Mutants of the Cu_A Site in Cytochrome *c* Oxidase of *Rhodobacter sphaeroides*: II. Rapid Kinetic Analysis of Electron Transfer†

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ABSTRACT: The function of the binuclear Cu_A center in cytochrome *c* oxidase (CcO) was studied using two *Rhodobacter sphaeroides* CcO mutants involving direct ligands of the Cu_A center, H260N and M263L. The rapid electron-transfer kinetics of the mutants were studied by flash photolysis of a cytochrome *c* derivative labeled with ruthenium trisbipyridine at lysine-55. The rate constant for intracomplex electron transfer from heme c to Cu_A was decreased from 40 000 s^{-1} for wild-type CcO to 16 000 s^{-1} and 11 000 s^{-1} for the M263L and H260N mutants, respectively. The rate constant for electron transfer from Cu_A to heme a was decreased from 90 000 s^{-1} for wild-type CcO to 4000 s^{-1} for the M263L mutant and only 45 s^{-1} for the H260N mutant. The rate constant for the reverse reaction, heme a to Cu_A, was calculated to be 66 000 s^{-1} for M263L and 180 s^{-1} for H260N, compared to 17 000 s^{-1} for wild-type CcO. It was estimated that the redox potential of Cu_A was increased by 120 mV for the M263L mutant and 90 mV for the H260N mutant, relative to the potential of heme a. Neither mutation significantly affected the binding interaction with cytochrome *c*. These results indicate that His-260, but not Met-263, plays a significant role in electron transfer between Cu_A and heme a.

Cytochrome *c* oxidase (CcO)[1] is a redox-linked proton pump that transfers electrons from ferrocytochrome *c* to molecular oxygen to form water (*1*, *2*). Three of the redox-active centers, heme a, heme a_3, and Cu_B, are located in subunit I, while Cu_A is located in subunit II. Our understanding of the structure and function of the Cu_A redox site has evolved considerably over the last several decades (*3*). For many years Cu_A was thought to consist of a single copper atom with a structure analogous to that found in blue copper proteins such as plastocyanin (*4*). EPR and electron nuclear double resonance studies of yeast CcO containing ^{15}N His or ^{2}H Cys indicated that Cu_A is ligated by at least one His and one Cys (*5*, *6*), while a selective Cu_A extraction study demonstrated that the Cys ligand(s) to Cu_A are located in subunit II (*7*). A crucial change occurred when it was recognized that Cu_A has many properties in common with the binuclear copper center A in nitrous-oxide reductase, which has a mixed valence Cu(1.5)···Cu(1.5) spin $^1/_2$ configuration (*8*, *9*). Four different models for a binuclear Cu_A center were proposed based on analysis of spectroscopic information (*10*−*13*). These models all have essential elements of the structure of Cu_A determined by X-ray crystallography of bovine CcO (*14*, *15*), *Paracoccus denitrificans* CcO (*16*), and a Cu_A domain reconstituted into a quinol oxidase fragment (*17*). In the crystallographic structures, the sulfur atoms of Cys-252 and Cys-256 serve as bridging ligands to the two copper atoms, while His-217 and Met-263 are terminal ligands to one copper atom and His-260 and the backbone carbonyl of Glu 254 are terminal ligands to the other copper atom (the sequence numbering of *R. sphaeroides* CcO subunit II is used throughout).

The reaction between ferrocytochrome *c* and CcO involves formation of a 1:1 complex between the two proteins, intracomplex electron transfer from heme c to the initial acceptor in CcO, and dissociation of the product complex. Stopped flow spectroscopy has been used to measure the rate of complex formation, but does not have sufficient time resolution to measure intracomplex electron transfer (*18*, *19*). A new ruthenium photoreduction technique has been developed to study electron transfer within a complex between CcO and ruthenium-cytochrome *c* (Ru−Cc) derivatives labeled with ruthenium trisbipyridine (*20*, *21*). The Ru(II) group is photoexcited to a metal-to-ligand charge-transfer state, Ru(II*), which rapidly transfers an electron to heme c (*22*). Reduced heme c was then found to transfer an electron to Cu_A in beef CcO with a rate constant of 6×10^4 s^{-1}, followed by electron transfer from Cu_A to heme a with a rate constant of 2×10^4 s^{-1} (*20*, *21*). This technique identified Cu_A to be the initial electron entry site in CcO, in agreement with a previous flow-flash study using CO-inhibited reduced CcO (*23*).

In this paper, the role of Cu_A in mediating electron transfer from Cc to heme a is investigated using mutants in which

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[1] Abbreviations: Cc, cytochrome *c*; CcO, cytochrome *c* oxidase; Ru-55-Cc, (dimethylbipyridine)(bpy)$_2$ruthenium-Lys-55-cytochrome *c*; bpy, bisbipyridine; Ru_2C, $[Ru(bpy)_2]_2(bphb)(PF6)_4$; bphb, 1,4-bis[2-(4′-methyl-2, 2′-bipyrid-4-yl)ethenyl]benzene; 3CP, carboxyl-2,2,5,5-tetramethyl-1-pyrolidinyloxy free radical; EPR, electron paramagnetic resonance.

