Evidence for Electron Equilibrium between the Two Hemes $b_L$ in the Dimeric Cytochrome $bc_1$ Complex*

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Structural analysis of the dimeric mitochondrial cytochrome $bc_1$ complex suggests that electron transfer between inter-monomer hemes $b_L$ may occur during $bc_1$ catalysis. Such electron transfer may be facilitated by the aromatic pairs present between the two $b_L$ hemes in the two symmetry-related monomers. To test this hypothesis, R. sphaeroides mutants expressing His$_n$-tagged $bc_1$ complexes with mutations at three aromatic residues (Phe-195, Tyr-199, and Phe-203), located between two $b_L$ hemes, were generated and characterized. All three mutants grew photosynthetically at a rate comparable to that of wild-type cells. The $bc_1$ complexes prepared from mutants F195A, Y199A, and F203A have, respectively, 78%, 100%, and 100% of ubiquinol-cytochrome $c$ reductase activity found in the wild-type complex. Replacing the Phe-195 of cytochrome $b$ with Tyr, His, or Trp results in mutant complexes (F195Y, F195H, or F195W) having the same ubiquinol-cytochrome $c$ reductase activity as the wild-type. These results indicate that the aromatic group at position195 of cytochrome $b$ is involved in electron transfer reactions of the $bc_1$ complex. The rate of superoxide anion ($O_2^-$) generation, measured by the chemiluminescence of 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo[1,2-c]pyrazin-3-one, hydrochloride; MCLA, 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo[1,2-a]pyrazin-3-one hydrochloride-O$_2^-$ adduct during oxidation of ubiquinol, is 3 times higher in the F195A complex than in the wild-type or mutant complexes Y199A or F203A. This supports the idea that the interruption of electron transfer between the two $b_L$ hemes enhances electron leakage to oxygen and thus decreases the ubiquinol-cytochrome $c$ reductase activity.

The cytochrome $bc_1$ complex (also known as ubiquinol-cytochrome $c$ reductase or Complex III) is an essential segment of the electron transfer chains of mitochondria and many respiratory and photosynthetic bacteria (1). It catalyzes electron transfer from ubiquinol to cytochrome $c$. The electron of ubiquinol is passed through the “low potential chain” consisting of heme $b_L$ and heme $b_H$, both housed in the cytochrome $b$ subunit.

Recently, mitochondrial cytochrome $bc_1$ complexes from beef (4, 5), chicken (6), and yeast (7) were crystallized, and their three-dimensional structures were determined. The structural information not only supports the Q-cycle mechanism but also suggests the complex functioning as a dimer. This suggestion stems from the following structural data (4–7): (i) the intertwining of ISPs in the two $bc_1$ monomers such that the head domain of ISP in one monomer is physically close to and interacting with the cytochrome $b$ and cytochrome $c_1$ in the 2-fold symmetry-related other monomer; (ii) the presence of two apparently non-communicating cavities in the dimeric complex, each connecting the Qo pocket of one monomer to the Qi pocket of the other; and (iii) the distance between the Fe atoms of the two hemes $b_i$ is only 21 Å, which is approximately the same as that between heme $b_L$ and heme $b_H$ in one monomer (Fig. 1). The short distance between the two hemes $b_L$ and the presence of several aromatic amino acid residues at the interface of the two cytochrome $b$ proteins has promoted investigator to speculate the existence of electron transfer or equilibrating between the two hemes $b_{i}$ (8–10). Recently the existence of an intertwined dimer in solution was confirmed (11) in the Rhodobacter sphaeroides $bc_1$ complex through the formation of a four-subunit (two ISPs and two cytochrome $b$s) adduct by two inter-subunit disulfide bonds between two engineered cysteine pairs: one at cytochrome $b$ and the head domain of ISP and the other at cytochrome $b$ and the tail domain of ISP. However, evidence for inter-monomer $b_L$-$b_H$ electron transfer during $bc_1$ catalysis is still missing, due to the lack of a suitable assay method.

