On the Mechanism of Quinol Oxidation at the Q_p Site in the Cytochrome bc_1 Complex

STUDIED USING MUTANTS LACKING CYTOCHROME b_L OR b_H

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To elucidate the mechanism of bifurcated oxidation of quinol in the cytochrome bc_1 complex, Rhodobacter sphaeroides mutants, H198N and H111N, lacking heme b_L and heme b_H, respectively, were constructed and characterized. Purified mutant complexes have the same subunit composition as that of the wild-type complex, but have only 9–11% of the electron transfer activity, which is sensitive to stigmatellin or myxothiazol. The \( P_m \) values for hemes b_L and b_H in the H111N and H198N complexes are −95 and −35 mV, respectively. The pseudo first-order reduction rate constants for hemes b_L and b_H in H111N and H198N, by ubiquinol, are 16.3 and 12.4 s\(^{-1}\), respectively. These indicate that the Q_p site in the H111N mutant complex is similar to that in the wild-type complex. Pre-steady state reductive rates of heme c_1 by these two mutant complexes decrease to a similar extent of their activity, suggesting that the decrease in electron transfer activity is due to impairment of movement of the head domain of reduced iron-sulfur protein, caused by disruption of electron transfer from heme b_L to heme b_H. Both mutant complexes produce as much superoxide as does antimycin A-treated wild-type complex. Ascorbate eliminates all superoxide generating activity in the intact or antimycin inhibited wild-type or mutant complexes.

The cytochrome bc_1 complex, also known as complex III or ubiquinol-cytochrome c oxidoreductase, is an essential segment of the electron transfer chain in mitochondria and photosynthetic bacteria (1). The complex catalyzes electron transfer from quinol to cytochrome c (c_2 in some bacteria) with concomitant translocation of protons across the inner membrane of mitochondria or cytoplasmic membrane of bacteria. Intensive biochemical and biophysical studies on this complex (2–4) have led to the formulation of the “protonmotive Q-cycle” mechanism for electron and proton transfer in this complex (5–7). The key step of the Q-cycle mechanism is the bifurcated oxidation of quinol at the quinol oxidation site (Q_p). In the Q-cycle mechanism, it was postulated that the first electron of quinol is transferred to the “high potential chain,” consisting of iron-sulfur protein (ISP)\(^2\) and cytochrome c_1. Then the second electron of quinol, via a transient semiquinone, is passed through the “low potential chain” consisting of cytochromes b_L and Q_p to reduce ubiquinone or ubisemiquinone bound at the quinol reduction site (Q_p). One drawback of this sequential scheme is the lack of a “functional” semiquinone at the Q_p site (8–10), even though some radicals have been reported under abnormal conditions (11, 12). Recently, pre-steady state kinetic analysis of the reduction of cytochrome b_L and ISP in a same sample using fast quenching coupled with EPR (13) indicates that both iron-sulfur cluster (ISC) and heme b_L are reduced by quinol at the same rate, suggesting a concerted scheme for the bifurcated oxidation of quinol at the Q_p site (13, 14).

Although the concerted mechanism explains why the proposed semiquinone at the Q_p site is not detected (13), the proponents of sequential mechanism argue that similar reduction rates observed in heme b_L and ISC is due to the low (60 \( \mu \)s) time resolution of the instrument used. They attribute the missing semiquinone to its low stability and the fast electron transfer to heme b_L (15). One way to confirm the existence of semiquinone at the Q_p site is using a mutant complex lacking heme b_L. If the sequential mechanism exists, one should expect to see some Q_p site semiquinone in this mutant complex, due to the lack of its electron acceptor, heme b_L.

The first cytochrome bc_1 complex crystallographic structure from bovine heart mitochondria was reported in 1997 (16). Since then, more x-ray crystallographic structures of bc_1 complexes from different species have become available (17–20). Based on the poor electron density of ISP and the larger than expected distance between ISC and heme c_1 in the first crystallographic structure, a need for head domain movement and flexibility of the neck region of ISP were proposed (16–18) and confirmed experimentally (21–27). The head domain of ISP is considered to have two docking positions: b-position and c_1-position (17). Reduction of ISP by quinol takes place when the head domain of ISP is located at the b-position. It then moves to the c_1-position to reduce cytochrome c_1. Although few investigators question the requirement for movement of the ISP head domain during bc_1 catalysis (17, 21–27), there is no