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the Cu_A ligands His-260 and Met-263 are substituted with Asn and Leu, respectively. Met-263 ligates the copper that presumably accepts an electron from Cc, while His-260 ligates the copper that is linked by a hydrogen-bond network to heme a. These mutants retain the binuclear Cu_A center, but the symmetry of the two copper atoms is altered and the redox potential of Cu_A is significantly increased (*24*). The ruthenium photoreduction technique is used to measure the rate constants for electron transfer from Cc to Cu_A and from Cu_A to heme a, as well as the relative redox potentials of Cu_A and heme a.

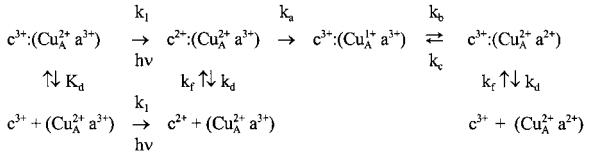
EXPERIMENTAL PROCEDURES

Materials. Horse heart cytochrome *c* (Type VI) and lauryl maltoside were obtained from Sigma Chemical Co. The Ru-55-Cc derivative was prepared as described by Liu et al. (*25*). The *R. sphaeroides* cytochrome oxidase wild-type and mutants were prepared as described by Zhen et al. in the preceding paper in this issue (*24*).

Flash Photolysis Experiments. Transient absorbance measurements were carried out as described by Geren et al. (*21*) by flash photolysis of 300 μL solutions contained in a 1 cm quartz semimicrocuvette. The excitation pulse was provided by a Phase R Model DL1400 flash lamp-pumped dye laser using coumarin 480 to produce a 480 nm light flash of <0.5 μs duration. The reaction of cytochrome *c* was monitored at 550 nm using an extinction coefficient of $\Delta\epsilon_{550} = 18.5$ mM^{-1} cm^{-1} (*26*). The reduction of heme a was measured at 605 nm using $\Delta\epsilon_{605} = 16$ mM^{-1} cm^{-1} and at 444 nm using $\Delta\epsilon_{444} = 59$ mM^{-1} cm^{-1} (*27*). The reaction of Cu_A was monitored at 830 nm using $\Delta\epsilon_{830} = 2.0$ mM^{-1} cm^{-1} (*28*). The extinction coefficients for the heme in Ru-55-Cc at 605 and 830 nm were measured to be $\Delta\epsilon_{605} = 1.2$ mM^{-1} cm^{-1} and $\Delta\epsilon_{830} = 0.15$ mM^{-1} cm^{-1}. The transients for heme a and Cu_A at 605 and 830 nm, respectively, were corrected for the small contribution from heme c at these wavelengths. Reaction solutions typically contained 3−10 μM Ru−Cc, 5−20 μM CcO, 10 mM aniline, 1 mM 3CP in 5 mM TrisCl, pH 8.0, at 22 °C. The aniline and 3CP functioned as sacrificial electron donors to reduce Ru(III) and prevent the back reaction with heme Fe(II). The ionic strength was adjusted by adding sodium chloride. The transients were fitted to appropriate theoretical equations as described by Geren et al. (*21*), and the reported errors are the estimated standard deviations. Ru_2C was a generous gift from Russell H. Schmehl (Departnemt of Chemistry, Tulane University), prepared as described by Baba et al. (*29*).

Ultracentrifuge Experiments. The equilibrium dissociation constant of the high affinity complex between horse cytochrome *c* and *R. sphaeroides* CcO was measured using the Beckman XL-A analytical ultracentrifuge. Samples typically contained 5 μM horse Cc, 6 μM CcO, in 5 mM TrisCl, pH 8, 0.1% lauryl maltoside, and 0 to 250 mM NaCl. The ultracentrifuge was run in the sedimentation velocity mode at a speed of 48 000 rpm. Absorbance scans were recorded every 15 min at a wavelength of 410 nm. Free Cc sedimented at a sedimentation velocity of 1.74 S, while CcO sedimented at 9.7 S. The concentration of free Cc in the sample was obtained from the absorbance in the free Cc plateau region, using an extinction coefficient of 106 mM^{-1} cm^{-1}. The concentration of the Cc:CcO complex was calculated from the difference between total Cc and free Cc. At zero NaCl, no free Cc was observed in the ultracentrifuge scan, while at 250 mM NaCl, the concentration of free Cc was equal to the total Cc, and there was no complex. The dissociation constant was calculated from the equation $K_D = [Cc][CcO]/[Cc:CcO]$.

