Effect of Famoxadone on Photoinduced Electron Transfer between the Iron-Sulfur Center and Cytochrome $c_1$ in the Cytochrome $bc_1$ Complex*

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Famoxadone is a new cytochrome $bc_1$, $Q_o$ site inhibitor that immobilizes the iron-sulfur protein (ISP) in the $b$ conformation. The effects of famoxadone on electron transfer between the iron-sulfur center (2Fe-2S) and cyt $c_1$ were studied using a ruthenium dimer to photoinitiute the reaction. The rate constant for electron transfer in the forward direction from 2Fe-2S to cyt $c_1$ was found to be $16,000 \text{s}^{-1}$ in bovine cyt $bc_1$. Binding famoxadone decreased this rate constant to $1,420 \text{s}^{-1}$, consistent with a decrease in mobility of the ISP. Reverse electron transfer from cyt $c_1$ to 2Fe-2S was found to be biphasic in bovine cyt $bc_1$, with rate constants of 90,000 and 7,300 $\text{s}^{-1}$. In the presence of famoxadone, reverse electron transfer was monophasic with a rate constant of 1,420 $\text{s}^{-1}$. It appears that the rate constants for the release of the oxidized and reduced ISP from the $b$ conformation are the same in the presence of famoxadone. The effects of famoxadone binding on electron transfer were also studied in a series of Rhodobacter sphaeroides cyt $bc_1$ mutants involving residues at the interface between the Rieske protein and cyt $c_1$ and/or cyt $b$.

The cytochrome (cyt)$^{1}$ $bc_1$ complex (ubiquinol:cytochrome $c$ oxidoreductase) is an integral membrane protein in the electron transport chains of mitochondria and many respiratory and photosynthetic prokaryotes (1). The complex translocates four protons to the positive side of the membrane per two electrons transferred from ubiquinol to cyt $c$ in a Q-cycle mechanism (2). A key-bifurcated reaction occurs at the $Q_o$ site in which the first electron is transferred from ubiquinol to ubiquinone in the Rieske iron-sulfur center (2Fe-2S) and then to cyt $c_1$ and cyt $c$ (1–3). The second electron is transferred from semiquinone in the $Q_o$ site to cyt $b$, and then to cyt $b_1$ and ubiquinone in the $Q_o$ site. Site inhibitors can be divided into three classes. Class Ia inhibitors such as myxothiazol and $(E)-\beta$-methoxyacrylate-stilbene alter the heme spectrum of cyt $b_1$, class Ib inhibitors such as UHDBT alter the EPR spectrum of 2Fe-2S, and class Ic inhibitors such as stigmatellin alter both (1–3). X-ray crystallographic studies have revealed that these different classes of inhibitors occupy different subsites in the $Q_o$ pocket and have different effects on the mobility of the extramembrane domain of the Rieske iron-sulfur protein (ISP) (4–8). Stigmatellin binding increases the midpoint redox potential ($E_m$) of 2Fe-2S by $-200$ to $-250 \text{mV}$ (9) and immobilizes the ISP in the $b$ conformation. In contrast to stigmatellin, famoxadone increases the $E_m$ of 2Fe-2S by only 26 $\text{mV}$ and immobilizes both the oxidized and reduced ISP in the $b$ conformation. This is consistent with the finding that famoxadone is more deeply buried in the $Q_o$ site than stigmatellin and that it does not form a hydrogen bond with the His-161 ligand on reduced 2Fe-2S (5, 8, 23). Azoxystrobin also immobilizes the ISP in the $b$ conformation but has only a minor effect on the $E_m$ of 2Fe-2S, decreasing it by 24 $\text{mV}$.

In this paper, the effects of famoxadone and azoxystrobin on electron transfer between the Rieske iron-sulfur center and cyt $c_1$ are studied using the buninuclear ruthenium complex, Ru$_2$D, to rapidly photooxidize or photoreduce cyt $c_1$ (10). Binding famoxadone to the $Q_o$ site of bovine cyt $bc_1$ decreased the rate constant for electron transfer from 2Fe-2S to cyt $c_1$ from 16,000 $\text{s}^{-1}$ to 1,480 $\text{s}^{-1}$, consistent with a decrease in the mobility of

