Photoinduced Electron Transfer between Cytochrome *c* Peroxidase and Horse Cytochrome *c* Labeled at Specific Lysines with (Dicarboxybipyridine)(bisbipyridine)ruthenium(II)†

Seung Hahm, Bill Durham,* and Francis Millett*
Department of Chemistry and Biochemistry, University of Arkansas, Fayetteville, Arkansas 72701
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ABSTRACT: The reactions of yeast cytochrome *c* peroxidase with horse cytochrome *c* derivatives labeled at specific lysine amino groups with (dicarboxybipyridine)(bisbipyridine)ruthenium(II) [Ru(II)] were studied by flash photolysis. All of the derivatives formed complexes with cytochrome *c* peroxidase compound I (CMPI) at low ionic strength (2 mM sodium phosphate, pH 7). Excitation of Ru(II) to Ru(II*) with a short laser flash resulted in electron transfer to the ferric heme group in cytochrome *c*, followed by electron transfer to the radical site in CMPI. This reaction was biphasic and the rate constants were independent of CMPI concentration, indicating that both phases represented intracomplex electron transfer from the cytochrome *c* heme to the radical site in CMPI. The rate constants of the fast phase were 5200, 19 000, 55 000, and 14 300 s^{-1} for the derivatives modified at lysines 13, 25, 27, and 72, respectively. The rate constants of the slow phase were 260, 520, 200, and 350 s^{-1} for the same derivatives. These results suggest that there are two binding orientations for cytochrome *c* on CMPI. The binding orientation responsible for the fast phase involves a geometry that supports rapid electron transfer, while that for the slow phase allows only slow electron transfer. Increasing the ionic strength up to 40 mM increased the rate constant of the slow phase and decreased that of the fast phase. A single intracomplex electron transfer phase with a rate constant of 2800 s^{-1} was observed for the lysine 72 derivative at this ionic strength. When a series of light flashes was used to titrate CMPI to CMPII, the reaction between the cytochrome *c* derivative and the Fe(IV) site in CMPII was observed. The rate constants for this reaction were 110, 250, 350, and 140 s^{-1} for the above derivatives measured in low ionic strength buffer.

The cytochrome *c*–cytochrome *c* peroxidase system is well-suited in many respects for investigating fundamental questions about biological electron transfer. Extensive structural data is available for cytochrome *c* isolated from several different organisms, while the X-ray crystal structure of yeast cytochrome *c* peroxidase has been determined for both the resting ferric form of the enzyme, CcP (Finzel et al., 1984), and the hydrogen peroxide oxidized form, CMPI (Edwards et al., 1987). In addition, site-directed mutants of both cytochrome *c* and cytochrome *c* peroxidase have been prepared and structurally characterized (Louie et al., 1988; Wang et al., 1990). Cytochrome *c* forms a complex with cytochrome *c* peroxidase at low ionic strength which is stabilized by electrostatic interactions (Mochan, 1970; Kang et al., 1977; Erman & Vitello, 1980). Poulos and Kraut (1980) proposed a model for the complex in which the positively charged amino groups on lysines 13, 27, 72, 86, and 87 surrounding the heme crevice of cytochrome *c* interact electrostatically with the carboxylate groups on Asp 34, 37, 79, and 216 on cytochrome *c* peroxidase. The complex has been crystallized, but no diffraction due to cytochrome *c* was observed, suggesting the presence of multiple binding orientations (Poulos et al., 1987). This explanation is also consistent with the observation of multiple phases for intracomplex electron transfer from ferrocytochrome *c* to the zinc porphyrin cation of zinc cytochrome *c* peroxidase (Wallin et al., 1991).

In the catalytic mechanism for cytochrome *c* peroxidase, CcP is oxidized by hydrogen peroxide to CMPI, which is then sequentially reduced to CMPII and CcP by two molecules of ferrocytochrome *c* (Kim et al., 1990). The two oxidized sites in CMPI are the oxyferryl heme Fe(IV) and a radical cation on Trp 191 (Mauro et al., 1988; Sivaraja et al., 1989). The single oxidized site in CMPII can be either the oxyferryl heme or the radical cation (Coulson et al., 1971; Ho et al., 1983). Summers and Erman reported that in stopped-flow experiments at low ionic strength horse ferrocytochrome *c* reduced the heme Fe(IV) site in CMPI with a rate constant of 450 s^{-1}. A flash photolysis technique has been used to measure a rate constant of 750 s^{-1} for intracomplex electron transfer from horse cytochrome *c* to CMPI at low ionic strength (Hazzard et al., 1987, 1988a–c).