Scheme 1



RESULTS

Reaction of Ru-55-Cc with Native Rb. sp. CcO. The reaction between Ru-55-Cc and wild-type *Rb. sphaeroides* CcO has been previously shown to occur according to Scheme 1 (*30*). Laser flash photolysis of a 1:1 complex between Ru-55-Cc and wild-type *Rb. sphaeroides* CcO at low ionic strength results in intracomplex electron transfer from photoreduced heme c to Cu_A with a rate constant of $k_a = 4 \times 10^4$ s^{-1}, followed by electron transfer from Cu_A to heme a with a rate constant of $k_b = 9 \times 10^4$ s^{-1} (*30*). The kinetics of the reaction remain the same up to 50 mM ionic strength, and then the complex begins to dissociate, and a slow phase appears due to the second-order reaction of uncomplexed Ru-55-Cc with CcO. The rate constant of the slow phase reaches a maximum of 7900 s^{-1} at 75 mM ionic strength and then decreases with further increases in ionic strength.

Reaction of Ru-55-Cc with Rb. sp. CcO Cu_A Ligand Mutant M263L. Flash photolysis of a solution containing 10 μM Ru-55-Cc and 16 μM M263L CcO in 5 mM TrisCl, pH 8.0, 20 mM NaCl, resulted in rapid photoreduction of heme c followed by biphasic reoxidation with rate constants of 16 000 ± 4000 s^{-1} and 330 ± 100 s^{-1}, as monitored at 550 nm (Figure 1). An additional very slow phase in the 550 nm transient with a rate constant of 130 s^{-1} is due to the back reaction of heme c Fe(II) with the sacrificial electron donor system. The 830 nm transient indicated that reduction of Cu_A is biphasic with rate constants of 17000 ± 5000 s^{-1} and 370 ± 100 s^{-1} (Figure 1). The 605 nm transient indicated that reduction of heme a is also biphasic with rate constants of 15000 ± 5000 s^{-1} and 270 ± 80 s^{-1} (Figure 1). The relative amplitude of the 605 nm transient is much smaller than for wild-type CcO, indicating a low ratio of heme a reduced to Cu_A reduced. Photoreduction of heme c makes a negative contribution to the 605 nm absorbance, and thus the fast phase of the 605 nm transient starts below the baseline (Figure 1). The contribution of heme c to the 605 nm transient was taken into account in the kinetic analysis. The fast phase at all three wavelengths is consistent with intracomplex electron transfer from heme c to Cu_A with a rate constant of $k_a = 16000 \pm 4000$, followed by electron transfer from Cu_A to heme a with an apparent rate constant greater than 16 000 s^{-1}. The fast phase has a relative amplitude of 30 ± 10%. The slow phase observed at all three wavelengths is apparently due to a fraction of Ru-55-Cc that is bound in a configuration that is very poorly oriented for electron transfer to Cu_A, such that the rate constant is only

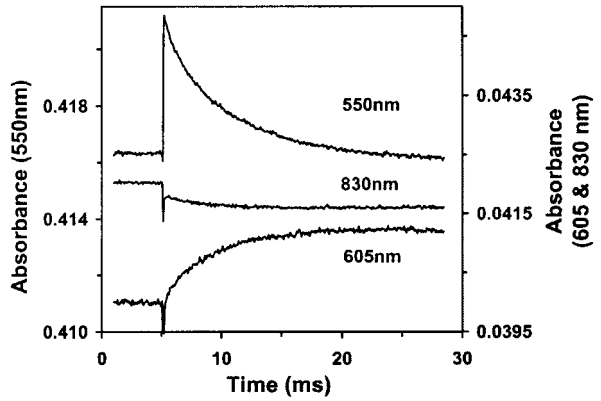


FIGURE 1: Photoinduced electron transfer from Ru-55-Cc to M263L *Rb. sphaeroides* CcO at 25 mM ionic strength. The solution contained 10 μM Ru-55-Cc and 16 μM M263L CcO in 5 mM TrisCl, pH 8.0, 20 mM NaCl, 10 mM aniline, 1 mM 3CP. The 550 nm transient indicated reoxidation of heme c in three phases with rate constants of 16000 ± 4000 s^{-1}, 330 ± 100 s^{-1}, and 130 ± 30 s^{-1} and relative amplitudes of 30, 50, and 20%. The fast phase was not resolved on the time scale shown, and the slow phase is due to the back reaction of heme c Fe(II) with the sacrificial electron donor system. The 830 nm transient indicated that reduction of Cu_A was biphasic with rate constants of 17000 ± 5000 s^{-1} and 370 ± 100 s^{-1} and relative amplitudes of 40% and 60%. The 605 nm transient indicated that reduction of heme a was biphasic with rate constants of 15000 ± 5000 s^{-1} and 270 ± 80 s^{-1} and relative amplitudes of 30% and 70%.