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The abbreviations used are: cyt, cytochrome; $Q_o$, quinol reduction site $o$; UHDBT, 6-n-undecyl-6-hydroxy-4,7-dioxobenzothiazole; ISP, Rieske iron-sulfur protein; $E_m$, midpoint redox potential; 2Fe-2S, Rieske iron-sulfur center; Ru$_2$D, [Ru(bpy)$_3$]$_2^+$/aqy(PF$_6$)$_2$; qpy, 2,2’-4’,4’-2’-quaterpyridine; Q$_1$C$_5$Br$_2$, 2,3-dimethyl-5-methyl-6-(1-bromodecyl-1,4-benzoquinol; TMPD, tetramethylphenylenediamine; SCR, succinate cytochrome $c$ reductase; 3CP, carboxyl-2,2,5,5-tetramethyl-1-pyrrolidinoyl free radical; Q$_{H_2}$, ubiquinol.

1 Z. L. Zhang, D. Xiao, Q. Byron, C.-H. Tai, B. D. Jordan, L. Yu, and C.-A. Yu, unpublished results.
ISP. Famoxadone binding also decreased the rate constant for reverse electron transfer from cyt c1 to 2Fe-2S to 1,420 s−1, indicating that the rate constants for the release of oxidized and reduced ISP from the cyt b conformation are the same in the presence of famoxadone. Famoxadone binding was also found to decrease the rate of electron transfer from 2Fe-2S to cyt c1 in R. sphaeroides cyt bc1, as well as mutants involving residues at the interface between the Rieske protein and cyt c1 and/or cyt b.

**EXPERIMENTAL PROCEDURES**

**Materials**—Ru1,D was prepared by a modification of the method of Downard et al. (24). Bovine cyt bc1 was purified as described by Yu et al. (25). Wild-type and mutant R. sphaeroides cyt bc1 were prepared as described by Tian et al. (11). Paraquat, succinate, TMPD, p-benzoquinone, and antimycin A were obtained from Sigma; stigmatellin was purchased from Fluka; and [Co(NH3)5Cl]2− was synthesized (26). N-Decyl-β-D-maltoside was obtained from Anatrace. Succinate cytochrome c reductase (SCR) was purified as described by Xiao et al. (28). The identity of the mutations was confirmed by DNA sequencing before and after photosynthetic or semi-aerobic growth of the cells as described previously (12). Mutant cytochrome bc1 was purified as described by Xiao et al. (12).

![Top, x-ray crystal structure of bovine cyt bc1, in the presence of famoxadone (23). Famoxadone is colored purple, the cyt c1 and cyt bc1 hemes are red, and the 2Fe-2S center is represented by a Corey-Pauling-Koltun model. The ISP is blue, and cyt b is gray. Residues 252–268 in the ef loop, which change conformation as a result of famoxadone binding are colored orange. Residues 163–171 in the neck-contacting domain are colored yellow. The residues in the ISP and cyt b that were mutated are shown as sticks. Bottom, x-ray crystal structure of bovine cyt bc1 P62.22 crystals (c1, conformation) (6). ISP is blue, cyt c1 is yellow, and the residues that were mutated are shown as sticks. R. sphaeroides numbering is used.](image-url)
Determination of Enzyme Activity and Redox Potential of the 2Fe-2S Cluster in Mutant Cyt bc₁.—The cyt bc₁ activity was determined in an assay mixture containing 100 mM Na²⁻K⁺ phosphate buffer, pH 7.4, 300 μM EDTA, 100 μM cyt c, and 25 μM 2,3-dimethoxy-5-methyl-6-(10-bromodecyl)-1,4-benzoquinol (Q₁C₁BrH₂) at 23°C using the method described by Xiao et al. (12). The redox potentials of 2Fe-2S in cyt bc₁ mutants were determined as described previously (12). The reduction of cyt c₁ was followed by measuring the increase of the absorbance at 555–545 nm in a Shimadzu UV2101 PC spectrophotometer. The reduction of 2Fe-2S was followed by measuring the negative CD peak at 500 nm of partially reduced complex minus fully oxidized complex in a JASCO J-715 spectropolarimeter (29–31). The same samples were used for the absorption and CD measurements. The redox potentials of 2Fe-2S were calculated from the redox states of heme c₁ and 2Fe-2S at pH 8.0 using 280 mV for the midpoint redox potential of heme c₁ (32).

