Intracomplex Electron Transfer between Ruthenium–Cytochrome *c* Derivatives and Cytochrome *c* Oxidase†

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ABSTRACT: The reactions of bovine cytochrome *c* oxidase with horse cytochrome *c* derivatives labeled at specific lysine amino groups with (dicarboxybipyridine)bis(bipyridine)ruthenium(II) were studied by laser flash photolysis. All of the derivatives form complexes with cytochrome *c* oxidase at low ionic strength (5 mM sodium phosphate, pH 7). Excitation of Ru(II) to Ru(II*) with a short laser flash resulted in rapid electron transfer to the ferric heme group of cytochrome *c*, followed by electron transfer to cytochrome *c* oxidase. The photoreduced heme Fe(II) in the cytochrome *c* derivative modified at lysine 25 on the periphery of the heme crevice domain transferred an electron to Cu_A with a rate constant of 1.1×10^4 s^{-1}. Cu_A then transferred an electron to cytochrome *a* with a rate constant of 2.3×10^4 s^{-1}. The derivatives modified at lysines 7, 39, 55, and 60 remote from the heme crevice domain of cytochrome *c* have nearly the same kinetics. The rate constant for electron transfer from the cytochrome *c* heme to Cu_A is greater than 10^5 s^{-1}, and the rate constant for electron transfer from Cu_A to cytochrome *a* is 2×10^4 s^{-1}. The cytochrome *c* derivatives modified at lysines 13 and 27 in the heme crevice domain react much more slowly than the other derivatives, with intracomplex rate constants for oxidation of cytochrome *c* ranging from 1000 to 6000 s^{-1}. The bulky ruthenium group at the heme crevice domain of these derivatives apparently alters the binding orientation, leading to smaller electron-transfer rates. At 200 mM ionic strength the complexes of all the derivatives are fully dissociated, and second-order kinetics are observed. The derivatives modified at lysines 7, 25, 39, 55, and 60 have nearly the same second-order rate constants as native cytochrome, while the rate constants of the derivatives modified at lysines 13 and 27 are considerably smaller.

Cytochrome *c* oxidase is a redox-linked proton pump that uses electrons from ferrocytochrome *c* to reduce molecular oxygen to water (Wikstrom et al., 1981; Chan & Li, 1990). It consists of three large subunits synthesized inside the mitochondria and 10 smaller subunits synthesized in the cytoplasm. Each monomer contains two heme groups, cyt a and cyt a_3, and two redox-active copper atoms, Cu_A and Cu_B. A number of studies have indicated that cyt a, cyt a_3, and Cu_B are located in subunit I (Winter et al., 1980; Ludwig, 1980), and mutagenesis techniques have been used to identify the ligands of these metals in subunit I of *Rhodobacter sphaeroides* cytochrome *c* oxidase (Shapleigh et al., 1992). Cu_A is located in subunit II and is ligated to Cys 196, Cys 200, and one or two histidine residues (Martin et al., 1988; Stevens et al., 1982; Hall et al., 1988). Extensive chemical modification studies have shown that seven highly conserved lysine amino groups surrounding the heme crevice of cytochrome *c* are involved in the formation of an electrostatic complex with cytochrome *c* oxidase (Smith et al., 1977, 1981; Ferguson-Miller et al., 1979; Rieder & Bosshard, 1980). A carbodiimide modification technique has been used to identify four carboxylate groups on subunit II that are involved in binding cytochrome *c* (Millett et al., 1983). One of these, Glu 198, is located between Cys 196 and Cys 200, which are proposed to ligand Cu_A, suggesting that Cu_A might accept electrons directly from cytochrome *c*.

The reaction between ferrocytochrome *c* and cytochrome *c* oxidase has been studied extensively by the stopped-flow technique (Wilson et al., 1975). Antalis and Palmer (1982) found that the second-order rate constant was 3×10^7 M^{-1} s^{-1} at low ionic strength and that it decreased as the ionic strength was increased. cyt a and Cu_A were reduced to the same extent and at the same rate, indicating rapid equilibrium between these species. Since cytochrome *c* binding is probably the rate-limiting step in the stopped-flow experiment, recent investigations have focused on methods to measure intracomplex electron-transfer rates. Veerman et al. (1982) studied the reaction using pulse radiolysis, but found that complex formation at low ionic strength prevented the reduction of cytochrome *c* by the solvated electron. Hazzard et al. (1991) used flavin flash photolysis to measure an intracomplex rate constant of 630 s^{-1} for electron transfer from cytochrome *c* to cyt a at low ionic strength, which increased to a maximum value of 1470 s^{-1} at 110 mM ionic strength.

In a different approach, Hill (1991) carried out flash photolysis studies of a complex between ferrocytochrome *c* and reduced, CO-inhibited cytochrome *c* oxidase in the presence of oxygen. The data are consistent with electron entry from cytochrome *c* to Cu_A with a rate constant greater than 70 000 s^{-1}, followed by electron transfer from Cu_A to cyt a with a rate constant of 6000 s^{-1}. The rate constant for electron transfer from Cu_A to cyt a was measured to be 20 000 s^{-1} in fully oxidized cytochrome *c* oxidase using pulse radiolysis (Kobayashi et al., 1989) and flash photolysis (Nilsson, 1992) and in three-electron-reduced, CO-inhibited cytochrome *c* oxidase using a perturbation method (Morgan et al., 1989).

We have recently developed a new technique to study electron transfer that utilizes proteins covalently labeled with a tris(bipyridine)ruthenium(II) complex (Pan et al., 1988, 1990; Durham et al., 1989; Geren et al., 1991; Hahm et al., 1992; Willie et al., 1992). The Ru(II) group can be photoexcited to a metal-to-ligand charge-transfer state, Ru(II*), which is a strong reducing agent, and can transfer an electron to a protein redox center on a nanosecond time

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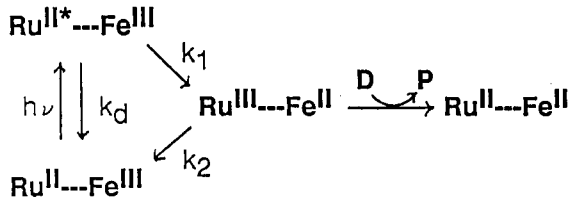
Scheme I



scale (Durham et al., 1989). In the present article, we have used this technique to measure electron transfer in 1:1 complexes between ruthenium–cytochrome c derivatives and cytochrome c oxidase.