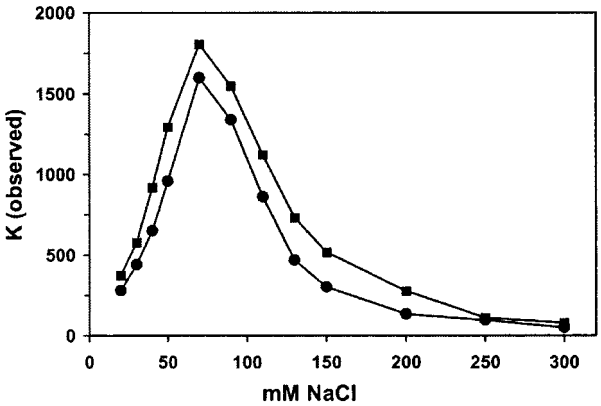


FIGURE 2: Ionic strength dependence of the slow phase of electron transfer from Ru-55-Cc to M263L CcO measured at 830 nm (■) and 605 nm (●). The solutions contained 10 μM Ru-55-Cc and 16 μM M263L CcO in 5 mM TrisCl, pH 8.0, NaCl, 10 mM aniline, 1 mM 3CP. The rate constants at 830 and 605 nm were the same within experimental error.

350 ± 100 s^{-1}. As the ionic strength was increased above 20 mM, the rate constant of the fast phase remained constant but the amplitude decreased until it reached zero at 100 mM NaCl, indicating that the complex was completely dissociated. The rate constant of the slow phase increased to a maximum of 1800 s^{-1} at 75 mM ionic strength and then decreased with further increases in ionic strength (Figure 2). At 75 mM ionic strength, the rate constants of the slow phases were 1840 ± 300 s^{-1}, 1800 ± 300 s^{-1}, and 1600 ± 300 s^{-1} for the 550, 830, and 605 nm transients, respectively (Figure 3). The slow phase is due to the bimolecular reaction of solution-phase Ru-55-Cc with M263L CcO and is rate-limited by complex formation. Since the rate of reduction of heme a is the same as that of Cu_A, within experimental error, the rate constant for electron-transfer equilibrium between Cu_A and heme a is fast compared to the rate of

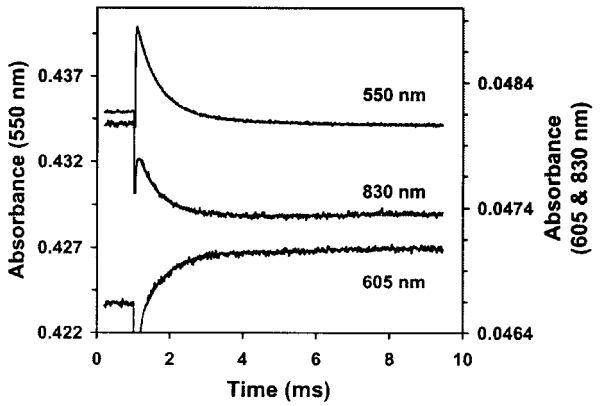


FIGURE 3: Photoinduced electron transfer from Ru-55-Cc to M263L *Rb. sphaeroides* CcO at 75 mM ionic strength. The solution contained 10 μM Ru-55-Cc and 16 μM M263L CcO in 5 mM TrisCl, pH 8.0, 70 mM NaCl, 10 mM aniline, 1 mM 3CP. The rate constants of the slow phases were 1840 ± 300 s^{-1}, 1800 ± 300 s^{-1}, and 1600 ± 300 s^{-1} for the 550, 830, and 605 nm transients.

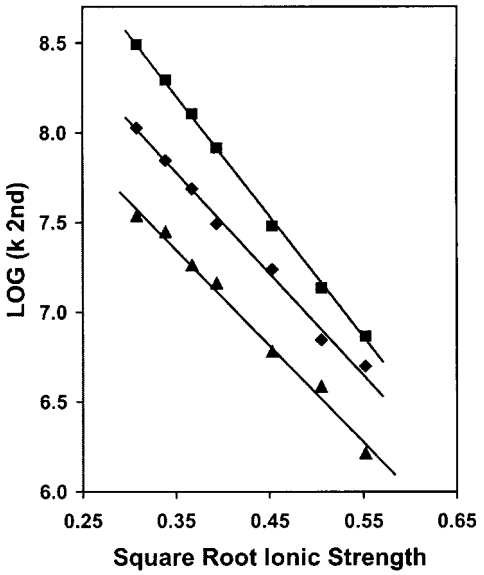


FIGURE 4: Ionic strength dependence of the second-order rate constants for the reaction from Ru-55-Cc to Cu_A in the CcO Mutants. The second-order rate constants were measured in 5 mM TrisCl, pH 8.0, NaCl, 10 mM aniline, and 1 mM 3CP. Wild-type (■); M263L mutant (◆); H260N mutant (▲).