Flash Photolysis Experiments—Transient absorbance measurements were carried out by flash photolysis of 300-μl solutions contained in a 1-cm glass semi-microcuvette. The excitation light flash was provided by a Microkinetics Phase R model DL1400 flash lamp-pumped dye laser using coumarin LD 490 to produce a 480-nm light flash of 0.3-μs duration. The detection system has been described by Heacock et al. (33). Samples typically contained 5 μM cyt bc₁ in a buffer with 0.01% dodecylmaltoside. In photoreduction experiments, 10 μM aniline and 1 mM 3CP were used as sacrificial donors, and catalytic concentrations of horse cyt c were used as sacrificial acceptors, and Q₁C₁BrH₂ was used to reduce cyt bc₁. To regenerate reduced quinol throughout the experiments, 1 mM 3CP was included. Redox mediators included p-benzoquinone (εₘₐₓ = 280 mV) and TMPD (εₘₐₓ = +275 mV) used in concentrations of 10 and 2 μM, respectively. The experiments were carried out aerobically to rapidly reoxidize the highly absorbing reduced paraquat.

RESULTS AND DISCUSSION

Effects of Famoxadone and Azoxystrobin on Electron Transfer between 2Fe-2S and Cyt c₁ in Bovine Cyt bc₁.—An important goal toward understanding the mechanism of electron transfer in cyt bc₁ is to determine what factors control the conformation of the ISP in each state of the complex, the dynamics of the changes between the different conformations, and the rate of electron transfer in each of the conformations. X-ray crystallographic studies are providing valuable information on the conformations of the ISP including the b conformation (Fig. 1, top), the c₁ conformation (Fig. 1, bottom), and intermediate and mobile conformations (4–9). However, it has been difficult to determine the kinetics of electron transfer from 2Fe-2S to cyt c₁ (34, 35) as well as the dynamics of ISP conformational changes. The development of the ruthenium photoreduction method provides an opportunity to measure electron transfer between 2Fe-2S and cyt c₁ in both the forward and reverse directions and thus provides kinetic information on two different initial redox states of cyt bc₁ (10). Moreover, it is becoming clear that the measured rates of electron transfer are probably rate-limited by conformational changes in the ISP (36). The binuclear complex Ru₂D contains the 2,2':4',4'–tetrapyridine ligand, which bridges the two ruthenium atoms (Fig. 2A, inset) (24). Ru₂D has a charge of +4, which allows it to bind with high affinity to the negatively charged domain on cyt c₁ (10). The electrochemical properties of Ru₂D are similar to those of the widely used ruthenium tris-bipyridine complex.
The metal-to-ligand excited state of Ru$_2$D with a lifetime of 0.5 s is both a strong oxidant and a strong reductant and can rapidly oxidize or reduce cyt$c_1$ in the presence of appropriate sacrificial electron acceptors or donors (10).

To study electron transfer in the forward direction, the ruthenium dimer Ru$_2$D was used to rapidly photooxidize cyt$c_1$ in bovine cyt$bc_1$ with cyt$c_1$ and 2Fe-2S was initially reduced (Fig. 2A). The excited state of Ru$_2$D oxidizes cyt$c_1$ within 1 s according to Scheme 1. Only one of the two ruthenium centers in Ru$_2$D is photoexcited in this experiment, and this one is represented in Scheme 1. The sacrificial electron acceptor paraquat was present in the solution to oxidize Ru(II*) and/or Ru(I).

The rapid photooxidation of cyt$c_1$ shown by the initial decrease in 552-nm absorbance was followed by biphasic reduction of cyt$c_1$ with rate constants of $k_1$ = 90,000 s$^{-1}$ and $k_2$ = 7,300 s$^{-1}$ and relative amplitudes of 57 and 43%. (Fig. 2B, famoxadone (30 μM) was added to the solution in A and subjected to flash photolysis. The monophasic transient has a rate constant of 1,420 ± 200 s$^{-1}$.)

The rate constant $k_2$ thus represents electron transfer of the first electron from cyt$c_1$ to 2Fe-2S and cyt$c_1$ followed rapidly by the transfer of the second electron from the semiquinone to cyt$b_L$ and cyt$b_H$ (10).

