Intracomplex Electron Transfer between Ruthenium-65–Cytochrome b_5 and Position-82 Variants of Yeast Iso-1-cytochrome c[†]

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Received February 22, 1993; Revised Manuscript Received April 29, 1993

ABSTRACT: We tested the idea that the aromatic ring on the invariant residue Phe-82 in cytochrome c acts as an electron-transfer bridge between cytochrome c and cytochrome b_5. Ru-65–cyt b_5 was prepared by labeling the single sulfhydryl group on T65C cytochrome b_5 with [4-(bromomethyl)-4′-methylbipyridine][bis(bipyridine)] ruthenium^{2+} as previously described [Willie, A., Stayton, P. S., Sligar, S. G., Durham, B., & Millett, F. (1992) *Biochemistry 31*, 7237–7242]. Laser excitation of the complex formed between Ru-65–cyt b_5 and *Saccharomyces cerevisiae* iso-1-cytochrome c at low ionic strength results in rapid electron transfer from the excited-state Ru(II*) to the heme group of Ru-65–cyt b_5 followed by biphasic electron transfer to the heme group of cytochrome c with rate constants of $(1.0 \pm 0.2) \times 10^5$ s^{-1} and $(2.0 \pm 0.4) \times 10^4$ s^{-1}. Variants of iso-1-cytochrome c substituted at Phe-82 with Tyr, Gly, Leu, and Ile have fast-phase rate constants of 0.4, 1.9, 2.1, and 2.0×10^5 s^{-1} and slow-phase rate constants of 5.3, 3.5, 2.4, and 2.0×10^3 s^{-1}, respectively. Increasing the ionic strength to 50 mM results in single-phase intracomplex electron transfer with rate constants of 3.8, 3.1, 3.0, 5.0, and 4.5×10^4 s^{-1} for the wild-type, Tyr, Gly, Leu, and Ile variants, respectively. These results demonstrate that an aromatic side chain at residue 82 is not needed for rapid electron transfer with cytochrome b_5. Furthermore, two conformational forms of the complex are present at low ionic strength with fast and slow electron-transfer rates. When the ionic strength is increased to 50 mM, interconversion between the two forms of the complex becomes rapid, resulting in a single phase for electron transfer with a rate constant that is intermediate between the slow and fast rates. At still higher ionic strength the complex dissociates, and second-order kinetics are observed.

The reaction between cytochrome b_5 and cytochrome c has become a paradigm for biological electron transfer. Ferrocytochrome b_5 rapidly reduces ferricytochrome c in a reaction that proceeds through a bimolecular complex stabilized by electrostatic interactions (Strittmatter, 1964; Ng et al., 1977; Stonehuerner et al., 1979). A molecular model for this complex was first constructed by Salemme (1976) using the X-ray crystal structures of the individual proteins (Takano & Dickerson, 1981; Argos & Mathews, 1975). In this model, the complex is stabilized by four charge-pair interactions between amino groups on cytochrome c Lys-13, -27, -72, and -79 and carboxylate groups on Glu-48, Glu-44, Asp-60, and the exposed heme propionate of cytochrome b_5, respectively. Chemical modification and site-directed mutagenesis studies have resulted in the characterization of these four charge-pair interactions and have also indicated that one or two additional charge-pairs are involved in complex formation (Ng et al., 1977; Stonehuerner et al., 1979; Smith et al., 1980; Mauk et al., 1982, 1986; Reid et al., 1984; Eltis et al., 1988; Rodgers et al., 1988; Rodgers & Sligar, 1991). The electrostatic interaction between the two proteins has been further characterized by pH dependence and proton linkage studies (Mauk et al., 1991). NMR and molecular dynamics studies have indicated that the complex is not static, as in the original Salemme model, but rather exists in a dynamic equilibrium between several different conformations (Ely & Moore, 1983; Burch et al., 1990; Whitford et al., 1990; Wendoloski et al., 1987).

The electron-transfer reaction between ferrocytochrome b_5 and ferricytochrome c has been studied using a number of different techniques. Stopped-flow studies indicated that the reaction is second-order above 0.1 M ionic strength and that the rate constant rapidly decreases with increasing ionic strength (Strittmatter, 1964; Eltis et al., 1991). McLendon and Miller (1985) used pulse radiolysis to measure a rate constant of 1600 ± 700 s^{-1} for intracomplex electron transfer at low ionic strength. Concar et al. (1991) and Whitford et al. (1991) have used nuclear magnetic resonance saturation transfer to measure electron transfer in the reverse direction, reporting a rate constant of 1 s^{-1}. Very recently, Meyer et al. (1993) studied electron transfer from ferrocytochrome b_5 to ferricytochrome c in a 1:1 complex with ferricytochrome b_5 and reported that the intracomplex rate constant is greater than 10^4 s^{-1}.