EXPERIMENTAL PROCEDURES

Materials. Horse heart cytochrome c (type VI) and lauryl maltoside were obtained from Sigma Chemical Co. The (dicarboxybipyridine)bis(bipyridine)ruthenium–cytochrome c (Ru_c-cyt c)[1] derivatives were prepared as described by Pan et al. (1988) and Durham et al. (1989). The (dimethylbipyridine)bis(bipyridine)ruthenium–cytochrome c (Ru_m-cyt c) derivatives were prepared by treating horse heart cytochrome c with (4-(bromomethyl)-4′-methylbipyridine)-bis(bipyridine)ruthenium^{2+} as previously described (Geren et al., 1991; Liu et al., 1992; R.-Q. Liu and B. Durham, manuscript in preparation). Beef heart cytochrome c oxidase was prepared by the methods of Capaldi and Hyashi (1972) and Hartzell and Beinert (1974). Enzyme preparations had a heme content of 9–11 nmol/mg of protein and a turnover number of 400 s^{-1}. No differences in the reduction kinetics of the two preparations were observed.

Flash Photolysis. Transient absorbance measurements were carried out as described by Geren et al. (1991) by flash photolysis of 300-μL solutions contained in a 1-cm glass semi-microcuvette. The excitation pulse was provided by a Phase R Model DL1400 flash lamp–pumped dye laser using coumarin 450 to produce a 450-nm light pulse of <0.5 μs duration. The detection system has been described by Durham et al. (1989). A number of different wavelengths were used to measure the reaction kinetics. The reaction of cytochrome c was detected at 550 nm using an extinction coefficient of $\Delta\epsilon_{550}$ = 18.6 mM^{-1} cm^{-1} (Margoliash & Frohwirt, 1959). The reduction of cyt a was measured at 604 nm using $\Delta\epsilon_{604}$ = 16 mM^{-1} cm^{-1} and at 444 nm using $\Delta\epsilon_{444}$ = 59 mM^{-1} cm^{-1}. These extinction coefficients for the reduction of cyt a were measured by Blair et al. (1982) using cyanide or formate to stabilize cyt a_3 and Cu_B in their oxidized states. The absorbance ratio $\Delta A_{604}/\Delta A_{444}$ was in the range of 0.28 ± 0.04 for the transients observed in the present studies, confirming that the redox center reduced was cyt a rather than cyt a_3. The $\Delta A_{604}/\Delta A_{444}$ ratio would be 0.07 for the reduction of cyt a_3.

The reaction of Cu_A was monitored at 830 nm using $\Delta\epsilon_{830}$ = 2.00 mM^{-1} cm^{-1} (Blair et al., 1983). The detection system for this wavelength consisted of a tungsten lamp, an 830-nm interference filter with a 10-nm bandpass, and a high-sensitivity photodiode detector. The absorbance transients were analyzed with the KINFIT kinetics program supplied by On-Line Instrument Systems, Inc. The transients were fit to either a single-exponential equation or the two-exponential equation, $A = A_0\{f\exp(-k_f t) + (1-f)\exp(-k_s t)\}$. The reaction solutions typically contained 5 μM ruthenium–cytochrome c derivative, 5–20 μM cytochrome c oxidase, 10 mM aniline, 1 mM 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy free radical (3CP), and 0.1% dodecyl maltoside in 5 mM sodium phosphate (pH 7.0) at 25 °C. Aniline and 3CP were included as electron donors to reduce Ru(III) and prevent the back reaction k_2 shown in Scheme I. The rate constants for electron transfer to cytochrome c oxidase were independent of the concentrations of aniline and 3CP. Other sacrificial donors, including EDTA and (*N,N*-dimethylamino)benzoate, could be used in place of aniline and 3CP and resulted in the same rate constants. The reactions were generally carried out aerobically using resting cytochrome c oxidase. No significant differences in the reduction kinetics were observed using pulsed cytochrome c oxidase that had been reduced and reoxidized (Colosimo et al., 1981). The ionic strength of the reaction solutions was changed by adding sodium chloride. Flash photolysis studies were also carried out using riboflavin to photoreduce cytochrome c, as described by Ahmad et al. (1982) and Hazzard et al. (1991). The reaction solutions contained 50 μM riboflavin, 5 mM EDTA, 1 μM cytochrome c, 0–5 μM cytochrome c oxidase in 0.1% dodecyl maltoside, and 100 mM sodium phosphate buffer (pH 7.0).

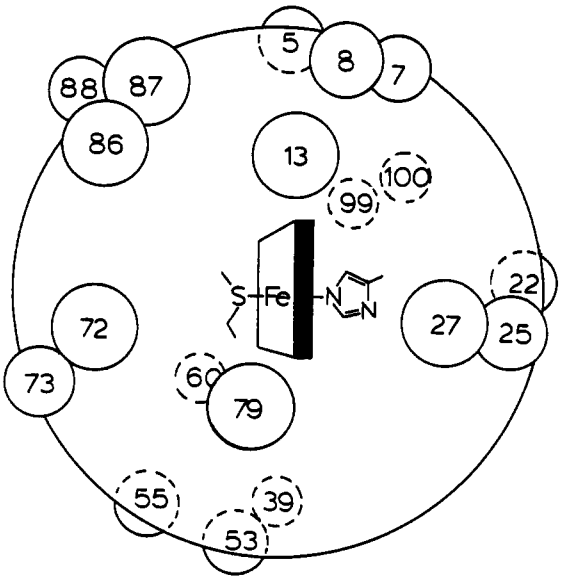


FIGURE 1: Schematic diagram of horse heart cytochrome c viewed from the front of the heme crevice. The approximate positions of the β carbons of the lysine residues are indicated by closed circles and dashed circles for lysines located toward the front and back of cytochrome c, respectively.

