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# Effect of subunit IV on superoxide generation by *Rhodobacter sphaeroides* cytochrome $bc_1$ complex

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ABSTRACT

Previous studies indicate that the three-subunit cytochrome $bc_1$ core complex of *Rhodobacter sphaeroides* contains a fraction of the electron transfer activity of the wild-type enzyme. Addition of subunit IV to the core complex increases electron transfer activity to the same level as that of the wild-type complex. This activity increase may result from subunit IV preventing electron leakage, from the low potential electron transfer chain, and reaction with molecular oxygen, producing superoxide anion. This suggestion is based on the following observations: (1) the extent of cytochrome *b* reduction in the three-subunit core complex, by ubiquinol, in the presence of antimycin A, never reaches the same level as that in the wild-type complex; (2) the core complex produces 4 times as much superoxide anion as does the wild-type complex; and (3) when the core complex is reconstituted with subunit IVs having varying reconstitutive activities, the activity increase in reconstituted complexes correlates with superoxide production decrease and extent of cytochrome *b* reduction increase.



## 1. Introduction

The cytochrome $bc_1$ complex from *Rhodobacter sphaeroides* catalyzes electron transfer from ubiquinol to cytochrome $c_2$ in the cyclic photosynthetic electron transfer chain [1]. Oxidation of ubiquinol is coupled to the vectorial translocation of protons across the membrane to generate a pH gradient and membrane potential for ATP synthesis [1]. The purified complex contains four protein subunits: the cytochrome *b* subunit housing two *b*-type hemes ($b_{566}$ and $b_{562}$), the cytochrome $c_1$ subunit housing a *c*-type heme ($c_1$), a Rieske iron-sulfur protein housing a high potential [2Fe2S] cluster, and subunit IV containing no redox prosthetic group. The first three subunits are core subunits [2], and the last one is a supernumerary subunit [3,4].

While study of the core subunits has been intensive and a wealth of information has been obtained, study of the supernumerary subunit is rather limited. The $bc_1$ complexes from all sources contain three core subunits, but they vary significantly in the number of supernumerary subunits [4]. For example, cytochrome $bc_1$ complexes purified from mitochondria of bovine heart and yeast and from chromatophores of *R. sphaeroides* and *Rhodobacter capsulatus* by a single chromatographic procedure have 8, 7, 1 and no supernumerary subunits [4]. Since *R. sphaeroides* $bc_1$ complex has only one supernumerary subunit (subunit IV), it is an ideal system for studying the function of supernumerary subunit.

The gene for subunit IV of *R. sphaeroides* $bc_1$ complex has been cloned and sequenced [5]. A mutant lacking subunit IV (RSΔIV) has been constructed [6]. Cytochrome $bc_1$ complex purified from RSΔIV chromatophores contains only the three core subunits. This three-subunit core complex has a fraction of the wild-type complex activity with a 4-fold increase in the apparent $K_m$ for $Q_2H_2$ [6]. Subunit IV was over-expressed in *Escherichia coli* cells as a GST fusion protein using the constructed expression vector pGEX-IV and purified to homogeneity with a procedure involving glutathione agarose gel, thrombin digestion and gel filtration [7]. Addition of purified recombinant IV to the three-subunit core complex restores $bc_1$ activity to the level of the wild-type complex [7], indicating that both the three-subunit core complex and recombinant IV are reconstitutively active.

The availability of the reconstitutively active three-subunit core complex [6] and subunit IV [7] in our laboratory enabled us to use *in vitro* reconstitution to study the interaction between supernumerary and core subunits in this bacterial $bc_1$ system. By deletion and substitution [8,9] mutations, residues 81–84 and residues 86–109 of subunit IV were found to be required for interaction with the core complex to restore $bc_1$ activity. Residues 86–109, the only transmembrane helix of this protein, are required for incorporation of subunit IV into the $bc_1$ complex [8]. Residues 81–84, with a sequence of YRYR, are required for subunit IV interaction with the core subunits [9], after subunit IV is incorporated into the complex through the transmembrane helix, to restore $bc_1$

*Abbreviations:* DM, dodecylmaltoside; $Q_oC_{10}BrH_2$, 2,3-dimethoxy-5-methyl-(10-bromodecyl)1,4-benzoquinol; SOD, superoxide dismutase; MCLA, 2-Methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazol[1,2-α]pyrazin-3-one hydrocholoride; $O_2^{\cdot-}$, superoxide anion radicals; AA, antimycin A; Stig., stigmatellin

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activity. Why subunit IV increases $bc_1$ activity upon interaction with the core complex, however, remains a question.

