Effect of Mutations of Arginine 94 on Proton Pumping, Electron Transfer, and Superoxide Anion Generation in Cytochrome b of the bc$_1$ Complex from Rhodobacter sphaeroides*

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**Background:** The bc$_1$ complex catalyzes electron transfer from ubiquinol to cytochrome c and translocates protons across the membrane.

**Results:** Substitution of Arg-94 decreased the electron transfer activity and proton pumping capability and increased the superoxide production.

**Conclusion:** Arg-94 is an important amino acid for the proton pumping capability of the bc$_1$ complex.

**Significance:** The result is significant for understanding the catalytic mechanism of the bc$_1$ complex.

Proton transfer involving internal water molecules that provide hydrogen bonds and facilitate proton diffusion has been identified in some membrane proteins. Arg-94 in cytochrome $b$ of the *Rhodobacter sphaeroides* bc$_1$ complex is fully conserved and is hydrogen-bonded to the heme propionate and a chain of water molecules. To further elucidate the role of Arg-94, we generated the mutations R94A, R94D, and R94N. The wild-type and mutant bc$_1$ complexes were purified and then characterized. The results show that substitution of Arg-94 decreased electron transfer activity and proton pumping capability and increased O$_2^-$ production, suggesting the importance of Arg-94 in the catalytic mechanism of the bc$_1$ complex in *R. sphaeroides*. This also suggests that the transport of H$^+\cdot$O$_2$, and O$_2^-$ in the bc$_1$ complex may occur by the same pathway.

The cytochrome bc$_1$ complex (also known as ubiquinol-cytochrome c oxidoreductase or complex III) is a multisubunit dimeric integral membrane protein complex. It is an essential segment of cellular energy-conserving electron transport chains in animals, plants, and bacteria (1, 2). This complex catalyzes electron transfer from ubiquinol to cytochrome c (or cytochrome c$_1$ in bacteria) and concomitantly translocates protons across the membrane to generate a membrane potential and pH gradient for ATP synthesis. The cytochrome bc$_1$ complexes from all species contain three core subunits, cytochrome $b$, cytochrome c$_{1}$, and the Rieske iron-sulfur protein, which house two $b$-type cytochromes ($b_1$ and $b_2$), one $c$-type cytochrome ($c_1$), and a high potential Rieske [2Fe-2S] cluster, respectively. Depending on the source, the cytochrome bc$_1$ complexes contain various numbers (zero to eight) of non-redox group-containing subunits, also called supernumerary subunits (3–5).

Intensive biochemical and biophysical studies on this complex (6–8) have led to the formulation of the "protonmotive Q-cycle" mechanism for electron and proton transfer in this complex (9–11). In the Q-cycle mechanism, it is postulated that the first electron of ubiquinol is transferred to the "high potential chain," consisting of the Rieske iron-sulfur protein and cytochrome c$_{1}$. Then, the second electron of ubiquinol, via a transient semiquinone, is passed through the "low potential chain," consisting of cytochromes $b_1$ and $b_2$, to reduce ubiquinone or ubisemiquinone bound at the ubiquinol reduction site (Q$_0$). One drawback of this sequential scheme is the lack of a "functional" semiquinone at the ubiquinol oxidation site (Q$_0$) (12–14), even though some radicals have been reported under abnormal conditions (15, 16). Recently, pre-steady-state kinetic analysis of the reduction of cytochrome $b_1$ and the Rieske iron-sulfur protein in the same sample using fast quenching coupled with EPR (17) indicated that both the iron-sulfur cluster and heme $b_1$ are reduced by ubiquinol at the same rate, suggesting a concerted scheme for the bifurcated oxidation of ubiquinol at the Q$_0$ site (18, 19).

It has been established that for every electron transferred through the bc$_1$ complex, two protons are translocated across the membrane. This 2H$^+$/e$^-$ stoichiometry has been verified in a wide variety of species through *in vitro* studies of bc$_1$ complexes in phospholipid (PL)$_2$ vesicles.