Table I. Spectral and electrochemical properties of ruthenium complexes

| Complex      | $E^o$ (III/II)$^a$ | $E^o$ (II/I)$^a$ | $E^o$ (III/II*)$^b$ | $E^o$ (II*/I)$^b$ | $\lambda_{max}$(abs)$^c$ | $\lambda_{max}$(em)$^d$ |
|--------------|--------------------|------------------|---------------------|------------------|----------------------|----------------------|
| Ru(bpy)$_3$$^{2+}$ | 1.26               | -1.35            | -0.87               | 0.78             | 452                  | 582                   |
| Ru$_2$D       | 1.24               | -1.10            | -0.81               | 0.95             | 480                  | 605                   |

$^a$Determined by cyclic voltammetry at a platinum disc-working electrode in acetonitrile with respect to a saturated calomel reference electrode (24).

$^b$Calculated from the excited state energy and the ground state potential as described previously (39).

$^c$Recorded in air-saturated 1 mM aqueous phosphate buffer, pH 7.0

$^d$Recorded in 4:1 ethanol/methanol glass at 77 K.

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To study electron transfer in the forward direction, the ruthenium dimer Ru$_2$D was used to rapidly photooxidize cyt$c_1$ in bovine cyt$bc_1$, with cyt$c_1$ and 2Fe-2S was initially reduced (Fig. 2A). The excited state of Ru$_2$D oxidizes cyt$c_1$ within 1 μs according to Scheme 1. Only one of the two ruthenium centers in Ru$_2$D is photoexcited in this experiment, and this one is represented in Scheme 1. The sacrificial electron acceptor paraquat was present in the solution to oxidize Ru(II*) and/or Ru(I).

The rapid photooxidation of cyt$c_1$ shown by the initial decrease in 552-nm absorbance was followed by biphasic reduction of cyt$c_1$ with rate constants of $k_1 = 16,000 ± 3,000$ s$^{-1}$ and $k_2 = 250 ± 50$ s$^{-1}$ (Fig. 2A). The rate constant $k_1$ has been assigned to electron transfer from reduced 2Fe-2S to photooxidized cyt$c_1$, whereas $k_2$ is correlated with the oxidant-induced reduction of cyt$b_H$ (Scheme 2) (10). The rate constant $k_2$ thus represents electron transfer of the first electron from cyt$c_1$ to 2Fe-2S and cyt$c_1$ followed rapidly by the transfer of the second electron from the semiquinone to cyt$b_L$ and cyt$b_H$ (10). The experimental rate constant $k_1$ is much smaller than the theoretical value predicted for electron transfer between 2Fe-2S and cyt$c_1$ in the crystallographic c$_1$ state, $1 × 10^6 - 2 × 10^7$ s$^{-1}$ (5, 10). Therefore, it appears that the measured rate constant for electron transfer is “gated” by changes in the conformation of the ISP (10). This interpretation is consistent with previous studies showing that $k_1$ has a large temperature dependence with an activation energy of 59 kJ/mol (10). X-ray diffraction studies have indicated that the ISP is largely in the b conformation when both cyt$c_1$ and 2Fe-2S are reduced (37). Therefore, the rate constant...
of 16,000 s⁻¹ could represent the rate of the conformational change from the b state of the ISP to the c₁ state where rapid electron transfer can occur.

The addition of 30 μM famoxadone to bovine cyt bc₁ led to a single phase of reduction of photooxidized cyt c₁ with a rate constant of 1,480 ± 250 s⁻¹, and no reduction of cyt bc₁ was observed at 562 nm (Fig. 2B). The rate constant was independent of the concentration of famoxadone over the range of 10–100 μM, consistent with a large binding constant. These results indicate that famoxadone binds strongly to the Q₁ site and decreases the rate constant for electron transfer from 2Fe-2S to cyt c₁ from 16,000 to 1,480 s⁻¹. It appears that famoxadone binding significantly decreases the rate constant for the conformational change from the b state to the c₁ state. The amplitude of the 552-nm transient for cyt c₁ reduction in the presence of famoxadone is 31% of the amplitude of the initial photooxidation. It is quite possible that the ISP is the best fit to the Eyring equation: 

\[ \ln(kT) = -\Delta H^*/RT + \Delta S^*/R + \ln(\beta/k_bT) \]

with \( \Delta H^* = 19.1 \pm 1.8 \) kJ/mol and \( \Delta S^* = -121 \pm 6 \) J/mol·K.

