# Single Electron Reduction of Cytochrome *c* Oxidase Compound F: Resolution of Partial Steps by Transient Spectroscopy†

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ABSTRACT: The final step of the catalytic cycle of cytochrome oxidase, the reduction of oxyferryl heme $a_3$ in compound F, was investigated using a binuclear polypyridine ruthenium complex ($Ru_2C$) as a photoactive reducing agent. The net charge of +4 on $Ru_2C$ allows it to bind electrostatically near $Cu_A$ in subunit II of cytochrome oxidase. Photoexcitation of $Ru_2C$ with a laser flash results in formation of a metal-to-ligand charge-transfer excited state, $Ru_2C^*$, which rapidly transfers an electron to $Cu_A$ of cytochrome oxidase from either beef heart or *Rhodobacter sphaeroides*. This is followed by reversible electron transfer from $Cu_A$ to heme *a* with forward and reverse rate constants of $k_1 = 9.3 \times 10^4\ s^{-1}$ and $k_{-1} = 1.7 \times 10^4\ s^{-1}$ for *R. sphaeroides* cytochrome oxidase in the resting state. Compound F was prepared by treating the resting enzyme with excess hydrogen peroxide. The value of the rate constant $k_1$ is the same in compound F where heme $a_3$ is in the oxyferryl form as in the resting enzyme where heme $a_3$ is ferric. Reduction of heme *a* in compound F is followed by electron transfer from heme *a* to oxyferryl heme $a_3$ with a rate constant of 700 $s^{-1}$, as indicated by transients at 605 and 580 nm. No delay between heme *a* reoxidation and oxyferryl heme $a_3$ reduction is observed, showing that no electron-transfer intermediates, such as reduced $Cu_B$, accumulate in this process. The rate constant for electron transfer from heme *a* to oxyferryl heme $a_3$ was measured in beef cytochrome oxidase from pH 7.0 to pH 9.5, and found to decrease upon titration of a group with a $pK_a$ of 9.0. The rate constant is slower in $D_2O$ than in $H_2O$ by a factor of 4.3, indicating that the electron-transfer reaction is rate-limited by a proton-transfer step. The pH dependence and deuterium isotope effect for reduction of isolated compound F are comparable to that observed during reaction of the reduced, CO-inhibited CcO with oxygen by the flow-flash technique. This result indicates that electron transfer from heme *a* to oxyferryl heme $a_3$ is not controlled by conformational effects imposed by the initial redox state of the enzyme. The rate constant for electron transfer from heme *a* to oxyferryl heme $a_3$ is the same in the *R. sphaeroides* K362M CcO mutant as in wild-type CcO, indicating that the K-channel is not involved in proton uptake during reduction of compound F.

Cytochrome *c* oxidase, (CcO[1]), the terminal component in the respiratory chain of both bacteria and eukaryotic mitochondria, reduces molecular oxygen using electrons transferred from cytochrome *c* (*1*). The high driving force of oxygen reduction is conserved by the formation of an electrochemical proton gradient as a result of two processes. First, since the electrons and protons originate from different sides of the coupling membrane, the chemical reaction itself causes electric charge separation and generation of a membrane potential. Second, the driving force of the chemical reaction is used to pump protons from the electronegative to the electropositive side of the membrane (*1*, *2*).

With fully oxidized enzyme as starting material, the first step of the cytochrome *c* oxidase reaction is the reduction of the binuclear center by two electrons, coupled with the uptake of two protons (*2*). This reaction is followed by the binding and two-electron reduction of molecular oxygen to form compound P, which has a major absorbance band at 607 nm (*3*). In successive one-electron reactions, compound P is reduced to compound F, with an absorbance band at 580 nm, followed by reduction of compound F to the oxidized form of the enzyme, O (ferric heme $a_3$) (*4*). Each of these one-electron reactions involves the uptake of one proton by the binuclear center, and is coupled to proton pumping (*5*, *6*).

Compound F is an oxyferryl heme $a_3$ ($Fe^{4+}{=}O^{2-}$) and is thought to be the last intermediate in the catalytic cycle of the enzyme before recovery of the resting ferric state (*4*). Since compound F can be obtained by treatment with excess hydrogen peroxide (*7*, *8*), the last electron-transfer step in the catalytic cycle can be investigated by injection of an electron into the system. Nilsson (*9*) introduced the use of the tris(bipyridyl)ruthenium$^{2+}$ complex to inject a single

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[1] Abbreviations: $Ru_2C$, $[Ru(bpy)_2]_2(bphb)(PF_6)_4$; bpy, bipyridine; bphb, 1,4-bis[2-(4′-methyl-2,2′-bipyrid-4-yl)ethenyl]benzene; $Ru_2D$, $[Ru(bpy)_2]_2(qpy)(PF_6)_4$; qpy, 2,2′:4′,4″:2″,2‴-quaterpyridine; 3-CP, 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy free radical; CcO, cytochrome *c* oxidase.

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electron into $Cu_A$ of cytochrome *c* oxidase in response to a short laser flash. The positive charge on the ruthenium complex allows it to bind to the cytochrome *c* binding site on cytochrome *c* oxidase, eliminating the need for cytochrome *c*.