RESULTS

The cytochrome c derivatives fall into two different classes on the basis of the location of the lysine labeled with the ruthenium complex (Figure 1). Previous chemical modification studies (Smith et al., 1977, 1981; Ferguson-Miller, 1978; Rieder & Bosshard, 1980) have shown that lysines 13, 27, and 72 are at the center of the binding domain for cytochrome c oxidase, lysine 25 is at the periphery of the binding domain, and lysines 7, 39, 55, and 60 are remote from the binding domain (Figure 1). Class I thus includes the derivatives modified at lysines 7, 39, 55, and 60 that are expected to react in a fashion similar to native cytochrome c, while class II includes the derivatives modified at lysines 13, 27, and 72 that are expected to react slower than native cytochrome c because of the steric effects of the bulky ruthenium complex. The derivatives modified at lysine 25 have kinetic properties similar to those of the class I derivatives and will be considered first.

[1] Abbreviations: Ru_c, (dicarboxybipyridine)bis(bipyridine)ruthenium; Ru_m, (dimethylbipyridine)bis(bipyridine)ruthenium; EDTA, ethylenediaminetetraacetic acid; 3CP, 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy free radical.

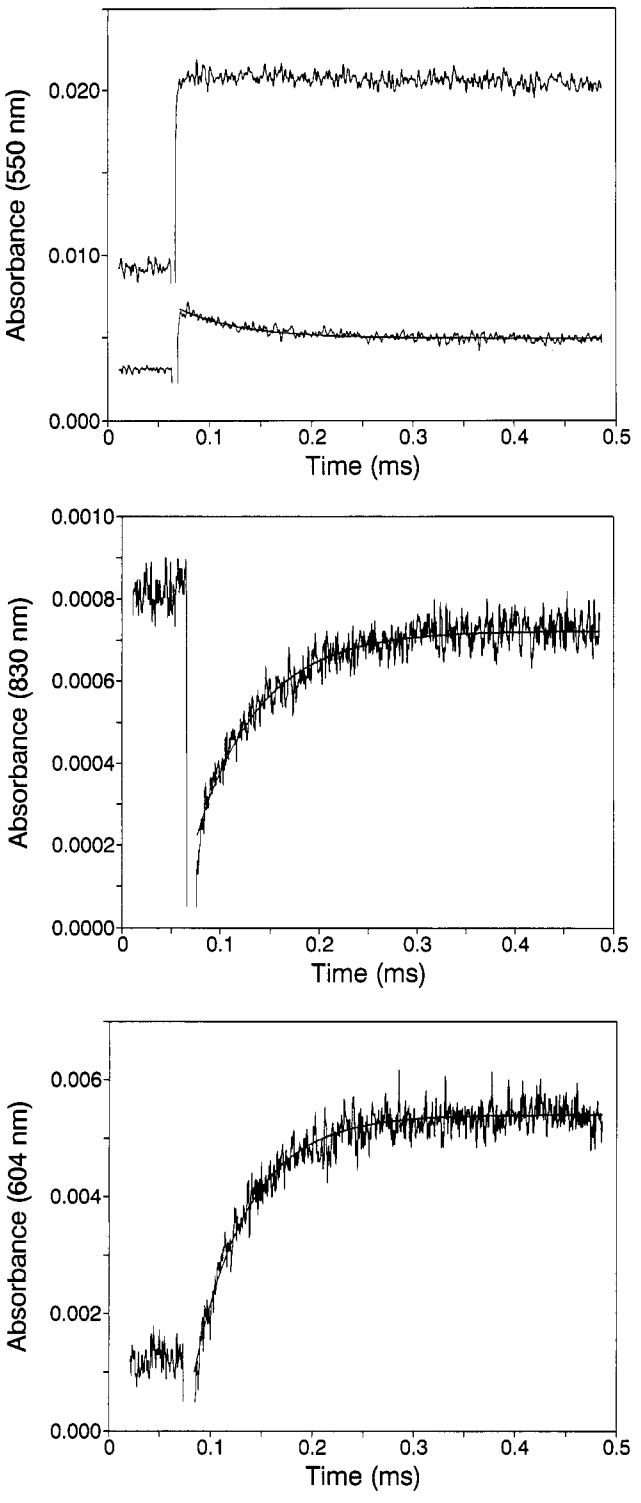


FIGURE 2: Photoinduced electron transfer from Ru_m-25-cyt c to cytochrome *c* oxidase. The solutions contained 10 mM MES (pH 6), 10 mM aniline, and 1 mM 3CP. (A, top) 550-nm transient of 10 μM Ru_m-25-cyt c in the absence of cytochrome *c* oxidase following a single laser flash (top spectrum); 550-nm transient of 10 μM Ru_m-25-cyt c and 20 μM cytochrome *c* oxidase (bottom spectrum). The smooth line is a single exponential with $k = 1.6 \times 10^4$ s^{-1} and $\Delta A = 0.0018$. (B, middle) 830-nm transient of the same solution as used in A; $k = 1.6 \times 10^4$ s^{-1} and $\Delta A = 0.0005$. (C, bottom) 604-nm transient of the same solution as used in A; $k = 1.9 \times 10^4$ s^{-1} and $\Delta A = 0.0044$.

Laser flash photolysis of Ru_m-25-cyt c resulted in rapid electron transfer from Ru(II*) to the heme group Fe(III) according to Scheme I (Figure 2A). Aniline and 3CP were used as electron donors to reduce Ru(III) and prevent the back reaction k_2 (Scheme I). The yield of photoreduced cyt c^{2+} calculated from the absorbance increase at 550 nm was 0.61 μM, corresponding to 6.1% of the 10 μM Ru_m-25-cyt c present in the solution (Figure 2A). When cytochrome *c* oxidase was added to the solution at low ionic strength, the yield of photoreduced cyt c^{2+} decreased from 0.61 to 0.19 μM (Figure 2A). The subsequent reoxidation of Ru_m-25-cyt c had a rate constant of $(1.6 \pm 0.3) \times 10^4$ s^{-1} and a magnitude of $\Delta[\text{cyt c}^{2+}] = 0.09 \pm 0.03$ μM (Figure 2A). The absorbance at 830 nm decreased within the deadtime of the instrument and then increased to nearly the original value (Figure 2B). This indicates that Cu_A is reduced rapidly by Ru_m-25-cyt c and is then reoxidized with a rate constant of $(1.6 \pm 0.3) \times 10^4$ s^{-1} and $\Delta[Cu_A^+] = 0.25 \pm 0.05$ μM. cyt a, monitored at 604 and 444 nm, was reduced with a rate constant of $(1.9 \pm 0.3) \times 10^4$ s^{-1} and $\Delta[\text{cyt a}^{2+}] = 0.28 \pm 0.05$ μM (Figure 2C). These results indicate that electron transfer follows the mechanism

$$c^{2+}\text{–}Cu_A^{2+}\text{–}a^{3+} \underset{k_r}{\overset{k_a}{\rightleftharpoons}} c^{3+}\text{–}Cu_A^{+}\text{–}a^{3+} \xrightarrow{k_b} c^{3+}\text{–}Cu_A^{2+}\text{–}a^{2+} \quad (1)$$