It has been reported that during electron transfer through the $bc_1$ complex, superoxide anion ($O_2^-$) is produced (12–18). This results from a leakage of the second electron of ubiquinol, from the low potential chain of the Q cycle electron transfer pathway, to interact with molecular oxygen. The electron-leaking site is thought to be located at the reduced cytochrome $b_i$ or ubisemiquinone of the Qo site (12–15, 17, 18). The amount of electron leakage is believed to be proportional to the concentration of reduced cytochrome $b_i$ or ubisemiquinone at the Qo site. Thus, if the inter-monomer $b_L$-$b_H$ electron transfer is interrupted, one should see an increase in the rate of $O_2^-$ generation, because electrons that normally shuttle between the two

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‡ The abbreviations used are: ISP, iron-sulfur protein; O$_2^-$, superoxide anion; MCLA, 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazol[1,2-a]pyrazin-3-one, hydrochloride; Q$_{10}$, BrH$_2$, 2,3-dimethoxy-5-methyl-6-(10-bromodecyl)-1,4-benzoquinol; MCLA, 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazol[1,2-a]pyrazin-3-one, hydrochloride; XO, xanthine oxidase unit(s).

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b1 hemes will accumulate at the b1 heme of one monomer, thus enhancing the chance for leakage and reaction with oxygen. In the three-dimensional structure of mitochondrial bc1 complex, several pairs of aromatic residues are located at the dimer interface between the two hemes b1 (4). These aromatic pairs may facilitate the inter-monomer heme b1-b1 electron transfer. If this is indeed the case the existence of inter-monomer heme b1-b1 electron transfer in the bc1 complex can be revealed by comparing the rates of O2 generation by the wild-type bc1 complex with those of mutant complexes having these aromatic pairs replaced with non-aromatic residues. An increase in O2 generation, as a result of increasing the concentration of reduced b1 or ubisemiquinone at the Qo site, by the mutant bc1 complex, would indicate inter-monomer heme b1-b1 electron transfer, involving aromatic amino acid residues.

Herein we report procedures for generating R. sphaeroides mutants expressing His8-tagged bc1 complexes with mutations at three highly conserved aromatic residues (Phe-195, Tyr-199, and Phe-203) located between the two b1 hemes of the dimeric complex. The rate of superoxide anion generation, the effect of oxygen on the activity, and the EPR characteristics of the cytochromes b1 and b1 in purified complexes from wild type and mutant strains are examined and compared.

EXPERIMENTAL PROCEDURES

Materials—Cytochrome c (horse heart, type III), hypoxanthine, superoxide dismutase, and xanthine oxidase were purchased from Sigma. N-Dodecyl-β-D-maltoside and N-octyl-β-D-glucoside were from Anatrace. Nickel nitrotriacetic acid gel and a Qiaprep spin Miniprep kit were from Qiagen. 2-Methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazol[1,2-a]pyrazin-3-one, hydrochloride (MCLA) was from Molecular Probes, Inc. 2,3-Dimethoxy-5-methyl-6-(10-bromodecyl)-1,4-benzoquinol (Q0C10BrH2) was prepared in our laboratory as previously reported (19).

Generation of R. sphaeroides Strains Expressing the His8-tagged Cytochrome bc1 Complexes with Mutations of Aromatic Residues Located at the Dimer Interface between Two Hemes b1—Mutations were constructed by the QuikChange site-directed mutagenesis kit from Stratagene using a supercoiled double-stranded pGEM7Zf (+)-fbcFB plasmid as template and a forward and a reverse primer for PCR amplification.

The pGEM7Zf (+)-fbcFB plasmid (20) was constructed by ligating the EcoRI-XbaI fragment from pSELNB3503 into EcoRI and XbaI sites of the pGEM7Zf (+) plasmid. The primers used are given in Table I.

The BstEI-XbaI fragment from the pGEM7Zf (+)-fbcFB1m plasmid was ligated into the pRKD418-fbcFBmChQ plasmid to generate the pRDK418-fbcFBmChQ plasmid. A plaque-purified procedure (21) was used to mobilize the pRDK418-fbcFBmChQ plasmid in Ercherichia coli S17–1 into R. sphaeroides BC17 cells. The presence of engineered mutations were confirmed by DNA sequencing of the 962-bp BstEI-XbaI fragment before and after photosynthetic growth of the cells as previously reported (21). DNA sequencing and oligonucleotide synthetesses were performed by the Recombinant DNA/Protein Core Facility at Oklahoma State University.