\(^{2}\) The abbreviations used are: ISP, iron-sulfur protein; Q_p, quinol; BrH_p, 2,3-dimethoxy-5-methyl-6-(10-bromodecy)-1,4-benzoquinol; DM, N-dodecyl-\(\beta\)-o-maltopyranoside; MCLA, 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo[1,2-c]pyrazin-3-one, hydrochloride; EPR, electron paramagnetic resonance; ISC, iron-sulfur cluster; ICM, intra-cytoplasmic membrane; P_m, site inhibitors that enhance the mobility of head domain of iron-sulfur protein; P_b, Q_p site inhibitors that fix the head domain of iron-sulfur protein at b-position.
Cytochrome bc₁ Complex

consensus for what the driving force for this movement is (8, 13, 28–31).

One proposed mechanism suggests that movement of reduced ISP head domain from the b- to c₁-position is regulated by protein conformational changes induced by electron transfer from heme b₁ to heme b₄ (13, 31). Recent results (32, 33) of analyses of the binding affinity and inhibitory efficacies of Pₐ, and Pᵢ-inhibitors, at different redox states of the cytochrome bc₁ complex, are consistent with this proposal. One way to further substantiate this proposal is to determine the electron transfer activity and pre-steady state reduction rates of hemes c₁, b₁, and b₄, by quinol, in the presence and absence of inhibitors, in mutant complexes lacking heme b₁ or heme b₄ and compared with those obtained from the wild-type complex. If this proposal is correct, one would expect to see a decrease in electron transfer activity and the rate of heme c₁ reduction in b₄ and b₁ knockout mutant complexes.

Formation of superoxide anion is a well established side reaction during the oxidation of quinol by cytochrome bc₁ complex. Addition of antimycin to the intact bc₁ complex increases superoxide production (34–36). The electron leakage (or superoxide production) site can be at the semiquinone (37) of the Qₚ site or reduced heme b₁ (34, 38), depending on the mechanism by which bifurcation of ubiquinol proceeds. If bifurcation of quinol at the Qₚ site proceeds by the sequential mechanism, semiquinone formed at the Qₚ site and reduced heme b₁ would both be the electron leakage sites during bc₁ catalysis. Thus, one would expect to see at least some increase in superoxide production in the mutant complex lacking heme b₁, due to the possible increase of semiquinone. If bifurcation of ubiquinol at the Qₚ site proceeds by the concerted mechanism, reduced heme b₁ would be the only electron leakage site, because there will be no semiquinone at the Qₚ site during bc₁ catalysis.

The mechanism for superoxide production by the bc₁ complex is unclear. Comparing superoxide production by wild-type and mutant complexes lacking either heme b₁ or heme b₄, under various conditions, should provide insight into the superoxide production mechanism.

Herein we report detailed procedures for generating Rhodobacter sphaeroides mutants expressing cytochrome bc₁ complex lacking either heme b₁ (H198N) or heme b₄ (H111N), purifying cytochrome bc₁ complexes from intra-cytoplasmic membranes (ICM) of both mutants, and characterizing the purified mutant complexes in subunit composition, electron transfer activity, absorption spectral properties, redox protential, pre-steady state reduction kinetics of hemes (b₁, b₄, and c₁), and superoxide production of purified complexes.

**EXPERIMENTAL PROCEDURES**

**Materials—**Cytochrome c (horse heart, type III), stigmatellin, myxothiazol, antimycin A, and xanthine oxidase were purchased from Sigma. N-Dodecyl-β-D-maltopyranoside (DM) and n-octyl-β-D-glucopyranoside were from Anatrace. Protease K was purchased from Invitrogen. Nickel-nitrilotriacetic acid gel and a Qiaprep spin Miniprep kit were from Qiagen. 2-Methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazol[1,2-α]pyrazin-3-one, hydrochloride (MCLA) was from Molecular Probes, Inc. 2,3-Dimethoxy-5-methyl-6-(10-bromodecyl)-1,4-benzoquinol (Q₆C₁₀BrH₂) was prepared as reported previously (39). All other chemicals were of the highest purity commercially available.