We recently reported a flash photolysis study of the reduction of cytochrome *c* peroxidase by yeast iso-1-cytochrome *c* derivatized at Cys 102 with 4-bromomethyl-4′-methylbipyridine(bisbipyridine)ruthenium(II) (Geren et al., 1991). The most surprising result was that the reduced heme group of cytochrome *c* transferred an electron to the radical cation in CMPI rather than to the oxyferryl heme Fe(IV). The intracomplex rate constant for this reaction was greater than 50 000 s^{-1} at low ionic strength. In the present paper we have extended this approach to the reaction of cytochrome *c* peroxidase with horse cytochrome *c* derivatives labeled at single lysines with (dicarboxybipyridine)(bisbipyridine)ruthenium(II) (Pan et al., 1988, 1990). The internal electron transfer reactions between the ruthenium and heme groups in these derivatives have been described in detail, with rate constants ranging from 1×10^5 to 2×10^7 s^{-1} (Durham et al., 1989). In the present study we have found that all of these derivatives transfer an electron to the radical cation in CMPI with biphasic kinetics suggesting two different binding orientations.

Experimental Procedures

Materials. Horse cytochrome *c* (Type VI) was obtained from Sigma Chemical Co. The Ru-cyt *c* derivatives were

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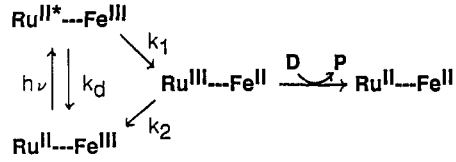
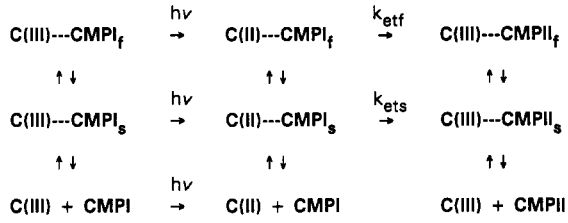
Scheme I



Scheme II



prepared as described by Pan et al. (1988) and Durham et al. (1989). Cytochrome *c* peroxidase was prepared from Red Star bakers' yeast as described by Geren et al. (1991).

Transient Absorption Kinetics. Transient absorbance measurements were carried out as described by Geren et al. (1991). Samples were prepared with the Ru-cyt *c* derivative and cytochrome *c* peroxidase in the indicated buffer containing either aniline or EDTA[1] as a sacrificial electron donor. The minimum amount of hydrogen peroxide needed to form CMPI was added, and then the sample was excited with a laser flash to photoreduce less than 10% of the heme in Ru-cyt *c*. Aniline reacted slowly with CMPI to form CcP, so it was necessary to initiate flash photolysis within 1 min of CMPI formation. The kinetic constants were independent of the concentration of aniline. A single sample could be subjected to at least 10 cycles of CMPI formation and photolysis back to CcP without affecting the kinetic parameters or spectral properties of CMPI. This indicated that no significant damage to cytochrome *c* peroxidase was induced by either aniline or photolysis. CMPI was stable for at least 10 min in the presence of EDTA, so this sacrificial electron donor was used for the complete flash titration studies.

Results

Excitation of Ru-72-cyt *c* with a laser flash resulted in formation of Ru(II*), which rapidly transferred an electron to the heme group according to the mechanism shown in Scheme I. Either EDTA or aniline was used as a sacrificial electron donor, D, to reduce Ru(III) to Ru(II) and prevent the back reaction k_2. The rate constant for heme reduction, k_1, was previously measured to be 1.4×10^7 s^{-1} for Ru-72-cyt *c* (Durham et al., 1989). The extent of heme reduction was not affected by complex formation with cytochrome *c* peroxidase in the resting Fe(III) state, CcP, at low ionic strength (Figure 1A). Excitation of Ru-72-cyt *c* in the presence of cytochrome *c* peroxidase compound I (CMPI) led to electron transfer from the photoreduced heme to CMPI according to Scheme II. Biphasic electron transfer kinetics were observed for a solution containing 5 μM Ru-72-cyt *c* and 6 μM CMPI in low ionic strength buffer (2 mM sodium phosphate, pH 7) (Figure 1B,C). The fast phase had a rate constant of k_{etf} = 14 300 ± 1500 s^{-1} and a relative amplitude of 0.57 ± 0.06, while the slow phase had k_{ets} = 350 ± 40 s^{-1} and an amplitude