Growth of Bacteria—E. coli cells were grown at 37 °C in LB medium (sodium chloride, SELECT peptone 140, and SELECT yeast extract, autozized low sodium). For photosynthetic growth of the bc1-complex-bearing R. sphaeroides BC17 cells an enriched Sistrom’s medium containing 5 mM glutamate and 0.2% casamino acids was used. Photosynthetic growth conditions for R. sphaeroides were essentially as described previously (21). Cells harboring the mutated cytochrome b gene on the pRDK418-fbcFBmChQ plasmid were grown photosynthetically for one or two serial passages to minimize any pressure for reversion. The inoculum volumes used for photosynthetic cultures were at least 5% of the total volume. Antibiotics were added to the following concentrations: 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 Activity Assay—Chromatophores were prepared, from which the His8-tagged cytochrome bc1 complexes were purified as previously reported (22). To assay cytochrome bc1 complex activity, chromatophores or purified cytochrome bc1 complexes were diluted with 50 mM Tris-Cl, pH 8.0, containing 200 mM NaCl and 0.01% dodecyl maltoside to a final concentration of cytochrome b of 3 μM. Appropriate amounts of the diluted samples were added to 1 ml of assay mixture containing 100 mM Na’/K’ phosphate buffer, pH 7.4, 300 μM EDTA, 100 μM cytochrome c, and 25 μM Q0C10BrH2. Activities were determined by measuring the reduction of cytochrome c (the onset of absorbance at 550 nm) in a Shimadzu UV 2101 PC spectrophotometer at 23 °C, using a millimolar extinction coefficient of 18.5 for calculation. The non-enzymatic oxidation of Q0C10BrH2 was subtracted from the assay.

To measure the effect of oxygen on bc1 activity, a Thunberg cuvette was used. 1 μl of assay mixture was placed in the main chamber, and a 10 μl aliquot of enzyme (5–10 pmol) was in the side arm. The cuvette was evacuated and flushed with argon five times. The reaction was started by mixing the bc1 complex solution and the assay mixture.

Measurement of Superoxide Anion Generation—Superoxide anion generation by the cytochrome bc1 complex was determined by measuring the chemiluminescence of MCLA-O2 adduct (23), in an Applied Photosynthesis stopped-flow reaction analyzer System SX.18M (Leatherhead, England), by leaving the excitation light off and registering light emission (24). Reactions were carried out at 23 °C by mixing 1:1 solutions A and B. Solution A contains 100 mM Na’/K’ phosphate buffer, pH 7.4, 1 mM EDTA, 1 mM KCN, 1 mM NaN5, 0.1% bovine serum albumin, 0.01% dodecyl maltoside, and an appropriate amount of wild-type or mutant bc1 complex. Solution B was the same as A with bc1 complex being replaced with 50 μM Q0C10BrH2 and 4 μM MCLA. O2 generation is expressed in xanthine oxidase (XO) units. One XO unit is defined as chemiluminescence (maximum peak height of light intensity) generated by 1 unit of XO, which equals 2.71 V from an Applied Photophysics stopped-flow reaction analyzer SX.18M, when solution A containing 100 mM Na’/K’ phosphate buffer, pH 7.4, 100 μM hypoxanthine, 4 μM MCLA, and 1 mM NaN5, is mixed with solution B containing 100 mM Na’/K’ phosphate buffer, pH 7.4, 1 mM NaN5, and 1 unit of XO.

Other Biochemical and Biophysical Techniques—Protein concentration was determined by the method of Lowry et al. (25). Cytochrome b (26) and cytochrome c1 (27) contents were determined according to reported methods. SDS-PAGE was performed according to Laemmli (28) using a Bio-Rad Mini-Protean dual slab vertical cell. Samples were digested with 10 mM Tris-C10缓冲, pH 6.8, containing 1% SDS, and 3% glycerol in the presence of 0.4% β-mercaptoethanol for 2 h at 37 °C before being subjected to electrophoresis.

