable experimental support for the promotomotive Q cycle, there are still some observations that are not necessarily compatible with this model (11–15). The oxidation of reduced QCR purified from yeast or bovine heart mitochondria by oxidants has been reported to occur even when the endogenous ubiquinone is depleted from the enzyme complex (11, 12). This result is apparently inconsistent with the proposed functional role of Q not only as a proton translocator but also as an electron mediator between b-type hemes and ISP. Also, it has been reported that no lag appears in the oxidation of b-type hemes when fully reduced yeast QCR is oxidized by ferricyanide (12), or of heme b in rat liver mitochondria by ferricyanide plus air (13). The latter result forms the basis for a proposed semiquinone cycle that assumes a shuttle of ubisemiquinone between center o and center i (8). According to the Q cycle mechanism, the oxidation of the b-type hemes initiated by pulsing an oxidant to the fully reduced QCR does not occur immediately; Q that is required to oxidize heme b$_{1/2}$ at center i is lacking because further oxidation of an intermediate SQ$_{c}$ by heme b$_{1}$ at center o is blocked as this heme is in the reduced state initially. Therefore, it is important to confirm experimentally whether the oxidation of b-type hemes accompanies a lag or not and to examine the obligatory role of ubiquinone in this oxidation (16–18).

In previous studies (11–13), nonphysiological oxidants such as ferricyanide and a cobalt chelate were used mostly as an electron acceptor for QCR. Although precautions to eliminate their direct interactions especially with the heme(s) in QCR were taken, the possibility has been pointed out that ferrous heme b$_{1}$ is accessible to ferricyanide at the outer side of submitochondrial particles (19). This problem would be resolved if we use cytochrome c plus cytochrome c oxidase (CCO) as the oxidizing system. On the other hand, spectral complexities that are introduced by use of this system and accordingly hamper the data analyses will be lessened by recording all the spectral changes with rapid-scan spectrophotometry. A series of absorption spectra collected during a reaction and displayed around 10 ms, and the end of this period was chosen as time 0 for recording the data. The time of the apparatus under the present experimental conditions was 12 ms, and usually 100 spectra were recorded with a length range of 12 ms. All spectra were recorded at room temperature.

**EXPERIMENTAL PROCEDURES**

Succinate-cytochrome c reductase (27), ubiquinol-cytochrome c reductase (6), and cytochrome c oxidase (20) were purified from beef heart muscle according to previously reported methods and stored at −80 °C until use. Succinate-cytochrome c reductase (SCR) contained about 3.5 nmol of heme b/mg of protein and exhibited the electron transfer activity of 70–90 nmol of cytochrome c reduced·min·mg−1·protein−1·mol·heme−1·min−1 in a medium containing 0.1 m sodium phosphate buffer (pH 7.4), 0.3 mM EDTA, 20 mM succinate, and 100 μM cytochrome c (28, 29). Purified preparations of ubiquinol-cytochrome c reductase (QCR) contained 4.2–4.5 nmol of heme c$_{1}$/mg of protein and exhibited the catalytic activity of 330–410 nmol of cytochrome c reduced·min·mol·c$_{1}$−1·mol·heme−1·min−1 when measured in an assay mixture containing 50 mM sodium phosphate buffer (pH 7.0), 0.3 mM EDTA, 50 μM cytochrome c, and 25 μM Q$_{10}$H$_{4}$ at 20 °C (6, 30). The initial rate of cytochrome c reduction was corrected against nonenzymatic reduction of cytochrome c by ubiquinol in the absence of QCR. The Q$_{c}$ content of QCR varied with enzyme preparations ranging from 0.1 to 0.36 mol of Q$_{c}$/mol of heme c$_{1}$. The purity and the enzyme activity of cytochrome c oxidase (CCO) were the same as reported previously (20).

Cytochrome c (horse heart, type III) and antimycin A were purchased from Sigma. Cholic acid was obtained from Wako Pure Chemicals (Tokyo, Japan). Other chemicals were obtained commercially at the highest purity.

Stopped-flow rapid-scan recordings with and without a laser flash were carried out on an apparatus as described previously (20–22). With this apparatus the recording time of a spectrum over a 200-nm wavelength range was 12 ms, and usually 100 spectra were recorded with a minimal time interval of 30 ms including the recording time. The dead time of the apparatus under the present experimental conditions was around 10 ms, and the end of this period was chosen as time 0 for displaying spectral changes.