In this paper we report on the use of two novel compounds as photoactive electron donors to cytochrome *c* oxidase, $Ru_2C$ and $Ru_2D$. These binuclear complexes of ruthenium have a net charge of +4, which allows them to bind strongly to cytochrome *c* oxidase at low ionic strength and donate electrons specifically to $Cu_A$ upon photolysis. The yield of reduced cytochrome *c* oxidase obtained upon a single flash is more than 5-fold higher than obtained with the tris(bipyridyl)ruthenium$^{2+}$ complex using the same laser power. These new binuclear compounds were used to study the kinetics of electron transfer from $Cu_A$ to the oxyferryl heme $a_3$ in compound F formed from both bovine and *Rhodobacter sphaeroides* CcO by treatment with hydrogen peroxide. The redox status of $Cu_A$, heme *a*, and oxyferryl heme $a_3$ were monitored at different wavelengths to investigate the possible occurrence of intermediates in the electron-transfer pathway. The pH dependence of the reaction between heme *a* and oxyferryl heme $a_3$, as well as the deuterium isotope effect in $D_2O$, was measured to determine the involvement of a proton-transfer step. The reaction was also studied in the K362M mutant of *R. sphaeroides* CcO to investigate the involvement of the K-channel in proton uptake. This electron-transfer reaction is of particular interest since it is coupled to proton pumping.

## EXPERIMENTAL SECTION

*Materials*. Bovine CcO was purified by the method of Capaldi and Hayashi (*10*) and had a heme content of 9−11 nmol/mg protein and a turnover number of 400 $s^{-1}$. The 6-His tagged wild-type and K362M mutant of *Rhodobacter sphaeroides* CcO was purified as previously described (*11*). Prior to kinetic experiments the enzyme was equilibrated with 5 mM Tris-HCl, pH 8, and 0.1% lauryl maltoside using three steps of dilution−concentration with Amicon-30 concentrators. The lauryl maltoside was obtained from Cal Biochem and the Tris-HCl was obtained from Sigma.

$Ru_2D$ was prepared by procedures described by Sadoski et al. (unpublished experiments). $Ru_2C$ was a generous gift from Russell H. Schmehl (Department of chemistry, Tulane University), prepared as described by Baba et al. (*12*).

*Ferryl Intermediate (F)*. The ferryl intermediate was prepared by adding up to 4 mM $H_2O_2$ to the oxidized enzyme (*7*). After a 5-min incubation period the characteristic peak at 583 nm in the difference (F − O) spectrum became evident. The yield of compound F was evaluated using extinction coefficients of $\Delta\epsilon_{583-630} = 5.3$ $mM^{-1}$ $cm^{-1}$ (*3*) and $\Delta\epsilon_{436-414} = 67$ $mM^{-1}$ $cm^{-1}$ (*8*).

*Flash Photolysis*. Absorbance transients were recorded using 300-$\mu$L samples in a 1-cm glass semimicrocuvette. Photolysis was carried out with a Phase R model DL 1400 flashlamp-pumped dye laser using coumarin 450 or LD 490 to produce a 450- or 480-nm light flash of 200-ns duration, respectively. The total energy per laser flash was approximately 100 mJ. The detection system was previously described by Pan et al. (*13*). Transients were recorded using a single laser flash, unless otherwise noted. $Cu_A$ was monitored at 830 nm using $\Delta\epsilon_{830} = 2$ $mM^{-1}$ $cm^{-1}$ (*14*). The reduction of heme *a* was measured at 605 nm using $\Delta\epsilon = 16$ $mM^{-1}$ $cm^{-1}$ (*15*). The reduction of oxyferryl heme $a_3$ was measured at 580 nm using $\Delta\epsilon = 5.3$ $mM^{-1}$ $cm^{-1}$ (*3*). The reaction samples typically contained 5 $\mu$M cytochrome *c* oxidase, 10 mM aniline, 1 mM 3-CP, and 0.1% lauryl maltoside in 5 mM Tris-Cl buffer, pH 8. Aniline and 3-CP were used as sacrificial electron donors to reduce Ru(III) and prevent the back-reaction between Ru(III) and $Cu_A$(I). All absorbance transients were analyzed using a KINFIT kinetics program obtained from On-line Instruments Systems Inc. The absorbance spectra were obtained with a Hewlett-Packard 8452A diode array spectrophotometer.

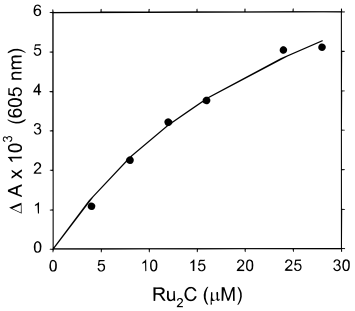


FIGURE 1: Effect of $Ru_2C$ concentration on the extent of photoreduction of heme *a* in *R. sphaeroides* cytochrome oxidase. A sample containing 5.0 $\mu$M *R. sphaeroides* CcO and the indicated concentration of $Ru_2C$ in 5 mM TrisCl, pH 8, 10 mM aniline and 1 mM 3-CP was subjected to a single 200-ns laser flash at 450 nm. The extent of photoreduction of heme *a* was measured at 605 nm 500 $\mu$s after the flash. The solid line is the best fit to the binding equation: $\Delta A = \Delta A_{max}[Ru_2C]/(K_d + [Ru_2C])$, with $K_d = 28$ $\mu$M, and $\Delta A_{max} = 1 \times 10^{-2}$ A.

Table 1: Yield of Reduced CcO Produced by Flash Photolysis of Ruthenium Complexes[a]

| ruthenium complex | cytochrome oxidase | $\Delta A_{605}$ | % yield |
|---|---|---|---|
| 25 $\mu$M $Ru_2C$ | 5.0 $\mu$M *Rb sp* CcO | 0.0050 | 6.2 |
| 25 $\mu$M $Ru_2C$ | 5.0 $\mu$M *Rb*. CcO + 5.0 $\mu$M Cc | 0.00062 | 0.8 |
| 22 $\mu$M $Ru_2C$ | 5.0 $\mu$M bovine CcO | 0.0051 | 6.3 |
| 60 $\mu$M $Ru(bpy)_3$ | 5.0 $\mu$M bovine CcO | 0.001 | 1.2 |

[a] Samples containing bovine or *R. sphaeroides* CcO and either $Ru_2C$ or $Ru(bpy)_3$ in 5 mM TrisCl, pH 8, 10 mM aniline, and 1 mM 3-CP were subjected to a single 200-ns laser flash at 450 nm. The extent of photoreduction of heme *a* was measured at 605 nm 500 $\mu$s after the flash. The percent yield of photoreduced heme *a* was calculated using $\Delta\epsilon_{605} = 16$ $mM^{-1}$ $cm^{-1}$. In the second experiment, 5 $\mu$M horse cytochrome *c* (Cc) was added to the CcO.