During electron transfer through the $bc_1$ complex, superoxide anion radicals ($O_2.^-$) are generated [10–12]. This is thought to result from the leakage of electrons, from the low potential electron transfer chain, to react with molecular oxygen. The electron leakage site is speculated to be ubisemiquinone radical of the $Q_P$ site [13–15] or reduced heme $b_L$ [16,17]. In fact, the electron leakage site (or superoxide generation site) in the $bc_1$ complex depends on which bifurcated quinol oxidation mechanism is functioning. At present, there are two popular mechanisms, the sequential and the concerted. Each has experimental support [18–22]. In the sequential mechanism, ubiquinol gives its first electron to ISP to form ubisemiquinone at the $Q_P$ site and then the electron from ubisemiquinone is transferred to heme $b_L$. In the concerted mechanism, the two electrons from ubiquinol are simultaneously transferred to ISP and heme $b_L$. If the sequential mechanism is functioning, the electron leakage sites are at ubisemiquinone of the $Q_P$ site and heme $b_L$. If the concerted mechanism is functioning, electron leakage site is only at heme $b_L$. Regardless which mechanism prevails, participation of the iron-sulfur cluster is mandated. In other words, electron that reacts with oxygen to generate superoxide is originated from the second electron of ubiquinol. Since mutant $bc_1$ complexes lacking heme $b_L$ or $b_H$ (17) can generate superoxide to the same level as that of the antimycin inhibited wild-type complex, heme $b_L$ is not an obligated component for superoxide generation. In the absence of heme $b_L$, molecular oxygen can act as a second electron acceptor during the bifurcated oxidation of $QH_2$ where the iron-sulfur cluster acts as the first electron acceptor at the hydrophobic $Q_P$ pocket of the $bc_1$ complex. Since there is no semiquinone radical detected, the molecular oxygen and iron-sulfur cluster must receive electrons from $QH_2$ at about the same rate. The failure to detect superoxide formation in a mutant, which has the head domain of ISP fixed at the *b*-position with an elevated redox potential (15), is likely due to the lack of an oxidized form of iron-sulfur cluster that is needed for the bifurcated oxidation of ubiquinol in order to produce superoxide. It is not due to the lack of mobility of the ISP head to reduce the accessibility of molecular oxygen.

Recently, the three-dimensional structure of the three-subunit core complex from *R. sphaeroides* has become available [23]. However, the loss of subunit IV during crystallization decreases the usefulness of this bacterial structure in the study of interaction with subunit IV. In the proposed structural model of *R. sphaeroides* $bc_1$ complex [8], subunit IV is near to cytochrome *b*, where Q binding sites and heme $b_L$ and $b_H$ reside. This, together with the observation of superoxide production during $bc_1$ catalysis, encouraged us to suggest that subunit IV stabilizes cytochrome *b*, preventing electron leakage from the low potential electron transfer chain, thus increasing $bc_1$ activity. To test this hypothesis, the rates of cytochromes *b* and $c_1$ reduction, by ubiquinol, and superoxide generation, by wild-type, three-subunit core and reconstituted complexes were determined and compared. Also, a reverse relationship between $bc_1$ activity and superoxide production was established.

## 2. Materials and methods

### 2.1. Materials

N-dodecyl-β-D-maltoside (DM) and N-octyl- β -D-glucoside were purchased from Anatrace. Ni-NTA resin was from Qiagen. Glutathione-agarose gel, superoxide dismutase (SOD), xanthine oxidase, and Mn-containing superoxide dismutase (SOD) were from Sigma. 2-Methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazol[1,2-α]pyrazin-3-one, hydrocholoride (MCLA) was from Molecular Probes. 2,3-dimethoxy-5-methyl-6-19′bromo-decyl-1, 4-benzoquinol ($Q_0C_{10}BrH_2$) was synthesized in our laboratory as previously described [24].

### 2.2. Enzyme preparations and activity assay

The $His_6$-tagged, wild-type and three-subunit core complexes were purified from chromatophores of BC17 carrying pRKD*fbc*$FBC_HQ$ [25] and RSΔIV carrying pRKD*fbc*$FBC_H$ [8], respectively. Recombinant wild-type and mutant subunit IVs were prepared as described previously [9]. Reconstituted $bc_1$ complexes were prepared by the addition of wild-type or mutant IVs to the three-subunit core complex at a 2:1 molar ratio and incubated for 1 h at 0 °C [9]. The concentrations of purified wild-type and mutant subunit IVs were determined by measuring the absorbance at 280 nm, using their respective millimolar extinction coefficients calculated according to the following equation [26]: $\varepsilon^{280}{}_{mM} = 5.50\ (n_{Trp}) + 1.49\ (n_{Tyr})$. $n$ = number of tryptophan or tyrosine residues present in wild-type or mutant subunit IVs. There are five tryptophan and three tyrosine residues in the wild-type IV. The values of $\varepsilon^{280}{}_{mM}$ used are 32 for wild-type and IV(R84E), 30.5 for IV(Y81A), and 29 for IV(81–84)A.

To assay electron transfer activity, purified cytochrome $bc_1$ 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 1 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, 1 mM EDTA, 100 μM cytochrome *c* and 25 μM $Q_{0C10BrH2}$. Activity was determined by measuring the reduction of cytochrome *c* (the increase in absorbance at 550 nm) in a Shimadzu UV-2101PC, at 23 °C, using a millimolar extinction coefficient of 18.5 for calculation. Non-enzymatic reduction of cytochrome *c*, determined under the same conditions in the absence of enzyme, was subtracted from the assay.