Two different methods were employed to prepare sample solutions for stopped-flow and rapid-scan measurements as follows. Method 1: purified QCR was diluted with 50 mM sodium phosphate buffer (pH 7.4) containing 0.25% sodium cholate to give a final concentration of 6–7 μM heme c$_{1}$ and placed in one of the reservoirs of the stopped-flow apparatus. The solution was bubbled with N$_{2}$ gas for 15 min at 20 °C to attain anaerobic conditions. The fully reduced form of QCR was prepared by addition of a slight excess of sodium dithionite, and allowed to stand for another 15 min at 20 °C under N$_{2}$ atmosphere. Then the temperature was lowered to 10 °C. The other reservoir contained cytochrome c (1.0 μM) and CCO (6–7 μM aa$_{3}$) in an air-saturated solution consisting of 50 mM sodium phosphate buffer (pH 7.4) and 0.25% sodium cholate. After incubation for 10 min at 10 °C, the solutions in both reservoirs were

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mixed at a 1:1 ratio to start the reaction, and spectral changes were recorded by rapid-scan spectrophotometry at an appropriate time interval. Method 2: a mixture containing QCR (6–7 μM cytochrome c1), 1 μM cytochrome c, and CCO (6–7 μM aa3) in a 0.25% sodium cholate, 50 mM sodium phosphate buffer (pH 7.4) was placed in one reservoir and bubbled with a gas mixture of CO and N2 (1:4) for 10 min at 20 °C. A slight excess of sodium dithionite was added to reduce all redox components before further incubation for 15 min at 20 °C. Then the temperature was brought to 10 °C. In the other reservoir, an air-saturated solution containing 50 mM sodium phosphate buffer (pH 7.4), 0.25% sodium cholate, and no protein components was placed, and the temperature was equilibrated at 10 °C. The two solutions in the reservoirs were mixed to flow at a 1:1 ratio into the observation cell of the stopped-flow apparatus, and the mixture in the cell was irradiated with a laser flash of 587 nm to initiate the redox reaction by photolyzing the cytochrome c oxidase–CO complex. The two methods gave essentially the same result in terms of the oxygen reaction.

Partially reduced QCR was prepared by using a catalytic amount of SCR (0.5 μM cytochrome b) and 10 mM sodium succinate as a reducing system. After reduction of QCR with succinate for 30 min at 20 °C, sodium malonate was added (12 mM, final) to block electron transfer to the air-saturated solution containing cytochrome c. The concentration of respiratory enzymes and the concentration of cytochrome components before further incubation for 15 min at 20 °C. Then the temperature was brought to 10 °C. In the other reservoir, an air-saturated solution containing 50 mM sodium phosphate buffer (pH 7.4), 0.25% sodium cholate, and no protein components was placed, and the temperature was equilibrated at 10 °C. The two solutions in the reservoirs were mixed to flow at a 1:1 ratio into the observation cell of the stopped-flow apparatus, and the mixture in the cell was irradiated with a laser flash of 587 nm to initiate the redox reaction by photolyzing the cytochrome c oxidase–CO complex. The two methods gave essentially the same result in terms of the oxygen reaction.

Results and Discussion

Reaction of Fully Reduced QCR with Dioxygen in the Presence of Cytochrome c Plus CCO—Fig. 1A shows three-dimensional display of spectral changes recorded on mixing dithionite-reduced QCR (0.36 mol of Q10/mol of heme c1) with an air-saturated solution containing cytochrome c and CCO in the stopped-flow rapid-scan apparatus. The concentration of cytochrome c was 6–7-fold lower than that of CCO or of QCR to minimize its spectral contribution to the absorbance change of heme c1 around 553 nm and to control the rate of its oxidation. An excess of CCO is expected to keep cytochrome c almost in the oxidized state during the redox reaction. The first spectrum taken immediately after the mixing, or at time 0, shows that QCR is almost in the reduced state with a peak of heme b components at 562 nm and a shoulder of heme c1 at 553 nm. A broad absorption band around 600 nm is due to the oxidized form of CCO. As the reaction proceeds, the 562-nm peak becomes smaller after a short lag period with a growth of the 604-nm peak. This result indicates that mixing of fully reduced QCR with cytochrome c and CCO under the air brought about the oxidation of hemes b in QCR and electron transfer to CCO. It should be noted that a fairly large portion of heme b still remained in the reduced state at 3 s (Fig. 1A and B).