![Temperature dependence of rate constant for electron transfer from cyt c₁ to 2Fe-2S in bovine cyt bc₁, inhibited with famoxadone.](Image)

**FIG. 4.** Temperature dependence of rate constant for electron transfer from cyt c₁ to 2Fe-2S in bovine cyt bc₁, inhibited with famoxadone. The 552-nm transients were recorded under the same conditions as in Fig. 3B. The solid line is the best fit to the Eyring equation: 

\[ \ln(kT) = -\Delta H^*/RT + \Delta S^*/R + \ln(\beta/k_bT) \]

with \( \Delta H^* = 19.1 \pm 1.8 \) kJ/mol and \( \Delta S^* = -121 \pm 6 \) J/mol·K.

and 3CP were present in the solution to reduce Ru(III). The photoreduction of cyt c₁ was followed by biphasic oxidation with rate constants of 90,000 ± 15,000 s⁻¹ and 1,500 ± 300 s⁻¹, respectively (Fig. 3A). The difference in kinetics compared with forward electron transfer is apparently the result of the initial redox states of the enzyme. X-ray diffraction studies have indicated that a smaller fraction of ISP is in the b conformation in the fully oxidized complex than in the complex with cyt c₁ and that 2Fe-2S is reduced (37). It is reasonable to assign the fast phase to the mobile conformation of the ISP and the slow phase to the conformation initially in the b state (10). With this assumption, the fast phase would be gated by fluctuations in conformation between the mobile state and the c₁ state, whereas the slow phase would be gated by the conformational change from the b state to the mobile state. The addition of famoxadone resulted in a single phase of cyt c₁ reduction with rate constants of 90,000 s⁻¹. It is interesting that the rate constants for the change in conformation from the b state to the mobile state must therefore be much smaller than the rate constant \( k_f \) from the mobile state to the b state in the presence of famoxadone. The rate constant \( k_f \) is 1420 s⁻¹ in the presence of famoxadone, whereas the rate constant \( k_f \) could be 90,000 s⁻¹ or even higher. It is interesting that the rate constants for...
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**TABLE II**

| Mutant     | Subunit | Enzymatic activity | $k_1^b$ | $k_2^c$ |
|------------|---------|--------------------|---------|---------|
| Wild type  |         |                    | 2.5     |         |
| K70C       | Rieske  |                    | 1.40    | 70,000  |
| D143C      | Rieske  |                    | 0.98    | 20,000  |
| F148C      | Rieske  |                    | 2.20    | 60,000  |
| P150C      | Rieske  |                    | 0.23    | 50,000  |
| G153C      | Rieske  |                    | 0.78    | 13,000  |
| K164C      | Rieske  |                    | 0.98    | 82,000  |
| P166C      | Rieske  |                    | 0.08    | 0       |
| V64C       | Cyt b   |                    | 2.5     | 58,000  |
| G98C       | Cyt b   |                    | 2.4     | 59,000  |
| M92C       | Cyt b   |                    | 2.5     | 82,000  |
| A158C      | Cyt b   |                    | 2.08    | 60,000  |
| Y302C      | Cyt b   |                    | 0.98    | 12,000  |
| I326C      | Cyt b   |                    | 0.80    | 86,000  |
| K70C:A185C | Cross-link |                | 0       | 0       |
| P33C:G89C  | Cross-link |                | 1.3     | 48,000  |
| N36C:G89C  | Cross-link |                | 2.5     | 64,000  |

$^a$ Enzymatic activity is expressed as micromole of cyt c-reduced/min/mmol cyt b at 25 °C. The error limits are ± 15%.

$^b$ $k_1$ is the experimental rate constant for electron transfer from 2Fe-2S to cyt c₁ measured from the 552-nm transient at pH 9.0, 25 °C. The error limits are ± 20%.

$^c$ $k_2$ is the rate constant for electron transfer from Q-H₂ to 2Fe-2S measured from the rate of cyt b₃ reduction at 562 nm in the presence of antimycin A at pH 9.0. The error limits are ± 20%.