### 2.3. Measurement of pre-steady state reduction rates of cytochromes b and $c_1$

Measurements were performed in a stopped-flow apparatus, Applied Photophysics SX.18MV spectrometer (Leatherhead, England) with a photodiode array detector. The reaction was started by mixing equal volumes of solution A and solution B. Solution A contained 100 mM Na/K phosphate, pH 7.4, 1 mM KCN, 0.1%BSA, 0.01% DM and 12.0 μM of fully oxidized cytochrome $bc_1$ complex (based on cytochrome $c_1$) and solution B contained 5 mM $NaH_2PO_4$ 1 mM KCN, 0.1%BSA, 0.01% DM and 240 μM $Q_0C_{10}BrH_2$, at 23 °C. The fully oxidized cytochrome $bc_1$ complexes were prepared by the treatment of isolated complexes with catalytic amounts of cytochrome *c* and cytochrome *c* oxidase, overnight, at 0 °C. The fully oxidized state was confirmed by absorption spectral analysis. To determine the reduction rates of cytochromes *b* and $c_1$, a spectrum from 600 nm to 500 nm, with a resolution of 2.17 nm was recorded every 2.08 ms. The dead time of the instrument was about 2 ms. Reductions of cytochromes *b* and $c_1$ were determined from the absorption changes at 560 nm–580 nm and 551 nm–539 nm, respectively. Time traces of the reaction were fitted with a first order rate equation to obtain the pseudo first order rate constants $k_1$ by Kaleidagraph.

To measure the effect of oxygen on pre-steady state reduction of cytochrome *b*, oxygen was removed from solution A and solution B before mixing to start the reaction. Two test tubes containing solution A and solution B were placed in a sealed, three-arm bottle containing some water. Solution A was connected to syringe A by a flexible tubing with a T-joint through the first arm; solution B was connected to syringe B with a similar manner as in solution A through the second arm; and the air space in the bottle was connected to a vacuum pump and argon gas through the third arm. The system was vacuumed then followed by flashing with argon, repeatedly, for 1 min to replace oxygen in these two solutions with argon. After removing oxygen, solutions were forced into their respective syringes. The stopped-flow experiments were carried out in an Applied Photophysics stopped-flow reaction analyzer SX.18MV.

### 2.4. Measurement of superoxide anion

Superoxide anion generation was determined by measuring the chemiluminescence of MCLA-$O_2.^-$ adduct [27–29], in an Applied Photophysics stopped-flow reaction analyzer SX.18MV by leaving the excitation light off and registering light emission, as previously reported [29]. 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, 0.1% BSA, 0.01% DM and 3 μM of wild-type or mutant $bc_1$ complex. Solution B was the same as A with $bc_1$ complex replaced with 50 μM $Q_0C_{10}BrH_2$ and 4 μM MCLA. The cytochrome $bc_1$ complexes used were in completely oxidized form before mixing. $O_2.^-$ generation is expressed in XO units. One XO unit is defined as chemiluminescence (maximum peak height of light intensity) generated by 1 U of xanthine oxidase which equals 2.0 V from an Applied Photophysics stopped-flow reaction analyzer SX.18MV.

### 2.5. Other biochemical methods

Cytochromes *b* [30] and $c_1$ contents [31] were determined as previously described.

## 3. Results and discussion

### 3.1. Effect of subunit IV on the activation energy barrier of the cytochrome $bc_1$ complex

While the restoration of activity to the three-subunit core complex upon the addition of subunit IV is well established, the nature of this activation is not yet understood. It is important to know whether or not activation is due to a decrease of the activation energy barrier caused by interaction between the core complex and subunit IV. The activation energies of the wild-type and three-subunit core complexes were determined by Arrhenius plots of $bc_1$ activity in these two complexes (see Fig. 1). An activation energy of 21.8 kJ/mol is obtained for the wild-type complex and 24.5 kJ/mol for the three-subunit core complexes at pH 7.4. Although the presence of subunit IV does somewhat decrease the activation energy of the $bc_1$ complex, it may not account for all of the activity enhancement; other factors may also be involved.

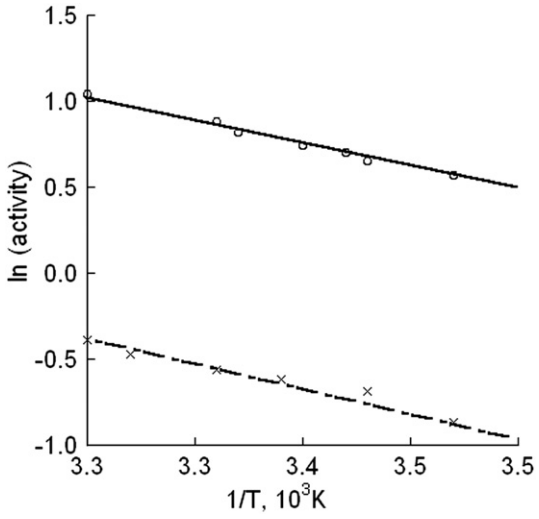


**Fig. 1.** Arrhenius plots for the electron transfer activities of wild-type and three-subunit core complexes. The ubiquinol-cytochrome *c* activities in the wild-type (-o-) and three-subunit core complex (-x-) were assayed at every 2° from 30 °C to 12 °C. The natural logarithms of the activities were plotted against the reciprocal of absolute temperature in an Arrhenius plot. The slopes of the Least Squares fitting straight lines were used to estimate their activation energies.

### 3.2. Effect of subunit IV on cytochrome b reduction by $Q_0C_{10}BrH_2$

Fig. 2 shows time course traces of heme *b* reduction by $Q_0C_{10}BrH_2$, in wild-type (red) and three-subunit core (blue) $bc_1$ complexes, in the absence (A and D) and presence of antimycin (B and E) or stigmatellin (C and F), at 1 and 50 s time scales. Experiments were performed with a stopped-flow apparatus. $Q_0H_{10}BrH_2$ was added in a 20-fold molar excess of the enzyme complex. Antimycin A is a $Q_N$ site inhibitor which blocks electron transfer from heme $b_H$ to ubiquinone. Stigmatellin is a $Q_P$ site inhibitor whose binding to cytochrome *b* fixes the ISP head at the *b*-position. The final concentration of antimycin A was 30 μM and of stigmatellin was 20 μM.