Based on the ubiquinone content of the samples employed, we simply assume that maximally 36% QCR in the purified preparation contains a full set of redox centers; that is, 1 mol each of Q10 (2), heme b1(1), heme b2(1), heme c1(1), and ISP(1). In parentheses the maximal number of reducing equivalents that can be retained is given. Accordingly, fully reduced QCR contains six reducing equivalents. It is proposed that center a accommodates two ubiquinone molecules at domains Q1aa and Q1low that have a strong and weak interaction with ubiquinone, respectively (24, 36). The stoichiometry of the present preparation shows that only one of them is retained probably at Q1aa. On reaction with the oxidizing system, QCR equipped with a full set of redox centers can be oxidized rapidly compared with the incomplete complex. This is in accord with...
the experimental result indicating that a fairly large portion of heme b remains in the reduced state after the rapid oxidation (Fig. 1, A and B) as described above. According to the Q cycle mechanism ubiquinone is supposed to be released from QCR to move from center o to center i or vice versa (2, 3). But it is less likely that a limited amount of ubiquinone-10 found in the purified preparation of QCR swims around in a detergent medium to mediate translocation of protons between centers o and i. Rather, it is more likely for ubiquinone to mediate proton translocation without being released from the QCR complex. Therefore, we postulate in QCR a big ubiquinone pocket that contains centers i and o, as assumed by Wikström and Krab (8). Ubiquinone and ubiquinol (not ubisemiquinone) may be able to make contact alternatively with o and i domain in the ubiquinone pocket through a “flip-flop” motion. Such a pocket may be provided by a dimeric structure of this complex as described previously. Further studies are required to find out whether this kind of mechanism is functioning in the catalytic reaction of QCR in phospholipid membranes. Also the x-ray crystallographic studies that are in progress will soon reveal these essential features (4, 37–39).

The time courses of absorbance difference at 566–575 nm, 554–540 nm, and 604–630 nm that monitor the redox change of hemes b, heme c1, and CCO, respectively (Fig. 1B), were derived from the data shown in Fig. 1A. Heme b components in QCR were oxidized with an initial lag, and a half-time for the oxidation was about 150 ms. Electron transfer to CCO occurred in the same time range as shown by an initial absorbance increase at 604–630 nm. The oxidation of heme c1 was triphasic; the rapid oxidation and re-reduction that occurred within 300 ms after initiation of the reaction was followed by a slow decrease in absorbance for heme c1. A decline of the absorbance of CCO was slower than heme c1. As the oxidation of heme b with a lag and the triphasic oxidation of heme c1 were already observed by de Vries et al. (40) who employed yeast mitochondrial membranes, the present result confirms their finding.

Fig. 1C shows the time difference spectra derived from the data in Fig. 1A. A difference spectrum between 0 and 150 ms shows a peak at 565 nm and a deep trough at 605 nm (spectrum 1 in Fig. 1C), indicating that heme b1 in QCR is oxidized first accompanied by electron transfer to CCO. Tsai et al. (12) demonstrated, by using a rapid freezing EPR technique, that low potential heme b2 was oxidized more rapidly than high potential heme b1 during reaction of fully reduced yeast QCR with Q1. The peak of heme b1 shifts to 563 nm in the difference spectra of 0.15–0.3 and 0.3–0.6 s (spectra 2 and 3), indicating that the oxidation of heme b11 dominates after heme b2 has been oxidized to an appreciable extent. The oxidation of heme c1 in a later stage is apparent as indicated by the peak at 553 nm in the difference spectra of 0.6–1.5 and 1.5–3 s (spectra 4 and 5).

Fig. 2 shows the effect of antimycin A on the oxidation of QCR (0.36 mol of Q10/mol of heme c1). Fully reduced QCR was prepared in the presence of antimycin A and mixed with an air-saturated solution containing cytochrome c plus CCO to initiate the reaction. There was no sign for the oxidation of b-type hemes, and the oxidation of heme c1 was biphasic. When the reduction of hemes b was incomplete by chance, an oxidant-induced reduction of heme b1 occurred although its extent relative to the total heme b was less than 5% (Fig. 2). An absorbance increase of CCO at 604 nm due to electron transfer from QCR proceeded along with the oxidation of heme c1, followed by its gradual decay as in Fig. 1.