Arg-79 in cytochrome $b$ of the yeast bc$_1$ complex is hydroxymethylamine- and bicarbonate-bonded to the heme propionate and provides a proton exit pathway to the bulk solvent mediated by a chain of hydrogen-bonded water molecules. Arg-79 is fully conserved in cytochrome $b$, indicating its importance for the catalytic mech-
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**TABLE 1**

| Primer | Sequence |
|--------|----------|
| R94A Forward | GCTTCATGCTGGCTTACCTGGCATCG |
| R94A Reverse | CCGATCGAGGTACGACGAGTC |
| R94N Forward | GCTTCATGCTGACCTGGCATCG |
| R94N Reverse | CCGATCGAGGTACGACGAGTC |
| R94D Forward | GCTTCATGCTGACCTGGCATCG |
| R94D Reverse | CCGATCGAGGTACGACGAGTC |

nism (20). Arg-94 in cytochrome b of *Rhodobacter sphaeroides* corresponds to Arg-79 in yeast. To further elucidate the role of this important amino acid in cytochrome b of *R. sphaeroides*, we report herein detailed procedures for generating mutations of Arg-94 in cytochrome b and characterize the electron transfer activity, proton pumping capability, pre-steady-state reduction kinetics of hemes (b and c₃), and superoxide anion production in purified bc₁ complexes. We found that substitution of Arg-94 decreased electron transfer activity and proton pumping capability and increased superoxide anion production. The transport of H⁺, O₂, and O₂⁻ may follow the same pathway.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cytochrome c (horse heart, type III), stigmatellin, antimycin A, carbonyl cyanide m-chlorophenylhydrazone (CCCP), and valinomycin were purchased from Sigma. 8-Hydroxyquinoline-1,3,6-trisulfonic acid trisodium salt (pyrazine-1-carboxylic acid) was from Acros Organics. Asolectin was obtained from Invitrogen. 2,3-Dimethoxy-5-methyl-6-(10-bromodecyl)-1,4-benzoquinol hydrochloride (MCLA) was from Molecular Probes. 2,3-Dimethoxy-5-methyl-6-(10-bromodecyl)-1,4-benzoquinol (Q₀C₁₀BrH₂) was prepared in our laboratory as reported previously (22). All other chemicals were of the highest purity commercially available.

**Generation of R. sphaeroides Cytochrome bc₁ Mutants**—Mutations were constructed using the QuikChange site-directed mutagenesis kit (Stratagene) with supercoiled double-stranded pGEM7Zf(+) -fbcB as a template. Forward and reverse primers were used for PCR amplification (Table 1). The pGEM7Zf(+) -fbcB plasmid (23) was constructed by ligating the NsiI-XbaI fragment from pRKD418-fbcFBKmC6HQ into the NsiI and XbaI sites of the pGEM7Zf(+) plasmid. The NsiI-XbaI fragment of the pGEM7Zf(+) -fbcB plasmid was ligated into the NsiI and XbaI sites of the pRDK418-fbcFBKmC6HQ plasmid to generate the pRDK418-fbcFBKmC6HQ plasmid. This plasmid was used to transform *E. coli* DH5α, and a plasmid containing the mutation was isolated from a single colony. The mutation sequence was amplified from the isolated plasmids by PCR. The PCR products were purified with an extraction kit from Sigma and then sequenced. DNA sequencing was carried out at the Recombinant DNA/Protein Core Facility of Oklahoma State University.

**Growth of Bacteria**—*E. coli* cells were grown at 37 °C in LB medium. *R. sphaeroides* BC17 cells (24) were grown photosynthetically at 30 °C in enriched Siström’s medium containing 5 mM glutamate and 0.2% casamino acids. Photosynthetic growth conditions for *R. sphaeroides* were essential as described previously (25). The concentrations and antibiotics used were as follows: ampicillin, 125 μg/ml; kanamycin sulfate, 30 μg/ml; tetracycline, 10 μg/ml for *E. coli* and 1 μg/ml for *R. sphaeroides*; and trimethoprim, 100 μg/ml for *E. coli* and 30 μg/ml for *R. sphaeroides*.

**Enzyme Preparations and Specific Electron Transfer Activity Assay**—Chromatophore membranes were prepared from frozen cell paste, and cytochrome bc₁ complexes with a His₆ tag placed at the C terminus of cytochrome c₁ were purified from chromatophores as described previously (25) and stored at -80 °C in the presence of 10% glycerol. Protein concentrations were determined by absorbance at 280 nm using a converting factor of 1 A₂₈₀ = 0.56 mg/ml. The concentrations of cytochromes b and c₁ were determined spectrophotometrically using published molar extinction coefficients (26–28).