In the absence of inhibitor (see Fig. 2A), the reduction kinetics of heme *b* in the wild-type and three-subunit core complexes are similar. They both are biphasic: a fast reduction phase followed by a slow reduction phase. The rate constants for the fast reduction phase of heme *b* in the wild-type and three-subunit core complexes are 16.3 $s^{-1}$ and 13.5 $s^{-1}$, respectively. The extent of heme *b* reduction is much less in the three-subunit complex (0.027 O.D. unit) than in the wild-type complex (0.055 O.D. unit). The less than 20% decrease in the rate of heme *b* reduction in the three-subunit core complex does not account for the 50% decrease in the extent of heme *b* reduction. These results seem to suggest that, in the absence of subunit IV, some electrons may deviate from their normal pathway, before they reach heme $b_H$ during $bc_1$ *catalysis*. However, it is difficult to assess the site of electron leakage during $bc_1$ catalysis by comparing reduction kinetics of hemes *b* in these two complexes because heme $b_H$ reduction by quinol 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 $b_L$ and then to $b_H$; (ii) the re-oxidation of reduced heme $b_H$ by ubiquinone; and (iii) the "back door" reduction through the $Q_N$ site. Therefore, the reduction kinetic of heme *b* was examined with the aid of $Q_N$ and $Q_P$ site inhibitors.

Addition of antimycin A (see Fig. 2B) increases the extent and decreases the rate of heme *b* reduction in the wild-type and three-subunit core complexes. However, the degree of effect varies in these two complexes. In the wild-type complex, the extent of heme *b* reduction increases by 27% (from 0.055 O.D. unit to 0.070 O.D. unit) while the reduction rate constant decreases by 44% (from 16.3 $s^{-1}$ to 9.1 $s^{-1}$). In the three-subunit core complex, the extent of heme *b* reduction increases by 50% (from 0.030 to 0.045) and the reduction rate decreases by 67% (from 13.5 $s^{-1}$ to 4.4 $s^{-1}$). Antimycin A blocks electron transfer from reduced heme $b_H$ to ubiquinone and prevents reduction of heme $b_H$ by $Q_0C_{10}BrH_2$ through the $Q_N$ site. Thus, the rate and extent of heme *b* reduction by $Q_0C_{10}BrH_2$ in the presence of antimycin A is through the $Q_P$ site via heme $b_L$. In the presence of antimycin A, the extent of heme *b* reduction in the three-subunit core complex (0.045 O.D. unit) is about 36% less than that in the wild-type complex (0.070 O.D. unit). This result further suggests that the absence of subunit IV facilitates electron leakage from the low potential electron transfer chain, reduced heme $b_L$ or ubisemiquinone at the $Q_P$ site, during $bc_1$ catalysis. When the wild-type, three-subunit core, and reconstituted complexes were assayed for ubiquinol-cytochrome *c* reductase activity, about 9.5%, 13.1%, and 9.8% of their respective activities are insensitive to antimycin A. The increase in antimycin A-insensitive activity in the three-subunit core complex is in line with the suggestion of more electron leakage in this complex. Because the portion of cytochrome *c* reduced by superoxide produced from the electrons linked from the $bc_1$ complex at the $Q_P$ site during catalysis is expected to be insensitive to antimycin A.

To further confirm this suggestion, the time course traces of heme *b* reductions in the wild-type and three-subunit core complexes, by $Q_0C_{10}$ $BrH_2$, were extended to 50 s (see Fig. 2D and E). If this suggestion is correct, the extent of heme *b* reduction in the core complex should never reach the same level as that in the wild-type complex. This is indeed the case. In fact, the heme *b* reduction by