**Generation of R. sphaeroides Cytochrome bc₁ Mutants—**Mutants were constructed by the QuikChange site-directed mutagenesis kit from Stratagene using a supercoiled double-stranded pGEM7Zf(+)–fbcB as template. Forward and reverse primers were used for PCR amplification (Table 1). The pGEM7Zf(+)–fbcB plasmid (40) was constructed by ligating the NsiI-XbaI fragment from pRKDfbcFBKmC6HQ into NsiI and XbaI sites of the pGEM7Zf(+) plasmid. The NsiI-XbaI fragment from the pGEM7Zf(+)–fbcB plasmid was ligated into the NsiI and XbaI sites of the pPRD418–fbcFBKmC6HQ plasmid to generate the pPRD418–fbcFBKmC6HQ plasmid. A plate-mating procedure (41) was used to mobilize the pPRD418–fbcFBKmC6HQ plasmid in Escherichia coli S17 cells into R. sphaeroides BC17 cells. The presence of engineered mutations was confirmed by DNA sequencing of the NsiI-XbaI fragment as previously reported (41). DNA primers were purchased from Invitrogen. DNA sequencing was performed by the Recombinant DNA/Protein Core Facility at Oklahoma State University.

**Enzyme Preparations and Activity Assays—**Chromato-

| Table 1 | Oligonucleotides used for site-directed mutagenesis |
|--------|--------------------------------------------------|
| **Sequence** |
| H198N(F)* | CGGTTCTTCTCCCTGATCACTGTGCCTGTTTG |
| H198N(R) | CCGAGGCGACAGGTCGTTCAGCAGAAGACCCG |
| H111N(F) | CGCGGCTTCTACGACATTCGCCGGCTCC |
| H111N(R) | GAGCGGCGGGAAGAGTTCTCTAGATAGACCG |

* F and R in parentheses denote forward and reverse primers, respectively.

**Table 1**

**Oligonucleotides used for site-directed mutagenesis**

The underlined bases correspond to the genetic codes for the amino acid(s) to be mutated.
grains) was then added, and several successive spectra of the reduced pyridine hemochromes were recorded (every 20 s) until there were no significant differences between two consecutive spectra. Table 2 lists the extinction coefficients (42) of pyridine hemochromes used to calculate the concentration of hemes b and c1. To calculate the concentration of hemes b and c1 in a bc1 sample, the equation pair below was employed. In these equations e stands for the extinction coefficient. The subscript numbers of e indicate the corresponding wavelengths. C stands for concentration.

\[
C_{c1} \times e_{549-540} + C_b \times e_{549-540} = A_{549-540} \quad \text{(Eq. 1)}
\]

\[
C_{c1} \times e_{558-580} + C_b \times e_{558-580} = A_{558-580} \quad \text{(Eq. 2)}
\]

**Potentiometric Titrations of the Cytochrome b of Mutant Cytochrome bc1 Complexes**—Redox titrations of cytochromes b in wild-type and mutant bc1 complexes were essentially according to the published method (43, 44). 3-ml aliquots of the bc1 complex (2 μM cytochrome b) in 0.1 M Na+/K+ phosphate buffer, pH 7.0, containing 25 μM 1,4-benzoquinone (Em1, 293 mV), 2,3,5,6-tetramethyl-p-phenylenediamine (Em1, 260 mV), 1,2-naphthoquinone (Em1, 143 mV), phenazine methosulfate (Em1, 80 mV), phenazine ethosulfate (Em1, 55 mV), 1,4-naphthoquinone (Em1, 36 mV), duroquinone (Em1, 5 mV), pyocyanine (Em1, −34 mV), indigo carmine (Em1, −125 mV), and anthraquinone-2-sulfonic acid (Em1, −225 mV) were used. Reductive titrations were carried out by addition of sodium dithionite solution to the ferricyanide-oxidized samples and oxidative titrations by addition of ferricyanide solution to the dithionite-reduced samples. At indicated t values during the redox titration, absorption spectra from 600 to 500 nm were taken. The optical absorbance at 560 nm, minus that at 580 nm, was used for determination of cytochrome b reduction. Midpoint potentials of cytochrome bL and bH were calculated by fitting the redox titration data, using the Nernst equation for a one-electron reaction (n = 1), by Kaleidagraph (44).