## RESULTS

*Reduction of Cytochrome Oxidase by $Ru_2C$*. Flash photolysis of a sample containing 25 $\mu$M $Ru_2C$ and 5.0 $\mu$M *R. sphaeroides* cytochrome oxidase in 5 mM Tris-Cl, pH 8, 10 mM aniline, and 1 mM 3CP results in rapid electron injection into the enzyme, as monitored at 605 nm. The amount of heme *a* reduced in a single flash has a hyperbolic dependence on the concentration of $Ru_2C$, with an apparent dissociation constant of $K_d = 28$ $\mu$M (Figure 1). This indicates that $Ru_2C$ forms a complex with cytochrome *c* oxidase at low ionic strength that is active in electron transfer. The yield of reduced heme *a* obtained with a single flash was 6.2% using an $Ru_2C$ concentration of 25 $\mu$M (Table 1). The yield decreased 8-fold upon addition of horse cytochrome *c* equal

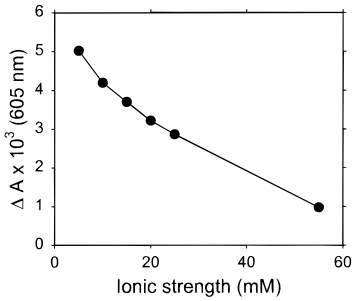


FIGURE 2: Effect of ionic strength on photoreduction of *R. sphaeroides* CcO by $Ru_2C$. A sample containing 5.0 $\mu$M CcO and 32 $\mu$M $Ru_2C$ in 5 mM TrisCl, pH 8, 0 to 100 mM NaCl, 10 mM aniline, 1 mM 3-CP was subjected to flash photolysis as described in Figure 1, and the extent of photoreduction of heme *a* was measured at 605 nm.

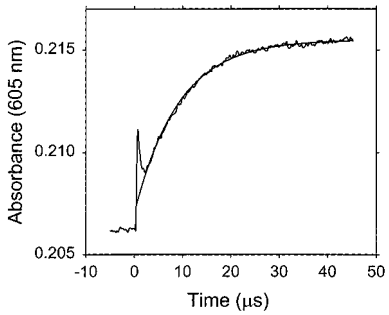


FIGURE 3: Kinetics of photoreduction of *R. sphaeroides* CcO by $Ru_2C$. A sample containing 10 $\mu$M CcO and 25 $\mu$M $Ru_2C$ in 5 mM TrisCl, pH 8, 10 mM aniline, 1 mM 3-CP was photoexcited with a 200-ns laser flash at 480 nm, and the reduction of heme *a* followed at 605 nm. The initial rapid increase and decrease in absorbance is due to the excited state of $Ru_2C$*. The major exponential increase in absorbance has a rate constant of (1.1 ± 0.2) × $10^5$ $s^{-1}$.

in concentration to that of cytochrome oxidase (Table 1), indicating that $Ru_2C$ and cytochrome *c* compete for the same binding site on cytochrome oxidase. The yield of reduced heme *a* also decreased significantly with increasing ionic strength (Figure 2), consistent with an electrostatic complex between $Ru_2C$, with a charge of +4, and the negatively charged binding site on cytochrome *c* oxidase. The photoreduction properties of $Ru_2D$ were essentially the same as those of $Ru_2C$.

The kinetics of the reaction between $Ru_2C$ and cytochrome oxidase were studied using time-resolved absorption spectroscopy (Figure 3). Photoexcitation of $Ru_2C$ with a 480-nm laser flash results in the formation of a metal-to-ligand charge transfer state, $Ru_2C$*, which has a luminescence band at 600 to 650 nm, and a broad absorption band from 520 to 650 nm (Sadoski et al., unpublished experiments). The lifetime of $Ru_2C$* is 1.0 ± 0.1 $\mu$s as determined from both the luminescence and absorbance transients. The initial rapid increase and decrease in the 605-nm absorbance observed upon flash photolysis of a mixture of $Ru_2C$ and *R. sphaeroides* cytochrome oxidase is due to the excited state of $Ru_2C$*, and has nearly the same lifetime as in the absence of cytochrome oxidase, 1.0 ± 0.1 $\mu$s (Figure 3). Electron transfer from $Ru_2C$* to the initial acceptor(s) in cytochrome oxidase must be complete within the lifetime of $Ru_2C$*. $Cu_A$ is the primary initial electron acceptor, but a small net increase in the 605-nm absorbance within 2 $\mu$s suggests a small amount of direct electron transfer from $Ru_2C$* to heme *a* (Figure 3). The major exponential increase in absorbance at 605 nm with a rate constant of $k_{obs}$ = (1.1 ± 0.2) × $10^5$ $s^{-1}$ is due to electron transfer from $Cu_A$ to heme *a*. It was not possible to measure the 830-nm absorbance of $Cu_A$ at times less than 20 $\mu$s with our detector because of light-scattering problems, so the initial reduction and reoxidation of $Cu_A$ could not be measured for this sample. However, $Cu_A$ is the only possible redox intermediate between $Ru_2C$* and heme *a* in this enzyme.