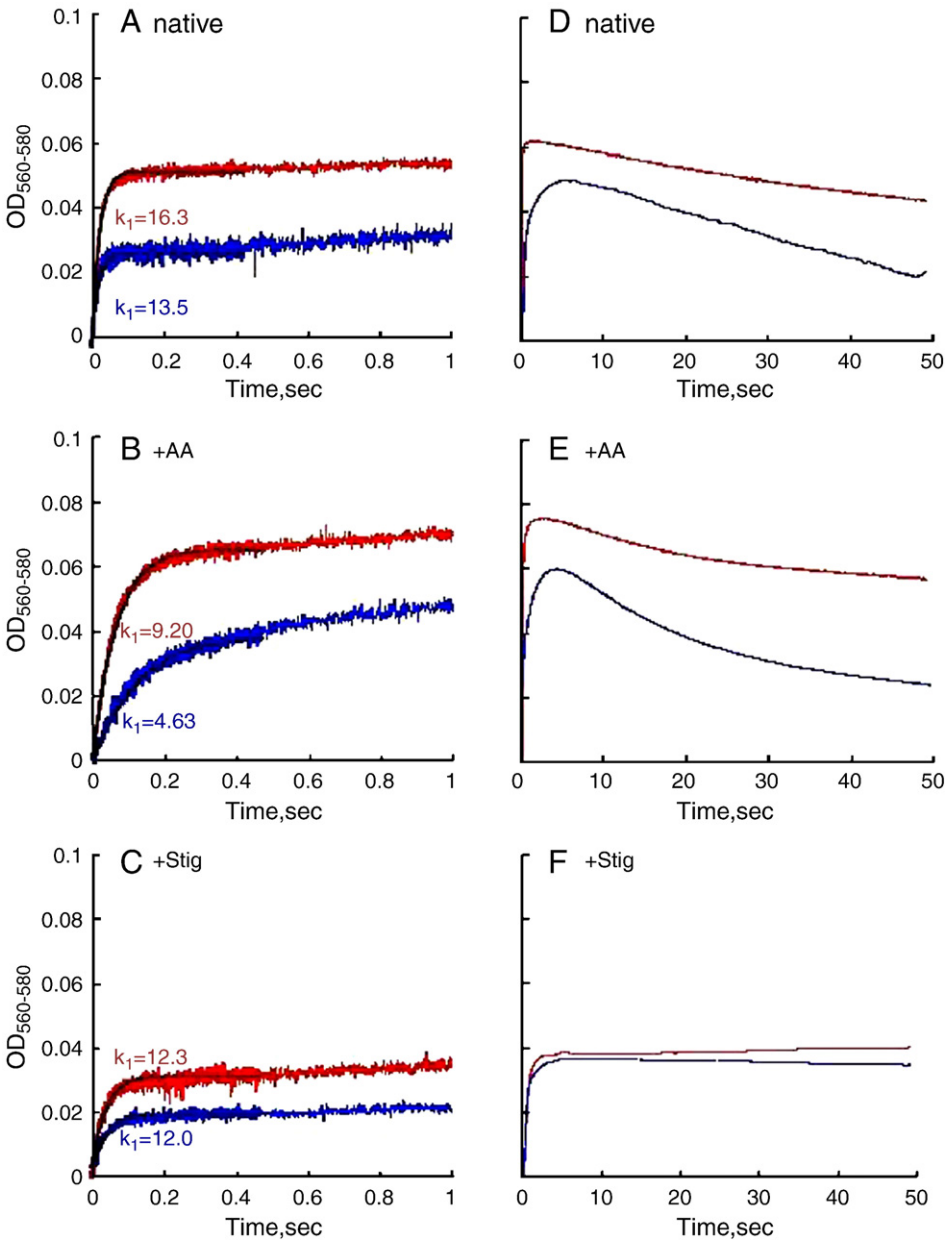


**Fig. 2.** Time courses of cytochrome *b's* reduction by $Q_0C_{10}BrH_2$. Wild-type (red) and three-subunit core (blue) complexes in the absence of inhibitors (panels A and D), and in the presence of antimycin A (panels B and E), or stigmatellin (panels C and F ). Experimental conditions were as described in the Materials and methods. The concentration of antimycin used was 30 μM and that of stigmatellin was 20 μM. The reduction of cytochrome *b* was followed by $A_{560nm}$–$A_{580nm}$. Solid lines represent fitted curves.

quinol in both complexes, in the absence and presence of antimycin A, is biphasic: a fast reduction phase followed by a slow re-oxidation phase. This re-oxidation phase is not due to substrate limitation or to electron transfer from reduced heme $b_H$ to ubiquionone because the concentration of $Q_0C_{10}BrH_2$ is 20 times that of cytochrome *b* and the system contains antimycin A (Fig. 2E). The rate of re-oxidation in the three-subunit core complex is faster than that in the wild-type complex. This re-oxidation may result from electron leakage from the low potential electron transfer chain. Possibly the difference in the extent of heme *b* reduction in the wild-type and three-subunit core complexes results from the difference in the rate of electron leakage in these two complexes.

If the differences in the extents of heme *b* reduction and in the re-oxidation kinetics on the extended (50 s) timescale originate from the leaks to oxygen, one would expect to see the diminishing of these differences (partly or completely) upon removal of oxygen. This is indeed the case. When reduction of cytochrome *b* in the wild-type and three-subunit core complexes, by $Q_0C_{10}BrH_2$, were determined under anaerobic conditions, the difference in the maximum extent of heme *b* reduction between these two complexes is 0.012 O.D. unit, a decrease of 20% compared to that obtained under aerobic conditions (0.015 O.D. unit). The re-oxidation phases observed during cytochrome *b* reduction in the wild-type and three-subunit core complexes are completely diminished.

Addition of stigmatellin (Fig. 2C) decreases the extent and rate of heme *b* reduction in the wild-type and three-subunit core complexes. The effects are similar in both complexes. In the presence of stigmatellin, heme *b* reduction in the three-subunit core complex (0.02 O.D. unit) is about 43% less than that in the wild-type complex (0.035 O.D. unit). Reduction rate constants are 12.3 $s^{-1}$ and 12.0 $s^{-1}$, respectively. These results are as expected, since heme *b* reductions by $Q_0C_{10}BrH_2$, in the presence of stigmatellin, are through the $Q_N$ site. No re-oxidation phase appears in the 50 s time trace indicating that there was no electron leakage in this electron transfer pathway.

To be sure that these observed differences in heme *b* reduction between the wild-type and three-subunit core complexes are indeed due to the lack of subunit IV, time course tracing of heme *b* reduction by $Q_0C_{10}BrH_2$ in reconstituted complex formed from recombinant wild-type IV and the three-subunit core complex were determined. The rate and extent of heme *b* reduction in the reconstituted complex are the same as in the wild-type complex (data not shown), confirming that subunit IV decreases electron leakage during $bc_1$ catalysis.