**Fast Kinetics Study**—To determine electron transfer rates between the quinol and heme b or heme c1, the cytochrome bc1 complex was mixed with ubiquinol (Q0C10BrH2) in equal volume at room temperature in an Applied Photophysics stopped-flow reaction analyzer SX.18MV (Leatherhead, United Kingdom). The concentration of bc1 complex was 12 μM (based on cytochrome c1) in 50 mM Tris-Cl, pH 8.0, at 4 °C, containing 200 mM NaCl and 0.01% DM. For use in the stopped-flow, Q0C10BrH2 in ethanol was diluted to 240 μM in the same buffer. Reductions of cytochrome b and cytochrome c1 in wild-type were monitored by the increase in absorption difference of A560–580 and A551–539 respectively, with a photodiode array scan between 600 and 500 nm. Reductions of cytochrome bH in H111N and bH in H198N were determined from the increase in A565–580 and A560–580. When an inhibitor was used, the cytochrome bc1 complex was treated with 5-fold molar excess of inhibitor over heme c1, for 5 min at 4 °C, prior to the experiment. Because the concentration of ubiquinol used was 20 times higher than that of the cytochrome bc1 complex, the reactions between bc1 and quinol were treated as pseudo first-order reactions. Time traces of the reaction were fitted with a first-order rate equation to obtain the pseudo first rate constants k1 by Kaleidagraph.

Detection of Q Radical at the Q0 Site with EPR—300 μl of 150 μM purified cytochrome bc1 complexes were treated with 10-fold excess of ubiquinol to fully reduce cytochrome c1 in 10 s and frozen in liquid nitrogen. EPR spectra were recorded at −170 °C with the following instrument settings: microwave frequency, 9.4 GHz; microwave power, 2.2 milliwatts; modulation amplitudes, 6.3 G; modulation frequency, 100 kHz; time constant, 655.4 ms; sweep time, 167.8 s; conversion time, 163.8 ms.

**RESULTS AND DISCUSSION**

**Characterization of Mutants Lacking Either Heme bL (H198N) or Heme bH (H111N)***—In the cytochrome b subunit of cytochrome bc1 complex from the *R. sphaeroides*, His198 and His111 are the ligands of heme bL, whereas His311 and His212 are the ligands of heme bH. Two mutants, H198N and H111N, in which histidine 198 and histidine 111 of cytochrome b were, respectively, substituted with Asn were constructed and selected for the present study. The H198N mutant knocks out heme bL, whereas the H111N mutant knocks out heme bH. Because the cytochrome bc1 complex is absolutely required for photosynthetic growth of this bacterium, and hemoglobin provides no energy, these two mutants can support photosynthetic growth. Cultures of wild-type and mutants were placed in a light tank after 4 h of dark grow. Photosynthetic growth was followed by the increase of cell intensity. None of the mutants show any evidence of growth in 6–8 days. To grow cells for the preparation of mutated bc1 complexes, H198N and H111N were grown semi-aerobically. These two mutants can grow semiaerobically at a rate comparable with that of the wild-type cells. ICM were prepared from semiaerobically grown cells and used for preparation of corresponding mutant complexes.

ICMs prepared from mutants H198N and H111N contain subunits cytochrome b, cytochrome c1, ISP, and subunit IV in the same concentrations as those detected in the wild-type ICM, determined by Western blot using antibodies against...
these four individual proteins. This result indicates that lacking heme $b_4$ in the H198N mutant and lacking heme $b_1$ in the H111N mutant does not impair the complex assembly into the ICM membrane. This finding is contradictory to the previous report (48) that ICMs from mutants lacking heme $b_1$ (H111N, H111D, and H212D) have subunits of cytochromes $b$ and $c_1$, whereas no such subunits were detected in ICMs from mutants lacking heme $b_4$ (H97N, H97D, H198N, and H198Y). While constructing mutants lacking heme $b_4$ or heme $b_1$, we observed that some heme $b_4$ knocked out mutants, such as H97F and H97N, are unstable, purification attempts were not successful. The structural stability of H198N and H111N mutant complexes in their ICMs enables us to purify and characterize these two mutant complexes with methods similar to those used for the wild-type complex.

Fig. 1 compares SDS-PAGE patterns of purified wild-type and mutant cytochrome $bc_1$ complexes. The purification procedure involves DM solubilization followed by nickel-affinity gel column chromatography (21). The yields and subunit compositions of purified mutant complexes are comparable with those of the wild-type.

