Use of a Photoactivated Ruthenium Dimer Complex To Measure Electron Transfer between the Rieske Iron−Sulfur Protein and Cytochrome c_1 in the Cytochrome bc_1 Complex†

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ABSTRACT: Electron transfer between the Rieske iron−sulfur protein (Fe_2S_2) and cytochrome c_1 was studied using the ruthenium dimer, Ru_2D, to either photoreduce or photooxidize cytochrome c_1 within 1 μs. Ru_2D has a charge of +4, which allows it to bind with high affinity to the cytochrome bc_1 complex. Flash photolysis of a solution containing beef cytochrome bc_1, Ru_2D, and a sacrificial donor resulted in reduction of cytochrome c_1 within 1 μs, followed by electron transfer from cytochrome c_1 to Fe_2S_2 with a rate constant of 90 000 s^{-1}. Flash photolysis of reduced beef bc_1, Ru_2D, and a sacrificial acceptor resulted in oxidation of cytochrome c_1 within 1 μs, followed by electron transfer from Fe_2S_2 to cytochrome c_1 with a rate constant of 16 000 s^{-1}. Oxidant-induced reduction of cytochrome b_H was observed with a rate constant of 250 s^{-1} in the presence of antimycin A. Electron transfer from Fe_2S_2 to cytochrome c_1 within the *Rhodobacter sphaeroides* cyt bc_1 complex was found to have a rate constant of 60 000 s^{-1} at 25 °C, while reduction of cytochrome b_H occurred with a rate constant of 1000 s^{-1}. Double mutation of Ala-46 and Ala-48 in the neck region of the Rieske protein to prolines resulted in a decrease in the rate constants for both cyt c_1 and cyt b_H reduction to 25 s^{-1}, indicating that a conformational change in the Rieske protein has become rate-limiting.

The cytochrome (cyt)[1] bc_1 complex is an essential component of the energy-conserving electron-transfer chains of mitochondria and many respiratory and photosynthetic prokaryotes (*1*). The complex contains two b hemes, b_L and b_H, located in a single polypeptide; the Rieske iron−sulfur protein; and cyt c_1 (*1, 2*). It is generally accepted that electron transfer through the complex involves a Q-cycle mechanism in which four protons are translocated to the positive (P) side of the membrane per two electrons transferred from ubiquinol to cyt *c* (*2*). A key bifurcated reaction occurs at the Q_o site where the first electron is transferred from QH_2 to the Rieske iron−sulfur center and then to cyt c_1 and cyt *c* (*1*−*3*). Following proton release, the second electron is transferred from semiquinone in the Q_o site to heme b_L and then to heme b_H and ubiquinone in the Q_i site. Three different groups (*4*−*6*) have recently determined the X-ray crystal structure of the cyt bc_1 complex from beef and chicken mitochondria. An unexpected result of these studies is that the Rieske iron−sulfur protein is found in several different conformations, depending on the crystal form and the presence of inhibitors at the quinol oxidation (Q_o) site. In the original bovine bc_1 $I4_122$ crystals, an anomalous signal for Fe_2S_2 is found close to cyt b_L, but its intensity is small, suggesting conformational mobility (*4, 7*). Addition of the Q_o inhibitors UHDBT or stigmatellin significantly increased the intensity of Fe_2S_2, suggesting that the Rieske protein underwent a transition to an immobilized state near the surface of cyt b_L (*7*). In contrast, addition of MOA-stilbene abolished the signal for the iron−sulfur center in the native crystals and gave rise to a new low-amplitude signal closer to cyt c_1, suggesting increased mobility (*7*). In native chicken (*5*) or beef $P6_522$ bc_1 crystals (*5, 6*), the Rieske protein is in a conformation with Fe_2S_2 close to cyt c_1 (c_1 state, Figure 1), which would support very rapid electron transfer between Fe_2S_2 and cyt c_1 (*5, 6*). In chicken bc_1 crystals grown in the presence of stigmatellin, the Rieske protein is in a conformation with Fe_2S_2 close to the Q_o site in cyt *b* (b state, Figure 1), which would allow rapid electron transfer from quinol to Fe_2S_2 (*5*). One puzzling aspect of the Q-cycle mechanism is how QH_2 at the Q_o site can deliver electrons sequentially to the high and low potential chains, even though thermodynamics would favor delivery of both electrons to the high potential chain (*1*−*3*). The X-ray crystallographic studies have led to several conformational explanations of this paradox (*4*−*7*). It has been proposed that the extrinsic domain of the Rieske protein changes conformation from the b state where oxidized Fe_2S_2 accepts an electron from QH_2 to the c_1 state where reduced Fe_2S_2 transfers an electron to cyt c_1 (Figure 1) (*4*−*7*). Conformational and kinetic factors could prevent oxidized Fe_2S_2 from ever getting close enough to semiquinone to accept an electron before electron transfer from semiquinone to cyt b_L.

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[1] Abbreviations: cytochrome, cyt; Ru_2D, $[Ru(bpy)_2]_2(qpy)(PF_6)_4$; qpy, 2,2′:4′,4″:2″,2‴-quaterpyridine; 3-CP, 3-carboxy-2,2,5,5,tetramethyl-1-pyrrolidinyloxy free radical; $Q_0C_{10}Br$, 2,3-dimethoxy-5-methyl-6-(10-bromodecyl)-1,4-benzoquinol; MOA-stilbene, (*E*)-β-methoxyacrylate-stilbene; UHDBT, 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole.