Ru_m-25-cyt c transfers an electron to Cu_A with a rate constant (k_a) greater than the response rate of the instrument, which was limited to 1×10^5 s^{-1} by light scattering artifacts. Cu_A then transfers an electron to cyt a with a rate constant $k_b = (1.8 \pm 0.3) \times 10^4$ s^{-1}. A small amount of reduced Ru_m-25-cyt c would remain in rapid equilibrium with reduced Cu_A after the initial fast phase if the kinetic constants satisfied the following relation: $k_a > k_r \gg k_b$. Under these conditions, the reduced Ru_m-25-cyt c would be reoxidized at the same rate as Cu_A, accounting for the 550-nm transient in Figure 2A. The rate constants were independent of the concentrations of Ru_m-25-cyt c and cytochrome *c* oxidase over the range 5–20 μM as long as the concentration of cytochrome *c* oxidase was equal to or greater than that of Ru_m-25-cyt c. This indicates that a 1:1 complex was fully formed at this ionic strength. There was no significant difference in the kinetics at pH 6 and pH 7 (Table I).

The reaction of Ru_c-25-cyt c with cytochrome *c* oxidase was slower than that of Ru_m-25-cyt c, allowing resolution of the reduction of Cu_A (Figure 3). The transients at the three wavelengths were fit to the kinetic equations for mechanism 1, assuming that k_r was small compared to k_b (Demas, 1983):

Table I: Intracomplex Rate Constants for Reduction of cyt a in Cytochrome *c* Oxidase by Ru-cyt c Derivatives[a]

ruthenium label	pH	k_f (s^{-1})	k_s (s^{-1})	*f*
		Class I		
Ru_c-7	7	2.0×10^4	2.2×10^3	0.7
Ru_m-25	6	1.7×10^4		1.0
Ru_m-25	7	1.9×10^4		1.0
Ru_c-25	7	1.0×10^4		1.0
Ru_m-39	7	2.2×10^4		1.0
Ru_m-55	6	2.2×10^4		1.0
Ru_m-55	7	2.1×10^4		1.0
Ru_m-60	7	2.1×10^4		1.0
		Class II		
Ru_c-13	7	1.1×10^3	1.9×10^2	0.4
Ru_m-13	7	6.0×10^3	1.5×10^3	0.5
Ru_c-27	7	6.6×10^3	2.1×10^3	0.4
Ru_c-27	6	3.0×10^3		1.0
Ru_m-27	7	5.0×10^3	1.5×10^3	0.7
Ru_c-72	7	2.2×10^4	6.0×10^2	0.4
Ru_m-72	7	2.2×10^4	3.0×10^3	0.4

[a] The reduction of cyt a was measured at 604 nm as described in the text. The pH 7 buffer contained 5 mM sodium phosphate, 10 mM aniline, and 1 mM 3CP. The pH 6 buffer contained 5 mM MES, 10 mM aniline, and 1 mM 3CP. The concentration of Ru-cyt c was 5–10 μM, and that of cytochrome *c* oxidase was 10–20 μM. *f* is the fraction of the fast phase. The error in each parameter is ±20%.