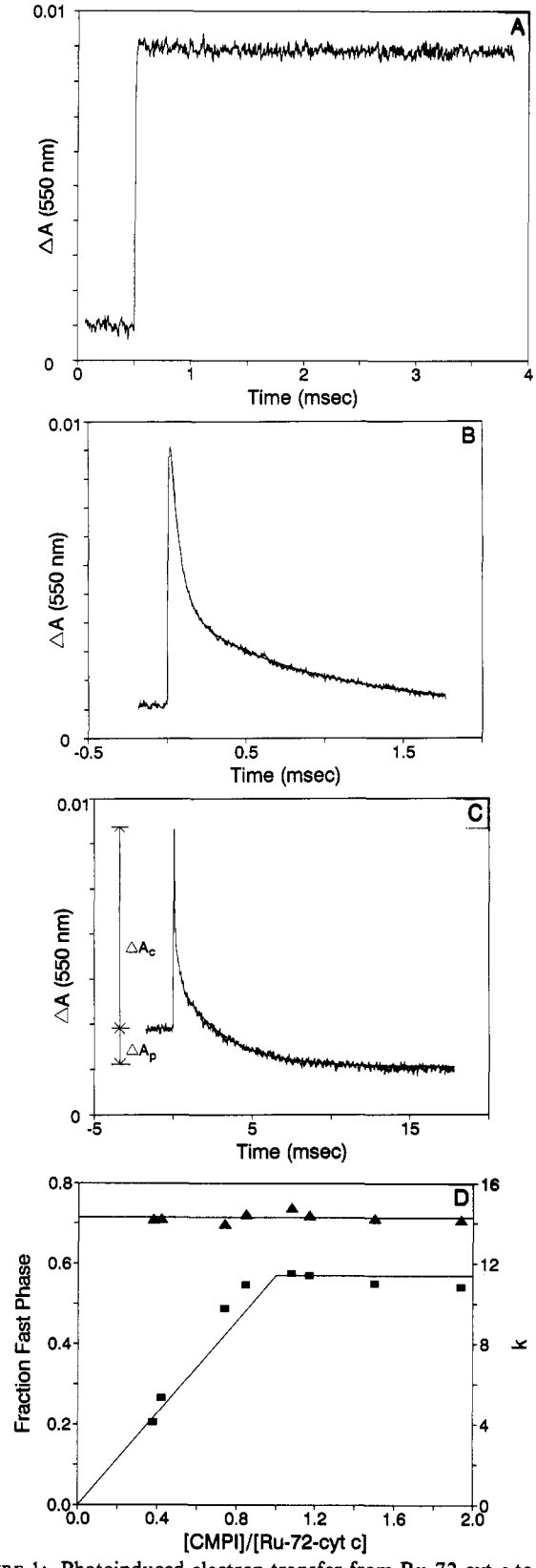


FIGURE 1: Photoinduced electron transfer from Ru-72-cyt *c* to cytochrome *c* peroxidase. The solutions contained 5 μM Ru-72-cyt *c* in a buffer of 2 mM sodium phosphate, pH 7, and 10 mM aniline. (A) A 550-nm transient of 5 μM Ru-72-cyt *c* and 6 μM CcP (III,R) following a single laser flash. (B) A 550-nm transient of 5 μM Ru-72-cyt *c* and 6 μM CMPI after a single laser flash. The smooth line represents the best fit to the function $A[f \exp(-k_{etf}t) + (1-f)\exp(-k_{ets}t)]$ with k_{etf} = 14 300 s^{-1}, k_{ets} = 350 s^{-1}, and f = 0.57. (C) Same solution as in (B) with a different time scale. (D) Dependence of the rate constant k (in 1000 s^{-1}) (▲) and amplitude f (■) of the fast phase on the ratio of CMPI to Ru-72-cyt *c*. [Ru-72-cyt *c*] was fixed at 5 μM.

[1] Abbreviations: bpy, 2,2′-bipyridine; dcbpy, 4,4′-dicarboxybipyridine; Ru-72-cyt *c*, bipyridine(4,4′-dicarboxybipyridine-Lys-72-cytochrome *c*)-ruthenium(II); EDTA, ethylenediaminetetraacetic acid.

Table I: Intracomplex Rate Constants for Electron Transfer from Ru-cyt *c* Derivatives to CMPI[a]

derivative	buffer	k_{etf}	k_{ets}	f
Ru-72-cyt *c*	2 mM P_i	14300	350	0.57
	2 mM P_i + 5 mM NaCl	14600	890	0.44
	2 mM P_i + 10 mM NaCl	9500	1250	0.37
	2 mM P_i + 25 mM NaCl	7500	2800	0.37
	5 mM P_i + 30 mM NaCl		2800	1.0
	0.5 mM P_i + 2 mM EDTA	18000	750	0.59
Ru-13-cyt *c*	2 mM P_i	5200	260	0.64
	2 mM P_i + 5 mM NaCl	4600	500	0.53
	2 mM P_i + 10 mM NaCl	4500	1100	0.50
Ru-25-cyt *c*	2 mM P_i	19000	520	0.50
	2 mM P_i + 5 mM NaCl	18000	714	0.50
	2 mM P_i + 10 mM NaCl	17500	974	0.42
	2 mM P_i + 15 mM NaCl	17400	1500	0.40
Ru-27-cyt *c*	2 mM P_i	55000	200	0.67
	2 mM P_i + 5 mM NaCl	38000	250	0.55
	2 mM P_i + 10 mM NaCl	23600	470	0.39