To assay ubiquinol-cytochrome c reductase activity, purified cytochrome bc₁ complexes were diluted with 50 mM Tris-Cl (pH 8.0) containing 200 mM NaCl and 0.01% n-dodecyl-β-D-maltosylcyanide to a final cytochrome bc₁ concentration of 1 μM unless specified otherwise. Appropriate amounts of the diluted samples were added to 1 ml of assay mixture containing 100 mM Na⁺/K⁺ phosphate buffer (pH 7.4), 0.3 mM EDTA, 100 μM cytochrome c, and 25 μM Q₀C₁₀BrH₂. The specific electron transfer activities were determined by measuring the reduction of cytochrome c (the increase in the absorbance at a wavelength of 550 nm) with a Shimadzu UV-2401 PC spectrophotometer at 23 °C using a millimolar extinction coefficient of 18.5 for calculation. The non-enzymatic oxidation of Q₀C₁₀BrH₂, determined under the same conditions in the absence of enzyme, was subtracted during specific activity calculations. Although the chemical properties of Q₀C₁₀BrH₂ and Q₀C₁₀H₂⁺ are comparable with those of Q₀C₁₀H₂⁺, the former is a better substrate for the cytochrome bc₁ complex (22).

**Preparation of Electron Transfer Complex-inlaid PL Vesicles**—Protein-PL vesicles were prepared by the cholate dialysis method of Kagawa and Racker (21). The wild-type or mutant bc₁ complex was mixed with 1 ml of asolectin micellar solution to give an asolectin (milligrams)/protein (milligrams) ratio of 40. The asolectin micellar solution was prepared by sonicating 200 mg of acetone-washed asolectin in 4 ml of 50 mM sodium phosphate buffer (pH 7.4) containing 2% sodium cholate and 100 mM KCl in an ice-water bath. Sonication was performed in an aliquot of semi-aerobically grown culture using the Qiagen plasmid miniprep kit. Because *R. sphaeroides* cells contain four types of endogenous plasmids, the isolated plasmids lacked the purity and concentration needed for direct sequencing. Therefore, a 2-kilobase pair DNA segment containing the mutation sequence was amplified from the isolated plasmids by PCR. The PCR products were purified with an extraction kit from Sigma and then sequenced. DNA sequencing was carried out at the Recombinant DNA/Protein Core Facility of Oklahoma State University.
an anaerobic environment by continually passing argon into the vessel. The bc1 complex/PL mixtures were incubated at 0 °C for 30 min before overnight dialysis at 4 °C against 100 volumes of 50 mM sodium phosphate buffer (pH 7.4) containing 100 mM KCl with three changes of buffer. The mixture was then dialyzed against 100 volumes of 150 mM KCl for 3–4 h.

**Determination of Proton Translocation Activity of bc1 Complex-PL Vesicles**—Proton translocation coupled to electron flow through the bc1 complex-PL vesicles was measured at room temperature using an Accumet Model 10 pH meter and Model 13-620-96 combination pH electrode. 25 nmol of Q0C10BrH2 was added to the 1.6-mL reaction mixture containing 150 mM KCl, 4 μM ferricytochrome c, 1 μM valinomycin, and an appropriate amount of bc1 complex-PL vesicles (30–50 μl). Electron flow was initiated by the addition of 5 nmol of ferricyanide, which oxidizes the cytochrome c and thus provides an electron acceptor for the complex. Electron flow under conditions in which no transmembrane ΔpH formed was measured in an identical manner except that the protonophore CCCP was present at a concentration of 2 μM to make the vesicles permeable to protons. Proton pumping (H+ /e−) was calculated as the ratio of the decrease in pH upon ferricyanide addition to bc1 complex-PL vesicles before and after treatment with CCCP.

**Fast Kinetics Study**—Measurements were performed in an Applied Photophysics SX.18MV stopped-flow spectrometer with a photodiode array scan between 600 and 500 nm. The reaction was started by mixing equal volumes of solutions A and B at room temperature. For the experiment to determine electron transfer rates between ubiquinol and heme b or c, solution A contained 50 mM Tris-Cl (pH 8.0) at 4 °C, 200 mM NaCl, 0.01% n-dodecyl-β-d-maltopyranoside, and 12.0 μM cytochrome bc1 complex (based on cytochrome c1). Solution B was the same as solution A except that the bc1 complex was replaced with 240 μM Q0C10BrH2. Reductions of cytochromes b and c1 were determined from the absorption changes at 560–580 nm and at 551–539 nm, respectively. When an inhibitor was used, the cytochrome bc1 complex was treated with a 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 much higher than that of the cytochrome bc1 complex, the reactions between bc1 and ubiquinol were treated as pseudo first-order reactions. Time traces of the reaction were fitted using a first-order rate equation to obtain the pseudo first-order rate constants (k1) by KaleidaGraph.