Fig. 2 shows absorption spectra of purified wild-type (A) and mutant complexes of H198N (B) and H111N (C). The presence of cytochromes $b_4$ and $b_1$ in the wild-type complex is revealed by a difference spectrum of dithionite-partially reduced minus ascorbate-reduced and a difference spectrum of dithionite-fully reduced minus dithionite-partially reduced, respectively (49). If a catalytic amount of succinate-Q reductase is added to a purified wild-type complex, $b_4$ is observed from a difference spectrum of succinate-reduced minus ascorbate-reduced and $b_1$ from a difference spectrum of dithionite-reduced minus succinate-reduced (50). Ascorbate reduces cytochrome $c_1$; succinate reduces cytochromes $c_1$ and $b_4$; dithionite reduces cytochromes $c_1$, $b_4$, and $b_1$. Cytochrome $b_4$ has an absorption peak at 560 nm, whereas cytochrome $b_1$ has a double-$\alpha$ peak with absorption at 565 nm and a shoulder at 558 nm (see Fig. 2A). The $\alpha$ absorption peak of cytochrome $b$ in the H198N mutant complex (Fig. 2B), which is obtained from a difference

![FIGURE 1. SDS-PAGE of purified cytochrome $bc_1$ complexes from wild-type and mutants H198N and H111N. Lanes 1–4 are for polypeptide standard, wild-type, mutant H198N, and mutant H111N, respectively. Aliquots of purified $bc_1$ complexes were incubated with 1% SDS and 0.4% $\beta$-mercaptoethanol at 37°C for 20 min. Digested samples containing about 200 pmol of cytochrome $c_1$ were subjected to electrophoresis. The molecular masses of standard polypeptides are: 10, 15, 20, 25, 30, 40, 50 and 60 kDa.]

![FIGURE 2. Absorption spectra of wild-type and mutant $bc_1$ complexes. A–C are for the wild-type (WT), mutants H198N and H111N complexes, respectively. The cytochrome $c_1$ concentration of complexes used was 1 $\mu$M for A, 1.5 $\mu$M for B and C. The gray spectrum stands for ascorbate-reduced sample; and black solid spectrum for the dithionite-fully reduced sample. The black dotted and black dot-dash spectra in B and C, respectively, are difference spectra of the dithionite-fully reduced minus ascorbate-reduced samples. Black dotted and black dot-dash spectra in A are the difference spectra of the dithionite-partially reduced (— — 50 mV) minus ascorbate-reduced and of the dithionite-fully reduced minus the dithionite-partially reduced, respectively.}
that the H111N mutant complex contains only heme absorbance of 28.5/mM cm at 539 nm. Because the extinction coefficient of individual heme absorbance is the same as the difference spectrum obtained from the first dithionite reduced minus ascorbate reduced samples. The difference extinction coefficient of difference absorbance spectra are identical to those of heme H111N, suggesting that the H111N mutant complex contains only heme H111. This result is consistent with the observation that the H111N mutant complex is at 565 nm with a shoulder at 558 nm (Fig. 3), indicating that only cytochrome b is present in this mutant complex.

To detect the presence of trace amounts of other cytochrome b in a given mutant, the complex was titrated with dithionite solution to reduce cytochromes b gradually (data not shown). In the mutant H198N complex, the last reduced heme b showed a heme b reduction spectrum, the same as that of the very first reduced one, suggesting that the H198N mutant complex contains only heme b. If there is some heme b in this mutant complex, the difference spectrum of the last dithionite reduced minus the second to last dithionite reduced should differ from that of the first dithionite reduced minus ascorbate reduced, because heme b is expected to be reduced last due to its low redox potential. In the H111N mutant complex, the difference spectrum of the first dithionite reduced minus ascorbate reduced is the same as the difference spectrum obtained from the last dithionite reduced minus the second to last dithionite reduced. These two difference absorption spectra are identical to those of heme b in the wild-type complex, suggesting that the H111N mutant complex contains only heme b.

In general, for bc1 complexes, hemes b, including heme b1 and b2, are calculated from the difference extinction coefficient of 28.5/mM cm between 560 and 580 nm in the difference spectrum of dithionite reduced minus ascorbate reduced samples. The heme c1 is calculated from a difference spectrum of ascorbate reduced minus ferricyanide-oxidized complex using a difference extinction coefficient of 17.5/mM cm at 561 and 539 nm. Because the extinction coefficient of individual heme b and heme b1 are not firmly established, we used the alkaline pyridine hemochromogen spectrum to determine the concentrations of hemes b and c1 in mutant complexes of H198N and H111N. The equations used for calculation are listed under “Experimental Procedures.” In the wild-type bc1 complex the b/c1 molar ratio is about 1.65. In mutant complexes of H198N and H111N, b/c1 molar ratios are close to 1.0 (see Table 3). Based on the heme b contents determined by pyridine hemochromogen and the difference absorption spectra of dithionite reduced minus ascorbate reduced mutant complexes, the difference extinction coefficients for hemes b and b1 were calculated to be 12.0/mM cm between 565 and 580 nm and 24.5/mM cm between 560 and 580 nm, respectively. The lower than expected b/c1 ratio in the wild-type complex is probably due to the presence of excess cytochrome c1 in the complex, as the His tag is located at the C terminus of cytochrome c1 protein. The presence of excess cytochrome c1 in the wild-type complex was confirmed by protein crystallization (51, 52). In the crystalline bc1 complex, the b/c1 molar ratio is 2. The excess cytochrome c1 is found in the mother liquid after crystallization.