[a] Transients were obtained as described for Figure 1 and fit with the function $A[(f \exp(-k_{etf}t) + (f - 1) \exp(-k_{ets}t)]$. The rate constants were measured as a function of CMPI and Ru-cyt *c* concentration to obtain the concentration-independent rate constants (in reciprocal seconds). The buffer contained the indicated concentrations of sodium phosphate, pH 7 (P_i), NaCl, and 10 mM aniline. The EDTA experiment contained no aniline. The error limits are ±10%.

of 0.43 ± 0.04. The amplitude of the fast phase increased as the ratio of CMPI to Ru-72-cyt *c* was increased from 0 to 1.0 and then remained constant at higher ratios (Figure 1D). The rate constant of the fast phase remained constant throughout this range. The slow portion of the transient displayed concentration-dependent kinetics at ratios below 1.0, consistent with the presence of both complexed and uncomplexed Ru-72-cyt *c*. At ratios of 1.0 and higher, the rate constant of the slow phase remained constant.

Ionic strength had a significant effect on the kinetics of the reaction between Ru-72-cyt *c* and CMPI. The kinetics were measured as a function of CMPI concentration at each ionic strength to obtain the limiting concentration-independent rate constants shown in Table I. As the concentration of NaCl was increased from 0 to 25 mM, the rate of the fast phase decreased from 14300 to 7500 s^{-1}, while the rate of the slow phase increased from 350 to 2800 s^{-1}. The dissociation constant was less than 5 μM at ionic strengths up to 25 mM. This is comparable to the dissociation constant for native horse cytochrome *c*, which ranged from 0.01 to 2 μM at ionic strengths from 10 to 50 mM (Kang et al., 1977; Vitello & Erman, 1987). At a somewhat higher ionic strength (5 mM sodium phosphate and 30 mM NaCl), only a single phase was observed, with a rate constant that had a hyperbolic dependence on CMPI concentration. The data were analyzed as described by Geren et al. (1991) to obtain a concentration-independent rate constant of 2800 ± 300 s^{-1} and a dissociation constant of 7 ± 3 μM. These are comparable to the values of 3220 s^{-1} and 15 μM for native horse cytochrome *c* at 30 mM ionic strength (Hazzard et al., 1988B). At ionic strengths above 50 mM, a single slow phase was observed that was linearly dependent on the concentration of CMPI, indicating second-order kinetics. The second-order rate constant decreased as the ionic strength was increased further (Figure 2). Biphasic kinetics were also observed when EDTA was used as a sacrificial electron donor instead of aniline (Table I). The rate constants were 18000 ± 1800 s^{-1} and 750 ± 70 s^{-1} in a buffer of 2 mM EDTA and 0.5 mM sodium phosphate, which are similar to those in a buffer of 2 mM sodium phosphate, 5 mM NaCl, and 10 mM aniline.

In order to determine whether Ru-72-cyt *c* transfers an electron to the free radical site or to the oxyferryl heme Fe(IV) site in CMPI, the kinetics were measured at different wavelengths as described by Geren et al. (1991). The extinction coefficient change for the reduction of CMPI by Ru-cyt *c* was calculated using the formula $\Delta\epsilon_p = \Delta\epsilon_c \Delta A_p / \Delta A_c$ mM^{-1} cm^{-1}, where $\Delta\epsilon_c$ is the difference extinction coefficient for cytochrome *c*, ΔA_c is the absorbance change due to oxidation of ferrocytochrome *c*, and ΔA_p is the absorbance change due to reduction of CMPI (see Figure 1C). The value of $\Delta\epsilon_p$ was found to be 2 ± 0.5 mM^{-1} cm^{-1} at 550 nm for the reaction between Ru-72-cyt *c* and CMPI (Figure 1C). However, it was not possible to determine from these data whether Ru-72-cyt *c* transferred an electron to the heme Fe(IV) group or to the $R^{\bullet +}$ in CMPI, since the 550-nm difference extinction coefficients for these two reactions are very small and nearly the same (2 mM^{-1} cm^{-1} for $R^{\bullet +}$ vs 1.5 mM^{-1} cm^{-1} for Fe(IV); Coulson et al., 1971; Ho et al., 1983). In contrast, at 420 nm the Fe(IV) to Fe(III) reaction has $\Delta\epsilon$ = 31 mM^{-1} cm^{-1}, whereas the $R^{\bullet +}$ to R reaction has a $\Delta\epsilon$ of nearly zero) Coulson et al., 1971; Ho et al., 1983). The 420-nm transient for the reaction between 5 μM Ru-72-cyt *c* and 7 μM CMPI in a buffer of 5 mM sodium phosphate, pH 7, and 10 mM aniline was biphasic, as in Figure 1C, and returned to a value close to that of the initial absorbance, with $\Delta\epsilon_p = -2.0 \pm 1.0$ mM^{-1} cm^{-1}. This indicates that both phases involved electron transfer to the radical site in CMPI according to reaction 1:

$$\text{cyt } c^{2+} + \text{CMPI(IV,R}^{\bullet +}) \rightarrow \text{cyt } c^{3+} + \text{CMPII(IV,R)} \quad (1)$$

$$\text{cyt } c^{2+} + \text{CMPII(IV,R)} \rightarrow \text{cyt } c^{3+} + \text{CcP(III,R)} \quad (2)$$