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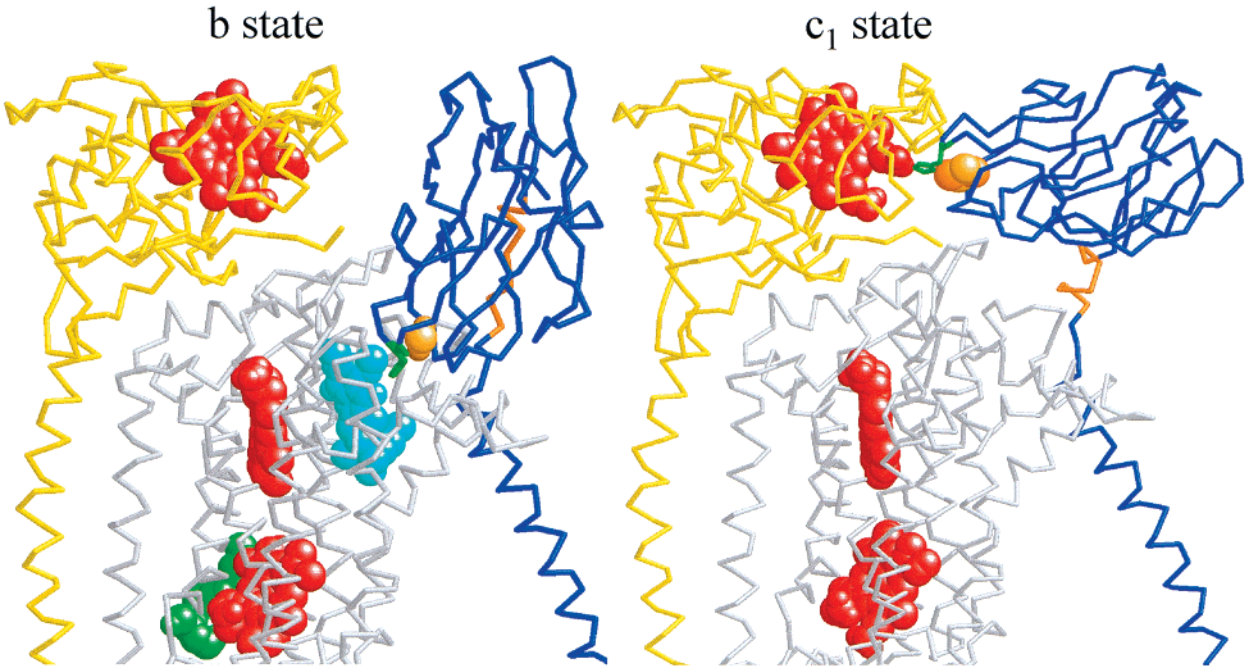


FIGURE 1: X-ray crystal structures of cytochrome bc_1 from chicken in the presence of stigmatellin and antimycin (b state) (*5*) and in the beef $P6_522$ crystals (c_1 state) (*6*). The Rieske, cyt c_1, and cyt *b* subunits are colored blue, yellow, and gray, respectively. The hemes, the Fe_2S_2 center, stigmatellin, and antimycin are represented by space-filling models colored red, yellow, cyan, and green, respectively. The Rieske neck region residues 66−72 are colored orange, and His-161 is colored green. Bovine Rieske residues 66−72 correspond to *R. sphaeroides* residues 42−48.

An important goal is to determine the conformation of the Rieske protein in each state of the bc_1 complex, the rate of electron transfer in each of the conformations, and the kinetics of change between the different conformations. However, very limited kinetic information is currently available on the intracomplex electron-transfer reactions involving the Rieske center, cyt c_1, and cyt *c*. The kinetics have previously been studied with the greatest time resolution in chromatophores from *Rhodobacter sphaeroides*, where the electron-transfer cycle can be initiated by a short light flash (*8*−*10*). Experiments using this system are limited by the rate of diffusion of photooxidized cyt c_2 from the reaction center to the bc_1 complex ($k_{diff} \approx 5000\ s^{-1}$) (*8*, *10*). The rate constant for intracomplex electron transfer between the Rieske center and cyt c_1 has been estimated to be much larger than 5000 s^{-1} using this method (*10*). A ruthenium photoexcitation method has been developed to measure the rate constant for intracomplex electron transfer between cyt *c* and cyt c_1 to be 60 000 s^{-1} (*11*, *12*).

In this paper, we report the use of a novel binuclear ruthenium complex, Ru_2D, to directly add or remove an electron from cyt c_1 in the cyt bc_1 complex upon flash photolysis in the presence of an appropriate sacrificial electron donor or acceptor. Ru_2D has a net charge of +4, which allows it to bind strongly and selectively to the negatively charged domain on the surface of cyt c_1. Photooxidation or photoreduction of cyt c_1 is complete within 1 μs, the lifetime of the metal-to-ligand excited state of Ru_2D. This new method has been used to measure electron transfer between the Rieske iron−sulfur protein and cyt c_1 in both the forward and the reverse directions as well as oxidant-induced reduction of cyt b_H. Experiments are reported with both bovine and *R. sphaeroides* cyt bc_1 complexes as well as *R. sphaeroides* mutants that affect the flexibility of the neck region of the Rieske iron−sulfur protein.