Oxidation of QCR with a Low Q10 Content—Fig. 3A shows the redox behavior of fully reduced QCR (0.1 mol of Q10/mol of heme c1) upon reaction with oxygen in the presence of cytochrome c and CCO. When the reaction was initiated by the flow-flash method (20, 41), CCO was oxidized completely within a few milliseconds at 10 °C (20). Since this oxidation is much faster than that of fully reduced QCR so far examined, no change was seen in QCR during the complete oxidation of CCO. Then, the shoulder at 553 nm disappeared accompanying an increase in absorbance at 604 nm, whereas the peak at 562 nm remained unchanged (Fig. 3A). This result indicates that hemes b in QCR with a low Q10 content were not oxidized by the cytochrome c/CCO system in the time range examined. However, it should be noted that QCR with the low Q10 content is catalytically active enough to exhibit the electron transfer activity of about 350 nmol of cytochrome c reduceds−1 (mol heme c1)−1 when assayed in the presence of 25 μM QH2 and 50 μM cytochrome c as described under “Experimental Procedures.” In accordance with this, when QCR (0.1 mol of Q10/mol of heme c1) was supplemented with exogenous ubiquinone-2 (Q2) prior to reduction with sodium dithionite, the oxidation of hemes b was restored to some extent (Fig. 3B). At the same time, re-reduction of heme c1 was recognized in a later stage. Antimycin A inhibited this oxidation. Therefore, it seems that the exogenous Q2 fulfills the role of intrinsic Q10 to a certain extent. These results clearly indicate that ubiquinone is an essential component for electron transfer from hemes b of QCR to CCO via cytochrome c. Previously, we reported that in ascorbate-reduced QCR reconstituted into potassium-loaded phospholipid vesicles, a potassium diffusion potential induced the

![Graph](image-url)
reduction of heme components and that ubiquinone was required for reversed electron transfer from heme c1 to heme b (42). These findings were confirmed recently (43), again supporting this essential role of ubiquinone.

The above conclusion is inconsistent with the previous reports that the oxidation of fully reduced QCR by oxidants was not affected even when the endogenous ubiquinone was depleted from the enzyme (11, 12). It should be noted, however, that either ferricyanide or cobalt chelate was used in these studies. In fact, using the QCR preparation that contained 0.1 mol of Q10/mol of heme c1 we also observed that both heme b and heme c1 were oxidized rapidly when the fully reduced form was reacted with 250 eq of ferricyanide (data not shown). This result contrasts with the non-oxidizability of hemes b when cytochrome c plus CCO was the oxidizing system (Fig. 3A) but agrees with the oxidation of these components in yeast QCR that was brought about by 500 eq of ferricyanide (12). Thus, the discrepancy between our results and previous ones (11, 12) would be ascribed to the direct oxidation of heme b components in QCR by nonphysiological oxidants, as pointed out previously (19).

FIG. 3. Oxidation of fully reduced QCR with a lower Q10 content by oxygen. A, the aerobic oxidation of Na₂S₂O₄-reduced QCR (0.1 mol of Q₁₀/mol of heme c₁) by oxygen in the presence of cytochrome c, and CCO was measured according to Method 2 as described under "Experimental Procedures." The concentrations of QCR, cytochrome c, and CCO after mixing were 3.0, 0.55, 3.0 μM, respectively. Initial 50 spectra are presented at the time interval of 60 ms. B, the enzymes were reduced completely with Na₂S₂O₄ in the presence of exogenously added Q₉ (Q₉/heme c₁ = 5.0). Other conditions were as in A.

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Summary of Experimental Observations—Stopped-flow rapid-scan spectrophotometric studies on the oxidation kinetics of reduced QCR have revealed characteristic behavior of the re-

FIG. 4. Oxidation of partially reduced QCR. A, QCR (6.0 μM heme c₁) containing 0.36 Q₁₀/mol of heme c₁ was reduced partially with succinate in the presence of cytochrome c and CCO under aerobic conditions. Partially reduced QCR was prepared by incubation of QCR with succinate in the presence of a catalytic amount of succinate-cytochrome c reductase (SCR). Under this condition heme c₁, iron-sulfur protein, and most of heme bₜₜ were reduced but not heme b₁ as reported previously (44). No initial lag was observed during oxidation of heme bₜₜ as shown by a trace at 562–575 nm (C, hemes b), and 554–564 nm (C, heme c₁) were obtained.