In order to study both reactions 1 and 2, 5 μM Ru-72-cyt *c* and 6 μM CMPI in a buffer of 4 mM EDTA and 5 mM sodium phosphate were subjected to a series of xenon flashes at 1-min intervals. The initial transient was monophasic with k_{obs} = 1700 s^{-1} and $\Delta A_p = -1.5 \pm 0.5$ mM^{-1} cm^{-1} (Figure 3A). With subsequent flashes the transients became biphasic and began to go below the baseline (Figure 3B), indicating the occurrence of reaction 2 as well as 1. The slow phase had a rate constant of 140 ± 20 s^{-1}, and its amplitude increased until it became the dominant phase at flash number 4 (Figure 3B). A plot of absorbance at 434 nm (a cytochrome *c* isosbestic point) vs flash number was consistent with the reaction sequence 1 and 2 (Figure 3C) (Coulson et al., 1971). The wavelength dependence of ΔA_p after the fourth flash halfway through the titration (Figure 3D) was in good agreement with the absorbance difference spectrum for CMPII(IV,R) − CcP(III,R) (Coulson et al., 1971; Ho et al., 1983). Reaction 2 could be observed without interference from reaction 1 at

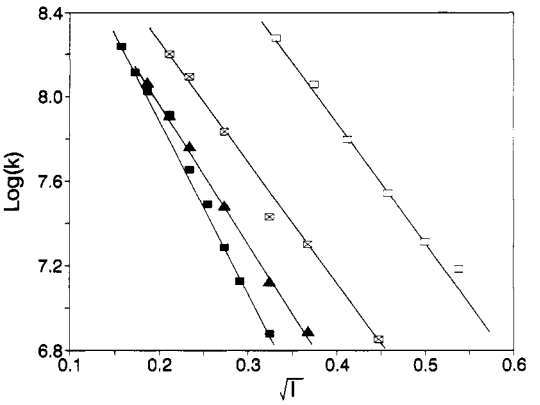


FIGURE 2: Ionic strength dependence of the second-order rate constants for electron transfer from Ru-cyt *c* derivatives to CMPI. The solutions contained 2 mM sodium phosphate, 30–200 mM NaCl, and 10 mM aniline. Ru-13-cyt *c* (▲); Ru-25-cyt *c* (▣); Ru-27-cyt *c* (□); Ru-72-cyt *c* (■). *k* is in units of M^{-1} s^{-1}.