EXPERIMENTAL PROCEDURES

Materials. Ru_2D was prepared by a modification of the method of Downard et al. (*13*). Bovine cyt bc_1 was purified as described by Yu et al. (*14*). Native and mutant *R. sphaeroides* cyt bc_1 were prepared as described by Tian et al. (*15*). Paraquat and antimycin A were obtained from Sigma, stigmatellin was purchased from Fluka, and $[Co(NH_3)_5Cl]^{2+}$ was synthesized (*16*).

Flash Photolysis Experiments. Transient absorbance measurements were carried out by flash photolysis of 300 μL of solutions contained in a 1-cm glass semi-microcuvette. The excitation light flash was provided by a Phase R model DL1400 flash lamp-pumped dye laser using coumarin LD 490 to produce a 480-nm light flash of <0.5 μs duration. The detection system has been described by Heacock et al. (*11*). Samples typically contained 7 μM cyt bc_1 in a buffer with 0.01% lauryl maltoside. In photoreduction experiments, 10 mM aniline and 1 mM 3CP were used as sacrificial donors, and catalytic concentrations of horse cyt *c* and bovine cyt oxidase (20 nM) were present to maintain cyt bc_1 in the oxidized state. In photooxidation experiments, paraquat or $[Co(NH_3)_5Cl]^{2+}$ were used as sacrificial acceptors, and 2,3-dimethoxy-5-methyl-6-(10-bromodecyl)-1,4-benzoquinol ($Q_0C_{10}Br$) was used to reduce cyt bc_1. The experiments were carried out aerobically to rapidly reoxidize the highly absorbing reduced paraquat.

RESULTS

Flash photolysis of a sample containing purified beef cyt c_1 and Ru_2D results in rapid reduction of cyt c_1 by the excited-state Ru(II*) as shown in Figure 2. Aniline and 3CP serve as sacrificial electron donors to reduce Ru(III) and to prevent the back reaction. The reduction of cyt c_1 is complete in 1 μs, the lifetime of the excited state of Ru_2D. The amount of cyt c_1 reduced in a single flash increases hyperbolically

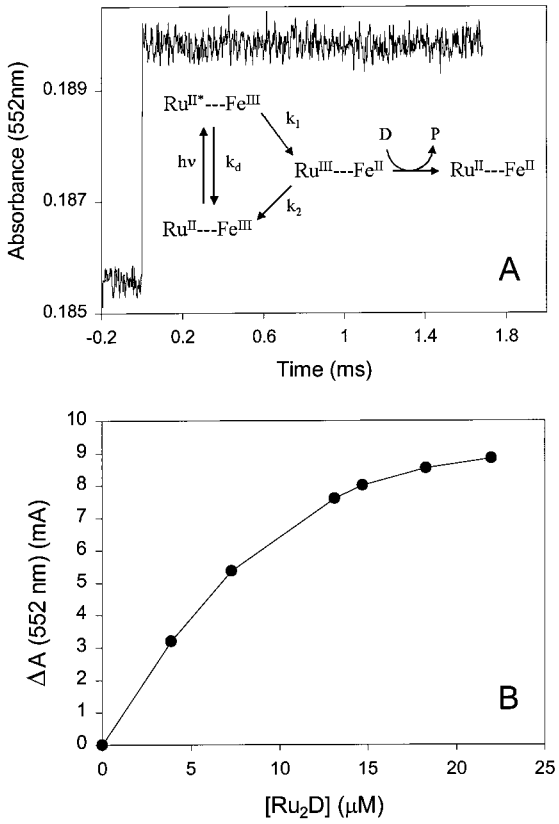


FIGURE 2: Photoinduced reduction of bovine cyt c_1 by Ru_2D. (A) A solution containing 6 μM bovine cyt c_1 and 7 μM Ru_2D in 5 mM Tris-HCl, pH 8.0, 10 mM aniline, and 1 mM 3CP at 25 °C was excited with a single 480-nm laser flash of 300 ns duration. (B) The absorbance change ΔA at 552 nm is plotted vs Ru_2D concentration.

as the concentration of Ru_2D increases, consistent with the formation of a complex between Ru_2D and cyt c_1 with a dissociation constant of 7 μM (Figure 2B). The yield of reduced cyt c_1 obtained with a single flash is 8% of the initial concentration present, with an Ru_2D concentration of 20 μM. The yield decreased to 2% upon addition of 100 mM NaCl, indicating dissociation of the complex.

Flash photolysis of a solution containing bovine cyt bc_1, Ru_2D, and aniline results in rapid reduction of cyt c_1, followed by biphasic reoxidation of cyt c_1 with rate constants of 90 000 and 7300 s^{-1} and relative amplitudes of 57% and 43% (Figure 3). When stigmatellin is added to the solution, the reoxidation of cyt c_1 is eliminated after several flashes (Figure 3). Stigmatellin binds preferentially in the presence of reduced Fe_2S_2 and locks the Rieske protein in the b conformation (*1*−*6*). These results indicate that photoreduced cyt c_1 transfers an electron to the Rieske iron−sulfur center in a biphasic reaction with rate constants of 90 000 and 7300 s^{-1}. The rate constant of the fast phase was independent of temperature down to 5 °C, within an error limit of ±20%. The slow phase decreased in amplitude and could not be measured reliably at low temperatures.