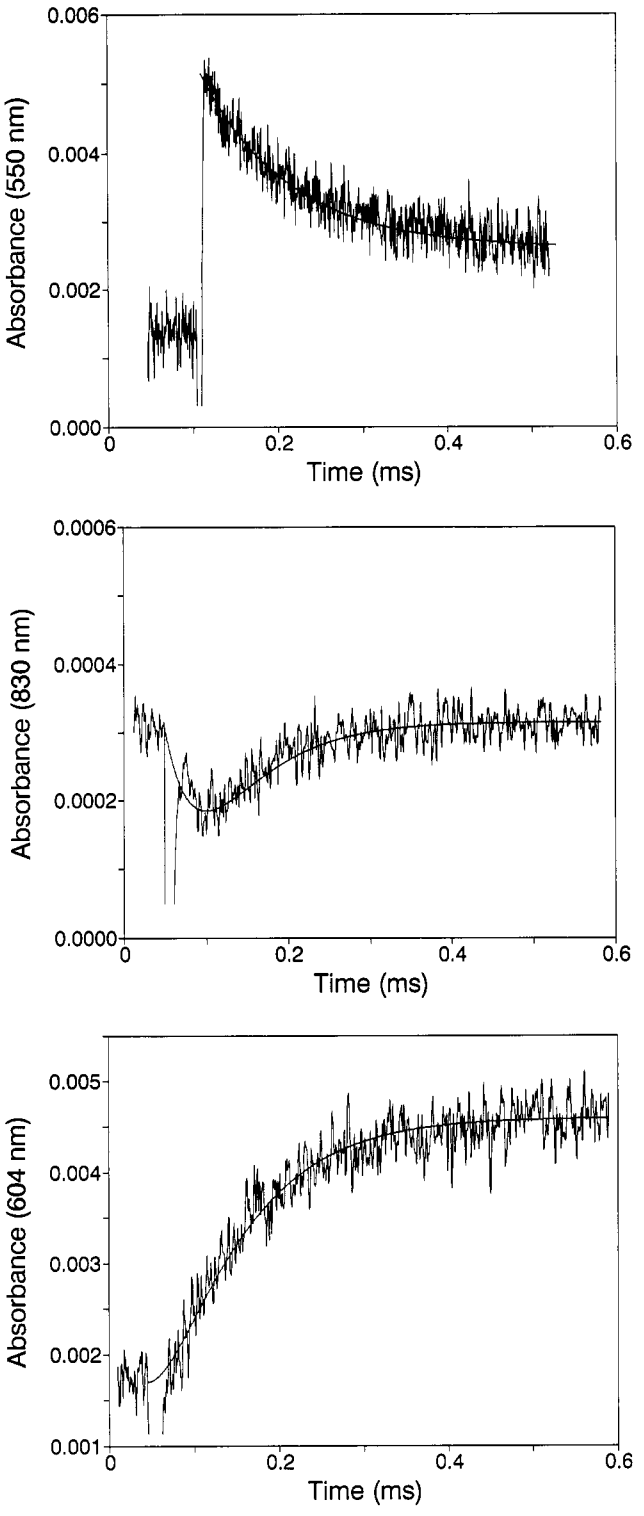


FIGURE 3: Photoinduced electron transfer from Ru_c-25-cyt c to cytochrome *c* oxidase. The solution contained 10 μM Ru_c-25-cyt c and 20 μM cytochrome *c* oxidase in 5 mM sodium phosphate (pH 7), 10 mM aniline, and 1 mM 3CP: (A, top) 550-nm transient; (B, middle) 830-nm transient; (C, bottom) 604-nm transient. The solid lines are the best fits to eqs 2–4 described in the text.

$$\Delta A_{550} = A_{550} \exp(-k_a t) \quad (2)$$

$$\Delta A_{830} = k_a A_{830}\{\exp(-k_a t) - \exp(-k_b t)\}/(k_b - k_a) \quad (3)$$

$$\Delta A_{604} = A_{604}\{k_b - k_a - k_b \exp(-k_a t) + k_a \exp(-k_b t)\}/(k_b - k_a) \quad (4)$$

The reoxidation of Ru_c-25-cyt c measured at 550 nm had $k_a = (1.0 \pm 0.2) \times 10^4$ s^{-1} and Δ[cyt c^{2+}] = 0.14 ± 0.04 μM.

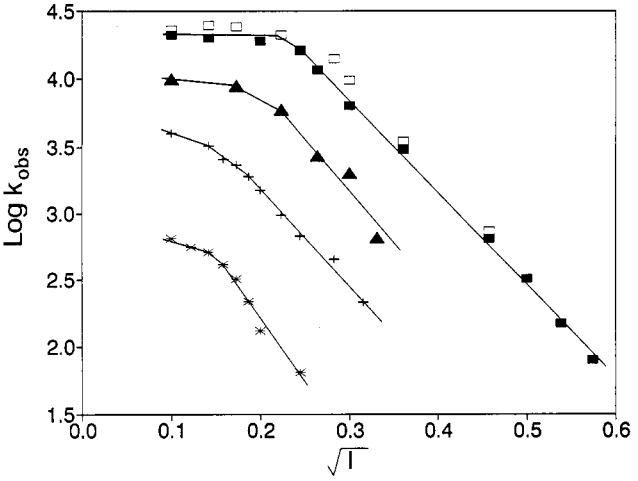


FIGURE 4: Ionic strength of the rate constant k_{obs} for reduction of cyt a in cytochrome *c* oxidase by Ru-cyt c derivatives. k_{obs} was obtained from the best fit of a single exponential to the 604-nm transient, even if it was biphasic. The solutions contained 10 μM Ru-cyt c and 20 μM cytochrome *c* oxidase in 5 mM sodium phosphate (pH 7), 10 mM aniline, and 0–500 mM NaCl. The square root of the ionic strength *I* is given on the horizontal axis: Ru_c-25, ▲; Ru_c-13, *; Ru_m-25, ■; Ru_c-27, +; Ru_m-55, □.

The 830-nm transient had $k_a = (1.3 \pm 0.5) \times 10^4$ s^{-1}, $k_b = (2.3 \pm 0.7) \times 10^4$ s^{-1}, and $\Delta A_{830} = 0.004 \pm 0.0015$, which corresponds to 0.23 ± 0.10 μM electrons transferred through Cu_A. The reduction of cyt a measured at 604 nm had $k_a = (1.2 \pm 0.3) \times 10^4$ s^{-1}, $k_b = (2.3 \pm 0.6) \times 10^4$ s^{-1}, and Δ[cyt a^{2+}] = 0.18 ± 0.04 μM. Although the error limits are somewhat large, particularly for the 830-nm transient, the results are consistent with mechanism 1.

The effects of ionic strength on the kinetics of solutions containing 11 μM Ru-cyt c and 20 μM cytochrome *c* oxidase are summarized in Figure 4. For the Ru_m-25-cyt c derivative, the rate constant for reduction of cyt a remained constant at $(1.8 \pm 0.4) \times 10^4$ s^{-1} as the ionic strength was increased from 5 to 50 mM, but further increases in ionic strength resulted in a decrease in the amplitude of the fast phase and the appearance of a slower phase. At 100 mM ionic strength, the extent of photoreduction of 11 μM Ru_m-25-cyt c was nearly as large in the presence of 20 μM cytochrome *c* oxidase as in its absence. The reoxidation of Ru_m-25-cyt c was biphasic, with rate constants of $(2.6 \pm 0.4) \times 10^3$ (Δ[cyt c^{2+}] = 0.26 ± 0.03 μM) and $(1.2 \pm 0.3) \times 10^2$ s^{-1} (Δ[cyt c^{2+}] = 0.18 ± 0.02 μM). The reduction of cyt a monitored at 604 nm was monophasic with nearly the same rate constant and amplitude as the fast phase of Ru_m-25-cyt c oxidation, $(2.9 \pm 0.4) \times 10^3$ s^{-1} (Δ[cyt a^{2+}] = 0.26 ± 0.04 μM). No significant reduction of Cu_A was observed at 830 nm. The slow phase of Ru_m-25-cyt c oxidation without a corresponding phase of cyt a reduction is similar to that observed by other workers (Hazzard et al., 1991; Larson et al., 1992). Further increases in ionic strength led to a decrease in the rate constant of cyt a reduction (Figure 4). At ionic strengths above 150 mM the reaction was second order, with a linear dependence of the rate constant on the concentration of cytochrome *c* oxidase over the range 5–20 μM. The ionic strength dependence of Ru_c-25-cyt c was similar to that of Ru_m-25-cyt c, except that the rate constants were smaller.