reduction of Cu_A, and $k_b + k_c$ is much larger than 1800 s^{-1}. The ratio of reduced heme a^{2+} to Cu_A^{1+} formed upon completion of the reaction was calculated to be 0.06 ± 0.01 using extinction coefficients of $\Delta\epsilon_{605} = 16$ mM^{-1} cm^{-1} for heme a and $\Delta\epsilon_{830} = 1.03$ mM^{-1} cm^{-1} for Cu_A (*24*). This indicates that the equilibrium constant for electron transfer between Cu_A and heme a is $K = k_b/k_c = 0.06 \pm 0.01$, corresponding to a difference in redox potentials of −72 ± 5 mV, compared to +46 mV for wild-type. The transients at all three wavelengths were fitted to the complete equations for Scheme 1, with $k_{obs} = 1800 \pm 300$ s^{-1}, $k_b > 1500$ s^{-1} and $k_c > 25\,000$. It was thus possible to measure the ratio k_b/k_c, but only set lower limits to the values of k_b and k_c. The second-order rate constant k_{second} decreased with increasing ionic strength above 100 mM ionic strength, consistent with an electrostatic interaction between the two proteins (Figure 4). The value of k_{second} for the M263L mutant was 3.2-fold smaller than that of wild-type CcO at 95 mM ionic

Table 1: Reaction of Ru-55-Cc and Ru_2C* with *R. Sphaeroides* CcO Mutants[a]

mutant	k_a (s^{-1})	K	k_b (s^{-1})	k_c (s^{-1})	k_{second} (μM^{-1} s^{-1})
wild-type	38 000	6.1	93 000	17 000	310
M263L	16 000	0.06	>1500	>25 000	96
			4000*	66 000*	
H260N	11 000	0.20	36	184	41
			6	29	

[a] The kinetic parameters were measured as described in the text in 5 mM TrisCl, pH 8.0, 10 mM aniline, 3 mM 3CP, and 0−300 mM NaCl. k_a, the fast intracomplex rate constant for electron transfer from Ru-55-Cc to Cu_A was measured at ionic strengths from 5 mM to 75 mM. The rate constants k_b and k_c for electron transfer between Cu_A and heme a were calculated over the ionic strength range 30−100 mM. The second-order rate constant k_{second} was measured at 95 mM ionic strength. Rates obtained by reaction with Ru_2C are marked by an asterisk (*). The error limits in the rate constants are ±30% or less.

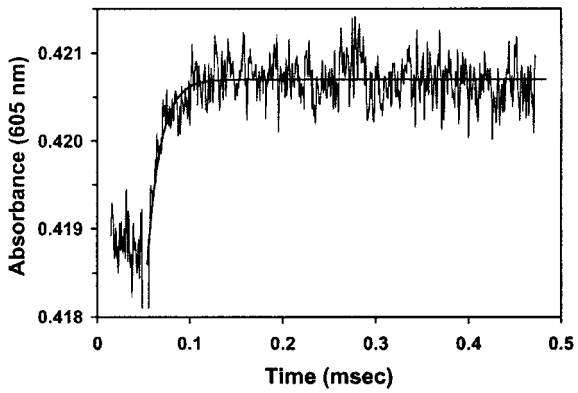


FIGURE 5: Photoinduced electron transfer from Ru_2C to M263L *Rb. sphaeroides* CcO. Photolysis of a solution containing 21 μM Ru_2C and 16 μM M263L in 10 mM aniline, 1 mM 3CP and 5 mM TrisCl, pH 8.0 resulted in a 605 nm transient with a rate constant of 70000 ± 20000 s^{-1}.

strength (Table 1), but the ionic strength dependence was very similar, indicating that the electrostatic interaction was similar (Figure 4) (*31*).

Reaction of Ru_2C with Rb. sp. CcO Cu_A Ligand Mutant M263L. The kinetics of the Cu_A mutant M263L were studied further by use of the Ru dimer Ru_2C which has previously been used by Zaslavsky et al. (*32*) to investigate the reduction of heme a by the Cu_A center in native Rb. sp. CcO. This dimer has a charge of +4 and binds specifically to the Cc binding site on CcO. The photolysis of 21 μM Ru_2C with 16 μM M263L CcO in 5 mM TrisCl, pH 8.0, resulted in rapid reduction of Cu_A within 1 μs, followed by electron transfer from Cu_A to heme a with an apparent rate constant of k_{app} = 70 000 s^{-1}, as measured at 605 nm (Figure 5). It was not possible to measure the 830 nm absorbance of Cu_A at times less than 20 μs with our detector, as is the case for wild-type enzyme (*32*), so the initial rate constant for reduction of Cu_A could not be measured. However, the lifetime of the Ru_2C excited state is 1.0 μs, and Cu_A is the only possible redox intermediate between Ru_2C* and heme a in this enzyme. Using the k_b/k_c value of 0.06 obtained from the Ru-55-Cc data and the relation $k_{app} = k_b + k_c$ (Figure 5), k_b = 4000 ± 1000 s^{-1} and k_c = 66000 ± 15000 s^{-1}.