To study electron transfer from the Rieske iron−sulfur center to cyt c_1 (the physiological direction), Ru_2D was used to photooxidize cyt c_1 in the presence of the sacrificial electron acceptor paraquat. Purified, reduced cyt c_1 was photooxidized within 1 μs by Ru_2D in the presence of paraquat (data not shown). The mechanism may involve either of the pathways shown in Scheme 1. Flash photolysis

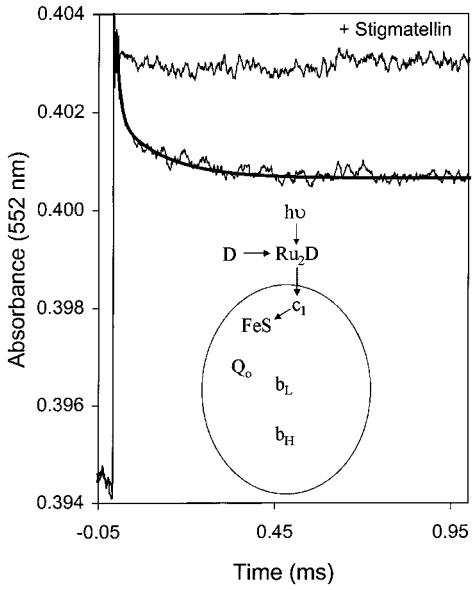
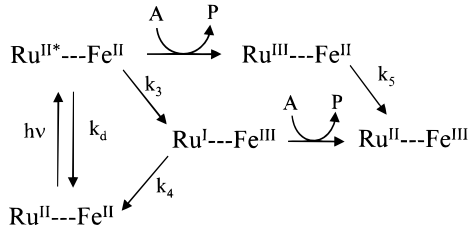


FIGURE 3: Electron transfer between Fe_2S_2 and cyt c_1 following photoreduction of bovine cyt bc_1. A sample containing 8 μM bovine cyt bc_1 and 25 μM Ru_2D in 5 mM Tris-HCl, pH 8, 10 mM aniline, 1 mM 3CP, and 0.01% lauryl maltoside was excited with a 480-nm laser flash. The 552-nm transient is the average of 10 single transients. Catalytic concentrations (50 nM) of cyt *c* and cyt oxidase are present in the solution to slowly reoxidize cyt bc_1 between flashes. The smooth curve is a biphasic fit with rate constants of 9 × 10^4 and 7.3 × 10^3 s^{-1} and relative amplitudes of 57% and 43%. The top curve was recorded following addition of stigmatellin and flashing several times to reduce the Rieske protein.

Scheme 1



of a solution containing reduced bovine cyt bc_1, Ru_2D, and paraquat leads to rapid oxidation of cyt c_1, followed by biphasic reduction with rate constants of 16 000 and 250 s^{-1} and relative amplitudes of 66% and 34% (Figure 4A). Addition of stigmatellin completely eliminates the reduction of cyt c_1, consistent with locking the reduced iron−sulfur center in the b state. These results indicate that the fast phase is due to electron transfer from the Rieske iron−sulfur center to cyt c_1. The rate constant of the fast phase has a large temperature dependence, consistent with an activation energy of 59 kJ/mol. The rate constant is also sensitive to the ionic strength of the buffer with values of 6000 s^{-1} at 3 mM ionic strength, 16 000 s^{-1} at 15 mM ionic strength, and 4700 s^{-1} at 53 mM ionic strength, all at 25 °C.

The oxidant-induced reduction of cyt b_H was studied in the presence of antimycin A to inhibit reduction of quinone at the Q_I site. Sufficient $Q_0C_{10}Br$ was added to completely reduce the Rieske center and cyt c_1 and to reduce cyt b_H by about 20%. Rapid photooxidation of cyt c_1 by Ru_2D was followed by biphasic reduction with rate constants of 4700 and 180 s^{-1} at 53 mM ionic strength, as monitored at 552 nm (Figure 4B). The absorbance at 562 nm increased in a step function due to the contribution of cyt c_1 oxidation at