The equilibrium constant for electron transfer from $Cu_A$ to heme *a* in *R. sphaeroides* cytochrome oxidase was obtained by measuring the residual decrease in absorbance at 830 nm and the increase in absorbance at 605 nm after the reaction was complete (at 500 $\mu$s), using extinction coefficients of $\Delta\epsilon_{830}$ = 2 $mM^{-1}$ $cm^{-1}$ for $Cu_A$ (*14*) and $\Delta\epsilon_{605}$ = 16 $mM^{-1}$ $cm^{-1}$ for heme *a* (*15*). The equilibrium constant is $K_{eq}$ = 5.4 ± 1.0, which corresponds to a 43 ± 5 mV difference between the midpoint potentials of $Cu_A$ and heme *a*. By using $K_{eq} = k_1/k_{-1}$ = 5.4 and $k_{obs} = k_1 + k_{-1}$ = 1.1 × $10^5$ $s^{-1}$, the values of the forward and reverse electron-transfer rate constants are $k_1$ = 9.3 × $10^4$ $s^{-1}$ and $k_{-1}$ = 1.7 × $10^4$ $s^{-1}$.

The photoexcited state of $Ru_2C$* also rapidly injected an electron into bovine cytochrome oxidase. The rate constant for electron transfer from $Cu_A$ to heme *a* is (2.2 ± 0.5) × $10^4$ $s^{-1}$ for the bovine enzyme, which is smaller than for the bacterial enzyme, and could be measured at both 605 and 830 nm (data not shown). The equilibrium constant for electron transfer between $Cu_A$ and heme *a* is 8 ± 3, which corresponds to a redox potential difference of 53 ± 8 mV. Flash photolysis of a sample containing 5 $\mu$M bovine CcO and 22 $\mu$M $Ru_2C$ gave a yield of reduced heme *a* of 6.3% (Table 1). By comparison, the yield obtained using 60 $\mu$M ruthenium trisbipyridine instead of $Ru_2C$ was 1.2% using identical conditions of laser power and buffer (Table 1).

*Electron Transfer to the Binuclear Center*. Injection of one electron into resting cytochrome oxidase from either bovine heart or *R. sphaeroides* does not exhibit further electron transfer after the rapid reduction of $Cu_A$ and heme *a*. To investigate electron transfer to the heme $a_3$−$Cu_B$ binuclear center, compound F was formed by treatment of cytochrome oxidase with 4 mM hydrogen peroxide. The difference spectrum relative to oxidized CcO displays the characteristic peak at 580 nm, and the maximum and minimum in the Soret region at 436 and 414 nm (Figure 4). The yield of compound F was 82% as measured from the 583−630 nm absorbance band using an extinction coefficient of $\Delta\epsilon_{583-630}$ = 5.3 $mM^{-1}$ $cm^{-1}$ (*3*), and 87% from the 436−414 nm absorbance band using $\Delta\epsilon_{436-414}$ = 67 $mM^{-1}$ $cm^{-1}$ (*8*). Flash photolysis of $Ru_2C$ and bovine compound F was carried out using a single laser flash immediately after formation of compound F in order to avoid reduction of the intermediate with multiple laser flashes used for signal averaging. The rate constant for electron transfer from photoreduced $Cu_A$ to heme *a* in compound F is (2.0 ± 0.5) × $10^4$ $s^{-1}$ for the bovine enzyme, as monitored by the 605- and 830-nm transients. This is essentially the same as the value for the resting oxidized enzyme, indicating that the rate constant for electron transfer from $Cu_A$ to heme *a* is not affected by whether heme *a* is ferric or oxyferryl. Following reduction by photoreduced $Cu_A$, reduced heme *a* transfers an electron to oxyferryl heme $a_3$ (Figure 5). The reoxidation

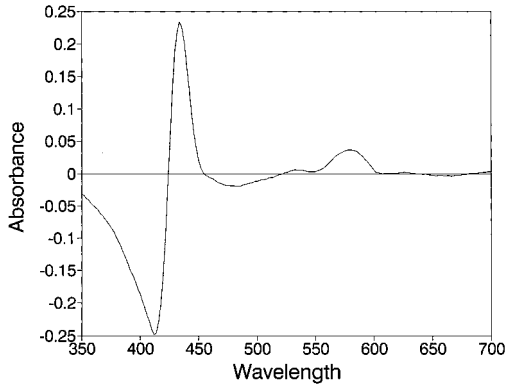


FIGURE 4: Difference spectrum of bovine compound F prepared by hydrogen peroxide treatment. Resting bovine CcO (8.3 $\mu$M) was treated with 4 mM $H_2O_2$ in 5 mM TrisCl, pH 8.0, containing 22 $\mu$M $Ru_2C$, 10 mM aniline, and 1 mM 3CP. After 5-min incubation at 25 °C, the spectrum was recorded and subtracted from the spectrum of the resting CcO solution.

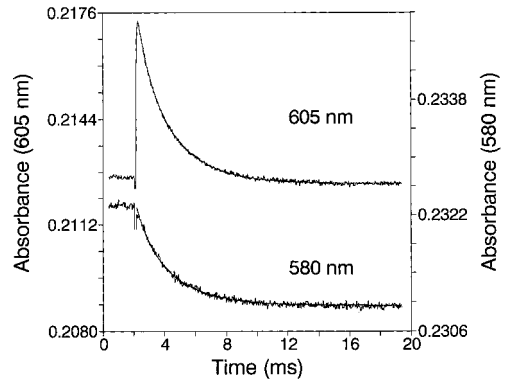


FIGURE 5: Kinetics of electron transfer from photoreduced heme *a* to oxyferryl heme $a_3$ in compound F. A sample containing 22 $\mu$M $Ru_2C$ and 8.3 $\mu$M bovine CcO in 5 mM TrisCl, pH 8.0, 10 mM aniline, and 1 mM 3-CP was treated with 4 mM $H_2O_2$ and incubated for 5 min to form compound F as described in Figure 4. The sample was photoreduced with a single laser flash at 480 nm, and the reduction and reoxidation of heme *a* was measured at 605 nm. The reduction of oxyferryl heme $a_3$ was followed at 580 nm. Both the 605 and 580 nm transients were biphasic, with a major phase with a rate constant of $(4.7 \pm 1.0) \times 10^2$ $s^{-1}$ and relative amplitude of 82%, and a minor phase with rate constant $(1.5 \pm 0.3) \times 10^3$ $s^{-1}$ and relative amplitude 18%.