### 3.3. Effect of subunit IV on the reduction of cytochrome $c_1$ in the $bc_1$ complexes

Fig. 3 shows time traces of cytochrome $c_1$ reduction in the wild-type (red) and three-subunit core (blue) complexes in the absence (A and D) and presence of antimycin A (B and E) or stigmatellin (C and F) in 1 s and 50 s time ranges. The heme $c_1$ reductions, in the absence of inhibitor, by $Q_0C_{10}BrH_2$ in the wild-type and three-subunit core complexes are biphasic: a fast reduction phase followed by a slow reduction phase (see Fig. 3A and B). The pseudo first order rate constants for the fast reduction phase are 14.2 $s^{-1}$ and 10.0 $s^{-1}$ for the wild-type and core complexes, respectively.

The addition of antimycin A decreases the rate of heme $c_1$ reduction by 49% (from 14.2 to 7.3 $s^{-1}$) in the wild-type complex and by 82% (from 10.0 to 1.8 $s^{-1}$) in the three-subunit core complex. These results are consistent with the previous report [17,32] that antimycin A has a significant effect on the reduction rate of heme $c_1$ in cytochrome $bc_1$ complexes. This inhibitor effect has been attributed to the long range effect of antimycin on the $Q_P$ site when binding to the $Q_N$ site. Subunit IV has little effect on the $Q_P$ site.

Although the reduction rate constants for heme $c_1$, upon addition of antimycin A, decrease drastically in both complexes, a maximum

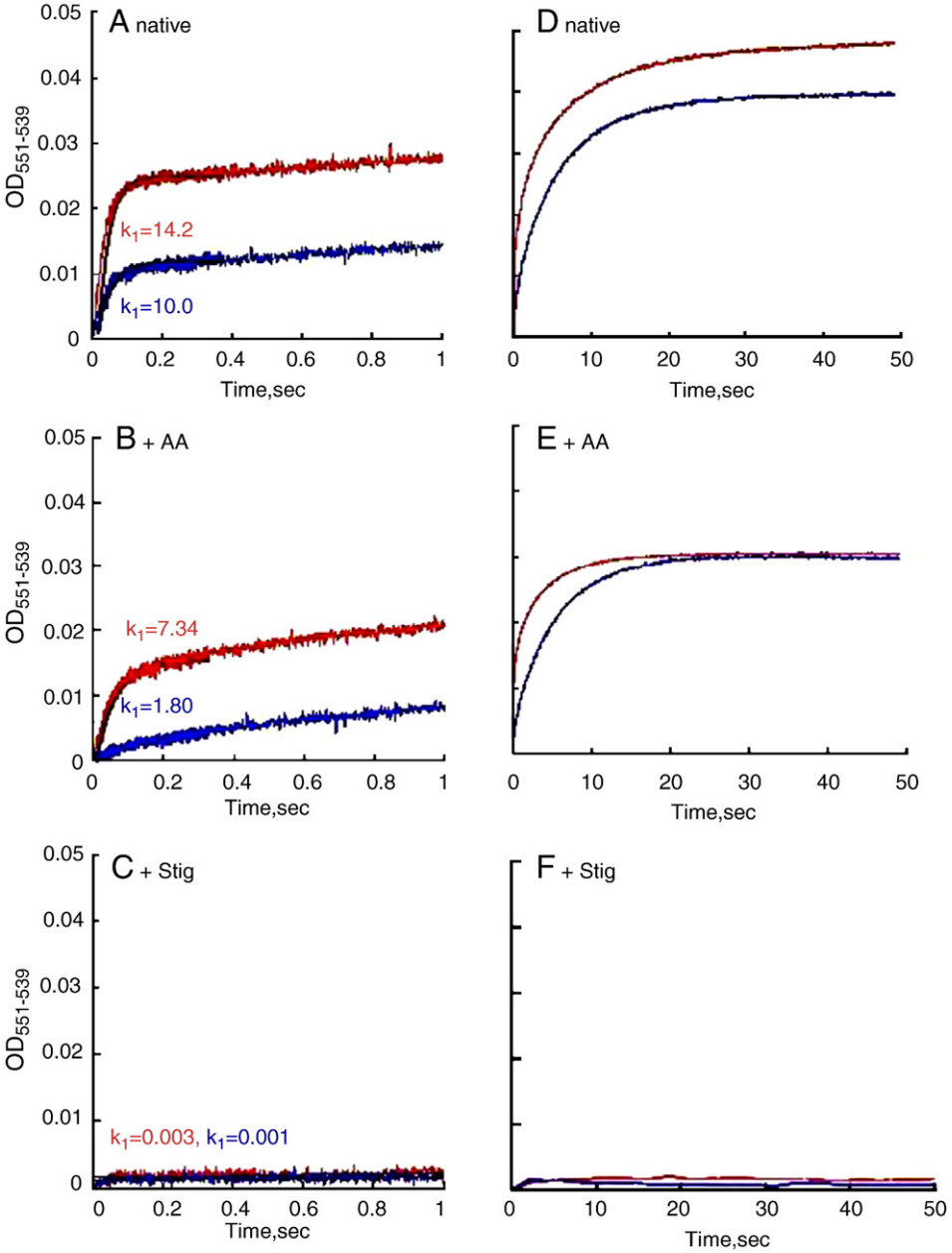


**Fig. 3.** Time courses of cytochromes $c_1$ reduction by $Q_0C_{10}BrH_2$. Wild-type (red) and three-subunit core (blue) complexes in the absence of inhibitors (panels A and D) and in the presence of antimycin A (panels B and E), or stigmatellin (panels C and F ). Experimental conditions were as described in Fig. 3, except the reduction of heme $c_1$ was followed by $A_{551nm}$-$A_{539nm}$. Solid lines represent fitted curves.