We have introduced a new method to study electron transfer that involves photoexcitation of a covalently attached tris(bipyridine)ruthenium complex (Pan et al., 1988, 1990; Durham et al., 1989; Geren et al., 1991; Hahm et al., 1992). Willie et al. (1992) carried out the *de novo* design and synthesis of Ru-65–cyt b_5,[1] in which the single sulfhydryl group on T65C cytochrome b_5 is labeled with [4-(bromomethyl)-4′-methylbipyridine][bis(bipyridine)]ruthenium^{2+}. The ruthe-

[†] This work was supported by NIH Grants GM20488 (F.M. and B.D.), GM33775 (S.S.), GM31756 (S.S.), and GM42501 (G.J.P.). S.H.W. was supported by a Department of Education Fellowship.

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[1] Abbreviations: Ru-65–cyt b_5, T65C cytochrome b_5 labeled with [4-(bromomethyl)-4′-methylbipyridine][bis(bipyridine)]ruthenium^{2+}; MES, 2-(N-morpholino)ethanesulfonic acid; ^{3}*Zn(porph)-cyt c, excited triplet state of Zn-porphyrin-cytochrome c.

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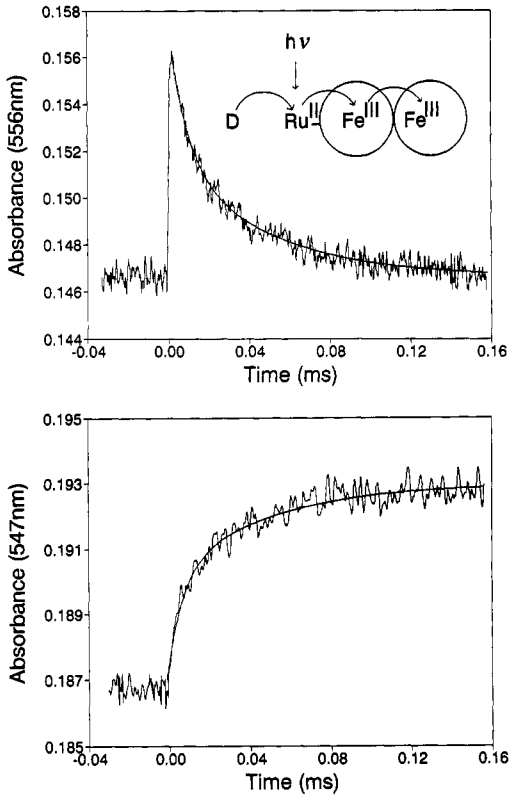

FIGURE 1: Transient kinetics following laser flash excitation of a solution containing 10 μM Ru-65–cyt b_5 and 10 μM of the C102T cytochrome *c* variant in 4 mM *N*-phenylglycine and 2 mM sodium phosphate, pH 7. The 556.5-nm transient (top) was fitted to the biexponential equation $A = A_a \exp(-k_a t) + A_b \exp(-k_b t)$ with $k_a = (1.1 \pm 0.2) \times 10^5\ s^{-1}$, $k_b = (2.1 \pm 0.4) \times 10^4\ s^{-1}$, $A_a = 0.005 \pm 0.001$, and $A_b = 0.004 \pm 0.001$. For the 547-nm transient (bottom), $k_a = (1.2 \pm 0.2) \times 10^5\ s^{-1}$, $k_b = (2.0 \pm 0.4) \times 10^4\ s^{-1}$, $A_a = 0.004 \pm 0.001$, and $A_b = 0.003 \pm 0.001$.

nium complex is only 12 Å from the heme group of cytochrome b_5, but does not interfere with cytochrome *c* binding. Laser excitation of the 1:1 complex formed bewteen Ru-65–cyt b_5 and horse cytochrome *c* at low ionic strength resulted in rapid electron transfer from Ru(II*) to the Ru-65–cyt b_5 heme group followed by biphasic electron transfer to the cytochrome *c* heme group with rate constants of $4 \times 10^5\ s^{-1}$ and $3 \times 10^4\ s^{-1}$. At high ionic strength the reaction is second-order, with the same rate constants as for the reaction between unmodified cytochrome b_5 and cytochrome *c* (Eltis et al., 1991). In the present paper the reactions between Ru-65–cyt b_5 and residue-82 variants of *Saccharomyces cerevisiae* iso-1-cytochrome *c* were studied in order to test the hypothesis of Wendoloski et al. (1987) that the aromatic ring on the invariant residue Phe-82 acts as an electron-transfer bridge between the heme groups of cytochrome b_5 and cytochrome *c*.