of heme *a* monitored at 605 nm consists of a major phase with a rate constant of $(4.7 \pm 1.0) \times 10^2$ $s^{-1}$ and relative amplitude of 82%, and a minor phase with rate constant $(1.5 \pm 0.3) \times 10^3$ $s^{-1}$ and relative amplitude 18%. Reduction of the oxyferryl heme $a_3$ in compound F is selectively monitored at 580 nm, where neither $Cu_A$ nor heme *a* contribute significantly (Figure 5). The 580 nm transient is also biphasic, with the same rate constants and relative amplitudes as the 605-nm transient. The total amplitude of the 605-nm transient is 3.5 ± 0.5 times that of the 580-nm transient, which is consistent with equimolar oxidation of heme *a* and reduction of oxyferryl heme $a_3$, using the extinction coefficients of heme *a* at 605 nm and oxyferryl heme $a_3$ at 580 nm (*3*, *15*). Most importantly, reduction of oxyferryl heme $a_3$ to the ferric form takes place simultaneously with reoxidation of heme *a*. This result demonstrates that an intermediate with an electron on $Cu_B$ does not transiently accumulate.

The pH dependence of electron transfer from heme *a* to oxyferryl heme $a_3$ was measured from pH 7.0 to pH 9.5

Table 2: pH Dependence of the Rate Constant for Electron Transfer from Photoreduced Heme *a* to Oxyferryl Heme $a_3$ in Compound F[a]

| pH | *k* (fast) | *K* (slow) | % slow |
|---|---|---|---|
| 7.0 | 1700 | 590 | 72 |
| 7.5 | 1500 | 520 | 81 |
| 8.0 | 1500 | 470 | 82 |
| 8.5 | 1200 | 420 | 70 |
| 9.0 | 1500 | 320 | 68 |
| 9.5 | 2100 | 220 | 86 |

[a] A sample containing 26 $\mu$M $Ru_2C$ and 10 $\mu$M bovine CcO in 10 mM aniline and 1 mM 3-CP was treated with 4 mM $H_2O_2$ and incubated for 5 min to form compound F. The sample was photoreduced with a single laser flash at 480 nm, and the reduction and reoxidation of heme *a* was measured at 605 nm. The reduction of oxyferryl heme $a_3$ was measured at 580 nm. The buffer consisted of 2.5 mM sodium phosphate (pH 7.0, 7.5), 5 mM TrisCl (pH 8.0, 8.5), or 5 mM sodium borate (pH 9.0, 9.5). The error limits are ±20% for the rate constants and the ratio of slow to fast phases.

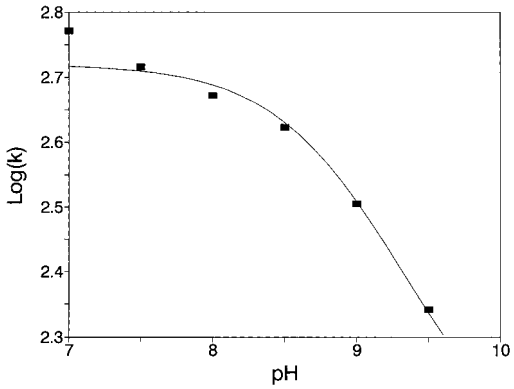


FIGURE 6: pH dependence of the rate constant for electron transfer from photoreduced heme *a* to oxyferryl heme $a_3$ in compound F. A sample containing 26 $\mu$M $Ru_2C$ and 10 $\mu$M bovine CcO in 10 mM aniline and 1 mM 3-CP was treated with 4 mM $H_2O_2$ and incubated for 5 min to form compound F. The sample was photoreduced with a single laser flash at 480 nm, and the reduction and reoxidation of heme *a* was measured at 605 nm. The reduction of oxyferryl heme $a_3$ was measured at 580 nm. The buffer consisted of 2.5 mM sodium phosphate (pH 7.0, 7.5), TrisCl (pH 8.0, 8.5), or 5 mM sodium borate (pH 9.0, 9.5). The solid line is an ionization curve with $pK_a = 9.0$, and limiting rate constants of 525 $s^{-1}$ at low pH and 120 $s^{-1}$ at high pH.

(Table 2; Figure 6). The rate constant of the major phase decreased from 590 $s^{-1}$ at pH 7.0 to 220 $s^{-1}$ at pH 9.5, and was fitted to an ionization curve with a $pK_a$ of 9.0 (Figure 6). The rate constant of the minor phase remained approximately constant and accounted for less than 30% of the reaction throughout the pH range (Table 2). The electron-transfer reaction was also measured in 95% $D_2O$ with an indicated pH meter reading of 7.6, which is equivalent to a pD of 8.0 (*16*). The 605-nm transient had a major phase with a rate constant of 110 ± 20 $s^{-1}$ and relative amplitude of 86%, and a minor phase with a rate constant of 770 ± 150 $s^{-1}$ and relative amplitude of 14% (Figure 7). The 580-nm transient was monophasic within experimental error, with a rate constant of 95 ± 20 $s^{-1}$. The major phase of electron transfer from heme *a* to oxyferryl heme $a_3$ thus has a deuterium kinetic isotope effect of 4.3 ± 1.0.