EPR spectra of bc complexes were recorded at 8.5 K on a Bruker EMX EPR spectrometer equipped with an Air Products flow cryostat. The instrument settings are detailed in the figure legend. Refined coordinates for the crystal structure of native bovine mitochondrial bc1 (29) (PDB code 1NTM) were used for distance determination.

FIG. 1. Distances between redox centers in bovine dimeric cytochrome bc1 complex. All distance measurements are made with native mitochondrial bc1 complex, refined to 2.4 Å resolution (29) (PDB code 1NTM). Superimposed on the ball-and-stick models of the redox centers (b1-b1, b1-ISP, and c1) are experimental electron densities of the anomalous diffraction signal from the iron atoms of the redox center contoured at 4 σ. The center to center distances are indicated by red arrowed lines and are as labeled; the edge to edge distances are shown with black arrowed lines and are as labeled.
RESULTS AND DISCUSSION

Involvement of the Phe-195 of Cytochrome b in Electron Transfer Activity of the Cytochrome bc1 Complex—Three aromatic amino acid residues in cytochrome b, Phe-195, Tyr-199, and Phe-203, were selected for mutation to test the hypothesis that inter-monomer b$_1$-b$_1$ electron transfer occurs during bc$_1$ catalysis, and such electron transfer is facilitated by aromatic residues located between the two b$_1$ hemes. Alignment of more than 40 sequences of cytochrome b reveals that Phe-195 is fully conserved except in Rhodopseudomonas viridis (in which Phe is replaced by Tyr); Tyr-199 is highly conserved, although it is replaced by Phe in many cases; and Phe-203 is less conserved, being substituted with Met, Ser, Leu, and Trp in some species (30). The selection of these three residues was based on the three-dimensional structure of the four-subunit cytochrome bc$_1$ complex of R. sphaeroides (Fig. 2A) constructed by using coordinates from bovine cytochromes b and c$_1$, ISP, and subunit VII (31). The distances between the symmetry pairs of aromatic residues Phe-195, Tyr-199, or Phe-203 are 4.5, 7.7, and 6.8 Å (Fig. 2B), respectively, when measured from edge to edge of the phenyl rings of the amino acid residues. They are, edge to edge, 3.8, 3.7, and 3.3 Å apart, respectively, in the corresponding residues in the bovine complex (Phe-197, Phe-183, and Phe-187 in bovine). The distances from heme b$_1$ to Phe-195, Tyr-199, or Phe-203 are 8.8, 4.9, and 10.3 Å, respectively, when measured from the iron center to the edges of the aromatic ring of the amino acid residues. They are 7.5, 8.6, and 10.2 Å, respectively, in the corresponding bovine enzyme. Recently a relative low resolution structure of cytochrome bc$_1$, complex from Rhodobacter capsulatus was reported (32). The distances obtained among these aromatic amino acid pairs are surprisingly close to those deduced from the model of the complex of R. sphaeroides.

When each of these three aromatic residues was replaced with alanine, the resulting mutants (F195A, Y199A, and F203A) grew photosynthetically at a rate comparable to that of the complement (wild-type) cells. Chromatophores prepared from these mutants have, respectively, 80, 100, and 100% of the bc$_1$ activity found in the complement chromatophores (Table II). When cytochrome bc$_1$ complexes prepared from these three mutant chromatophores were assayed for ubiquinol-cytochrome c reductase activity, the Y199A and F203A mutant complexes had the same activity as the complement complex, and the F195A mutant complex had about 78% (see Table II). These results indicate that Phe-195 of cytochrome b is involved in inter-monomer electron transfer in the dimeric bc$_1$ complex, but residues Tyr-199 or Phe-203 are not.