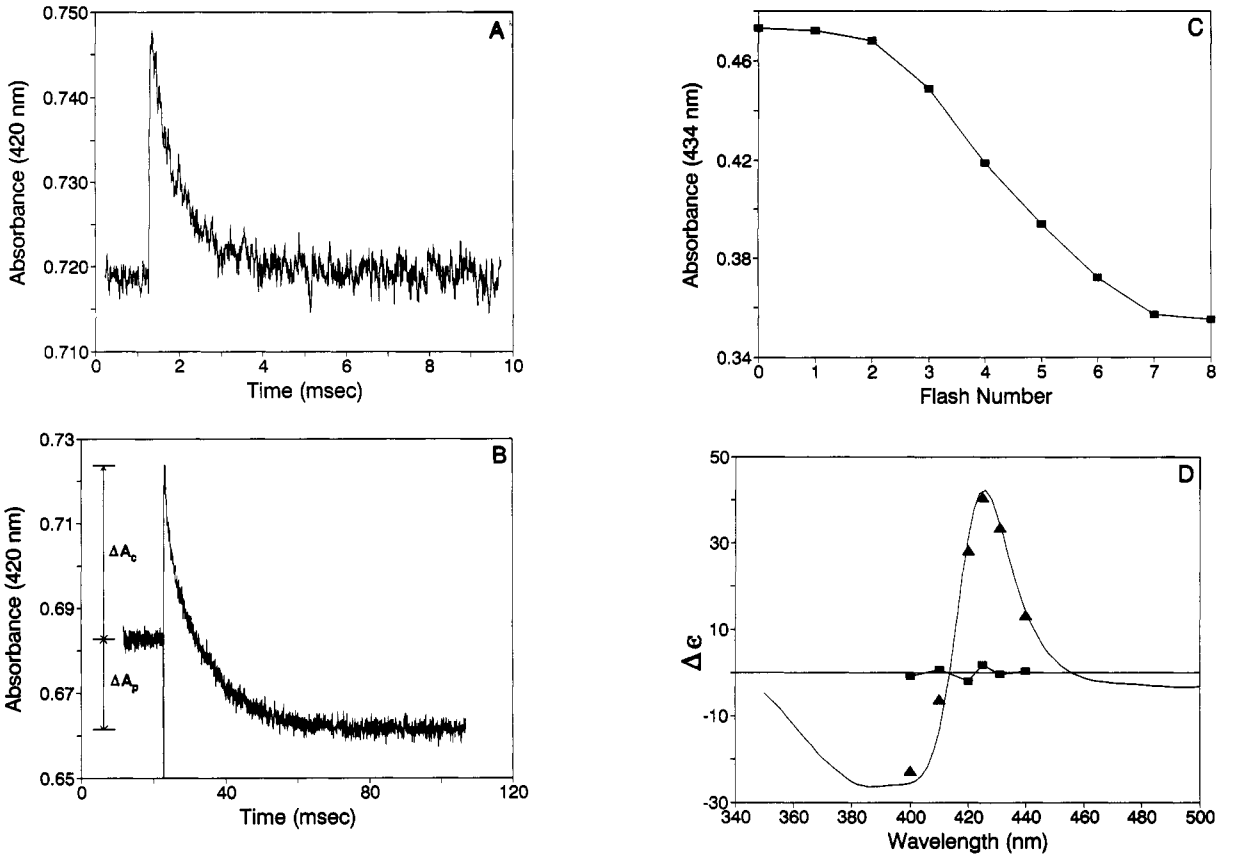


FIGURE 3: Multiple flash electron transfer from Ru-72-cyt c to cytochrome c peroxidase. The solutions contained 5 μM Ru-72-cyt c and 6 μM CMPI in a buffer of 5 mM sodium phosphate, pH 7, and 4 mM EDTA. (A) A 420-nm transient after the first xenon flash. (B) A 420-nm transient following the fourth xenon flash halfway through the titration from CMPI to CcP. (C) Flash titration measured at 434 nm. The solution was subjected to a series of xenon flashes separated by 1 min, and the 434-nm absorbance was measured 1 min after each flash. (D) Wavelength dependence of $\Delta\epsilon_p$ observed after the first flash (■) and after the fourth flash halfway through the titration (▲). $\Delta\epsilon_p$ was calculated from the formula $\Delta\epsilon_p = \Delta\epsilon_c\Delta A_p/\Delta A_c$ mM^{-1} cm^{-1}, where $\Delta\epsilon_c = 44.3$ mM^{-1} cm^{-1} is the extinction coefficient change for cytochrome c at 420 nm, ΔA_c is the absorbance transient observed at 420 nm for photoreduction of cytochrome c, and ΔA_p is the absorbance change due to reduction of cytochrome c peroxidase measured at the indicated wavelength. The solid line is ϵ(CMPI) − ϵ(CcP).

the cytochrome c isosbestic wavelength, 434 nm, and was first observed on the third flash, with a rate constant of k_{obs} = 140 s^{-1}. The value of k_{obs} remained constant through the fifth flash and then decreased because a fraction of the cytochrome c peroxidase was in the nonreactive CcP(III,R) state. The value of k_{obs} for the third through fifth flash was not affected by increasing the total concentration of cytochrome c peroxidase, indicating that it represented an intracomplex electron transfer reaction from Ru-72-cyt c to heme Fe(IV) in CMPII.

The derivatives labeled at lysines 13, 25, and 27 also displayed biphasic intracomplex electron transfer to CMPI at low ionic strength, with k_{etf} values ranging from 5200 s^{-1} for Ru-13-cyt c to 55000 s^{-1} for Ru-27-cyt c (Table I). Increasing the ionic strength decreased the rate of the fast phase and increased the rate of the slow phase (Table I). At higher ionic strengths, the reactions became second-order, and the rate constants decreased with increasing ionic strength (Figure 2). Flash titration experiments in the Soret region indicated that all of the derivatives obeyed the mechanism represented by reactions 1 and 2. The rate constants for reaction 2 were determined by titration studies at 434 nm (Table II).

The electron transfer kinetics of the Ru-cyt c derivatives and native horse cytochrome c were compared using the flash photolysis technique developed by Hazzard et al. (1987, 1988a–c). At high ionic strength (50 μM lumiflavin, 20 mM EDTA, and 25 mM sodium phosphate, pH 7), the k_{obs} values for native cytochrome c and all of the derivatives were linearly dependent on the concentration of CMPI over the range 0.5–5 μM, indicating second-order kinetics. The second-order rate constants shown in Table II indicate that Ru-13-cyt c and Ru-72-cyt c reacted more slowly than native cytochrome c, while Ru-27-cyt c actually reacted faster than native cytochrome c.