The same methods were used to study the photoreduction of *R. sphaeroides* compound F by $Ru_2C$. The rate constant for electron transfer from photoreduced $Cu_A$ to heme *a* is $(1.1 \pm 0.2) \times 10^5$, which is the same as for resting state *R. sphaeroides* CcO. The reoxidation of heme *a* monitored at

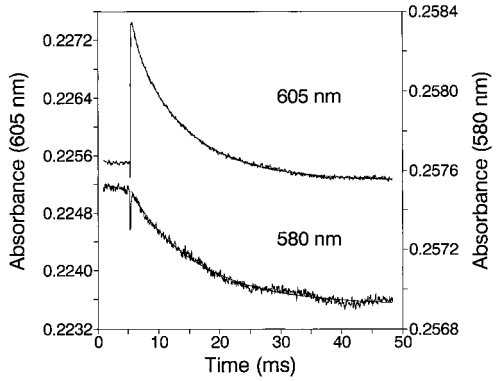


FIGURE 7: Deuterium isotope effect of the kinetics of electron transfer from photoreduced heme *a* to oxyferryl heme $a_3$. A sample containing 20 $\mu$M $Ru_2C$ and 11 $\mu$M bovine CcO in 95% $D_2O$ containing 5 mM TrisCl, pH 7.6, 10 mM aniline, and 1 mM 3-CP was treated with 4 mM $H_2O_2$ and incubated for 5 min to form compound F with 80% yield. The sample was photoreduced with a single laser flash at 480 nm. The reoxidation of heme *a* followed at 605 nm was biphasic, with a major phase with a rate constant of $110 \pm 20$ $s^{-1}$ and relative amplitude of 86%, and a minor phase with a rate constant of $770 \pm 150$ $s^{-1}$ and relative amplitude of 14%. The 580-nm transient was monophasic within experimental error, with a rate constant of $95 \pm 20$ $s^{-1}$.

Table 3: Electron Transfer from Heme *a* to Oxyferryl Heme $a_3$ in *R. sphaeroides* Wild-Type CcO and the K362M Mutant[a]

| | *k* | | |
|---|---|---|---|
| CcO | 605 nm | 580 nm | $\Delta A_{605}/\Delta A_{580}$ |
| wild-type CcO | $740 \pm 150$ | $720 \pm 150$ | $3.0 \pm 0.5$ |
| K362M CcO | $710 \pm 150$ | $690 \pm 150$ | $3.0 \pm 0.5$ |

[a] Samples containing 22 $\mu$M $Ru_2C$ and 9 $\mu$M *R. sphaeroides* CcO in 5 mM TrisCl, pH 8.0, 10 mM aniline, and 1 mM 3-CP were treated with 4 mM $H_2O_2$ and incubated for 5 min to form compound F. The sample was photoreduced with a single laser flash at 480 nm, and electron transfer from heme *a* to oxyferryl heme $a_3$ was monitored at 605 and 580 nm. The transients at both wavelengths were monophasic.

605 nm and reduction of oxyferryl heme $a_3$ monitored at 580 nm were both monophasic, with rate constants of $(7.3 \pm 1.5) \times 10^2$ $s^{-1}$ (Table 3). Therefore, it appears that heme *a* transfers an electron to oxyferryl heme $a_3$ without measurable buildup of any transient intermediate in the bacterial enzyme as well as in the bovine enzyme.

The reduction of compound F in the K362M mutant of *R. sphaerodies* CcO was studied to determine whether proton transfer through the K-channel was coupled to reduction of the oxyferryl heme. The 605- and 580-nm transients were both monophasic with rate constants of $(7.0 \pm 1.5) \times 10^2$ $s^{-1}$, indicating that the rate of electron transfer from heme *a* to oxyferryl heme $a_3$ is the same as in wild-type CcO (Table 3).

## DISCUSSION

*Efficiency of $Ru_2C$ Photoreduction of Cytochrome c Oxidase*. The photoinjection of an electron into oxidized states of cytochrome oxidase provides a method to study rapid internal electron transfer reactions that is complementary to the more commonly used technique of photolyzing the reduced CO-bound enzyme in the presence of oxygen. Several laboratories have been developing methods to improve both the rate constant and yield of the photoreduction technique (*9*, *13*, *17*−*20*). The +4 charge on the ruthenium dimer, $Ru_2C$, introduced in the present study, allows it to bind to the negatively charged cytochrome *c* binding site on subunit II of cytochrome oxidase. The lifetime of the metal-to-ligand charge transfer state, $Ru_2C^*$, is 1.0 $\mu$s, so the observed electron transfer from $Ru_2C^*$ to $Cu_A$ of cytochrome *c* oxidase must occur within this time scale. The hyperbolic dependence of the extent of cytochrome *c* oxidase reduction on the concentration of $Ru_2C$ indicates formation of a complex between $Ru_2C$ and cytochrome oxidase with a dissociation constant of $K_d = 28$ $\mu$M. The decrease in photoreduction yield with increasing ionic strength is consistent with an electrostatic interaction between $Ru_2C$ and cytochrome *c* oxidase. The specific 8-fold inhibition of electron transfer from $Ru_2C$ to cytochrome oxidase by the addition of 1 equiv of cytochrome *c* at low ionic strength indicates that $Ru_2C$ binds at the cytochrome *c* interaction domain. Of particular importance, the yield of photoreduction of cytochrome oxidase is about 5-fold larger with $Ru_2C$ than with $Ru(bpy)_3$ using the same laser power and buffer conditions (Table 1). This increased efficiency is probably due to the longer lifetime and stronger electrostatic binding of $Ru_2C$. The crystal structures of cytochrome *c* oxidase from both bovine heart (*21*) and *Paracoccus denitrificans* (*22*) reveal a cluster of carboxylates, including Asp 110 and Asp 158, that is close to the $Cu_A$ center and most likely forms the binding site for cytochrome *c* and $Ru_2C^{4+}$.