For the experiment to determine proton production rates, solution A contained 150 mM KCl, 30 μM Q0C10BrH2, 2 μM valinomycin, 100 μM pyranine, and bc1 complex-PL vesicles (35 μl/ml of solution A). Solution B contained 150 mM KCl and 8 μM ferricytochrome c. Solutions A and B were both adjusted carefully to pH 7.0 with 2 mM KOH. Proton pumping was determined from the absorption changes at 457 nm.

**Measurement of Superoxide Anion Generation**—Superoxide anion generation by the cytochrome bc1 complex was determined by measuring the chemiluminescence of the MCLA-O2 adduct (29) in an Applied Photophysics SX.18MV stopped-flow spectrometer by leaving the excitation light off and registering the light emission (30). Reactions were carried out at 23 °C by mixing solutions A and B at 1:1. Solution A contained 100 mM Na+ /K+ phosphate buffer (pH 7.4), 1 mM EDTA, 1 mM KCN, 1 mM NaN3, 0.1% bovine serum albumin, 0.01% n-dodecyl-β-d-maltopyranoside, and 5.0 μM wild-type or mutant bc1 complex. Solution B was the same as solution A except that the bc1 complex was replaced with 150 μM Q0C10BrH2 and 4 μM MCLA.

### RESULTS AND DISCUSSION

**Photosynthetic Growth Behaviors of the Wild-type and Mutant bc1 Complexes**—Because the cytochrome bc1 complex is absolutely necessary for photosynthetic growth of *R. sphaeroides*, a mutant with a substitution at a critical position will not grow photosynthetically, whereas mutants with substitutions at noncritical positions will grow. Thus, observing the photosynthetic growth behavior, one can determine whether substitutions are at critical positions. In mid-log phase, semi-aerobically dark-grown wild-type and mutant cells were inoculated into enriched Sistrom’s medium and subjected to anaerobic photosynthetic growth conditions. Table 2 shows that mutants could grow, indicating that substitutions of Arg-94 are noncritical to the complex. These mutants grew at a decreased rate compared with the wild-type bc1 complex, suggesting that substitutions of Arg-94 affect the function of the cytochrome bc1 complex and thus retard the growth of *R. sphaeroides*.

**Proton Translocation Activity of the Wild-type and Mutant bc1 Complexes**—The bc1 complex catalyzes electron transfer from ubiquinol to cytochrome c and concomitantly translocates protons of ubiquinol across the membrane. The two protons of ubiquinol are released via two pathways. The ejection of the first proton is controlled by the protonation and deprotonation of the histidine ligands of the [2Fe-2S] cluster. The histidine ligands take up a proton from the substrate, ubiquinol, upon reduction of the [2Fe-2S] cluster and release it to the intermembrane space when oxidized by cytochrome c1, as was suggested recently (31). Glu-295 in cytochrome b is important for the release of the second proton, as proposed previously (31–33). Glu-295 is completely conserved in mitochondrial cytochrome b (34), and the importance of the residue in proton transfer is indicated by mutagenesis studies because alteration of glutamine abolishes ubiquinol oxidation in *R. sphaeroides* (35). Additionally, recent kinetic studies showed that protons of a group with a pKα of 5.7 blocked catalysis, and this effect was attributed to Glu-295 (36). The second proton of ubiquinol is first transferred to Glu-295 to form the neutral acid and is then released and delivered to heme propionate A by rotation of the side chain of Glu-295. The subsequent proton release is mediated by a hydrogen-bonded water chain stabilized by cytochrome b residues (Fig. 1) (20, 31).