Reaction of Ru-55 with Rb. sp. CcO Cu_A Ligand Mutant H260N. The reaction between Ru-55-Cc and H260N CcO was also biphasic at low ionic strength, consistent with two

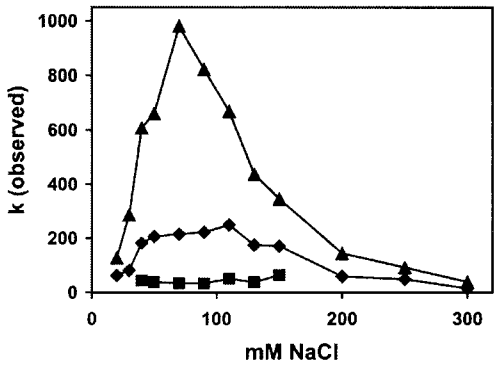


FIGURE 6: Ionic strength dependence of the slow phase of electron transfer from Ru-55-Cc to H260N CcO measured at 830 nm (▲) and 605 nm (◆, ■). The solutions contained 17 μM Ru-55-Cc and 24 μM M263L CcO in 5 mM TrisCl, pH 8.0, NaCl, 10 mM aniline, 1 mM 3CP.

configurations of the complex. At 25 mM ionic strength the reoxidation of heme c measured at 550 nm was biphasic with rate constants of 11000 ± 3000 s^1 and 90 ± 30 s^{-1}, while the reduction of Cu_A observed at 830 nm had rate constants of 12000 ± 5000, and 120 ± 40 s^{-1}. The fast phase had a relative amplitude of 25 ± 10%. The reduction of heme a measured at 605 nm did not have any fast phase, but only a slow phase with a rate constant of 63 ± 20 s^{-1}. As the ionic strength was increased, the amplitude of the fast phase in the 550 and 830 nm transients decreased until this phase disappeared above 75 mM ionic strength. The rate constant of the slow phase in the 550 and 830 nm transients increased until a maximum of 1000 s^{-1} was reached at 75 mM ionic strength and then decreased with further increases in ionic strength (Figure 6). In contrast, the 605 nm transient consisted of two phases of equal amplitude with rate constants of 220 ± 40 s^{-1} and 45 ± 20 s^{-1} over the ionic strength range from 45 mM to 155 mM (Figure 6). The transients at all three wavelengths for a sample in 75 mM ionic strength buffer are shown in Figure 7. These results are consistent with bimolecular electron transfer from Ru-55-Cc to Cu_A with an apparent rate constant of 1000 s^{-1}, followed by biphasic electron transfer from Cu_A to heme a with apparent rate constants of 220 s^{-1} and 35 s^{-1}. The ratio of heme a reduced to Cu_A reduced is $K = k_b/k_c$ = 0.20 ± 0.03, consistent with a redox potential difference of −41 ± 5 mV (wild-type = +46 mV). Using the ratio k_b/k_c = 0.20 and the relation $k_{app} = k_b + k_c$, the individual rate constants were calculated to k_b = 36 s^{-1} and k_c = 184 s^{-1} for the fast phase and k_b = 6 s^{-1} and k_c = 29 s^{-1} for the slow phase (Table 1). The biphasic reduction of heme a indicates that there are two slightly different conformations of the Cu_A center in the H260N CcO mutant. The second-order rate constant was smaller than that of wild-type CcO by a factor of 7.5 at 95 mM ionic strength (Table 1), but the ionic strength dependence was similar (Figure 4).

Measurement of Dissociation Constants by Ultracentrifuge Methods. The formation of a complex between native horse Cc and the mutants of CcO was studied using the Beckman XL-A analytical ultracentrifuge operating in the sedimentation velocity mode as previously described by Wang et al. (*30*). Under conditions of excess CcO, the dissociation constant of the high-affinity binding site was determined by measuring the concentration of complexed and uncomplexed Cc as a function of ionic strength. Both mutants formed a

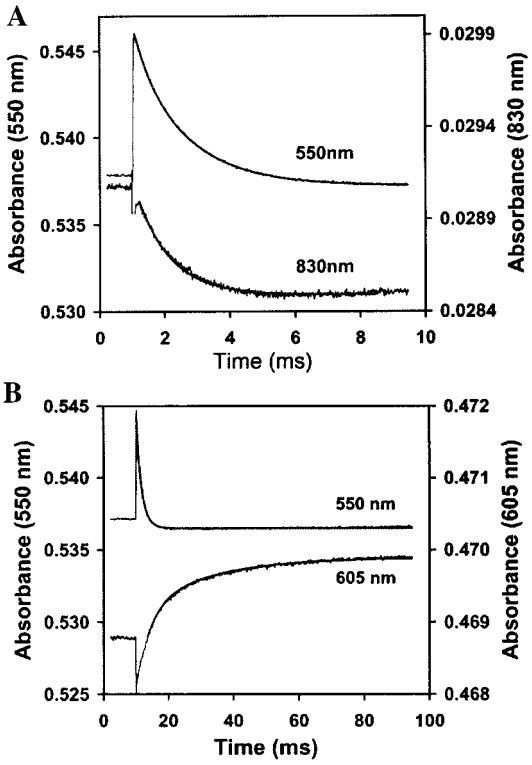


FIGURE 7: Photoinduced electron transfer from Ru-55-Cc to H260N *Rb. sphaeroides* CcO at 75 mM ionic strength. The solution contained 17 μM Ru-55-Cc and 24 μM M263L CcO in 5 mM TrisCl, pH 8.0, 70 mM NaCl, 10 mM aniline, 1 mM 3CP. The 550 and 830 nm transients each had a slow phase with a rate constant of 1000 $\pm$ 200 s^{-1}. The 605 nm transient consisted of two phases of equal amplitude with rate constants of 220 $\pm$ 40 s^{-1} and 34 $\pm$ 20 s^{-1}.