The kinetics of all of the class I derivatives are nearly the same as that of Ru_m-25-cyt c. The extent of photoreduction of the heme group in cytochrome *c* was greatly decreased upon complex formation with cytochrome *c* oxidase at low ionic strength, and rapid reduction of Cu_A was observed at 830 nm within the deadtime of the instrument. The rate

constant for reoxidation of Cu_A is the same as that for the reduction of cyt a measured at 604 nm, and it ranged from 1.9×10^4 to 2.2×10^4 s^{-1} for the various derivatives (Table I). A low-amplitude reoxidation of cytochrome *c* was observed at 550 nm with the same rate constant as at 604 and 830 nm. The fast phase was eliminated at ionic strengths above 100 mM, and the rate constant of the remaining slow phase decreased with further increases in ionic strength (Figure 4).

The kinetics of the class II derivatives modified at lysines 13 and 27 are quite different from those of the other derivatives. The reoxidation of Ru_c-27-cyt c was biphasic at low ionic strength, with rate constants of $(6.0 \pm 1.0) \times 10^3$ (Δ[cyt c^{2+}] = 0.22 ± 0.03 μM) and $(1.8 \pm 0.3) \times 10^3$ s^{-1} (Δ[cyt c^{2+}] = 0.41 ± 0.03 μM). This was accompanied by biphasic reduction of cyt a with rate constants of $(6.6 \pm 1.0) \times 10^3$ (Δcyt a^{2+} = 0.21 ± 0.03 μM) and $(2.1 \pm 0.3) \times 10^3$ s^{-1} (Δ[cyt a^{2+}] = 0.34 ± 0.04 μM) (Table I). No reduction of Cu_A was observed at 830 nm. The addition of higher concentrations of cytochrome *c* oxidase to the reaction mixture did not alter the rate constants for either cytochrome *c* oxidation or cyt a reduction, indicating that a 1:1 complex was fully formed at a concentration of 5 μM under these conditions. Increasing the ionic strength resulted in a decrease in k_{obs} (Figure 4). At ionic strengths above 100 mM, the reaction obeyed second-order kinetics. The reactions of Ru_m-27-cyt c, Ru_c-13-cyt c, and Ru_m-13-cyt c with cytochrome *c* oxidase are similar to that of Ru_c-27-cyt c, with the rate constants shown in Table I. No reduction of Cu_A was observed at 830 nm for these derivatives.

The reaction of Ru_c-72-cyt c with cytochrome *c* oxidase at low ionic strength was rather unusual. The extent of photoreduction of Ru_c-72-cyt c was decreased to 34% of its original value upon addition of cytochrome *c* oxidase, and rapid reduction of Cu_A was observed at 830 nm within the instrument deadtime. Reoxidation of Cu_A was accompanied by reduction of cyt a at 604 nm, each with a rate constant of 2.2×10^4 s^{-1} and extent of 0.51 ± 0.05 μM. In addition, a much slower phase of cyt a reduction was observed with a rate constant of 600 ± 100 s^{-1} and Δ[cyt a^{2+}] = 0.55 ± 0.05 μM. This derivative can apparently bind to cytochrome *c* oxidase in two different ways, one resembling the class I derivatives and the other the class II derivatives. The reaction of Ru_m-72-cyt c was similar to that of Ru_c-72-cyt c, except that the slow phase rate constant was $(3.0 \pm 0.05) \times 10^3$ s^{-1} (Table I).

In order to compare the reactions of all of the Ru-cyt c derivatives with that of native cytochrome *c*, the flavin flash photolysis method of Hazzard et al. (1991) was used to measure the second-order rate constants at high ionic strength (100 mM sodium phosphate (pH 7), 5 mM EDTA, and 50 μM riboflavin). The kinetic data are summarized in Table II. The rate constants of the derivatives modified at lysines 13, 27, and 72 were less than that of native cytochrome *c*, while those of the derivatives modified at lysines 39 and 60 were nearly the same. The rate constant of Ru_c-25-cyt c was 2-fold smaller than that of native cytochrome *c*, while that of Ru_m-25-cyt c was slightly larger. The rate constant of Ru_m-55-cyt c was over 2-fold greater than that of native cytochrome *c*. The same rate constants were obtained for the Ru-cyt c derivatives using either riboflavin excitation or ruthenium excitation.

Table II: Second-Order Rate Constants for the Reaction of Ru-cyt c Derivatives with Cytochrome *c* Oxidase at 200 mM Ionic Strength[a]

ruthenium label	k (10^6 M^{-1} s^{-1})
native horse cyt c	5.4
Class I	
Ru_c-7	6.0
Ru_c-25	2.3
Ru_m-25	7.0
Ru_m-39	6.4
Ru_m-55	13.6
Ru_m-60	6.6
Class II	
Ru_c-13	0.3
Ru_m-13	0.3
Ru_c-27	1.3
Ru_m-27	1.8
Ru_c-72	0.8
Ru_m-72	1.6

[a] The reaction solutions contained 1 μM cytochrome *c*, 0–5 μM cytochrome *c* oxidase, 0.1% dodecyl maltoside, 50 μM riboflavin, 5 mM EDTA, and 100 mM sodium phosphate (pH 7). The error is ±15%.

DISCUSSION

The ruthenium cytochrome *c* derivatives have several favorable properties for the study of intracomplex electron-transfer reactions (Pan et al., 1990; Hahm et al., 1992). The photoexcited ruthenium complex, Ru(II*), transfers an electron to the cytochrome *c* heme group Fe(III) in a very rapid and highly selective first-order reaction. The k_1 rate constants range from 2×10^7 s^{-1} for Ru_c-72-cyt c down to 1.5×10^5 s^{-1} for Ru_c-7-cyt c (Durham et al., 1989). The k_1 rate constants of the Ru_m-cyt c derivatives are nearly the same as those for the Ru_c-cyt c derivatives modified at the same lysine (Liu and Durham, manuscript in preparation). Ru_m-55-cyt c has $k_1 = 4.0 \times 10^5$ s^{-1}, Ru_m-25-cyt c has $k_1 = 3 \times 10^5$ s^{-1}, while Ru_m-39-cyt c and Ru_m-60-cyt c modified at the back of the molecule have $k_1 < 1 \times 10^5$ (Liu et al., manuscript in preparation).