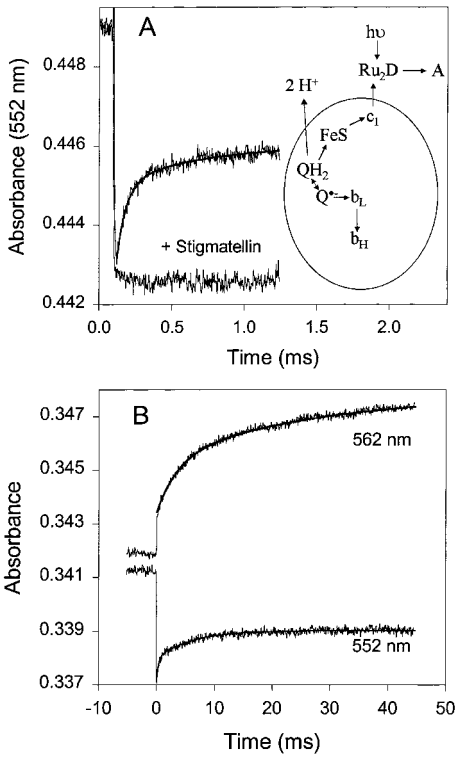


FIGURE 4: Electron transfer within the bovine cyt bc_1 complex following photooxidation of cyt c_1. (A) The 552-nm transient is shown for a solution containing 6 μM bovine cyt bc_1 and 20 μM Ru_2D in 20 mM Tris-HCl, pH 8, and 10 mM paraquat. 50 μM $Q_0C_{10}Br$ is added immediately before the flash to reduce cyt bc_1. The smooth curve is a biphasic fit with rate constants of 16 000 and 250 s^{-1} and relative amplitudes of 66% and 34%. The bottom transient was obtained after addition of 30 μM stigmatellin. (B) A sample containing 7 μM bovine cyt bc_1 and 19 μM Ru_2D in 5 mM Tris-HCl, pH 8.0, 50 mM NaCl, 10 mM paraquat, and 20 μM antimycin A was treated with sufficient $Q_0C_{10}Br$ to completely reduce the Rieske center and cyt c_1 and to reduce cyt b_H by about 20%. Rapid photooxidation of cyt c_1 by Ru_2D was followed by biphasic reduction with rate constants of 4700 ± 700 and 180 ± 40 s^{-1}, as monitored at 552 nm. The 562-nm transient indicated biphasic reduction of cyt b_H with rate constants of 250 ± 50 and 30 ± 10 s^{-1}.

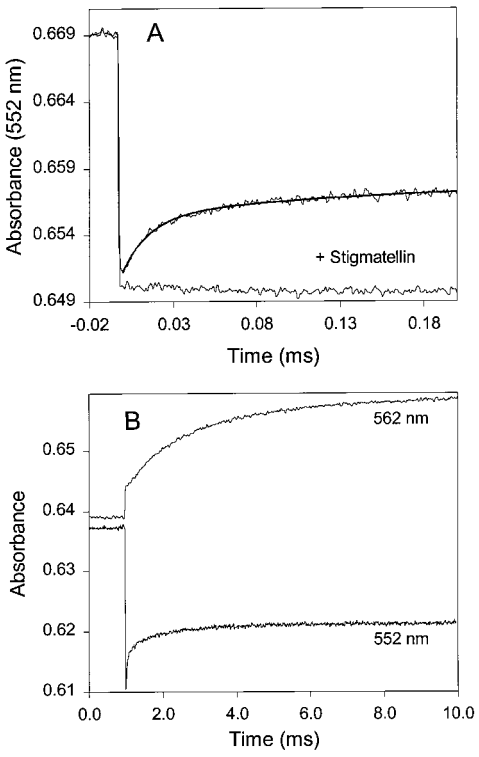


FIGURE 5: Electron transfer within *R. sphaeroides* cyt bc_1 following photooxidation of cyt c_1. (A) A solution containing 5 μM *R. sphaeroides* cyt bc_1 and 17 μM Ru_2D in 20 mM sodium borate, pH 9.0, and 5 mM $[Co(NH_3)_5Cl]^{2+}$ was treated with $Q_0C_{10}Br$ to reduce cyt bc_1 and excited with a laser flash. The smooth curve is a biphasic fit with rate constants of 61 000 and 1000 s^{-1}. The lower transient was obtained with the same sample after treatment with 20 μM stigmatellin. (B) The same solution in panel A was treated with antimycin A, and sufficient $Q_0C_{10}Br$ was added to completely reduce Fe_2S_2 and cyt c_1 and to reduce cyt b_H by about 20%. The 562-nm transient indicates biphasic reduction of cyt b_H with rate constants of 1000 and 260 s^{-1} and relative amplitudes of 50% each. The biphasic 552-nm transient has rate constants of 60 000 and 1000 s^{-1} and relative amplitudes of 60% and 40%.

this wavelength. This was followed by biphasic reduction of cyt b_H with rate constants of 250 and 30 s^{-1}. The small contribution of cyt c_1 to the 562-nm absorbance was taken into account in the kinetic analysis. Addition of MOA-stilbene completely eliminates the slow phase of cyt c_1 reduction and both phases of cyt b_H reduction. The rate constant for the fast phase of cyt b_H reduction is comparable to that for the slow phase of cyt c_1 reduction. This phase thus represents transfer of the first electron from QH_2 to Fe_2S_2 and cyt c_1, followed rapidly by transfer of the second electron from the semiquinone to cyt b_L and cyt b_H. The slow phase of cyt b_H reduction may be due to a small amount of the Ru(III) form of Ru_2D that is bound to a nonproductive site on the enzyme, slowly dissociates, binds to cyt c_1, and accepts an electron.

Photoinduced electron transfer in the *R. sphaeroides* cyt bc_1 complex was similar to that observed with the beef complex. It was found that $[Co(NH_3)_5Cl]^{2+}$ was a more efficient sacrificial electron acceptor than paraquat in this system, giving a photooxidation yield of 22%. Flash photolysis of a solution containing reduced *R. sphaeroides* cyt bc_1, Ru_2D, and $[Co(NH_3)_5Cl]^{2+}$ results in rapid photooxidation of cyt c_1, followed by biphasic reduction with rate constants of 61 000 and 1000 s^{-1} (Figure 5A). Both reduction phases were completely eliminated by addition of stigmatellin. Flash photolysis in the presence of antimycin A led to biphasic reduction of cyt b_H with rate constants of 1000 and 260 s^{-1} and relative amplitudes of 50% each (Figure 5B). Addition of MOA-stilbene eliminated both phases of reduction of cyt b_H and the slow phase of reduction of cyt c_1. The slow phase of reduction of cyt c_1 has the same rate constant as the fast phase of reduction of cyt b_H.