### TABLE 2

| Strain | Photosynthetic growth | Activitya |
|--------|-----------------------|-----------|
| WT     | + + + +               | 4.58 (100%) |
| R94A   | +                     | 0.18 (4%)   |
| R94D   | + +                   | 1.61 (35%)  |
| R94N   | + + +                 | 2.34 (51%)  |

*The enzyme-specific electron transfer activity of the purified bc1 complexes is expressed as micromoles of cytochrome c reduced per min/nmol of cytochrome b at room temperature.*
The proton pumping property has been well demonstrated in PL vesicles embedded with the purified mitochondrial bc₁ complex (37–40). Fig. 2 shows a typical proton translocation activity assay for PL vesicles embedded with the R. sphaeroides bc₁ complex. After pH equilibrium was reached for the mixture containing Q₀C₁0BrH₂, valinomycin, ferricytochrome c, and bc₁ complex-PL vesicles, an aliquot of ferricyanide solution (arrow 1) was added to initiate electron flow from Q₀C₁0BrH₂ to cytochrome c. After pH equilibrium was attained, the protonophore CCCP was added (arrow 2) to render the liposome membrane freely permeable to protons, and then a second aliquot of ferricyanide was added (arrow 3). The ratio of the pH changes produced by the addition of equal amounts of ferricyanide before (x) and after (y) the addition of CCCP was taken as a measure of the H⁺/e⁻ ratio for the proton translocation activity of the bc₁ complex. The protons released after CCCP addition were the “scalar” protons only, as no contribution from the accumulation of “vectorially” translocated protons was possible.

Table 3 shows the proton translocation activity (H⁺/e⁻) of the wild-type and mutant bc₁ complexes. The proton translocation activity of R94N showed a small decrease compared with the wild-type bc₁ complex, whereas that of R94A and R94D exhibited a significant decrease. H⁺/e⁻ reflects only the final ratio of proton translocation to electron transfer. To further study the proton translocation rate, a kind of dye, pyranine, was used in a stopped-flow experiment. Fig. 3 shows that the magnitude of proton translocation rate of the wild-type and mutant bc₁ complexes can be ranked as WT > R94N > R94D > R94A. These results indicate that a positive correlation exists between the changes in proton translocation activity (H⁺/e⁻) and the proton translocation rate.

Proton transfer pathways involving internal water molecules that provide hydrogen bonds and facilitate proton diffusion have been identified in other membrane proteins such as bacteriorhodopsin (41) and cytochrome c oxidase (42). Although the mechanisms for proton translocations are different for different proteins, the underlying principle for bringing protons to the active sites is similar. For Arg-94, the nitrogen atom of the guanidyl easily forms a hydrogen bond with H₂O, and the repulsion function of the positive charge to H⁺ is also favorable for H⁺ movement. For Ala, the carbon atom of the methyl does not easily form a hydrogen bond with H₂O, and there is no positive charge on its side chain. In addition, as a hydrophobic amino acid, the hydrophobic microenvironment is also unfavorable.
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A Native

![Graph](image)

B +AA

![Graph](image)

Effect of Mutations on Cytochrome b Reduction by Ubiquinol—Fig. 4 shows the time course traces of heme b reduction by $Q_{0,C_{10}}BrH_2$ in the wild type and mutants in the absence (panel A) and presence (panel B) of antimycin A. Antimycin A is a $Q_N$ site inhibitor that blocks electron transfer from heme $b_{14}$ to ubiquinone and prevents reduction of heme $b_{14}$ by $Q_{0,C_{10}}BrH_2$ through the $Q_N$ site.

In the case of the native complex (Fig. 4A), the extent of heme b reduction of mutants decreased relative to the wild type. It should be noted that heme b reduction is mostly heme $b_{14}$ (43). The heme $b_{14}$ reduction by ubiquinol in the $bc_1$ complex, according to the Q-cycle mechanism, is affected by (i) the forward reduction through the $Q_P$ site to heme $b_{14}$ and then to $b_{13}'$, (ii) the reoxidation of reduced heme $b_{14}$ by ubiquinone, and (iii) the “back door” reduction through the $Q_N$ site. Therefore, it is difficult to explain the differences in the extent of heme b reduction among the wild type and mutants.

In the presence of antimycin A (Fig. 4B), heme b reduction by $Q_{0,C_{10}}BrH_2$ occurred only through the $Q_P$ site via heme $b_{14}$, so this case is simplified. Relative to the wild type, the heme b reduction extent of R94N, R94D, and R94A decreased by 2.4, 77.71, and 84.93%, respectively. This decrease implies that some electrons may deviate from the low potential electron transfer chain and leak from the reduced heme $b_{14}$ or ubisemiquinone at the $Q_P$ site during $bc_1$ catalysis. The results also indicate that the extent of the electron leak is WT < R94N < R94D < R94A.