Oxidation of Partially Reduced QCR—Fig. 4A illustrates the redox behavior of partially reduced QCR (0.36 mol of Q₁₀/mol of heme c₁) upon reaction with cytochrome c plus CCO under aerobic conditions. Partially reduced QCR was prepared by incubation of QCR with succinate in the presence of a catalytic amount of succinate-cytochrome c reductase (SCR). Under this condition heme c₁, iron-sulfur protein, and most of heme bₜₜ were reduced but not heme b₁ as reported previously (44). No initial lag was observed during oxidation of heme bₜₜ as shown by a trace at 562–575 nm (C, hemes b), and 554–564 nm (C, heme c₁) were obtained.
dox components in the complex as follows. (i) Starting from the fully reduced QCR the oxidation of \( b \)-type hemes accompanies an initial lag; apparently heme \( b_L \) is oxidized first, followed by heme \( b_H \). Antimycin A inhibits the oxidation. The oxidation of heme \( c_1 \) is triphasic and becomes biphasic in the presence of antimycin A. (ii) Q 10 is essential for electron transfer from \( b \)-type hemes to heme \( c_1 \) and ISP. (iii) Starting from partially reduced QCR the oxidation of \( b \)-type hemes occurs immediately without a lag.

In the sections that follow we try through simulations to explain these observations based on the Q cycle mechanism that we propose in a mechanistic model. It is expected that the redox behavior of heme \( b_L \), heme \( b_H \), and heme \( c_1 \) as observed in the present study are reproduced well semiquantitatively, and the behavior of other components would be described too. This model also helps to test the validity of some ideas about the function of QCR and allows predictions.

**Reaction Model**—Fig. 5 illustrates the reaction scheme for simulating oxidation of QCR. Each box in this scheme represents a redox state of monomeric QCR that contains heme \( b_L \), heme \( b_H \), heme \( c_1 \), and ISP in addition to ubiquinone-10. The location of every species in the box reflects their physical arrangements in QCR; heme \( b_L \), heme \( c_1 \), and ISP are on the cytoplasmic side of the mitochondrial inner membrane, and heme \( b_H \) is inside the membrane; ubiquinone is situated on either center \( o \) near heme \( b_L \) or center \( i \) near heme \( b_H \). Thus, electron transfer is assumed to occur between one redox center and the nearest neighbor.

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**FIG. 5.** The mechanistic Q cycle model for simulating oxidation of QCR. Each box in this scheme represents a redox state of monomeric QCR that contains heme \( b_L \), heme \( b_H \), heme \( c_1 \), and ISP in addition to ubiquinone-10. The location of every species in the box reflects their physical arrangements in QCR; heme \( b_L \), heme \( c_1 \), and ISP are on the cytoplasmic side of the mitochondrial inner membrane, and heme \( b_H \) is inside the membrane; ubiquinone is situated on either center \( o \) near heme \( b_L \) or center \( i \) near heme \( b_H \). Thus, electron transfer is assumed to occur between one redox center and the nearest neighbor.
A decrease in an apparent rate constant for the oxidation of heme I. (i) The apparent rate constant for the oxidation of heme standing time by a mechanism that cannot be specified. 

b S53 to S56 still retain one reducing equivalent either on heme state, whereas the most oxidized states in the lower block from S53 to S56 still retain one reducing equivalent either on heme b or SQ, although actually this will be lost during a long standing time by a mechanism that cannot be specified.

Simulations of QCR Oxidation—Rate constants used for simulating the oxidation of fully reduced QCR are shown in Table I. (i) The apparent rate constant for the oxidation of heme c1, 40 s⁻¹, was estimated from three exponential analyses of the absorbance change at 554–540 nm in Fig. 1A. (ii) Rate constants for intramolecular electron transfer (ET) among redox centers in QCR were assumed to be in the order of 10⁵ s⁻¹ that belongs to the uppermost estimate in the previous studies (3, 24, 45–48). Simulations indicate, as will be discussed later, that the rate constants for ET can be in the orders lower than 10⁵ s⁻¹ because intramolecular ET is rarely rate-limiting. (iii) The potential differences for a redox pair exchanging an electron were derived from the values in Table I and used to estimate the relative rate constants for intramolecular ET. It is to be noted that no free energy change is assumed for the oxidation of ubiquinol by oxidized ISP (ΔEₘ = Eₘ, ISP - Eₘ, ISP = 0) (where Eₘ indicates midpoint redox potential). (iv) A decrease in an apparent rate constant for the oxidation of heme c₁ in later stages of the reaction was assumed appropriately to explain both a transient accumulation of reduced heme c₁ following the initial rapid oxidation and a slow oxidation that follows (Fig. 1). (v) Translocation of ubiquinone from center o to center i was equated with an appearance of a species with ubiquinone in center i and vice versa. Usually the rate constants for translocation of ubiquinone and ubiquinol, kₒ and kᵢₒ, were changed to give the satisfactory result. 

Fractional changes of ubiquinol, ubiquisemiquinone, ubiquinone, reduced ISP, reduced heme c₁, reduced heme b₁, and reduced heme b₁ were obtained by summing up every intermediate that contains either of these components (Table II).