The importance of flexibility in the neck region of the Rieske iron−sulfur protein was explored using two *R. sphaeroides* cyt bc_1 mutants that should increase the rigidity of the neck region. The ALA-PLP mutant has double proline substitution at Ala-46 and Ala-48 in the neck region, and the ADV-PPP mutant has triple proline substitution at residues 42−44 (*15*). Photooxidation experiments using Ru_2D in the presence of $Q_0C_{10}Br$ and antimycin A were carried out as described above for wild-type *R. sphaeroides* cyt bc_1. No fast phase was observed in the 552-nm transient for the reduction of photooxidized cyt c_1 in the ALA-PLP mutant. Instead, the reduction of cyt c_1 occurs in a single slow phase with a rate constant of 25 s^{-1}, which is the same as the rate constant for reduction of cyt b_H observed at 562 nm. These results suggest electron transfer from Fe_2S_2 to cyt c_1 becomes

rate limiting in the double mutant, and thus the rate constants for reduction of cyt c_1 and cyt b_H are the same. No reduction of cyt c_1 or cyt b_H was observed when the same experiment was carried out on the ADV-PPP mutant, indicating that electron transfer from Fe_2S_2 to cyt c_1 is completely inhibited.

DISCUSSION

Measurement of the kinetics of electron transfer between the Rieske iron−sulfur protein and cyt c_1 has been a difficult problem (*8*−*10*). The ruthenium photoexcitation technique provides a new way to rapidly oxidize or reduce cyt c_1 and initiate internal electron transfer in the cyt bc_1 complex. The +4 charge on Ru_2D allows it to bind selectively to the negatively charged cyt *c* binding site on cyt c_1 in the cyt bc_1 complex. The lifetime of the metal-to-ligand charge transfer state, Ru_2D^*, is 1.0 μs, so the observed electron transfer to or from cyt c_1 occurs within this time scale (*17*). Oxidized cyt c_1 is photoreduced by Ru_2D^* in the presence of the sacrificial electron donors aniline and 3CP, which reduce Ru(III) and prevent the back reaction as shown in Figure 2A. The dependence of photoreduction yield on Ru_2D concentration and ionic strength is consistent with formation of an electrostatic complex between Ru_2D and cyt c_1. Ru_2D has also been used to photoreduce Cu_A in cyt *c* oxidase (*17*). Reduced cyt c_1 is photooxidized by Ru_2D in the presence of the sacrificial electron acceptors paraquat or $[Co(NH_3)_5Cl]^{2+}$. Cyt c_1 could be oxidized by either Ru(II*) or Ru(III) as shown in Scheme 1. The detailed mechanism is currently under investigation. Upon receiving an electron, $[Co(NH_3)_5Cl]^{2+}$ rapidly decomposes to aqueous Co^{2+}, so the reaction is irreversible (*18*). Likewise, reduced paraquat is rapidly oxidized by oxygen, so this reaction is irreversible under aerobic conditions. The yield of photoreduction or photooxidation is between 8% and 22%, which is more than 10-fold larger than using ruthenium trisbipyridine under the same conditions.

Electron transfer between the Rieske iron−sulfur center and cyt c_1 was studied in both the forward and the reverse directions in bovine cyt bc_1. Photoreduction of cyt c_1 by Ru_2D in the completely oxidized bc_1 complex led to biphasic oxidation of cyt c_1 with rate constants of 90 000 and 7300 s^{-1}. It is important to note that the initial amplitude of photoreduction of cyt c_1 is the same in the presence and in the absence of stigmatellin; thus, there is no faster phase of electron transfer that was not detected. The total extent of cyt c_1 reoxidation is 30% of the amount photoreduced, consistent with the finding that the redox potentials of cyt c_1 and Fe_2S_2 are nearly the same at pH 8 (*19*). However, it is possible that a small fraction of cyt bc_1 molecules may have lost the Rieske protein during purification, so the extent of cyt c_1 reoxidation cannot be used to accurately measure the difference in redox potentials of the two centers. It is tempting to speculate that the fast phase represents electron transfer from cyt c_1 to the Rieske iron−sulfur center that is initially in the c_1 conformation, while the slow phase represents electron transfer to the Rieske center that is initially in the b conformation. In the latter case the Rieske center would have to rotate to the c_1 conformation before electron transfer could occur. However, the conformational status of the Rieske protein in oxidized bovine cyt bc_1 in the absence of inhibitors is unclear. In the $I4_122$ crystals, the Rieske protein is about 40% in the b state and about 60% in a mobile state (*4*). In contrast, the Rieske protein is largely in the c_1 state in the beef $P6_522$ and $P2_1$ crystals (*5*, *6*), while an intermediate conformation is found in the beef $P6_5$ crystals (*6*). The rate of electron transfer between cyt c_1 and Fe_2S_2 is expected to be very fast in the c_1 state but negligible in the b state.