EXPERIMENTAL PROCEDURES

Materials. Ru-65–cyt b_5 was prepared and characterized as described by Willie et al. (1992). Variants of yeast iso-1-cytochrome *c* (Pielak et al., 1985; Hilgen & Pielak, 1991) were extracted from yeast and concentrated by batch chromatography as described by Sherman et al. (1968). The variants were then purified on a Pharmacia FPLC system equipped with a UV-N detector and an LCC-500 controller. In all cases, pure protein was obtained after one passage over an S-Sepharose Fast Flow HR 10/10 column using 20 mM sodium phosphate, 1 mM ATP, and a NaCl gradient at pH 7.2 (iso-1-cytochrome *c* elutes at approximately 0.35 M NaCl).

Table I: Rate Constants for Electron Transfer between Ru-65–cyt b_5 and Cytochrome *c* Variants at Low Ionic Strength[a]

cytochrome *c*	pH	k_a (s^{-1})	*f*	k_b (s^{-1})
horse	7	4.0×10^5	0.80	3.0×10^4
iso-1	7	1.0×10^5	0.70	1.8×10^4
iso-1	6	1.0×10^5	0.70	2.0×10^4
C102T	7	1.1×10^5	0.60	2.0×10^4
F82Y;C102T	7	4.2×10^4	0.50	5.0×10^3
F82G;C102T	7	1.9×10^5	0.60	3.5×10^3
F82G;C102T	6	2.0×10^5	0.60	3.0×10^3
F82L;C102T	7	2.1×10^5	0.24	9.4×10^2 (0.34) 2.5×10^2 (0.42)
F82L;C102T	6	1.9×10^5	0.67	2.4×10^3
F82I;C102T	7	2.0×10^5	0.23	4.4×10^2 (0.48) 0.9×10^2 (0.29)
F82I;C102T	6	2.0×10^5	0.58	2.0×10^3

[a] The pH 7 buffer contained 2 mM *N*-phenylglycine and 1 mM sodium phosphate, while the pH 6 buffer contained 2 mM *N*-phenylglycine and 5 mM MES. The rate constants were measured at 25 °C and were independent of concentration over the range 5–20 μM Ru-65–cyt b_5 and cyt *c*. *f* is the fraction of the total amplitude due to the fast phase. The fractional amplitudes of the two slow phases for F82L-cyt *c* and F82I-cyt *c* at pH 7 are given in parentheses. The error of each parameter is ±20%.

Purity was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using Coomassie blue staining. Variants are denoted by their one-letter codes with the wild-type residue listed first, followed by the sequence position and the variant residue. Multiple changes are separated by a semicolon (e.g. F82Y;C102T). The C102T variant is used because removal of the sole cysteine sulfhydryl obviates formation of dimers (Cutler et al., 1987; Betz & Pielak, 1992). Seventy percent of all eukaryotic cytochromes *c*, including horse cytochrome *c*, possess the threonine at position 102. Wild-type yeast cytochrome *c* was obtained from Sigma (type VIIIB) and confirmed to be exclusively iso-1-cytochrome *c* as described by Geren et al. (1991). It was treated with dithiothreitol to reduce any disulfide cross-linked dimers, passed through a Biogel P2 column to remove excess reagent, and stored in the reduced form.