### Table 3: Characterization of mutant H198N and H111N bc1 complexes

| Characterization | Wild-type | H198N | H111N |
|------------------|-----------|-------|-------|
| Photosynthetic growth | Yes | No | No |
| Cytochrome b/cytochrome c1 ratio | 1.65 | 1.0 | 0.98 |
| Specific activity* | 3.5 | 0.4 | 0.32 |
| Antimycin sensitivity | Yes | 36.4% | No |
| Stigmatellin sensitivity | Yes | Yes | Yes |
| Myxothiazol sensitivity | Yes | Yes | Yes |

* The unit of specific activity is μmol of cytochrome c reduced/min/mmol of cytochrome c1.

The 36.4% means the loss of 36.4% of its bc1 activity upon antimycin treatment.

### Effect of Mutations on the Electron Transfer Activity and the Redox Potential of b Hemes—Specific activities of purified mutant complexes were determined and compared with the wild-type complex. Mutant complexes of H198N and H111N have low bc1 activities, about 9–11% of that in the wild-type complex (Table 3). The bc1 activity detected in these two mutant complexes is inhibited by stigmatellin and myxothiazol, indicating ubiquinol can bind and be oxidized at the Qb site in both complexes. As expected, the H111N mutant complex is completely resistant to antimycin. However, it is somewhat surprising that H198N is partially sensitive to antimycin.

To see if these substitutions have any effect on the E°m of heme b1 or heme b1 of mutants H198N and H111N were determined, respectively. As shown in Fig. 3, heme b1 in the H198N mutant complex has an E°m of −35 mV, significantly lower than the E°m of heme b1 in the wild-type complex (50 mV). The E°m of heme b1 in the H111N mutant complex is −95 mV, comparable with that in the wild-type complex (−93 mV). However, this value is lower than that reported by others (48) using ICM of the same mutant. Thus the substitution in mutant H198N has some effect on the E°m of heme b1, whereas the substitution in mutant H111N has little effect on the E°m of heme b1.

These results seem contradictory to the cumbic interaction between hemes bL and bH1 reported in the literature (15, 48).

### Effect of Mutations on the Rate of Heme c1 Reduction by Quinol—The loss of heme b1 and heme b1 in the mutant complexes of H198N and H111N, respectively, provides us an opportunity to study the effect of the low potential chain on c1 reduction and movement of the ISP head domain. The fast-kinetics study is carried out on the stop-flow instrument of Applied Photophysics Ltd. Fig. 4 shows the time traces of heme c1 reduction, for 1.2 s, in wild-type and mutant complexes of H198N and H111N. Heme c1 reduction rates in the two mutant complexes are much lower than that in the wild-type complex. Assuming heme c1 reduction is a pseudo first-order reaction, the k1 values are determined to be 11.6, 1.7, and 1.4 s−1 for the wild-type and mutant complexes of H198N and H111N, respectively. The heme c1 reduction rates in the H198N and H111N mutant complexes are only about 15–12% of that in the wild-type complex. Despite of low reduction rates of heme c1 in the two mutant complexes, maximum levels of heme c1 reduction are the same as that of the wild-type complex. 50% of the total heme c1 is reduced by ubiquinol as reported (53). Because the Qb sites in the H198N and H111N mutant complexes are similar to that in the wild-type complex, as both mutant complexes are sensitive to stigmatellin and myxothiazol (the Qb site inhibitor), the decrease in rate of heme c1 reduction is not due to the initial bifurcated oxidation of quinol. Likely, this decrease results from impairment of movement of the head domain of reduced ISP, from the b-position to c1-position.
caused by disruption of electron transfer from heme $b_L$ to heme $b_H$ in these mutant complexes.