Fig. 6 illustrates semiquantitative simulations of three cases, the oxidation of fully reduced QCR in the absence (Fig. 6A) and presence (Fig. 6B) of antimycin A and the oxidation of partially reduced QCR (Fig. 6C). Fig. 6A reproduces the triphasic oxidation of heme c₁, the oxidation of heme b₁ with a lag (Fig. 1B), and the oxidation of heme b₁ prior to heme b₁ (Fig. 1C) in the absence of antimycin A. The reaction model predicts that the oxidation of heme b₁ always accompanies a lag because either ubiquisemiquinone (SQ) or ubiquinone (Q) acts as a direct oxidant of reduced heme b₁ appears for the first time in the intermediates in R₄ (SQ₄) or in R₅ (Q₅).

In addition to the redox behavior of heme b₁, heme b₁, and heme c₁, Fig. 6A also simulates a rapid oxidation of ubiquinol with an apparent half-time of 100 ms. This is comparable to the oxidation of fully reduced QCR from yeast by ferricyanide (12). The oxidation profiles of ISP and heme c₁ are similar although heme c₁ is oxidized faster than ISP. The oxidation of heme b₁ starts with a lag and almost parallels that of ubiquinol. The oxidation of heme b₁ lags behind heme b₁. This order of oxidation is reverse to the initial oxidation of heme b₁ followed by heme b₁ that is assumed in the reaction scheme of Fig. 5. It is highly possible that the immediate filling of the oxidized heme b₁ with an electron from heme b₁ by rapid intramolecular ET gives a feature as if heme b₁ were oxidized first. The reduction level of heme b₁ stays around 20% for the rest of the reaction. In line with this, the SQ/Q ratio at 3 s after initiation of the reaction is about 4.

The effect of antimycin A on the oxidation of fully reduced QCR is simulated by assuming that the rate constant for the oxidation of reduced heme b₁ by ubiquinone in center i is zero (Fig. 6B). Although the oxidation of heme c₁ is simulated as a multiphasic change, it becomes apparently biphasic if the initial rapid changes, which proceed during the dead time of the stopped-flow apparatus, are neglected. The simulation satisfactorily reproduces the non-oxidizability of heme b₁ also. It is reported that inhibition of submitochondrial particles oxidizing superoxide anion by antimycin A induces superoxide anion generation, and the reaction of SQ with molecular oxygen is supposed to be the cause (49). This will occur competitively with the consecutive oxidation of SQ₄ through the high potential (ISP and heme c₁) pathway.

The reaction model also explains the oxidation of partially reduced QCR (Fig. 6C), which is equated to a mixture of S11 and S12. A model for this reaction consists of the intermediates
The reaction intermediates contributing to fractional populations of ubiquinol, ubisemiquinone, ubiquinone, reduced ISP, reduced heme c, reduced heme bL, and reduced heme bH are listed in Table I. Based on the common reaction model encourage us to explore the molecular characteristics of the Q cycle mechanism in more detail as follows.

Mechanistic Characteristics of the Q Cycle Model—The simulation shown by Fig. 6A can be resolved into the rise and decay of each intermediate in the reaction scheme (Fig. 5), and all the reaction intermediates are classified tentatively into six classes according to the maximal fraction that they attain in the time course (class 5 > class 4 > class 3 > class 2 > class 1 > class 0). Among 56 species, 46 intermediates belong to the lower five classes. The most significant 10 intermediates are S1, S2, S3, S4, S11, S18, S43, S44, S55, and S56 and cumulatively can explain most of the simulation result. Their fractional changes as a function of time are shown in Fig. 7A. Reaction intermediates on the upper block (Fig. 7A, top and middle) contribute to the change in the early stage of the oxidation, and those on the lower block (Fig. 7A, bottom) explain most of the later change. In addition to these intermediates, contributions to the reaction by the others are illustrated in Fig. 7B that will be called the reaction map hereafter. The intermediates on the upper block in the reaction scheme are funneled into the lower block through RS59 and RS67. A transient accumulation of S44 is most prominent approaching 0.64 in fraction, and this as well as S45 decays with time being replaced by a growth of S55 and S56 (Fig. 7A, bottom). An accumulation of SQ is mostly represented by S56, suggesting that SQ is stabilized in center i while heme bH remains in the oxidized state. On the contrary, S55 contains reduced heme bH. Contributions from more oxidized intermediates on the upper block (S39 and S40) to the spectral change are almost negligible, and the reduction of oxidized heme bH by SQ in center o that leads to RS59 (S27→S41) and RS67 (S34→S45) must play a crucial role in establishing the reaction pathways.