Flash Photolysis Experiments. Transient absorbance measurements were carried out by flash photolysis of 300-μL solutions contained in 1-cm glass semimicrocuvettes. The flash photolysis instrumentation has been previously described by Willie et al. (1992) and Durham et al. (1989). The response rate of the detector was set to be at least 10 times as fast as the fastest phase in the transient. Samples typically contained 1–40 μM Ru-65–cyt b_5, 1–40 μM cytochrome *c*, 2 mM *N*-phenylglycine, and either 1 mM sodium phosphate, pH 7, or 5 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6. The sample also contained a catalytic concentration of cytochrome oxidase (20 nM) to reoxidize cytochrome *c* between laser flashes. Complete visible spectra were obtained for each sample before and after flash photolysis. Single transients were generally adequate for kinetic analysis, but in some cases up to 10 transients were averaged. The transients were analyzed as described by Willie et al. (1992) using the OLIS KINFIT program with a monoexponential equation, the biexponential equation $A = A_a \exp(-k_a t) + A_b \exp(-k_b t)$, or a triexponential equation. The transient was fitted to the equation with the smallest number of exponentials that resulted in a fit with no systematic time dependence for the residuals. The need for biexponential fits was clearly apparent in the low ionic strength transients. Because of signal-to-noise limitations, it was not possible to resolve kinetic phases that had amplitudes less than 10% of the total amplitude. A single sample could be subjected to hundreds of laser flashes with no change in the rate constants of the transient or in the

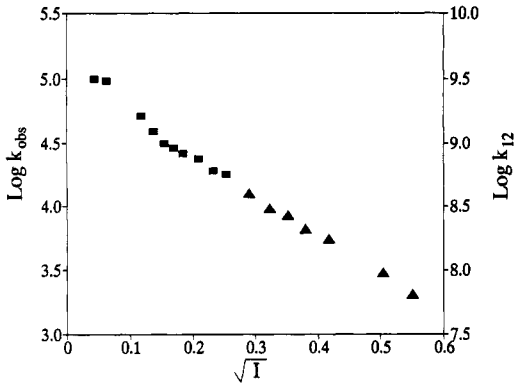

FIGURE 2: Ionic strength dependence of the reaction between Ru-65–cyt b_5 and the C102T cytochrome c variant. The rate constant k_{obs} (s^{-1}) (■) measured using 20 μM Ru-65–cyt b_5 and 20 μM C102T-cyt c is plotted against the left-hand axis. k_{obs} is for the fast phase at low ionic strength and the single phase at ionic strengths above 50 mM. The second-order rate constant k_{12} (M^{-1} s^{-1}) (▲) is plotted against the right-hand axis. The buffer contained 1 mM sodium phosphate, pH 7, 2 mM N-phenylglycine, and 0–500 mM sodium chloride. The horizontal axis is the square root of the ionic strength I (M).

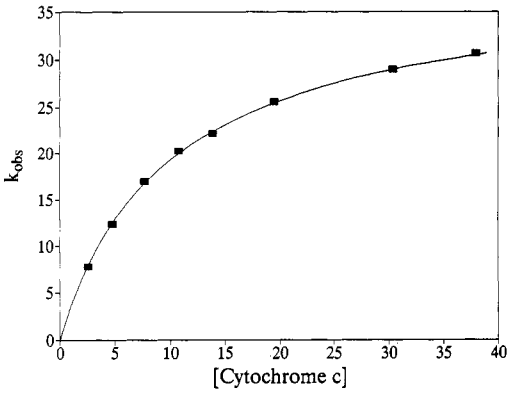

FIGURE 3: Concentration dependence of k_{obs} (in 10^3 s^{-1}) for the reaction between Ru-65–cyt b_5 and iso-1-cytochrome c at 50 mM ionic strength. The pH 7 buffer contained 5 mM N-phenylglycine, 2.5 mM sodium phosphate, and 40 mM NaCl at 25 °C. The smooth curve is the best fit to eq 1 with k_c = (3.8 ± 1.0) × 10^4 s^{-1} and K = 14 ± 3 μM.

absorption spectra, indicating no significant photolysis damage. Similar kinetics were observed using N-phenylglycine, aniline, or EDTA as the sacrificial electron donor, taking into account the contribution of the donor to the total ionic strength.