Fig. 5 shows the effect of antimycin A and stigmatellin on the reduction rates of heme $c_1$ in the wild-type and mutant complexes. Antimycin A is a $Q_N$ site inhibitor and stigmatellin is a $Q_P$ site inhibitor. In the wild-type complex antimycin A decreases heme $c_1$ reduction pseudo first-order rate constant from 11.6 to 4.7 s$^{-1}$ (Fig. 5A). This is consistent with the previous report (14) that antimycin A has a significant effect on the reduction rate of heme $c_1$ in cytochrome $bc_1$ complexes. In the H198N mutant complex, the rate constant decreases from 1.7 to 1.1 s$^{-1}$. In the H111N mutant complex, the rate constant decreases from 1.4 to 1.0 s$^{-1}$.

Because both mutant complexes, in which no intact low potential chain is available, also show a decreased rate of heme $c_1$ reduction in the presence of antimycin A, this inhibitor effect cannot be due to blocking of electron transfer in the low potential chain. It is probably due to the long range effect of antimycin on the $Q_P$ site binding to the $Q_N$ site. In other words, the effect of antimycin A on the heme $c_1$ reduction rate is not through the low potential redox component but through the cytochrome $b$ protein subunit.

Addition of stigmatellin to the wild-type and mutant complexes abolishes heme $c_1$ reduction. These results further confirm that the $Q_P$ site in these two mutant complexes is functional.

**Effect of Mutations on the Rates of Heme $b_L$ and Heme $b_H$ Reductions by Quinol**—Fig. 6 shows hemes $b$ reduction by quinol, in the presence and absence of antimycin and stigmatellin, in wild-type and mutant $bc_1$ complexes. In the H198N mutant complex (Fig. 6B), a small portion of heme $b_H$ is reduced by quinol, in the absence of inhibitors, and the reduction is biphasic. The rate constants for the fast and slow reduction phases are 12.4 and 0.86 s$^{-1}$, respectively. Fast phase reduction is abolished when antimycin is present. As
expected, the presence of stigmatellin has little effect because heme $b_H$ is reduced by quinol through the $Q_N$ site, not the $Q_P$ site.
In the H111N mutant complex (Fig. 6C), a small portion of heme $b_L$ is rapidly reduced by quinol, in the absence of inhibitors, with a reduction rate constant of about $16.3 \text{ s}^{-1}$, followed by a slow reoxidation. It is likely that the reoxidation of heme $b_L$ will lead to superoxide formation. The slower decay rate of superoxide formed in the H111N complex as compared with that formed in the H198N complex, as will be described in Fig. 8, B and C, supports this speculation. Addition of stigmatellin abolishes this reduction. Addition of antimycin affects the rate and extent of heme $b_L$ reduction: the rate constant decreases from $16.3 \text{ s}^{-1}$ and the extent of $b_L$ reduction decreases by 30%. In the wild-type complex antimycin also affects both the rate and extent of heme $b$ reduction by quinol (see Fig. 6A): it decreases the rate but increases the extent (14, 54–58). It should be noted that heme $b$ reduction observed in the wild-type complex, in the absence of inhibitor, is mostly heme $b_H$. The input of electron for reduction could come from the $Q_p$ site via heme $b_L$ and less likely, directly from quinol at the $Q_N$ site, because the reduction rate of heme $b_L$ ($k_1 = 16.3 \text{ s}^{-1}$) is larger than that of heme $b_H$ ($k_1 = 12.4 \text{ s}^{-1}$) (Fig. 6, B and C). These results seem contradictory to a report indicating that the rate of heme $b_H$ reduction is larger than that of heme $b_L$ in the yeast cytochrome $bc_1$ complex (59). The observation that the reduction rate of heme $b_L$ in the H111N mutant complex is similar to, albeit higher than, that of the wild-type complex (see Fig. 6A) indicates that the $Q_p$ site in this mutant complex is functional. This finding further supports the idea that antimycin has a long range effect on the $Q_p$ site to decrease the rates of heme $c_1$ and heme $b_L$ reduction.