The simulation results are insensitive to the rate of an elementary ET step as long as the rate constant is between 10^5 and 10^3 s^{-1}, and the ratio between the forward and backward rate constants is kept constant. The simulations are more sensitive to the reaction steps with rate constants around 100 s^{-1} or below that correspond to the oxidation of reduced heme c, and translocation of ubiquinone and ubiquinol between center o and center i. The rate of oxidation of heme c, was intentionally reduced in the present experiments by decreasing the amount of cytochrome c, an electron mediator between QCR and CCO, and by lowering the temperature to 10 °C. Under this specified condition, the translocation rates of ubiquinone and ubiquinol seemingly are most important in controlling the reaction.

Effects of Thermodynamic Barriers on the Oxidation Kinetics—The simulation shown in Fig. 6A and Fig. 7 was done...
successfully by assuming no thermodynamic barrier for the initial oxidation of ubiquinol in center $o$ by oxidized ISP. Here a positive thermodynamic barrier is supposed for an endergonic reaction, whereas the negative reaction is for an exergonic process. Ding et al. (24), however, assumed that a thermodynamic barrier imposed by the SQ$_o$ formation ($\Delta E_m = E_m, SQ_{QH} - E_m, ISP = 0.23$ V) was essential for the bifurcation (24). Thus, the effects of imposing either the positive or negative thermodynamic barrier on the oxidation kinetics were examined based on the present reaction model. When the thermodynamic barrier was $\Delta E_m = 0.1$ V, the oxidation of heme $b_L$ and heme $b_H$ slowed down slightly, and re-reduction of ISP and heme $c_1$ was also suppressed slightly (Fig. 8A). The others are almost the same as in the case of $\Delta E_m = 0$ (Fig. 6A).

When the imposed barrier was $\Delta E_m = 0.25$ V, which was close to 0.23 V as suggested by Ding et al. (24), the oxidation of all redox components was suppressed appreciably (Fig. 8B). Remarkably the oxidation of ubiquinol itself lagged behind that of heme $b_L$ and heme $b_H$. This is caused mainly by suppression of ET from ubiquinol to ISP, due to a high thermodynamic barrier, even when both ISP and heme $c_1$ are in the oxidized state. Such a high barrier cannot exist because the simulation deviates appreciably from the experimental result (compare Fig. 8B with Fig. 1).

When the negative thermodynamic barrier was imposed ($\Delta E_m = -0.1$ V), the oxidation of heme $b_L$ and heme $b_H$ were accelerated appreciably. In accordance with this, the re-reduction of ISP and heme $c_1$ was also promoted (Fig. 8C). Fig. 9 depicts how ubisemiquinone is generated depending on the thermodynamic barrier imposed. SQ$_o$ is generated only once in the initial stage of the reaction, and this is shown by the time course of S4 because contributions from S6, S7, and S14 containing SQ$_o$ are negligible. On the other hand, SQ$_i$ is generated twice as shown by transient formation of S11, S18, S24, and S31 and by S44, S48, S52, and S56, although the extents of contribution are variable. Thus, generation of SQ$_o$ in the initial stage of the reaction is appreciable with $\Delta E_m = -0.1$ V as expected (Fig. 9A). Even when $\Delta E_m = 0$, SQ$_o$ is generated transiently to a maximal fraction of above 0.1 (Fig. 9B). Only with the positive barrier the level of SQ$_o$ becomes negligibly low (Fig. 9C). In this case only SQ$_o$ is detected. Therefore, if the non-occurrence of SQ$_o$ is proved by the ESR measurement (17, 29), it is a sure sign for the existence of the positive thermodynamic barrier although its advantage in controlling the reaction is not clear.

Thermodynamic Barrier Versus Kinetic Control—We have
The production of superoxide anion from mitochondrial particles that oxidize succinate in the presence of antimycin A (49) may be taken as a sign for the incomplete oxidation of ubiquinol and, accordingly, to indicate that further oxidation of SQ to Q is suppressed. In fact, the simulation with $\Delta G_m = 0$ indicates that SQ$_i$ is generated transiently to a maximal fraction of 0.13, and this may have a chance to react with molecular oxygen to generate superoxide anion. If SQ$_i$ escapes the attack by oxygen, it will be oxidized to ubiquinone through the high potential (ISP and heme c$_1$) pathway.