*Electron Transfer from $Cu_A$ to Heme a*. The rate constant for electron transfer from $Cu_A$ to heme *a* in bovine heart CcO was measured to be $(2.2 \pm 0.5) \times 10^4$ $s^{-1}$ using the present method, which is in good agreement with previous studies using pulse radiolysis (*17*), ruthenium trisbipyridine (*9*), or ruthenium-labeled cytochrome *c* (*13*, *18*) to reduce $Cu_A$ in the fully oxidized enzyme, as well as in three-electron-reduced, CO-inhibited CcO using a perturbation method (*23*). The corresponding rate constant for *R. sphaeroides* CcO is $1.1 \times 10^5$ $s^{-1}$, which is significantly larger than that for beef CcO. This is comparable to a value of $6.7 \times 10^4$ $s^{-1}$ obtained for *R. sphaeroides* CcO using a time-resolved electrogenic method (*24*). The equilibrium constant for electron transfer between $Cu_A$ and heme *a* was found to be $8 \pm 3$ in bovine CcO and $5.4 \pm 1.0$ in *Rb. sphaeroides* CcO. By assuming a midpoint potential for $Cu_A$ of 245 mV (*25*), the midpoint potential of heme *a* is estimated to be $298 \pm 8$ mV for bovine CcO. This is in good agreement with that found for the bovine enzyme by Kobayashi et al. (*17*). In equilibrium redox experiments using the bovine enzyme, heme *a* manifests two-half-waves of reduction, with midpoint potentials of 362 mV and 238 mV (*26*−*29*). The apparent midpoint potential estimated in the present kinetic experiments thus appears to be intermediate between the high and low potential redox transitions of heme *a*. The midpoint potential of heme *a* is known to depend on both the redox and protonation states of the binuclear center, and these could be different in the kinetic and equilibrium experiments. In the present kinetic experiments on the resting enzyme, heme $a_3$ and $Cu_B$ are both oxidized and the binuclear center is probably not protonated.

The rate constant for electron transfer from $Cu_A$ to heme *a* is $(2.1 \pm 0.5) \times 10^4$ $s^{-1}$ in bovine compound F where heme $a_3$ is oxyferryl, the same as it is in the resting enzyme. The corresponding rate constant of $1.1 \times 10^5$ $s^{-1}$ for *Rb.*

*sphaeroides* CcO also does not depend on whether heme $a_3$ is in the oxyferryl or the ferric state. This is perhaps surprising, since the rate constant for electron transfer from $Cu_A$ to heme *a* is significantly slower, $8 \times 10^3$ $s^{-1}$, during reaction of oxygen with fully reduced CcO, and is controlled by proton uptake accompanying the transition from compound P to compound F (*29*, *30*). It has been proposed that there is an electrostatic coupling between proton uptake at the binuclear center and electron transfer from $Cu_A$ to heme *a* (*31*). The addition of a positive charge to the binuclear center would increase the redox potential on heme *a* and favor electron transfer from $Cu_A$ to heme *a*. The present results therefore suggest that the net charge at the binuclear site is approximately the same in compound F as in the oxidized state. This is supported by recent electrostatic calculations using the *Paracoccus* CcO crystal structure, which show that the additional negative charge on oxyferryl heme relative to ferric heme is balanced by protonation of the hydroxide ion bound to the binuclear center (*32*).

*Electron Transfer from Heme a to Oxyferryl Heme $a_3$.* The $Ru_2C$ photoreduction experiments on compound F demonstrate that photoreduced heme *a* transfers an electron to oxyferryl heme $a_3$ with a rate constant of 470 $s^{-1}$ for the major phase in bovine CcO, and 730 $s^{-1}$ in *sphaerodies* CcO. Most significantly, the kinetics for reduction of the oxyferryl heme $a_3$ are the same as that for reoxidation of heme *a*, clearly indicating that no intermediate with $Cu_B$ reduced is accumulated transiently upon reduction of compound F. The kinetics for electron transfer in compound F are comparable to previous photoreduction studies using time-resolved spectroscopy (*9*) or an electrogenic method (*24*, *33*). However, these earlier studies did not specifically examine the kinetics of reduction of oxyferryl heme $a_3$. The origin of the minor fast phase in the reaction is not known, but it was also observed in the earlier studies (*9*, *24*, *33*). The yield of compound F formed by the $H_2O_2$ treatment was estimated to be 82% as measured from the absorbance difference at 583−630 nm. The absence of a 607-nm peak in the difference spectrum indicated that there was not a significant amount of compound P present, and there was no phase in the 605-nm transient with a rate constant of about $10^4$ $s^{-1}$, which is characteristic of the reduction of compound P. It is also unlikely that there was a significant amount of resting oxidized CcO present, since this would have resulted in a transient that did not return to the preflash baseline. No error limits were given for the extinction coefficient for compound F, $\Delta\epsilon_{583-630} = 5.3$ $mM^{-1}$ $cm^{-1}$ (*3*), but a small variation in this value could account for the fact that the apparent yield of compound F is less than quantitative.