Table 2: Equilibrium Dissociation Constant K_D for the Binding of Horse Cc to the High Affinity Site on *Rb. Sp.* CcO

	K_D (in μM)				
mutant	0−30 mM I	45 mM I	65 mM I	85 mM I	105 mM I
wild-type	<0.2	1.0	5.6	18	>30
H260N	<0.2	0.8	5.0	19	>30
M263L	<0.2	1.7	9.0	22	>30

[a] K_D (in μM) was measured using the ultracentrifuge in the sedimentation velocity mode for 1 h at 48 000 rpm. Solutions contained 4 μM Cc and 7 μM CcO in 5 mM Tris-Cl, pH 8.0, 0.1% lauryl maltoside, and 1−250 mM NaCl to adjust the ionic strength I. The error limits are ±20%.

strong 1:1 complex with native horse Cc at 5 mM ionic strength with a K_D value less than 0.2 μM. The K_D values increased with increasing ionic strength until the complex was dissocated above 100 mM ionic strength (Table 2). The H260N CcO mutant has the same K_D value as wild-type CcO throughout the ionic strength range, while the K_D value of the M263L CcO mutant is about 1.7 times larger than that of wild-type CcO. The sedimentation coefficients of both mutants are 9.7 S, the same as wild-type CcO.

DISCUSSION

The structure of the C-terminal globular domain of subunit II consists of a 10-stranded β-barrel which is homologous to that of type 1 blue copper proteins such as plastocyanin (*14*−*17*). This raises the question of what advantages the mixed-valence binuclear Cu_A site found in CcO has compared with a mononuclear type 1 copper site. Larsson et al. (*12*) have proposed that the reorganization energy could be reduced significantly in the symmetric binuclear site since the bond-length changes upon electron transfer would be only half as large as for a mononuclear site. This could have a significant effect on the rates of electron transfer from Cc to Cu_A and from Cu_A to heme a because of the small driving forces involved in these reactions. An additional advantage of the binuclear Cu_A site is that different pathways for electron entry from Cc and electron exit to heme a can be utilized.

The electron-transfer reactions of Cu_A in wild-type *R. sphaeroides* CcO have been previously investigated using horse Ru-55-Cc, which is labeled with ruthenium trisbipyridine at lysine 55 on the bottom of Cc remote from the binding domain (*30*). The ruthenium complex on Ru-55-Cc does not interfere in the interaction with CcO, nor does it affect the binding constant (*30*). Kinetic studies of subunit II surface mutants indicated that the acidic residues Asp-214, Glu-157, Glu-148, and Asp-195 interact electrostatically with lysines surrounding the heme crevice of Cc (*30*). Mutational studies have also implicated the corresponding residues in *Paracoccus* CcO in the interaction with Cc (*33*). Mutation of Trp-143 to Phe or Ala dramatically decreased the rate constant for electron transfer from Ru-55-Cc to Cu_A by 450−1000-fold, without affecting the binding strength (*30*). Trp-143 is located on the surface of subunit II just above the copper atom that is ligated by Met-263 (*14*−*17*). It was proposed that Trp-143 could provide a pathway for electron transfer from the Cc heme group to this copper atom in the Cu_A binuclear center (*30*, *34*). Roberts and Pique (*35*) have used a computational docking program to determine a theoretical model for the complex between Cc and CcO that is fully consistent with the kinetic and binding studies of the *R. sphaeroides* CcO mutants (*30*, *36*).

To investigate the mechanism of electron transfer from Cc to heme a mediated by Cu_A, two *R. sphaeroides* CcO mutants were examined, M263L and H260N. Met-263 ligates the copper that presumably accepts an electron from Cc, while His-260 ligates the copper that is linked by a hydrogen-bond network to heme a. In both of these mutants, two copper atoms are retained at the Cu_A center, but the EPR signal is altered, and the 830 nm band is shifted and decreased in amplitude. In addition, the difference in redox potentials between heme a and Cu_A is decreased from +46 mV for wild-type CcO to −72 mV for M263L CcO and −41 mV for H260N CcO. The visible and EPR spectral properties of heme a are unaltered in M263L CcO (*24*), suggesting that the redox potential of heme a is unchanged, and that the redox potential of Cu_A is increased by 118 mV relative to that in wild-type CcO. Although the visible and EPR spectral properties of heme a are altered somewhat in the H260N mutant (*24*), it is likely that most of the change in the difference redox potential is due to an increase in the redox potential of Cu_A of approximately 87 mV. These results indicate that the mixed valence Cu(1.5)···Cu(1.5) state of the wild-type Cu_A center is converted to a state in which the two copper atoms are no longer equivalent, but still coupled (*24*). The spectroscopic and kinetic properties of the equivalent Cu_A mutant M227I of *Paracoccus denitrificans* have also been studied (*37*), giving similar results with respect to redox potential changes.