**TABLE III**

| Mutant     | Subunit | Enzymatic activity | $\Delta E_m^b$ | $k_1$ (without famoxadone) | $k_1$ (with famoxadone) |
|------------|---------|--------------------|----------------|---------------------------|-------------------------|
| Wild type  |         |                    | 2.5            | 80,000 s⁻¹              | 4,800 s⁻¹                |
| K70C       | Rieske  |                    | 2.5            | 70,000 s⁻¹              | 2,400 s⁻¹                |
| D143C      | Rieske  |                    | 2.4            | 59,000 s⁻¹              | 2,000 s⁻¹                |
| P150C      | Rieske  |                    | 2.5            | 82,000 s⁻¹              | 1,800 s⁻¹                |
| G153C      | Rieske  |                    | 2.08           | 60,000 s⁻¹              | 1,000 s⁻¹                |
| I326C      | Cyt b   |                    | 0.80           | 86,000 s⁻¹              | 1,200 s⁻¹                |
| K70C:A185C | Cross-link |                | 0              | 0                        | 0                       |
| P33C:G89C  | Cross-link |                | 1.3            | 48,000 s⁻¹              | 1,100 s⁻¹                |
| N36C:G89C  | Cross-link |                | 2.5            | 64,000 s⁻¹              | 1,100 s⁻¹                |

$^a$ Enzymatic activity is expressed as micromole of cyt c-reduced/min/mmol cyt b at 25 °C. The error limits are ± 15%.

$^b$ $\Delta E_m$ is the difference in redox potential between 2Fe-2S and cyt c₁ at pH 8.0, 25 °C. The error limits are ± 5 mV. ND, not determined.

$^c$ $k_1$ is the rate constant for electron transfer from 2Fe-2S to cyt c₁ measured from the 552-nm transient at pH 9.0, 25 °C. The error limits are ± 20%.

$^d$ $k_2$ is the rate constant for electron transfer from 2Fe-2S to cyt c₁ in the presence of 30 μM famoxadone at pH 9.0, 25 °C. The error limits are ± 20%.

forward and reverse electron transfer are the same in the presence of famoxadone. This indicates that the rate constant $k_2$ does not depend on the redox state of 2Fe-2S, consistent with the finding that famoxadone binding changes the redox potential of 2Fe-2S by only 26 mV. The temperature dependence of $k_d$ gives activation parameters of $\Delta H^\circ = 19.1 \pm 1.8$ kJ/mol and $\Delta S^\circ = -121 \pm 6$ J/mol·K for the conformational change from the b state to the mobile state in the presence of famoxadone (Fig. 4).

The present studies indicate that famoxadone binding significantly decreases the rate constant for the release of the ISP from the b conformation in cyt bc₁. X-ray crystallography studies have revealed that famoxadone binding leads to significant conformational changes in three domains on the surface of cyt b: residues 163–171 that contact the neck region of the ISP; residues 262–268 in the ef loop that are part of the ISP-docking crater; and residues 252–256 in the middle of the ef loop that connects the Q₈ site to the other two surface domains (Fig. 1, top) (23). The ef loop may play a role in relaying famoxadone-induced conformational changes in the Q₈ pocket to the ISP crater and the neck contact domain to decrease the rate of release of the ISP. The linkage between the conformation of the Q₈ site and the dynamics of the ISP movement could be a key to how the enzyme promotes the transfer of the first electron from Q-H₂ to 2Fe-2S but inhibits the transfer of the second electron from semiquinone to 2Fe-2S. The potential role of the ef loop in regulating the dynamics of ISP domain movement during the catalytic cycle is particularly intriguing.