The rate constant for electron transfer from heme *a* to oxyferryl heme $a_3$ in bovine compound F decreases with increasing pH (Table 2) and could be fitted to an ionization with a $pK_a$ of 9.0 and limiting rate constants of 525 and 120 $s^{-1}$ at low and high pH, respectively (Figure 6). This moderate pH dependence is more likely due to the deprotonation of a number of residues that indirectly affect the rate of proton transfer rather than to a single group that directly transfers a proton to the binuclear center. The deuterium isotope effect of 4.3 for this reaction is quite large, and indicates that electron transfer is coupled to proton uptake by the binuclear center. The photoreduction studies of isolated compound F reported in the present study are in general agreement with experiments involving flash photolysis of CO-bound fully reduced CcO in the presence of oxygen (*29*, *34*). The slowest phase of the reaction, associated with electron transfer from heme *a* to oxyferryl heme $a_3$, has a rate constant of 800 $s^{-1}$ at pH 7.0 which decreases to 300 $s^{-1}$ at pH 9.0 and could be fitted to an ionization curve with a $pK_a$ of 8.8 (*34*). The deuterium kinetic isotope effect for this reaction is 2.5 at pH 7.4 (*29*), which is somewhat smaller than observed by the present method. Since the CO flash photolysis studies and the ruthenium photoreduction studies are initiated from different redox states of the enzyme, the reasonably good agreement between the two methods suggests that electron transfer from heme *a* to oxyferryl heme $a_3$ is not controlled by conformational effects imposed by the initial redox state.

Several current models of proton pumping involve formation of reduced $Cu_B$ during reduction of compound F. In the histidine cycle mechanism proposed by Wikstrom et al. (*35*) and modified by Iwata et al. (*22*), reduction of $Cu_B$ in compounds P or F results in the protonation of His 325 as it is released from $Cu_B$ and moves to a different orientation. Recent electrostatic calculations based on the *Paracoccus* CcO crystal structure indicate that His 325 can indeed be protonated in the P1 state with $Cu_B$ reduced (*32*). The failure to detect the transient buildup of an intermediate with $Cu_B$ reduced during reduction of compound F indicates that such an intermediate, if it occurs at all, must be very short-lived.

Any mechanism for electron transfer from heme *a* to oxyferryl heme $a_3$ must account for the unusually slow rate of this reaction, and the coupling to proton uptake at the binuclear site. The rate of electron transfer between heme *a* and heme $a_3$ depends greatly on the redox state of the enzyme, ranging from less than 1 $s^{-1}$ in resting CcO (*36*) to $3 \times 10^5$ $s^{-1}$ in the mixed-valence enzyme (*37*). The X-ray crystal structures of CcO have revealed a direct covalent pathway for electron transfer between heme *a* and heme $a_3$ involving just three residues in helix X of subunit I, the heme *a* ligand His 378, Phe 377, and the heme $a_3$ ligand His 376 (*21*, *22*). The short length of this pathway would support the fast rate of electron transfer observed in the mixed-valence enzyme. Since this pathway is likely to be the same in all redox states, the slower rate observed in some states must be due to other factors. Verkhovsky et al. (*38*) have presented evidence that the slow rate of reduction of heme $a_3$ in the pulsed enzyme is not limited by the rate of electron transfer, but by the rate of proton uptake which shifts the redox equilibrium between heme *a* and heme $a_3$. A similar mechanism could hold for the reduction of compound F. Both $Cu_B$ and oxyferryl heme $a_3$ would be in rapid redox equilibrium with reduced heme *a*, but the redox potentials for both centers would be so low in the unprotonated state that only a small fraction of either center would be reduced. Protonation of His 325 would favor reduction of $Cu_B$ and the release of the His 325 ligand. The resulting change in electrostatics at the binuclear center would lead to protonation of the oxygen atom on the oxyferryl heme $a_3$ iron, which would dramatically increase the redox potential and allow electron transfer from $Cu_B$ to the oxyferryl heme to form $Fe^{3+}-OH^-$. The overall rate of reduction of oxyferryl heme $a_3$ is not controlled by electron transfer in this mechanism, but rather by the rate of the protonation steps. The protonation of the oxyferryl heme $a_3$ would have to be

controlled by the initial protonation of His 325 in order to explain the absence of a buildup of an intermediate with $Cu_B$ reduced. This control is also necessary to achieve coupling between reduction of compound F and proton pumping.

Of course, another interpretation of the failure to detect a redox intermediate during electron transfer from heme *a* to oxyferryl heme $a_3$ is that $Cu_B$ is never reduced during this reaction. In this case a "proton trap" mechanism might occur rather than the histidine cycle mechanism (*39*). According to this mechanism, protons taken up during formation of compound F in order to maintain electroneutrality would be released to the cytoplasm as the protons required for reduction of the oxyferryl heme $a_3$ were delivered to the binuclear center. The rate-limiting step in this mechanism would also be proton uptake by the binuclear center rather than electron transfer.

The identification of specific residues involved in the delivery of protons to the binuclear center is critical to an understanding of the mechanism. The rate constant for electron transfer from heme *a* to oxyferryl heme $a_3$ in compound F was found to be the same in the *R. sphaeroides* K362M CcO mutant as in wild-type CcO (Table 3), consistent with recent flow−flash (*40*) and photoelectric studies (*24*). Since the K362M mutation blocks the reduction of the oxidized enzyme to the R state, it has recently been proposed that the K-channel is required for the uptake of protons which are coupled to the initial electron transfer from heme *a* to the oxidized heme $a_3$ $Cu_B$ center (*40*−*42*). The slow phase of the electrogenic response during reduction of compound F is completely absent in the D132N mutant (*24*), suggesting that the D-channel is utilized for proton uptake that is coupled to reduction of oxyferryl heme $a_3$. The D-channel residue which is closest to the binuclear center, Glu-286, has also been implicated in proton uptake during reduction of both the P and F states (*43*). However, the mechanism by which specific residues control the delivery of chemical protons to the binuclear center and couple that process to vectorial proton pumping remains unknown.

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