Mutants with double and triple alanine substitutions in these three aromatic residues of cytochrome b were also generated and characterized. These are: F195A/Y199A, F195A/F203A, Y199A/F203A, and F195A/Y199A/F203A. All but the Y199A/F203A mutant complex have about 78% of the activity found in the complement complex (see Table II). The Y199A/F203A has the same activity as that of the complement complex. Because the extent of bc$_1$ activity decrease in the double or triple alanine substitution mutants containing the F195A mutation is the same as that observed for the F195A mutant complex, aromatic residues Tyr-199 and Phe-203 apparently play no complementary or auxiliary role to residue Phe-195 in inter-monomer electron transfer.

Because the extent of activity change upon the replacement Phe-195 with alanine is a relatively small, 22% decrease, special attention was paid during data collection. The experiments were not only repeated by three times but also repeated by different investigators. We are very confident that the difference in activity is real and not an experimental artifact. In fact the small change in activity observed upon replacement of aromatic amino acid with an alanine at residue position 195 was expected, because the inter-monomer electron transfer is not in the main path of electron transfer.

Absorption spectral analysis of mutant complexes of F195A, F195A/Y199A, F195A/F203A, and F195A/Y199A/F203A indicates that the amounts and absorption properties of cytochromes b and c$_1$ in these mutant complexes are the same in the complement complex. Western blot analysis using antibodies against R. sphaeroides ISP and subunit IV also indicate that these mutant complexes have the same amount of ISP and subunit IV as does the complement complex. Thus the decrease in bc$_1$ activity in the F195A mutant complex is not due to mutational effects on the assembly of the bc$_1$ protein subunits into the chromatophore membrane or to changes in the binding affinity of protein subunits in the complex.

Although the three pairs of aromatic residues are all located at the dimer interface of the cytochrome b subunits, they are not the only residues contributing to the stability of the dimer. As observed in the bovine complex, at least 23 residue pairs against R. sphaeroides ISP and subunit IV also indicate that these mutant complexes have the same amount of ISP and subunit IV as does the complement complex. Thus the decrease in bc$_1$ activity in the F195A mutant complex is not due to mutational effects on the assembly of the bc$_1$ protein subunits into the chromatophore membrane or to changes in the binding affinity of protein subunits in the complex.

The underlined bases correspond to the genetic codes for the amino acid(s) to be mutated.

| Table I | Oligonucleotides used for site-directed mutagenesis |
|---------|---------------------------------------------------|
| F195A (F) | 5'-GCGCGAGGCTCAACCGGTTGTCCTCGTGACTACGGTGGCGTTC-3' |
| F195Y (F) | 5'-GAAGGGCAGCAAGCAGTGGATCGAGAGGCGGCGAAGGCGGTTAGCGTGG-3' |
| F195Y (F) | 5'-GCGGAGGAGCGAGATGAGAGGCGGCGAAGGCGGTTAGCGTGG-3' |
| F195W (F) | 5'-GAAGGGCAGCAGGTGGATCGAGAGGCGGCGAAGGCGGTTAGCGTGG-3' |
| F195W (F) | 5'-GAGGAGGAGCGAGATGAGAGGCGGCGAAGGCGGTTAGCGTGG-3' |
| F195Y (F) | 5'-GAAGGGCAGCAGGTGGATCGAGAGGCGGCGAAGGCGGTTAGCGTGG-3' |
| F195Y (F) | 5'-GAGGAGGAGCGAGATGAGAGGCGGCGAAGGCGGTTAGCGTGG-3' |
| Y199A (F) | 5'-GAAGGGCAGCAGGTGGATCGAGAGGCGGCGAAGGCGGTTAGCGTGG-3' |
| Y199A (R) | 5'-GAAGGGCAGCAGGTGGATCGAGAGGCGGCGAAGGCGGTTAGCGTGG-3' |
| F203A (F) | 5'-GAAGGGCAGCAGGTGGATCGAGAGGCGGCGAAGGCGGTTAGCGTGG-3' |
| F203A (R) | 5'-GAAGGGCAGCAGGTGGATCGAGAGGCGGCGAAGGCGGTTAGCGTGG-3' |
| F195A/Y199A (F) | 5'-CTCAACCGGCGATCACGGCGGGCAGCAGGGCGTGCAGCGAGGCGAACCGGTTGAG-3' |
| F195A/Y199A (R) | 5'-CTCAACCGGCGATCACGGCGGGCAGCAGGGCGTGCAGCGAGGCGAACCGGTTGAG-3' |
| F195A/F203A (F) | 5'-CTCAACCGGCGATCACGGCGGGCAGCAGGGCGTGCAGCGAGGCGAACCGGTTGAG-3' |
| F195A/F203A (R) | 5'-CTCAACCGGCGATCACGGCGGGCAGCAGGGCGTGCAGCGAGGCGAACCGGTTGAG-3' |
| Y199A/F203A (F) | 5'-CTCAACCGGCGATCACGGCGGGCAGCAGGGCGTGCAGCGAGGCGAACCGGTTGAG-3' |
| Y199A/F203A (R) | 5'-CTCAACCGGCGATCACGGCGGGCAGCAGGGCGTGCAGCGAGGCGAACCGGTTGAG-3' |
| F195A/Y199A/F203A (F) | 5'-CTCAACCGGCGATCACGGCGGGCAGCAGGGCGTGCAGCGAGGCGAACCGGTTGAG-3' |
| F195A/Y199A/F203A (R) | 5'-CTCAACCGGCGATCACGGCGGGCAGCAGGGCGTGCAGCGAGGCGAACCGGTTGAG-3' |
stantial contributions to holding the dimer together. As the cytochrome b and ISP subunits are highly conserved, it is reasonable to believe that the interaction at the dimer interface would also be conserved. Because the structural effect of these mutations is probably a creation of small cavities, it is not surprising to find that even the triple mutation does not disturb the structural integrity of the bc₁ dimer.