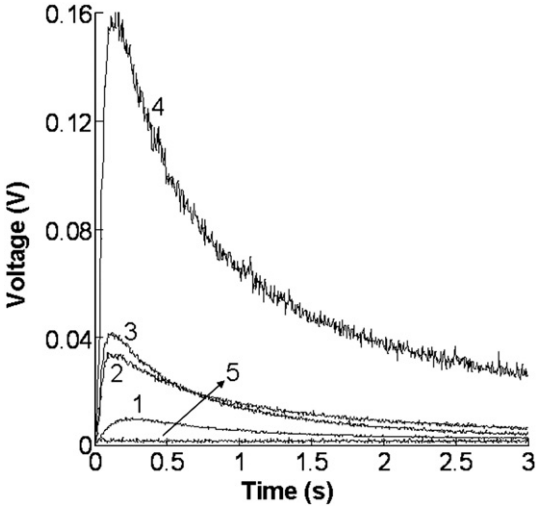


**Fig. 4.** Time traces of superoxide generation. The superoxide generation reactions were carried out at 23 °C in the Applied Photophysics stopped-flow reaction analyzer SX 18MV by mixing 1:1 solutions A and B containing enzyme complexes and substrate as detailed in Materials and methods. Curves 2 to 4 represent wild-type complex, the complex reconstituted from the core complex and recombinant wild-type IV, and the three-subunit core complex, respectively. Curve 1 shows superoxide generation by beef heart $bc_1$ complex under the same conditions. Curve 5 is for control experiments when no $bc_1$complexes or $Q_0C_{10}BrH_2$ was present in the system. A similar curve was obtained when 300 U/ml Mn-SOD was added to the complete system.

reduction of OD value of 0.03 is reached in both complexes (see Fig. 3 E). These results are consistent with the previous report [17,32] that antimycin A has a significant effect on the reduction rate of heme $c_1$ in cytochrome $bc_1$ complexes. This inhibitor effect has been attributed to the long range effect of antimycin on the $Q_P$ site when binding to the $Q_N$ site [17,32]. Subunit IV has little effect on the $Q_P$ site.

Since it is known that superoxide can reduce cytochrome *c*, it seems possible that part of this $c_1$ reduction results from heme $c_1$ reduced by superoxide. To test this possibility the extents of heme $c_1$ reduction in antimycin-treated intact, three-subunit core and reconstituted wild-type complexes were measured in the presence of SOD. The extents of heme $c_1$ reduction in these three antimycin-treated complexes decrease in the presence of SOD. It is as expected that the extent of decrease (compared to that in the absence of SOD) is larger in the three-subunit core complex than the wild-type and reconstituted wild-type complexes. These results are in line with the data obtained in the next section showing that the three-subunit core complex has higher superoxide generating activity than that of the wild-type or reconstituted wild-type complex.

The addition of stigmatellin to these two complexes abolishes heme $c_1$ reduction (see Fig. 3F). These results confirm that the $Q_P$ site in the three-subunit core complex is functional.

### *3.4. Effect of subunit IV on superoxide production by the cytochrome $bc_1$ complex*

What is the fate of the leaked electron from the low potential electron transfer chain? One of the pathways is reaction with molecular oxygen to form superoxide anion. If this is the pathway, one should see more superoxide production by the three-subunit core complex than the wild-type complex because more electrons leak from the former.

Production of superoxide by the cytochrome $bc_1$ complex can be determined by measuring the decease in rate of cytochrome *c* reduction in the presence of SOD under conditions of continuous turnover of the $bc_1$ complex. The small rate of cytochrome *c* reduction, compared with the normal rate of cytochrome *c* reduction, compromises the accuracy of this method. The MCLA-$O_2.^-$ chemiluminescence method, which has been wildly used to measure $O_2.^-$ production, is more sensitive than the cytochrome *c* method [27]. However, use of the MCLA-$O_2.^-$ chemiluminescence method to determine superoxide production during continuing turnover of the $bc_1$ complex (in the presence of ubiquinol and cytochrome *c*), encounters a high background rate of $O_2.^-$ production resulting from the non-enzymatic oxidation of ubiquinol by cytochrome *c*, making it difficult to unambiguously compare $O_2.^-$ production by various subunit IV mutant complexes. This difficulty has been overcome by measuring the chemiluminescence of the MCLA-$O_2.^-$ adduct during a single turnover of $bc_1$ complex using the Applied Photophysics stopped-flow reaction analyzer SX.18 MV by leaving the excitation light source off and registering light emission [29]. Because the system contains no cytochrome *c*, chemiluminescence of MCLA-$O_2.^-$ resulting from non-enzymatic oxidation of ubiquinol by cytochrome *c* is eliminated, enabling us to accurately evaluate changes in the rate of superoxide anion generation by various $bc_1$ complexes.