Dutton and co-workers (*20*) have found that a semiclassical relation for long-range electron transfer, developed by Marcus (*21*), applies to a wide range of biological systems:

$$k_{et} = k_o \exp[-\beta(r - r_o)] \exp[-((\Delta G^{\circ\prime} + \lambda)^2/4\lambda RT)] \quad (1)$$

where r is the distance between the closest macrocycle atoms in the two redox centers, the van der Waals contact distance $r_o = 3.6$ Å, β is taken to be 1.4 $Å^{-1}$, the nuclear frequency k_o is 10^{13} s^{-1}, λ is the nuclear reorganization energy, and $\Delta G^{\circ\prime}$ is the driving force (*20*). In the structure of the bovine $P6_522$ crystals reported by Zhang et al. (*5*), the Fe_2S_2 ligand His-161 is only 4.0 Å from the oxygen atom of heme c_1 propionate D and 8.2 Å from the heme c_1 macrocycle atom C3D. These distances are even closer in the bovine $P6_522$ structure reported by Iwata et al. (*6*), 3.8 and 7.8 Å, respectively, and His-161 forms a hydrogen bond with the heme propionate. The reorganization energy λ should be in the range of 0.7−1.0 eV for electron transfer between Fe_2S_2 and cyt c_1 (*22*), and $\Delta G^{\circ\prime}$ is close to zero. A rate constant of 1×10^6−2×10^7 s^{-1} is estimated from eq 1 assuming that $r = 8.2$ Å, $\Delta G^{\circ\prime} = 0$, and $\lambda = 1.0$−0.7 eV. Given the uncertainties in this calculation, the experimental value of 9×10^4 s^{-1} is in reasonable agreement with the smaller theoretical value and may represent the true rate of electron transfer in the c_1 conformation. It is also possible that the observed rate constant is affected by dynamic fluctuations in the conformation of the Rieske protein. If the fluctuations are slow as compared to electron transfer in the optimal c_1 state and the population of the c_1 state is small, then the observed rate constant is limited by the rate of the fluctuations and is "gated" (*23*, *24*). On the other hand, if the fluctuations are rapid as compared to electron transfer, then the observed rate constant is $k_{obs} = fk_{et}$, where f is the fraction of molecules in the c_1 state and electron transfer is "coupled" (*24*). A recent molecular dynamics simulation indicated that rotation of the soluble domain of the Rieske protein within 1 ns is feasible, consistent with the rapid fluctuation model (*25*, *26*). The small temperature dependence of the fast phase of electron transfer from cyt c_1 to Fe_2S_2 is consistent with a model in which the Rieske protein rapidly fluctuates within an ensemble of conformations that includes the c_1 state and various intermediate states. The slow phase with a rate constant of 7.3×10^3 s^{-1} could be controlled by the rate of dissociation of the Rieske protein from the b state to the ensemble of rapidly fluctuating conformations.

Electron transfer in the reduced bovine cyt bc_1 complex with cyt c_1 and Fe_2S_2 initially reduced and cyt b_L and cyt b_H initially oxidized was studied by rapidly photooxidizing cyt c_1 with Ru_2D in the presence of paraquat. The reduction of cyt c_1 has a fast phase with a rate constant of 16 000 s^{-1} due to electron transfer from initially reduced Fe_2S_2 to cyt c_1. Since the redox potentials of cyt c_1 and Fe_2S_2 are nearly the same at pH 8 (*19*), the two redox centers will approach equilibrium during the fast phase. The slow phase with a rate constant of 250 s^{-1} is due to electron transfer from QH_2

to Fe_2S_2, which then undergoes electron-transfer equilibration with cyt c_1. The fast phase rate constant is much slower than the predicted value in the c_1 state, suggesting that electron transfer is conformationally gated or coupled. The difference in rate constants in the oxidized and reduced complexes may be due to a difference in the dynamics of Rieske protein conformational changes or to the presence of quinol in the reduced complex. The rate constant in the reduced complex has a large temperature dependence, suggesting that the reaction could involve conformational changes in the Rieske protein with a significant energy of activation, 59 kJ/mol. The rate constant is also sensitive to ionic strength, suggesting that ionic interactions may affect the conformational changes. The slow phase of cyt c_1 reduction has nearly the same rate constant as reduction of cyt b_H in the presence of antimycin, 250 s^{-1}. This phase therefore represents transfer of the first electron from QH_2 to Fe_2S_2 and then to cyt c_1, followed rapidly by transfer of the second electron to cyt b_L and cyt b_H.

Electron transfer in the *R. sphaeroides* cyt bc_1 complex is similar to that in the bovine enzyme, except that the rate constants are larger. The reduction of photooxidized cyt c_1 has a fast phase with a rate constant of 60 000 s^{-1} due to electron transfer from Fe_2S_2 to cyt c_1 and a slow phase with a rate constant of 1000 s^{-1} due to electron transfer from QH_2 to Fe_2S_2 and then to cyt c_1. The slow phase of reduction of cyt c_1 has the same rate constant as the reduction of cyt b_H in the presence of antimycin A. This is consistent with a mechanism involving rate-limiting electron transfer from QH_2 to Fe_2S_2 and cyt c_1, followed by rapid electron transfer from the semiquinone to cyt b_L and cyt b_H. The observed rate constant for this process, 1000 s^{-1}, is comparable to that measured in flash photolysis studies of *R. sphaeroides* chromatophores (*10*). Hong et al. (*27*) have recently presented evidence that the activation barrier for this process occurs after formation of a complex between QH_2 and the oxidized, deprotonated Rieske center.

X-ray crystallography has revealed significant conformational changes in Rieske neck residues 66−72 (Figure 1). The role of conformational flexibility in this neck region has has been studied using mutants that should increase the rigidity of the neck (*15*). The steady-state turnover number of the double mutant ALA-PLP is 23 s^{-1} as compared with 83 s^{-1} for wild-type enzyme (*15*). In the present studies, the reduction of photooxidized cyt c_1 in the ALA-PLP mutant occurs in a single slow phase with a rate constant of 25 s^{-1}, which is the same as the rate constant for reduction of cyt b_H. These results indicate that for the ALA-PLP mutant the conformational change of the Rieske protein is rate-limiting for both reduction of oxidized cyt c_1 and reduction of cyt b_H as well as overall enzyme turnover. The large activation energy for the steady-state turnover of the ALA-PLP mutant, 69 kJ/mol, probably reflects the decreased flexibility of the Rieske protein. In contrast, the rate constant for reduction of cyt c_1 in the wild-type enzyme, 60 000 s^{-1}, is much faster than that for reduction of cyt b_H, 1000 s^{-1}, which in turn is much faster than steady-state turnover. It therefore appears that the conformational change of the Rieske protein is not rate-limiting in the wild-type enzyme.