Whether in the absence or presence of antimycin A, the rate constants of heme b reduction of the mutants all decreased relative to the wild type, and the extent of the decrease can be described as R94N < R94D < R94A (Fig. 4). The decrease in the rate constants of heme b reduction suggests that the transfer rate of the second electron of $Q_{0,C_{10}}BrH_2$ is decreased for mutants.

The addition of antimycin A (Fig. 4) increased the extent and decreased the rate of heme b reduction in the wild type and mutants, and these results are consistent with a previous report (44). However, the degree of this effect varied in these complexes. Relative to the native case (Fig. 4A), the rate of heme b reduction of the wild type, R94N, R94D, and R94A was decreased by 52, 43, 50, and 58%, respectively. Relative to the native case (Fig. 4A), the extent of heme b reduction of the wild type, R94N, R94D, and R94A was increased by 83, 255, 25, and 56%, respectively.

Effect of Mutations on Cytochrome c1 Reduction by Ubiquinol—Fig. 5 shows the time course traces of heme c1 reduction by $Q_{0,C_{10}}BrH_2$ in the wild type and mutants in the absence (panel A) and presence (panel B) of antimycin A. In both the absence and presence of antimycin A, the rate constants of heme c1 reduction of all mutants decreased relative to the wild type, and the extent of the decrease is R94N < R94D < R94A. The decrease in the rate constants of heme c1 reduction suggests that the transfer rate of the first electron of $Q_{0,C_{10}}BrH_2$ is decreased for mutants.

Relative to the values for the native case, the addition of antimycin A decreased the rate of heme c1 reduction by 18, 9, 9, and 73% in the wild type, R94N, R94D, and R94A, respectively (Fig. 5). These results are consistent with previous reports (18, 43) that antimycin A has a significant effect on the reduction rate of heme c1 in cytochrome $bc_1$ complexes. This inhibitive effect has
been attributed to the long-range effect of antimycin A on the QP site when binding to the QN site (18, 43). In other words, the effect of antimycin A on the heme $c_1$ reduction rate does not occur through the low potential redox component but through the cytochrome $b$ protein subunit.

In the Q-cycle mechanism, the oxidation of ubiquinol and reduction of ubiquinone or ubisemiquinone occur mainly in the QP and QN sites of cytochrome $b$. In the mutant $bc_1$ complexes, all of the mutant complexes were sensitive to antimycin A (Figs. 4B and 5B) and stigmatellin (a QP site inhibitor) (data not shown). The changes in electron transfer activity or the changes in heme $b$ and $c_1$ reduction should all be related to the mutation at Arg-94, but they are not related to the impairment of the QP or QN site.

Substitution of Arg-94 decreased the proton translocation rate, slowed down the decomposition rate of Q$_0$C$_{10}$BrH$_2$ by feedback inhibition, and then slowed down the transfer of the two electrons of ubiquinol. A decrease in the first electron transfer induced the heme $c_1$ reduction rate decrease and the electron transfer activity decrease (Table 2). A decrease in the second electron transfer induced the heme $b$ reduction rate decrease (Fig. 4).

The decreases in the proton translocation rate, the heme $b$ and $c_1$ reduction rate, and the electron transfer activity all changed in the same order of $R94N$/$H11021$ $R94D$/$H11021$ $R94A$ for the three mutants. This positive correlation also supports the relationship discussed above. In addition, the decreases in the heme $b$ reduction rate and the electron transfer activity are also related to the production of superoxide anion, as discussed below.

**Effect of Mutations on Superoxide Anion Production by the Cytochrome $bc_1$ Complex**—The above results show that some electrons may leak from the low potential electron transfer chain during $bc_1$ catalysis for mutants. What is the fate of these leaked electrons? One of the possible pathways is a reaction with molecular oxygen to form superoxide anions. If this is the pathway, one should see more superoxide anion production by mutants than the wild-type complex because more electrons leak from the mutant complexes.