**Effects of Famoxadone on Electron Transfer between 2Fe-2S and Cytochrome c₁ in R. sphaeroides**

Electron transfer in the forward direction was studied using Ru₂D to photooxidize cyt bc₁ in R. sphaeroides according to Scheme 1 in the presence of the sacrificial electron acceptor [Co(NH₃)₅Cl]²⁺. The reduction of photooxidized cyt c₁ was biphasic with rate constants of $k_1 = 80,000 \pm 15,000$ s⁻¹ and $k_2 = 1,500 \pm 300$ s⁻¹ (Fig. 5A). $k_1$ is attributed to electron transfer from 2Fe-2S to cyt c₁, whereas $k_2$ is due to subsequent electron transfer from Q-H₂ to 2Fe-2S and cyt c₁ followed by electron transfer from the semiquinone to cyt b₃ and cyt b₃ (Scheme 2) (10). Previous studies of the effects of temperature, pH, and redox potential demonstrated that $k_1$ is not rate-limited by true electron transfer in the c₁ state but rather is gated by conformational changes from the b state and the mobile state to the active c₁ state (36). According to this analysis, the population of the c₁ state is small but the rate constant for electron transfer from 2Fe-2S to cyt c₁ in the c₁ state is much larger than the observed value of $k_1 = 80,000$ s⁻¹. The addition of famoxadone leads to monophasic reduction of cyt c₁ with a rate constant of 4,800 ± 800 s⁻¹ (Fig. 5B).
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5B). There is no reduction of cyt b1 in the presence of famoxadone, consistent with displacement of Q-H2 from the Qc site. The most reasonable explanation of these results is that famoxadone binding stabilizes the b state of the ISP and that the observed rate constant k1 is gated by the rate constant kd for the release of the ISP from the b state to the mobile state and the c1 state. The rate constant k1 for the conformational change from the mobile state to the b state is expected to be much larger than k2 in the presence of famoxadone and could be 80,000 s−1 or larger. Electron transfer was also studied in the reverse direction using Ru3D to photoreduce cyt c1 in the oxidized complex according to Scheme 3 in the presence of the sacrificial electron donors aniline and 3CP. Oxidation of photoreduced cyt c1 was biphasic with rate constants of 8,400 ± 15,000 s−1 and 4,800 ± 800 s−1 and relative amplitudes of 60 and 40%, respectively. The addition of famoxadone led to monophasic oxidation of cyt c1 with a rate constant of 6,800 ± 1,200 s−1. It appears that the Rieske protein is largely in the b conformation in the presence of famoxadone and the rate of release to the c1 conformation is decreased. The fact that the rate constants for forward and reverse electron transfer are nearly the same in the presence of famoxadone indicates that the rate constant kd does not depend on the redox state of 2Fe-2S.

Effect of Mutations in the Iron-Sulfur Protein and cyt b on Electron Transfer in Cytochrome bc1—Mutations in R. sphaeroides cyt bc1 were generated by site-directed mutagenesis to characterize the interaction of the ISP with cyt c1 and with cyt b (Fig. 1). Flash photolysis studies were carried out to determine the effects of these mutations on the rate constants k1 for electron transfer from 2Fe-2S to cyt c1 and k2 for electron transfer from Q-H2 to 2Fe-2S as described above (Table II). The effects of famoxadone on the kinetics of selected mutants was also examined (Table III). The ISP mutant P166C is completely inactive in photoinduced electron transfer, suggesting that this mutation may have led to a critical alteration in the conformation of the ISP. The P150C mutation near the 2Fe-2S center dramatically decreases the rate constant k2 for electron transfer from Q-H2 to 2Fe-2S down to only 4 ± 1 s−1 but does not significantly affect the rate constant k1 for electron transfer from 2Fe-2S to cyt c1. It appears that the structural change caused by this mutation greatly affects the interaction of the ISP with the cyt b peptide but does not affect the dynamics of the interaction of the ISP with cyt c1. Famoxadone binding decreased the rate constant k1 somewhat more than for wild-type cyt bc1, indicating that the ISP was held more tightly in the b conformation (Table III). The K70C mutation on the ISP decreases k1 to 12,000 ± 2,000 s−1 but not greatly affecting k2. It appears that the ISP is held more tightly in the b conformation in this mutant than in wild-type enzyme. Earlier studies have shown that the rate constant k1 for electron transfer from 2Fe-2S to cyt c1 was not greatly affected by ISP mutations S154A and Y156W, which decrease the redox potential of 2Fe-2S significantly (36). These results provided evidence that k1 is not rate-limited by true electron transfer in the c1 state but is gated by conformational changes from the b state and from the mobile state to the c1 state. The effects of famoxadone binding on k1 in the S154A and Y156W mutants were comparable with the effect on wild-type cyt bc1. Xiao et al. (12) have previously shown that the formation of a disulfide cross-link between ISP and cyt b in the K70/C/A185C mutant led to a complete loss of steady-state activity, providing experimental evidence for the mobile shuttle mechanism of the ISP. The K70/C/A185C mutant was totally inactive in photoinduced electron transfer, providing further confirmation of the mobile shuttle mechanism. Xiao et al. (38) previously prepared the P33C/G89C and N36C/G89C mutants where each has a disulfide cross-link between the tail region of the ISP and cyt b. These mutants both have good steady-state enzyme activity, providing evidence for the intertwined dimer structure of cyt bc1. The photoinduced electron transfer kinetics is not greatly affected by cross-linking in these mutants, providing further evidence that the tail region of the ISP is not involved in the mobile shuttle mechanism.

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