In bovine cytochrome b the three interfacial aromatic pairs display three entirely different contact geometries: the Phe-179 pair (195 in R. sphaeroides bc₁) has an on-edge interaction with the two phenyl rings aligned roughly parallel to the membrane plane; with an angle between the two planes of 37°; the phenyl rings of the Phe-183 pair (199 in R. sphaeroides bc₁) are stacked on top of each other with an angle of 0°; and the Phe-187 pair (203 in R. sphaeroides bc₁) is partially stacked, and these two phenyl rings are oriented normally to the membrane plane with an angle between the two rings of 34.7°. In biological electron transfer complexes such as the cytochrome bc₁ complex (4) and photosynthetic reaction centers (33), donors and receptors of electron transfer machines are always observed in an on-edge arrangement, as determined by x-ray crystallography. Presumably the on-edge interaction between partners provides more efficient electron transfer than other orientations. The observed on-edge interaction for the Phe-179 pair in the bovine bc₁ structure strongly supports our mutational data indicating that this particular pair mediates lateral electron transport between bc₁ monomers. In addition, Phe-179 has the shortest distance to the Qo site, which may also be advantageous for its role as an electron transfer mediator between monomers.

Essentiality of the Aromatic Group in the Phe-195 of Cytochrome b—To establish that the loss of ubiquinol-cytochrome c reductase activity in the F195A mutant complex results from the loss of an aromatic ring at this position of cytochrome b, mutants with conservative substitution at Phe-195 (F195Y, F195W, and F195H), were generated and characterized. These three mutants grew photosynthetically at a rate comparable to that of the complement cells and, in bc₁ complexes in chromatophore membranes or in the purified state, have the same ubiquinol-cytochrome c reductase activity as that of the complement complex (see Table II). These results confirm the es-
Characterization of mutants in the cytochrome b of the bc1 complex

The data presented are mean values ± S.D. from three experiments.

| Mutants          | Photosynthetic growth | bc1 complex Activity |
|------------------|-----------------------|-----------------------|
| Wild type        | + +                   | 2.21 ± 0.03           | 2.50 ± 0.01 |
| F195A            | + +                   | 1.76 ± 0.02           | 1.94 ± 0.02 |
| Y199A            | + +                   | 2.21 ± 0.03           | 2.52 ± 0.03 |
| F203A            | + +                   | 2.20 ± 0.03           | 2.51 ± 0.02 |
| F195Y            | + +                   | 2.22 ± 0.03           | 2.50 ± 0.01 |
| F195W            | + +                   | 2.20 ± 0.02           | 2.49 ± 0.03 |
| F195H            | + +                   | 2.21 ± 0.01           | 2.50 ± 0.02 |
| F195AY199A       | + +                   | 1.76 ± 0.02           | 1.95 ± 0.02 |
| F195AF203A       | + +                   | 1.75 ± 0.01           | 1.93 ± 0.03 |
| Y199AF203A       | + +                   | 2.20 ± 0.02           | 2.50 ± 0.02 |
| F195AY199A/F203A | + +                   | 1.77 ± 0.01           | 1.96 ± 0.02 |