Reaction 2 occurs for S26 and S33 in which both ISP and heme b$_L$ are oxidized with ubiquinol being in center o. This is the situation that originated from ubiquinol bifurcation of electron flow into ISP and heme b$_L$ is expected to occur during turnover of QCR. First, starting from S26, RS38 proceeds because this is favorable thermodynamically. Then RS59, ET from SQ$_i$ to oxidized heme b$_L$, must follow as this is essential to explain the emergence of S43, S44, S55, and S56 on the lower block. It is to be noted that RS59 (and RS67) proceeds only when ISP is in the reduced state, and it is likely that this situation is brought about under a delicate kinetic control. Brandt et al. (25, 26) have proposed a catalytic switch mechanism as a prerequisite for the bifurcation of the electron pathway at center o and speculated that ISP in the reduced state directs the electron flow into heme b$_L$ stressing its important role in the timing of the reaction. Our analyses essentially support this proposal and furthermore suggest that the bifurcation can be purely a kinetic event. Because of this, we would like to propose a “kinetic switch” mechanism. Whether conformational changes are involved in the switching mechanism as assumed by Brandt et al. (25, 26) should be answered by further studies.

A concern of the kinetic control is shown by the following example with the previous case of $\Delta G_m = 0$ as a reference (Figs. 6A and 7B). The rate constant for ET from SQ$_i$ to heme b$_L$ in S27, $k_{45}$, was changed to 1 s$^{-1}$ to make this comparable to $k_{45} = 40/20$ s$^{-1}$ which is the rate constant for oxidation of heme c$_1$ in S27 (Table I). All the other constants were kept unchanged. Although the oxidation profiles of heme b$_L$ and heme b$_T$ were almost the same as in the reference, the oxidation of both ISP and heme c$_1$ slowed down appreciably. Apparently, the oxidation of heme c$_1$ is almost biphasic (Fig. 10A). At the same time, the oxidation of ubiquinol was rapid for the initial one-third but stalled temporarily and was followed by a slower oxidation. During the stall, the extent of SQ generation approached a maximal level transiently up to 0.3 in fraction and approached a constant level. Contrary to ubiquinol and SQ, ubiquinone was generated gradually with an initial lag. At 3 s the relative fraction of quinone to ubisemiquinone was about 4 contrary to 3/4 observed in the reference. On the other hand, during the turnover the transient appearance of S20, S26, and S27 became apparent in the reaction map (Fig. 10B) caused by the kinetic barrier imposed on RS59 and RS67. Consequently, S40, which is a fully oxidized species, was formed to an appreciable extent, and accordingly, an accumulation of S56 became less significant. These clearly indicate the significance of the kinetic control mechanism.

CONCLUSION

The simplified reaction scheme based on the Q cycle mechanism and shown in Fig. 5 explains satisfactorily the following experimental observations. (i) Starting from the fully reduced QCR the oxidation of 6-type hemes accompanies an initial lag; apparently heme b$_L$ is oxidized first, followed by heme b$_T$. Antimycin A inhibits the oxidation. The oxidation of heme c$_1$ is triphasic and becomes biphasic in the presence of antimycin A. (ii) The substoichiometric amount of Q$_{10}$ is essential for elec-
electron transfer from \(b\)-type hemes to ISP and heme \(c_1\). (iii) Starting from partially reduced QCR the oxidation of \(b\)-type hemes occurs immediately without a lag.

Based on the simulation studies the following mechanisms are proposed. When both heme \(b_1\) and heme \(b_h\) are in the reduced state ubiquinol in center \(o\) is oxidized consecutively by the high potential (ISP and heme \(c_1\)) pathway, providing ubiquinone in center \(i\). This initiates the delayed oxidation of heme \(b_1\) followed by heme \(b_h\). Even if the consecutive oxidation pathway does function, however, the kinetic control mechanism directs an electron flow preferentially into oxidized heme \(b_1\) when this becomes available to SQ. The key point of this mechanism is that the fate of SQ is determined by the redox state of ISP; only when ISP is in the reduced state SQ can act as an electron donor to heme \(b_1\). Thus, ISP is regarded as a redox-linked switch. No endergonic process is required for the bifurcation to occur.

The current mechanistic Q cycle model has proved its usefulness in allowing the semiquantitative analysis of the redox behavior of the metal centers and intrinsic ubiquinone in QCR, and in predicting the kinetic mechanism that controls the electron flow. This approach will become a powerful tool in studying the molecular mechanism of energy conservation if elaborated for more rigorous and quantitative treatment of the dynamic data.

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