The Ru_c-lysine group has a charge of 1+, while the Ru_m-lysine group has a charge of 2+, allowing all of the derivatives to form 1:1 complexes with cytochrome *c* oxidase at low ionic strength. The large size of the ruthenium complex (9-Å radius) is expected to alter the binding orientation of the class II cytochrome *c* derivatives modified at lysines in the interaction domain. The most direct indication of this effect is provided by the comparison of the second-order rate constants of the derivatives with that of native cytochrome *c* given in Table II. The decreased rate constants of the derivatives modified at lysines 13, 27, and 72 are consistent with previous chemical modification studies demonstrating the importance of these lysines in binding cytochrome *c* oxidase (Figure 1). It is perhaps surprising that these derivatives are as active as they are, considering the large size of the ruthenium complex. Evidently, a large change in cytochrome *c* binding orientation does not completely abolish electron transfer.

It is interesting that the second-order rate constant of Ru_c-25-cyt c is about 2-fold less than that of native cytochrome *c*, while that of Ru_m-25-cyt c is slightly larger. Previous chemical modification studies indicated that this lysine was on the periphery of the binding domain. The 2+ charge on the Ru_m-lysine 25 group apparently compensates for any unfavorable steric effects. The rate constants of the derivatives modified at lysines 7, 39, and 60 are nearly the same as that of native cytochrome *c*, consistent with their location far away from the binding domain. The 2.5-fold greater rate constant for Ru_m-55-cyt c is surprising given the location of this lysine on the bottom left side of cytochrome *c* remote from the binding domain. Henderson et al. (1977) have suggested that cytochrome *c* binds in a deep pocket in the cytochrome *c* oxidase dimer, and it is possible that the Ru_m-Lys 55 group makes a favorable interaction with the part of this pocket remote from the major binding site.

When a complex between Ru-cyt *c* and cytochrome *c* oxidase is excited with a laser flash, Ru(II*) rapidly transfers an electron to the cytochrome *c* heme to give Fe(II) with a yield of 3–10%, depending on the derivative. Since the ruthenium complex is covalently attached to cytochrome *c*, the yield of photoreduced Fe(II) is not expected to be greatly affected by complex formation. The photoreduced Fe(II) in Ru-cyt *c* then transfers an electron to Cu_A and/or cyt a in cytochrome *c* oxidase. The sequence of electron-transfer reactions will depend on the redox potentials of the metal centers, their relative reorganizational energies, and the electronic coupling between them. The redox potential of cytochrome *c* is 260 mV in solution, but is reported to decrease to as low as 220 mV when bound to cytochrome *c* oxidase (Schroedl & Hartzell, 1977). The redox potential of Cu_A has been measured to be 245 mV (Erecinska et al., 1971; Wilson et al., 1976), while that of cyt a has been reported to be 362 mV under conditions where cyt a_3 is oxidized (Wilson et al., 1972).

The kinetics for Ru_m-25-cyt c shown in Figure 2 is consistent with electron transfer according to mechanism 1. The photoreduced heme of Ru_m-25-cyt c transfers an electron to Cu_A with a rate constant k_a that is too large to be resolved by the spectrophotometer. Cu_A then transfers an electron to cyt a with rate constant $k_b = 1.8 \times 10^4\ s^{-1}$. The low-amplitude reoxidation of the Ru_m-25-cyt c heme suggests that it remains in rapid equilibrium with Cu_A during the reduction of cyt a. If the redox potential of bound cytochrome *c* were 30 mV more negative than that of Cu_A, then about 35% of its heme would remain reduced during rapid equilibrium with Cu_A, in agreement with the results of Figure 2. The rate constant for electron transfer from Cu_A to cyt a measured in the present studies is in agreement with previous measurements for this reaction in fully oxidized cytochrome *c* oxidase (Kobayashi et al., 1989; Nilsson, 1992) and in three-electron-reduced, CO-inhibited cytochrome *c* oxidase (Morgan et al., 1989). Cu_A is nearly completely reoxidized after the fast phase electron transfer to cyt a (Figure 2B). This indicates that the redox potential of cyt a is much more positive than that of Cu_A, consistent with equilibrium titration studies (Wilson et al., 1972) and previous kinetic studies (Pan et al., 1991; Larson et al., 1992). However, other workers have reported that Cu_A and cyt a are reduced to the same extent by ferrocytochrome *c*, indicating that they have similar redox potentials (Antalis & Palmer, 1982; Hazzard et al., 1991). The reasons for this discrepancy are unknown, but they may arise from differences in the redox or ligand binding state of cyt a_3, which is known to affect the redox potential of cyt a (Larson et al., 1992; Morgan et al., 1989)

The reaction of Ru_c-25-cyt c with cytochrome *c* oxidase is slower than that of Ru_m-cyt c, probably due to a change in orientation caused by the additional carboxylate group on the ruthenium complex. This decrease in the reaction rate allowed resolution of the reduction of Cu_A by the Ru_c-25-cyt c heme (Figure 3). The kinetics at all three wavelengths shown in Figure 3 are consistent with mechanism 1 involving electron transfer from the Ru_c-25-cyt c heme to Cu_A with a rate constant of $(1.1 \pm 0.3) \times 10^4\ s^{-1}$, followed by electron transfer to cyt a with a rate constant of $(2.3 \pm 0.6) \times 10^4\ s^{-1}$.

The class I cytochrome *c* derivatives modified at lysines 7, 39, 55, and 60 all have essentially the same kinetics as Ru_m-25-cyt c at low ionic strength (Table I). The failure to resolve the rapid electron transfer reaction from the photoreduced heme of these derivatives to Cu_A is most likely due to the response rate of the spectrophotometer detection system, which was limited to $1 \times 10^5\ s^{-1}$ by light scattering artifacts. The rate of this reaction is thus greater than $1 \times 10^5\ s^{-1}$. An alternative possibility that Ru(II*) transferred an electron directly to Cu_A rather than to the heme group of cytochrome *c* is very unlikely since these derivatives are modified at lysines far removed from the binding site (Figure 1). In addition, the rapid reduction and reoxidation of the cytochrome *c* heme would not be observed in that case.