* The purified bc1 complex was in 50 mM Tris-Cl, pH 8.0, containing 200 mM NaCl, 200 mM histidine, and 0.5% octyl-glucoside.

** Specific activity (S.A.) is expressed as micromoles of cytochrome c reduced/min/mmol of cytochrome b at room temperature.

+ +, cell growth rate is essentially the same as that of the wild-type cells.

The chemiluminescence of MCLA-O$_2^-$ generated when cytochrome bc$_1$ complex is mixed with ubiquinol and MCLA, is registered in light emission. Because the system contains no cytochrome c, chemiluminescence of MCLA-O$_2^-$ resulting from non-enzymatic oxidation of ubiquinol by cytochrome c, is eliminated. This method enables us to accurately evaluate changes in the rate of superoxide anion generation by various bc$_1$ complexes.

Fig. 3 shows actual tracings of superoxide generation by wild-type and F195A mutant bc$_1$ complexes. MCLA chemiluminescence induced by bc$_1$ complex reaches peak intensities after ~0.06 s at room temperature and then decays. No detectable luminescence is detected when the enzyme-containing solution or Q$_0$C$_{10}$BrH$_2$ is omitted from the above system, or 300 units/ml superoxide dismutase was added to the system.
Table III compares the rates of \( \text{O}_2 \) generation by the complement and mutant cytochrome \( \text{bc}_1 \) complexes. Oxidation of ubiquinol by complement and F195A mutant complexes produces 0.17 and 0.49 \( \text{XO} \) units per milligram of protein, respectively. A similar increase in the rate of \( \text{O}_2 \) production is observed in mutant complexes of F195A/Y199A, F195A/F203A, and F195A/Y199A/F203A. However, the rates of \( \text{O}_2 \) production by mutant complexes Y199A, F203A, and Y199A/F203A are similar to that of the complement complex. These results support the idea that the decrease in \( \text{bc}_1 \) activity in the Phe-195 mutant complex results from interruption of the inter-monomer \( \text{b}_1-\text{b}_1 \) electron transfer, facilitated by the aromatic group in position Phe-195.

**Effect of Oxygen on Cytochrome c Reduction by Cytochrome \( \text{bc}_1 \) Complex**—Table IV shows the effect of oxygen on the cytochrome \( \text{bc}_1 \) activity in purified complexes from wild type and mutants F195A, Y199A, and F203A. The complement, Y199A and F203A mutant complexes catalyzed electron transfer, from ubiquinol to cytochrome c, at a rate of 2.5 \( \mu \text{mol} \) of cytochrome c reduced per minute per nanomole of cytochrome \( b \) at 23 °C under aerobic conditions. Removal of oxygen from the assay system increases the activities of these four complexes by 4\% (2.6 \( \mu \text{mol} \) cytochrome c reduced per minute per nanomoles of cytochrome \( b \)). It should be noted that this activity increase is not due to the inhibition of the contaminated cytochrome c oxidase in the \( \text{bc}_1 \) preparation, because addition of sodium azide to the assay mixture, under aerobic conditions, does not increase the rate of cytochrome c reduction. Addition of superoxide dismutase to the assay mixture causes a 5.2\% activity decrease in the complement, Y199A and F203A cytochrome \( \text{bc}_1 \) complexes. Apparently the \( \text{O}_2 \) generated from the electron leak at the \( \text{Q}_0 \) site is capable of reducing cytochrome c at a slightly lower rate than normal electron transfer through \( \text{bc}_1 \) complex. Addition of superoxide dismutase to the assay system converts the \( \text{O}_2 \) into hydrogen peroxide, which reduces cytochrome c very slowly, and thus decreases the rate of cytochrome c reduction. From the activity of cytochrome \( \text{bc}_1 \) complex, determined by reduction of cytochrome c in the presence or absence of oxygen, one can estimate the rate of cytochrome c reduction by \( \text{O}_2 \). It is about three quarters of the rate of cytochrome c reduction by \( \text{bc}_1 \) complex.