Fig. 4 shows the tracings of $O_2.^-$ generation by wild-type complex (curve 2), three-subunit core complex (curve 4), and reconstituted complex (curve 3). MCLA-$O_2.^-$ chemiluminescence induced by $bc_1$ complex reaches peak intensity after about 0.06 s at room temperature, and then decays. Maximum peak height (0.158 V) induced by the three-subunit core complex (see curve 4) is about 4 times that of the wild-type complex (0.038 V) (see curve 2). It should be noted that maximum peak height was $bc_1$ complex concentration dependent. Addition of subunit IV to the core complex decreases $O_2.^-$ production to about the same level as that of wild-type complex (see curve 3). No luminescence was detected when $bc_1$ complex was omitted from the enzyme-containing solution or $Q_0C_{10}BrH_2$ is omitted from the substrate-containing solution (curve 5). Addition of 300 U/ml superoxide dismutase to either the substrate or enzyme solution completely abolishes luminescence (curve 5), indicating that $O_2.^-$ is responsible for the luminescence observed. These results clearly indicate that the presence of subunit IV decreases superoxide anion production by the $bc_1$ complex.

The decrease in superoxide production by the wild-type or reconstituted wild-type complexes may result from the slowed rate of superoxide releasing from its generating site, the hydrophobic $Q_P$ pocket, to the aqueous phase to react with MCLA. The $Q_P$ pocket is more insulated in the wild-type complex than that in the core complex, because subunit IV provides an additional physical barrier. This additional barrier may come from subunit IV itself or a minor structural change around the $Q_P$ site of cytochrome *b* protein induced by the presence of subunit IV. Therefore, the release of superoxide from the $Q_P$ pocket of the wild-type complex would be slower than that of the core complex. One can imagine that in the hydrophobic $Q_P$ pocket the reduced heme $b_L$ and superoxide are in equilibrium. If releasing of superoxide from the pocket proceeds with a smaller rate, a higher heme $b_L$ reduction will be resulted. Although the physical relationship between subunit IV and heme $b_L$ is not yet established, due to the unavailability of the 3-D structure of a four-subunit

**Table 1**
Summary of electron transfer and superoxide production by various *bc*1 complex preparations.

| Preparations | Electron transfer μmol *c*/min/nmol *b* XO unit/nmol *b* | Superoxide production XO unit/nmol *b* | |
|---|---|---|---|
| | | −AA | +AA |
| Wild-type | 2.42 ± 0.03 | 0.07 ± 0.01 | 0.23 ± 0.02 |
| Core complex | 0.63 ± 0.01 | 0.25 ± 0.02 | 0.42 ± 0.03 |
| [Core complex + wild type IV] | 2.32 ± 0.03 | 0.07 ± 0.01 | 0.23 ± 0.02 |
| [Core complex + IV(Y81A)] | 2.03 ± 0.02 | 0.09 ± 0.01 | 0.27 ± 0.03 |
| [Core complex + IV (R84E)] | 1.41 ± 0.02 | 0.16 ± 0.02 | 0.34 ± 0.03 |
| [Core complex + IV(81–84)A | 0.92 ± 0.01 | 0.21 ± 0.02 | 0.38 ± 0.03 |

The data represented were mean values ± standard deviations from three experiments.

complex. The closed relationship between cytochrome *b* subunit and subunit IV is, however, confirmed by the recovery of both subunits in the same fraction during subfractionation of the cytochrome $bc_1$ complex.

### 3.5. The relationship between electron transfer and supeoxide anion generation in the cytochrome $bc_1$ complex

If our suggestion that the presence of subunit IV decreases superoxide production, thus increasing electron transfer activity in the three-subunit core complex, is correct, one should see an inverse relationship between electron transfer activity and superoxide production activity in $bc_1$ complexes having various degrees of functionally active subunit IV. Functional activity of subunit IV refers to its ability to interact with the three-subunit core complex to restore $bc_1$ activity. This activity is also called reconstitutive activity. The availability of recombinant mutant subunit IVs with varying reconstitutive activity in our laboratory enables us to prepare $bc_1$ complexes with varying electron transfer activities. Table 1 summarizes electron transfer and superoxide anion production activities in various complexes in the presence and absence of antimycin A. The electron transfer activity, expressed as μmol cytochrome *c* reduced per min per nmol *b*, for the three-subunit core complex and reconstituted complexes formed from the core complexes and recombinant wild-type and mutant IVs, Y81A, IV(R84E), IV(81–84)A , are 0.6, 2.3, 2.0, 1.4, and 0.9, respectively. The $O_2.^-$ production by these five complexes, expressed as XO unit per nmol *b*, are 0.25, 0.067, 0.092, 0.155, and 0.206, respectively (Table 1). These results indicate that the superoxide production by the $bc_1$ complex is inversely proportional to its electron transfer activity. In other words, reconstituted complex, formed from three-subunit core complex and subunit IV with higher reconstitutive activity, has higher electron transfer activity but lower superoxide generation activity. All these results support the idea that subunit IV decreases electron leakage, thus increasing electron transfer activity of the $bc_1$ complex. They also explain the observation that more complicated $bc_1$ complexes, such as bovine heart $bc_1$ complex, which have more supernumerary subunits, are more stable, have higher electron transfer activity, and generate much less superoxide anion during normal electron transfer (see Fig. 4, curve 1).

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