Table II: Rate Constants for Electron Transfer from Ru-cyt c Derivatives to Cytochrome c Peroxidase

derivative	k_{et2}[a] (s^{-1})	k[b] (M^{-1} s^{-1})
Ru-13-cyt c	110	6.5×10^6
Ru-25-cyt c	250	3.7×10^7
Ru-27-cyt c	350	9.6×10^7
Ru-72-cyt c	140	1.4×10^7
native horse		5.0×10^7

[a] Samples containing 5 μM Ru-cyt c and 10 μM CMPI in a buffer of 5 mM sodium phosphate, pH 7, and 4 mM EDTA were subjected to a series of xenon flashes as described for Figure 3, and the 434-nm transients for the third through fifth flashes were fit with a single exponential to obtain the rate constant for reaction 2. Error limits are ±10%. [b] Samples containing 2 μM Ru-cyt c and 0.5–5 μM CMPI in a buffer of 25 mM sodium phosphate, pH 7, 20 mM EDTA, and 50 μM lumiflavin were subjected to a weak xenon flash, and k_{obs} was measured at 550 nm as a function of CMPI concentration to obtain the second-order rate constants. Error limits are ±10%.

Discussion

The Ru-cyt c derivatives have several favorable properties for the study of intracomplex electron transfer to cytochrome c peroxidase. First, the reduction of the cytochrome c heme Fe(III) by Ru(II*) is highly selective and well-defined, and the rate is rapid compared to subsequent electron-transfer reactions (Durham et al., 1989). No direct reduction of CMPI

by Ru(II*) was observed in the present experiments, since the amount of CMPI reduced was the same as the amount of ferrocytochrome *c* oxidized. Second, the derivatives have the same heme redox potentials and visible spectra as native cytochrome *c*, indicating a similar conformation in the heme crevice region (Pan et al., 1989). It is expected, however, that ruthenium modification might cause localized changes in conformation, as well as relatively small long-range conformational changes. Two-dimensional NMR studies (500 MHz) of the Ru-cyt *c* derivatives are currently in progress in our laboratory to address this question and will be reported elsewhere. Third, the Ru-lysine bears the same net charge, +1, as a native lysine, and all of the derivatives formed complexes with cytochrome *c* peroxidase at low ionic strength. However, the large size of the ruthenium complex (9-Å radius) will undoubtedly alter the binding orientation of the derivatives modified at lysines 13, 27, and 72, since these lysines are located at the binding domain. These derivatives can, therefore, provide information on how changes in binding orientation affect electron transfer kinetics.

One of the most significant results of the present studies is that each of the Ru-cyt *c* derivatives transferred an electron exclusively to the radical cation in CMPI(IV,R^+) to form CMPII(IV,R). The Ru-cyt *c* derivative then transferred an electron to the oxyferryl heme Fe(IV) in CMPII(IV,R) to form CcP(III,R). This reaction sequence was observed for all of the horse Ru-cyt *c* derivatives at both low and high ionic strength, as well as for the yeast iso-1-cytochrome *c* derivative labeled with $Ru(bpy)_2$(dimethylbipyridine) at Cys-102 on the back of the molecule (Geren et al., 1991). The reaction sequence 1, 2 was observed during equilibrium titration with ferrocyanide or ferrocytochrome *c* at pH values of 7 and above (Coulson et al., 1971). In contrast, Summers and Erman (1988) found in stopped-flow studies at low ionic strength that native horse cytochrome *c* reduced the Fe(IV) site in CMPI(IV,R^+) to form CMPII(III,R^+). They reported that the radical site was not reduced by cytochrome *c* and proposed a mechanism in which CMPII(III,R^+) was converted to the oxyferryl heme form CMPII(IV,R) before reaction with cytochrome *c* could occur.

Another surprising result of the present studies is that all of the derivatives displayed biphasic kinetics for electron transfer to the radical site in CMPI at low ionic strength. The rate constants were independent of the concentration of CMPI, indicating that both phases represented intracomplex reactions. The amplitude of the fast phase increased as the ratio of CMPI to Ru-72-cyt *c* increased to 1.0 and then remained constant (Figure 1D). This suggests that the two phases represent alternative binding orientations for Ru-72-cyt *c* with an overall stoichiometry of 1:1. It is unlikely that the two phases are due to the simultaneous binding of two molecules of Ru-72-cyt *c* to CMPI, since in that case the kinetic parameters would be expected to reach a plateau at a ratio of 0.5 CMPI to Ru-72-cyt *c* rather than 1.0 as observed. Steady-state kinetics and equilibrium binding studies have suggested the presence of multiple binding sites on cytochrome *c* peroxidase (Kang et al., 1977, 1978; Kornblatt & English, 1986). However, Vitello and Erman (1987) found that horse cytochrome *c* bound to porphyrin cytochrome *c* peroxidase with a stoichiometry of 1:1, while Kim et al. (1990) proposed that a single-binding-site, multiple-pathway mechanism is more compatible with the steady-state kinetics than a two-binding-site mechanism. Wallin et al. (1991) have recently observed three kinetic phases for the reaction between horse ferrocytochrome *c* and the zinc porphyrin radical cation in zinc cytochrome *c* peroxidase at low ionic strength. The rate constants were 2420, 38, and 8.8 s^{-1} with relative amplitudes of 0.76, 0.19, and 0.05. They proposed that the three phases represented dynamically interconverting conformational substates. Brownian dynamics simulations have identified two possible binding sites on cytochrome *c* proxidase in addition to the major site represented by the Poulos and Kraut model (Northrup et al., 1987, 1988).