The reactions of Ru-55-Cc with the M263L and H260N CcO mutants are biphasic at low ionic strength. Since a slow phase is not seen in wild-type CcO, it appears that the mutations alter the conformation of the binding site on the surface of subunit II such that Ru-55-Cc binds in two orientations at low ionic strength. The fast phase is due to electron transfer within a complex that is favorable for rapid electron transfer. Since the fast phase rate constants remain the same from 5 mM to 75 mM ionic strength, the binding orientation of Ru-55-Cc is independent of ionic strength, as is also observed with wild-type CcO (*30*). The slow phase at low ionic strength is due to Ru-55-Cc binding in a configuration that is very poorly oriented or inactive in electron transfer. In the latter case, Ru-55-Cc would have to reorient from the inactive to the active configuration for electron transfer to occur, an example of configurational gating (*38*). The rate constant of the slow phase of electron transfer increases with increasing ionic strength up to 75 mM, suggesting that a decrease in the strength of the electrostatic interaction allows Cc to dissociate from the inactive configuration and bind to the active configuration more readily (*30*).

The fast phase rate constants k_a for the M263L and H260N mutants are only 2.5- and 3.5-fold smaller than the intracomplex rate constant for wild-type CcO, $k_a = 4 \times 10^4\ s^{-1}$. This change in heme c to Cu_A rate may reflect a slight alteration in the orientation of Ru-55-Cc binding or an increase in the reorganization energy due to a less coupled binuclear site in these mutants. The latter would be expected to decrease the rate constant for electron transfer to Cu_A. However, the increase in redox potential of the Cu_A site in the M263L and H260N mutants by 118 mV and 87 mV, respectively, would increase the driving force of the reaction and partially offset the effect of the increase in reorganization energy. As a result, these two Cu_A ligand mutations do not appear to have a major effect on the fast, intracomplex phase of electron transfer from Ru-55-Cc to Cu_A.

The rate constants for electron transfer between Cu_A and heme a in the M263L CcO mutant are $k_b = 4000\ s^{-1}$ and $k_c = 66\,000\ s^{-1}$, compared to $93\,000\ s^{-1}$ and $17\,000\ s^{-1}$, respectively, for wild-type (*30*, *32*). The H260N mutation decreases the rate constants for electron transfer between Cu_A to heme a by over 4 orders of magnitude, to $k_b = 45\ s^{-1}$ and $k_c = 180\ s^{-1}$ for the fast phase. The increase in reorganization energy upon conversion of a mixed valence binuclear Cu_A center in wild-type CcO to a less-equivalent center in the mutants would be expected to decrease the rate constant for electron transfer to heme a. In addition, the large increase in the redox potential of Cu_A in the mutants would decrease the driving force of the forward reaction and decrease the rate constant k_b. However, this same factor would increase the driving force of the reverse reaction and be expected to increase k_c, as observed for M263L but not for H260N. If anything, these effects should be larger in the M263L mutant than in H260N because of the larger decrease in redox potential. It therefore appears that the large decrease in k_b and k_c for the H260N mutant is primarily due to a disruption in the structure between the centers. It is apparent that His-260 on the electron exit side of the Cu_A center plays a critical role in electron transfer to heme a.

It is expected that the M263L and H260N mutations would have different effects on electron entry and exit from Cu_A, since Met-263 ligates the copper that presumably accepts an electron from Cc, while His-260 ligates the copper that is linked by a hydrogen-bond network to heme a. On the basis of crystallographic information and theoretical calculations, it has been proposed that the pathway for electron transfer from Cu_A to heme a involves a hydrogen-bond network through the Cu_A ligand His-260, the peptide backbone, and the highly conserved subunit I residue Arg-482, to the heme a propionates (*14*−*16*, *39*−*43*). The large decrease in the rate constants for electron transfer between Cu_A and heme a in the H260N mutant suggests that this pathway is important for electron transfer. Medvedev et al. (*44*) carried out tunneling current calculations indicating that an alternative pathway involving the Cu_A ligand Cys-256, Arg-482, and the heme propionates is dominant, largely as a result of the large degree of delocalization of electron density in the Cu_A center on the sulfur of Cys-256. The present studies question the importance of this pathway, although it is not possible to rule out an effect of the H260N mutation on the ligation of Cu_A with Cys-256.

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