When \( \text{bc}_1 \) complex from mutant F195A was assayed in the presence and absence of oxygen, the activities were 1.94 and 2.39 \( \mu \text{mol} \) of cytochrome c reduced per minute per nanomole cytochrome \( b \), respectively. This amounts to a 23\% activity increase in the absence of oxygen. Under aerobic conditions, addition of superoxide dismutase to the assay mixture causes the activity of the F195A mutant complex to decrease from 1.94 to 1.70 \( \mu \text{mol} \) of cytochrome c reduced per minute per nanomole cytochrome \( b \). This indicates that 12\% of the observed activity (reduction of cytochrome c) is due to \( \text{O}_2 \). The disruption of electron transfer between the two \( \text{b}_1 \) hemes in dimeric cytochrome \( \text{bc}_1 \) complex enhances (from 5.2 to 12\%) the electron leakage and decreases (2.61 to 2.39 \( \mu \text{mol} \) of cytochrome c reduced per minute per nanomole cytochrome \( b \)) the normal electron transfer rate during oxidation of ubiquinol and reduction of cytochrome c. Because the activity of F195A mutant complex is not restored to the level of the activity of the complement complex under the aerobic conditions, activity loss due to disruption of electron transfer between the two \( \text{b}_1 \) hemes cannot be attributed entirely to electron leak to oxygen. About half of the activity loss is due to disruption of electron transfer between the two hemins \( \text{b}_1 \). In the presence of inter-monomer electron transfer, the electron at \( \text{Q}_0 \) (either at \( \text{b}_1 \) or ubisemiquinone) of one monomer can be oxidized by the two hemins \( \text{b}_1 \) of the complex. When inter-monomer electron transfer is interrupted, as in the F195A mutant complex, the electron at hemin \( \text{b}_1 \) can only be oxidized by hemin \( \text{b}_1 \) of the same monomer; this accounts for a 9\% decrease of activity. Another explanation for activity decrease in the F195A mutant complex is the relatively more oxidized state of hemins \( \text{b}_1 \) in dimeric \( \text{bc}_1 \) complex with inter hemin \( \text{b}_1 \) electron transfer than in those without. This more oxidized state of hemin \( \text{b}_1 \) would facilitate electron trans-
fer from ubisemiquinone at Qo site. The fact that disruption of electron transfer between two $b_1$ hemes of dimeric complex causes a decrease of activity indicates that both monomers are not function independently, some sort of negative cooperativity does exist (9).

**Effect of F195A on EPR Characteristics of the $b$ Cytochrome—** Although evidence presented above clearly demonstrates that the loss of $b_1$ complex activity in mutants, F195A, F195A/Y199A, F195A/F203A, and F195A/Y199A/F203A, correlates with the leakage of electrons that normally shuttle between the two $b_1$ hemes through Phe-195, whether or not mutation F195A affects the microenvironments of the cytochromes $b$ is unknown. To test this possibility, EPR characteristics of the $b$ cytochromes in cytochrome $b$ F195A and wild type were examined after samples were reduced with sodium ascorbate to eliminate the overlapping signal from cytochrome $c_1$. As shown in Fig. 4, this mutant has EPR characteristics identical to those of the wild-type complex, with features at $g = 3.53$ and $g = 3.77$ previously assigned to cytochrome $b_1$ and $b_1$, respectively, and a $g = 4.29$ signal thought to be due to nonspecifically bound iron (III) (40). These data indicate that substitution of Phe-195 with alanine has no significant effect on the environments of cytochrome $b$ heme. This mutation also has no effect on the midpoint potential of cytochromes $b$.

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