The effect of ionic strength on the kinetics of Ru-72-cyt *c* suggests the possibility of dynamic interconversion between the "fast" and "slow" binding orientations. However, ionic strength could affect the geometries of the two orientations as well as the rate of interconversion between them. At low ionic strength the binding interaction will be strong, and interconversion between the two orientations is expected to be slow. As the ionic strength is increased, faster interconversion is expected, consistent with the observed increase in the rate of the slow phase and decrease in the rate of the fast phase. At higher ionic strength the rate of interconversion is expected to become rapid compared to the rate of electron transfer. This is consistent with the observation of a single intramolecular rate constant of 2800 s^{-1} for Ru-72-cyt *c* at 40 mM ionic strength. Brownian dynamics simulations at still higher ionic strength (0.1 M) indicate that the reaction occurs through a large ensemble of encounter complexes (Northrup et al., 1987, 1988).

The 8-fold increase in the rate of the slow phase of Ru-72-cyt *c* with increasing ionic strength is particularly striking. It is possible that the binding orientation responsible for the slow phase is inactive in electron transfer, and the observed rate constant is governed by the rate of conversion to the fast orientation. This would be an example of "conformational gating", which has been invoked to explain a number of observations for the reaction between cytochrome *c* and cytochrome *c* peroxidase (Hazzard et al., 1988a,b; Cheung & English, 1988; Hoffman & Ratner, 1987). Indeed, the ionic strength dependence of the slow phase of Ru-72-cyt *c* closely resembles that for the single intracomplex reaction observed for native horse cytochrome *c* by flavin flash photolysis. The rate constant increased from 750 s^{-1} at 8 mM ionic strength to 3220 s^{-1} at 30 mM ionic strength (Hazzard et al., 1988b).

The second-order rate constants measured by flavin flash photolysis provide a useful comparison of the reactivities of the different Ru-cyt *c* derivatives with native horse cytochrome *c* (Table II). Ru-25-cyt *c* had nearly the same second-order rate constant as native horse cytochrome *c*, consistent with other evidence that lysine 25 is not directly involved in binding cytochrome *c* peroxidase (Poulos & Kraut, 1980; Kang et al., 1978; Smith & Millett, 1980). The decreased second-order rate constants for the Lys 13 and Lys 72 derivatives were expected in view of the proposed involvement of these residues in binding cytochrome *c* peroxidase according to the Poulos–Kraut model. The large intracomplex rate constants observed for these derivatives at low ionic strength indicate that stable complexes with favorable geometries for electron transfer can be formed that accommodate the bulky ruthenium group. This is a remarkable example of the plasticity of biological electron transfer reactions. The 2-fold increase in the second-order rate constant for the Ru-27-cyt *c* derivative compared to native cytochrome *c* was unexpected, since this residue was originally proposed to interact with Asp 79 of cytochrome *c* peroxidase. However, in the revised model Lys 27 is not actually in contact with Asp 79 but is still close to the interface between the two proteins (Finzel et al., 1984). Apparently, the ruthenium complex at this residue either increases the binding strength or allows more favorable encounter complexes to be formed.

The remarkably large intracomplex rate constant observed for this derivative at low ionic strength suggests that a very favorable complex can indeed be formed. In addition, higher ionic strengths were needed to reach second-order kinetics than for the other derivatives (Figure 2), indicating that this derivative bound more tightly to cytochrome *c* peroxidase.

All of the derivatives had much larger rate constants for electron transfer to the radical cation on Trp 191 than to the oxyferryl heme Fe(IV). This could be due to a lower reorganization energy for the radical or to better electronic coupling. Although the rate constants for the Ru-cyt *c* derivatives are considerably larger than those measured for native cytochrome *c* by the stopped-flow or flavin flash photolysis techniques (Summers & Erman, 1988; Hazzard et al., 1988a), they are comparable to rate constants for the reaction between ferrocytochrome *c* and the radical cation of zinc porphyrin cytochrome *c* peroxidase (Everest et al., 1991; Wallin et al., 1991). The kinetics of the present system are also reminiscent of the reaction of cytochrome c_2 with the photooxidized reaction center from *Rhodobacter sphaeroides*, which displayed biphasic kinetics with k_{et} values of 1.2×10^6 s^{-1} and 1.5×10^4 s^{-1} (Overfield et al., 1979; Long et al., 1989).

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