**Attempt to Detect $Q$ Radical at the $Q_p$ Site with Mutant H198N**—Because H198N has no heme $b_L$, and thus no electron acceptor for semiquinone, more semiquinone radical would have increased if the sequential mechanism for bifurcated quinol oxidation is functioning. Fig. 7 shows EPR spectra from wild-type and mutant $bc_1$ complexes. In the absence of antimycin a strong signal at $g = 2.00$ is observed in the quinol-reduced cytochrome $bc_1$ complexes from wild-type (Fig. 7A, curve 1) and mutant H198N (Fig. 7B, curve 1). This signal decreases significantly when antimycin is added (Fig. 7A and B, curve 2). Apparently the portion of signal that disappears (Fig. 7A and B, curve 4) is the signal of semiquinone radical at the $Q_N$ site. However, the signal portion that is insensitive to antimycin is also present in fully oxidized complexes of wild-type and mutant H198N (Fig. 7A and B, curve 3). It should be noted that this free radical, of unknown origin, is much more concentrated in the H198N complex than in the wild-type complex. Its origin is currently under investigation.

**FIGURE 7.** EPR spectra of ISP and free radical (semiquinone) under different conditions. Purified wild-type (WT) (A), H198N (B), and H111N (C) $bc_1$ complexes were treated with 10-fold excess of $Q_0C_{10}BrH_2$ solution, to fully reduce cytochrome $c_1$, and frozen in liquid nitrogen. EPR spectra were recorded at $-170^\circ\text{C}$ with the following instrument settings: microwave frequency, 9.4 GHz; microwave power, 2.2 milliwatts; modulation amplitudes, 6.3 G; modulation frequency, 100 kHz; time constant, 655.4 ms; sweep time, 167.8 s; conversion time, 163.8 ms. Curve 1 is the spectrum for cytochrome $bc_1$ complexes reduced by $Q_0C_{10}BrH_2$; curve 2 for those reduced by $Q_0C_{10}BrH_2$ in the presence of antimycin; curve 3 for ferricyanide-oxidized cytochrome $bc_1$ complexes; curve 4 for the spectrum derived from curve 1 minus curve 2. The absence of signals of reduced ISP defines the fully oxidized state of the complex. For mutant H111N, the signal intensities were reduced to one-third to fit in the figure.
The EPR spectra for mutant H111N bc₁ complex (Fig. 7C) were also determined. Oxidized H111N has an unusual EPR spectrum that is not due to contamination. This spectrum appears to be a characteristic of mutants lacking heme b₃, because another heme b₃-deficient mutant, H212N, has a similar EPR spectrum (data not shown). The concentrations of ISP in all three complexes were about the same, as indicated by the g = 1.89 signal. The g = 1.89 signal in Fig. 7C looks smaller because the intensity of the spectrum was reduced to one-third of the original to compare the signals in the g = 2.00 region, of the three complexes.

Superoxide Production in Mutant bc₁ Complexes during Catalysis—Because there is no heme b₁ in the H198N and no heme b₃ in the H111N, it should be easier for oxygen to get electrons, from either semiquinone or the reduced heme b₁, in these mutant complexes than in the wild-type. Fig. 8 shows superoxide production by mutant and wild-type complexes under different conditions. In the absence of antimycin (red tracings), production of superoxide anion by mutant complexes of H198N (Fig. 8B) and H111N (Fig. 8C) is much greater than by the wild-type (Fig. 8A). At the point of strongest chemiluminescence output, superoxide production by H198N and H111N is about 5 times that of the wild-type complex. Antimycin significantly increases superoxide production but slightly decreases its production rate in the wild-type complex (blue tracing in Fig. 8A). Antimycin has little effect on the superoxide production in mutants H198N and H111N (blue tracings in Fig. 8, B and C). This lack of effect of antimycin on superoxide production by H198N and H111N indicates that superoxide is produced at the Qₚ site, not at the Qₙ site. Thus during bc₁ catalysis, oxygen can only get electrons from reduced heme b₁ or from semiquinone at the Qₚ site.

In bc₁ complexes with fully reduced ISP and cytochrome c₁, no chemiluminescence (O₂) is observed upon the addition of quinol (black tracings in Fig. 8), indicating that superoxide production is dependent on ISP reduction by quinol. Therefore, quinol at the Qₚ site transfers its first electron to ISC; the second electron, either transferred to heme b₁ or retained as semiquinone, reacts with oxygen to produce superoxide. Because there is no detectable semiquinone radical at the Qₚ site, molecular oxygen may share quinol electrons with ISP when heme b₁ is not available. Normally reduced heme b₁ may leak its electron to oxygen. This leakage increases when the low potential electron transfer chain is blocked by antimycin. Because reduction of ISC is the first reaction in both superoxide generation and cytochrome c reduction catalyzed by bc₁ complex, the similar activation energy (60) for these reactions seems reasonable, if the reduction of ISC is rate-limiting.

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