In conclusion, a ruthenium photoexcitation method has been developed to measure the rate constant for a key step in the cyt bc_1 complex electron transfer between the Rieske iron−sulfur protein and cyt c_1. The rate constant depends on the redox state of the enzyme and, under some conditions, may be controlled by conformational changes in the Rieske protein. This new method can also be used to study the kinetics of electron transfer from quinol to the Rieske protein and heme b_L and b_H and can be applied to cyt bc_1 complexes isolated from different sources.

SUPPORTING INFORMATION AVAILABLE

Figure of the temperature dependence of electron transfer from Fe_2S_2 to cyt c_1 in bovine cyt bc_1. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Trumpower, B. L., and Gennis, R. B. (1994) *Annu. Rev. Biochem. 63*, 675−716.
2. Trumpower, B. L. (1990) *J. Biol. Chem. 265*, 11409−11412.
3. Brandt, U. (1998) *Biochim. Biophys. Acta 1365*, 261−268.
4. Xia, D., Yu, C.-A., Kim, H., Xia, J.-Z., Kachurin, A. M., Zhang, L., Yu, L, and Deisenhofer, J. (1997) *Science 277*, 60−66.
5. Zhang, Z., Huang, L., Shulmeister, V. M., Chi, Y.-I., Kim, K. K., Hung, L.-W., Crofts, A. R., Berry, E. A., and Kim, S.-H. (1998) *Nature 392*, 677−684.
6. Iwata, S., Lee, J. W., Okada, K., Lee, J. K., Wata, M., Rasmussen, B., Link, T. A., Ramaswamy, S., and Jap, B. K. (1998) *Science 281*, 64−71.
7. Kim, H., Xia, D., Yu, C.-A., Xia, J.-Z., Kachurin, A. M., Zhang, L., Yu, L., and Deisenhofer, J. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 8026−8033.
8. Meinhardt, S. W., and Crofts, A. R. (1982) *FEBS Lett. 149*, 223−227.
9. Snozzi, M., and Crofts, A. R. (1985) *Biochim. Biophys. Acta 809*, 260−270.
10. Crofts, A. R., and Wang, Z. (1989) *Photosynth. Res. 22*, 69−87.
11. Heacock, C., Liu, R., Yu, C., Yu, L, Durham, B., and Millett, F. (1993) *J. Biol. Chem. 268*, 27171−27175.
12. Tian, H., Sadoski, R., Zhang, L., Yu, C.-A., Yu, L., Durham, B., and Millett, F. (2000) *J. Biol. Chem.* (in press).
13. Downard, A. J., Honey, G. E., Phillips, L. F., and Steel, P. J. (1991) *Inorg. Chem. 30*, 2259−2260.
14. Yu, C.-A., Xia, J.-Z., Kachurin, A. M., Yu, L., Xia, D., Kim, H., and Deisnenhofer, J. (1996) *Biochim. Biophys. Acta 1275*, 47−53.
15. Tian, H., Yu, L, Mather, M., and Yu, C. A. (1998) *J. Biol. Chem. 273*, 27953−27959.
16. Moeller, T., Ed. (1957) *Inorganic Synthesis*, Vol. V, p 185, McGraw Hill Book Company, Inc., New York.
17. Zaslavsky, D., Sadoski, R. C., Wang, K., Durham, B., Gennis, R. B., and Millett, F. (1998) *Biochemistry 37*, 14910−14916.
18. Kalyanasundaram, K. (1992) *Photochemistry of Polypyridine and Porphyrin Complexes*, p 146, Academic Press, New York.
19. Zhang, L., Snyder, C., Trumpower, B. L., Yu, L., and Yu, C.-A. (1999) *FEBS Lett. 460*, 349−352.
20. Moser, C. C., Keske, J. M., Warncke, K., Farid, R. S., and Dutton, P. L. (1992) *Nature 355*, 796−802.
21. Marcus, R. A. (1956) *J. Chem. Phys. 24*, 966−978.
22. Scott, J. R., McLean, M., Sligar, S. G., Durham, B., and Millett, F. (1994) *J. Am. Chem. Soc. 116*, 7356−7362.
23. Hoffman, B. M., and Ratner, M. A. (1987) *J. Am. Chem. Soc. 109*, 6237−6243.
24. Davidson, V. L. (1996) *Biochemistry 35*, 14035−14039.
25. Izrailev, S., Crofts, A. R., Berry, E. A., and Schluten, K. (1999) *Biophys. J. 77*, 1753−1768.
26. Crofts, A. R., Guergova-Kuras, M., Huang, L., Kuras, R., Zhang, Z., and Berry, E. A. (1999) *Biochemistry 38*, 1579−15806.
27. Hong, S., Ugulava, N., Guerfova-Kuras, M., and Crofts, A. R. (1999) *J. Biol. Chem. 274*, 33931−33844.

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