Fig. 6 shows the tracings of $O_2^-$ generation by the wild type and mutants. MCLA-$O_2^-$ chemiluminescence induced by the $bc_1$ complex reached peak intensity after $\sim 0.1$ s at room temperature and then decayed. Relative to the wild type, $O_2^-$ generation increased by 18, 36, and 61% for R94N, R94D, and R94A, respectively. No luminescence was detected when the $bc_1$ complex was omitted from the enzyme-containing solution. This result suggests that some electrons do leak from the low potential electron transfer chain to $O_2$ and then form $O_2^-$ during $bc_1$
catalysis. The result also indicates that the extent of the electron leak is WT < R94N < R94D < R94A, which is the same as the result from the heme b reduction analysis above. There is an inverse relationship between O$_2^\text{e}^-$ production and the heme b reduction rate (Figs. 4 and 6). The heme b reduction rate indicates the second electron transfer rate. It can therefore be deduced that another reason for the decreased second electron transfer rate in the hydrophobic QP pocket for mutants is the decreased O$_2^\text{e}^-$ production. More superoxide anions released means a lower electron transfer rate.

In addition, the generation of more O$_2^\text{e}^-$ implies that more electrons are consumed by O$_2$ in the bc$_1$ complex, which would result in decreased electron transfer activity. Thus, the decreased activity of mutants is ascribed to the increased O$_2^\text{e}^-$ generation as well as to the decreased proton pumping (discussed above). Under anaerobic conditions, there is no O$_2^\text{e}^-$ generation. Thus, one can deduce that the difference between activities in a mutant and the wild type under anaerobic conditions should be less than that under aerobic conditions. This is indeed the case. Fig. 7 shows that, compared with the wild type, the electron transfer activities of R94N, R94D, and R94A decreased by 49, 65, and 96%, respectively, under aerobic conditions, whereas they decreased by 41, 61, and 94%, respectively, under anaerobic conditions. The difference is significant ($p < 0.05$, Student’s t test) between aerobic and anaerobic conditions for every mutant complex.

Transport Pathway of H$^+$, O$_2^\text{e}^-$ and O$_2^\text{e}^-$ in the bc$_1$ Complex—It is known that O$_2^\text{e}^-$ is generated inside the bc$_1$ complex, perhaps in the hydrophobic environment of the Q$_\text{P}$ pocket through bifurcated oxidation of ubiquinol by transfer of its two electrons to a high potential electron acceptor, an iron-sulfur cluster, and a low potential heme b$_1$ or molecular oxygen. The protein subunits, at least those surrounding the Q$_\text{P}$ pocket, play a role either in preventing the release of O$_2^\text{e}^-$ from its production site to aqueous environments or in preventing O$_2$ from getting access to the hydrophobic Q$_\text{P}$ pocket (45). The increase in superoxide anion production by mutants (Fig. 6) may result from the rapid transport of O$_2$ and O$_2^\text{e}^-$ between the hydrophobic Q$_\text{P}$ pocket and the aqueous phase because the O$_2$ concentrations in the aqueous phase of the wild type and mutants are different. 

Substitution of Arg-94 changes the proton pumping and O$_2^\text{e}^-$ generation simultaneously. This suggests that the transport of H$^+$, O$_2^\text{e}^-$ and O$_2^\text{e}^-$ may occur by the same pathway as described in Figs. 1 and 8. Arg-94 of cytochrome $b$ may function as a gate for the transport of H$^+$, O$_2^\text{e}^-$ and O$_2^\text{e}^-$. However, the factors that influence the transport of H$^+$, O$_2^\text{e}^-$ and O$_2^\text{e}^-$ may be different. H$^+$ transport is related mainly to the hydrogen bond and charge as described above, whereas that of O$_2$ and O$_2^\text{e}^-$ may be affected mainly by the side chain size of Arg-94 or its substitute. The long side chain, e.g. that of Arg-94 in the wild type, is unfavorable for the movement of O$_2$ and O$_2^\text{e}^-$ and this results in low O$_2^\text{e}^-$ production. However, a short side chain, e.g. that of Ala-94, is favorable for the movement of O$_2$ and results in high O$_2^\text{e}^-$ production. In addition, as a hydrophobic amino acid of Ala, the hydrophobic environment is also favorable to the solubility of O$_2$ and generation of O$_2^\text{e}^-$ (45). More O$_2^\text{e}^-$ production by R94D than R94N may be related to the negative charge, which has a repulsive force to O$_2^\text{e}^-$ providing a favorable condition for O$_2^\text{e}^-$ movement. In this study, only one residue was mutated and studied, so this suggestion remains to be further investigated.

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