The small intracomplex rate constants observed at low ionic strength for the class II cytochrome *c* derivatives modified at lysines 13 and 27 (Table I) are consistent with the small second-order rate constants for these derivatives at high ionic strength (Table II). The bulky ruthenium complex on these derivatives is expected to alter the binding orientation to cytochrome *c* oxidase, thus decreasing the rate of electron transfer. In terms of current electron-transfer theory, the decrease in rate constant from $\sim 10^5$ to $\sim 10^3\ s^{-1}$ would require an increase of about 2.5 Å in the separation between the redox centers (Durham et al., 1989). It is not possible to tell whether these derivatives initially transferred an electron to Cu_A or cyt a, since no reduction of Cu_A was observed. If Cu_A was the initial site of reduction, then the much more rapid electron-transfer reaction from Cu_A to cyt a would prevent any transient absorbance change at 830 nm. The biphasic kinetics observed for these derivatives suggests that they have two different binding orientations, both involving relatively slow electron transfer. The unusual biphasic kinetics observed for Ru_c-72-cyt c suggests that it can bind to cytochrome *c* oxidase in two substantially different orientations, one similar to the class I derivatives and the other similar to the class II derivatives. The length and flexibility of the lysine side chain might allow the ruthenium complex to be folded toward the back of the molecule, allowing class I type binding and kinetics.

At low ionic strength, the rate constants for all of the derivatives were independent of cytochrome *c* oxidase concentration above 5 μM, indicating that the dissociation constants were less than about 2 μM. The kinetics of the class I derivatives remained unchanged up to about 50 mM ionic strength, but further increases in ionic strength led to a decrease in the amplitude of the fast phase and the appearance of a slower phase (Figure 4). This indicates that complexed cytochrome *c* is replaced by uncomplexed cytochrome *c* as the ionic strength is increased from 50 to 100 mM. Above 150 mM ionic strength, the rate constants were linearly dependent on the concentration of cytochrome *c* oxidase, indicating second-order kinetics. Thus, the plots in Figure 4 provide an indication of the minimum ionic strength needed to dissociate the complex. The plots for all of the class I derivatives were quite similar to one another. The class II derivatives modified at lysines 13 and 27 required significantly lower ionic strengths to begin the decrease in the rate constant, indicating weaker binding. In no case did any of the rate constants increase as the ionic strength was increased.

The very rapid electron transfer between the class I cytochrome *c* derivatives and Cu_A in the complex with fully oxidized cytochrome *c* oxidase at low ionic strength is in agreement with previous studies by Hill (1991) on the fully reduced complex. He estimated that the rate constant for electron transfer from native horse ferrocytochrome *c* to Cu_A is greater than $7 \times 10^4\ s^{-1}$ in flash photolysis experiments on the complex between ferrocytochrome *c* and fully reduced, CO-inhibited cytochrome *c* oxidase. The fact that the class I cytochrome *c* derivatives have nearly the same second-order rate constants as native cytochrome *c* at high ionic strength (Table II) suggests that their kinetics at low ionic strength should be representative of native cytochrome *c*. However, Hazzard et al. (1991) observed a rate constant of only 630 s^{-1} for oxidation of bovine cytochrome *c* by cytochrome *c*

oxidase at low ionic strength. In these experiments, solution phase photoreduced flavin semiquinone was used to reduce about 5% of the cytochrome *c* present in the solution. It is possible that cytochrome *c* bound in the active, rapidly reacting orientation to cytochrome *c* oxidase is not accessible for reduction by the solution phase flavin semiquinone. Instead, the flavin semiquinone could preferentially reduce a small fraction of cytochrome *c* that is bound in an inactive orientation where its heme is accessible to the solution phase. This inactive orientation would then have to be converted to the active orientation before electron transfer could occur, in a conformational gating mechanism (Hoffman & Ratner, 1987). This mechanism is supported by the finding that the rate constant increased from 630 to 1470 s^{-1} as the ionic strength was increased from 10 to 110 mM, since higher ionic strength would be expected to weaken binding and increase the rate of interconversion between the inactive and active forms of the complex. At high ionic strength, where no complexes are present, there is complete agreement between the rate constants determined by the flavin flash photolysis method and the ruthenium photoexcitation method (Table II).

A number of recent studies have been carried out using external reducing agents at high ionic strength. Pan et al. (1991) found that bovine cytochrome *c* reduced Cu_A-depleted cytochrome *c* oxidase with a rate constant of 740 s^{-1} at 110 mM ionic strength, compared to 2580 s^{-1} for native cytochrome *c* oxidase. They concluded that Cu_A either is the primary electron acceptor or regulates the rate of direct electron transfer from cytochrome *c* to cyt a. Hazzard et al. (1992) recently found that a series of position 82 variants of yeast iso-1-cytochrome *c* reduces cytochrome *c* oxidase with rate constants of about 2000 s^{-1} at 110 mM ionic strength. This study indicates that the aromatic side chain at Phe 82 of cytochrome *c* is not required for electron transfer. Larsen et al. (1992) used a uroporphyrin/NADH flash photolysis system to study the reaction between horse cytochrome *c* and cytochrome *c* oxidase, and they obtained a rate constant of 1829 s^{-1} at 100 mM ionic strength.

CONCLUSIONS

The ruthenium photoexcitation technique has been used to measure rapid intracomplex electron transfer between Ru-cyt *c* derivatives and cytochrome *c* oxidase at low ionic strength. The mechanism for Ru-cyt *c* derivatives modified at lysines remote from the heme crevice involves initial electron transfer to Cu_A with a rate constant greater than 10^5 s^{-1}. Cu_A then transfers an electron to cyt a with a rate constant of 2×10^4 s^{-1}. At high ionic strength the complexes are dissociated, and the second-order rate constants for the Ru-cyt c derivatives are comparable to that for native horse cytochrome *c*.

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