RESULTS

Photoexcitation of a solution containing Ru-65–cyt b_5 and the C102T variant of iso-1-cytochrome c in low ionic strength buffer (1 mM sodium phosphate, pH 7) resulted in rapid electron transfer from Ru(II*) to heme Fe(III) in Ru-65–cyt b_5, followed by electron transfer to heme Fe(III) in cytochrome c (Figure 1). N-Phenylglycine was used as a sacrificial electron donor, D, to reduce Ru(III) to Ru(II) and prevent the back reaction between Ru(III) and heme Fe(II). The oxidation of Ru-65–cyt b_5 Fe(II) was measured at 556.5 nm (a cytochrome c isosbestic), while the reduction of cytochrome c Fe(III) was measured at 547 nm (a cytochrome b_5 isosbestic). The transients at the two wavelengths are biphasic, each with a fast phase rate constant of (1.1 ± 0.2) × 10^5 s^{-1} and a relative amplitude of 60 ± 10% and a slow-phase rate constant of (2 ± 0.5) × 10^4 s^{-1} and an amplitude of 40 ± 10% (Figure 1; Table I). The amount of cytochrome b_5 oxidized in each phase of the transient is the same as the amount of cytochrome c reduced, within error limits of ±10%. The two rate constants were unchanged as the concentrations of Ru-65–cyt b_5 and cytochrome c were both increased from 5 to 20 μM, consistent with electron transfer within a 1:1 complex with a dissociation constant less than 5 μM. The kinetics were also unchanged when the concentration of cytochrome c was increased from 5 to 20 μM, holding the Ru-65–cyt b_5 concentration fixed at 5 μM. This indicates that no higher-order complexes were formed. The kinetic constants are nearly the same at pH 6 as at pH 7 (Table I). There is no significant difference between the kinetics of wild-type iso-1-cytochrome c and the C102T variant under any conditions (Table I).

As the ionic strength was increased, the observed rate constants decreased and the transients became monophasic (Figure 2). At 50 mM ionic strength k_{obs} had a hyperbolic dependence on the concentration of wild-type iso-1-cytochrome c (Figure 3) and could be fitted to the equation

$$k_{obs} = k_c[\text{cyt } c]/(K + [\text{cyt } c]) \quad (1)$$

with k_c = (3.8 ± 1) × 10^4 s^{-1} and K = 14 ± 3 μM (Table II).

Table II: Rate Constants and Dissociation Constants for the Reactions of Ru-65–cyt b_5 with Cytochrome c Variants at 50 mM Ionic Strength[a]

cytochrome c	pH	k_c (s^{-1})	K (μM)
iso-1	7	3.8 × 10^4	14
F82Y;C102T	7	3.1 × 10^4	16
F82G;C102T	7	1.9 × 10^4	13
F82G;C102T	6	3.5 × 10^4	10
F82L;C102T	7	1.2 × 10^4	9
F82L;C102T	6	5.0 × 10^4	12
F82I;C102T	7	1.2 × 10^4	13
F82I;C102T	6	4.5 × 10^4	11

[a] The pH 7 buffer contained 2.5 mM sodium phosphate, 5 mM N-phenylglycine, and 40 mM NaCl, and the pH 6 buffer contained 5 mM MES, 5 mM N-phenylglycine, and 40 mM NaCl. The observed rate constants, k_{obs}, were fitted to eq 1 as described in Figure 2 to obtain k_c and K. The error in each parameter is ±20%.

Table III: Second-Order Rate Constants for the Reactions between Ru-65–cyt b_5 and Cytochrome c Variants at 106 mM Ionic Strength[a]

cytochrome c	pH	k (M^{-1} s^{-1})
horse	7	3.8 × 10^8
iso-1	7	3.3 × 10^8
C102T	7	3.0 × 10^8
F82Y;C102T	7	3.8 × 10^8
F82G;C102T	7	3.2 × 10^8
F82G;C102T	6	4.1 × 10^8
F82L;C102T	6	3.5 × 10^8
F82I;C102T	6	3.1 × 10^8

[a] The pH 7 buffer contained 2 mM sodium phosphate, 2 mM N-phenylglycine, and 100 mM NaCl, and the pH 6 buffer contained 5 mM MES, 2 mM N-phenylglycine, and 100 mM NaCl. The error limits are ±15%.

At ionic strengths above 100 mM k_{obs} was linearly dependent on the concentration of cytochrome c up to 20 μM, giving the second-order rate constants shown in Figure 2 and Table III. The kinetic behavior of iso-1-cytochrome c is similar to that previously observed for horse cytochrome c, except that the transition between first- and second-order kinetics occurs at a much higher ionic strength. The highest ionic strength at which saturation kinetics could be observed for horse cytochrome c was 10 mM. Under these conditions, the amplitude of the fast phase increased hyperbolically with increasing concentration, while the rate constant of the fast phase remained constant at (3 ± 1) × 10^5 s^{-1} (Figure 4). A dissociation constant of K_d = 11 ± 2 μM was calculated from these data (Figure 4).

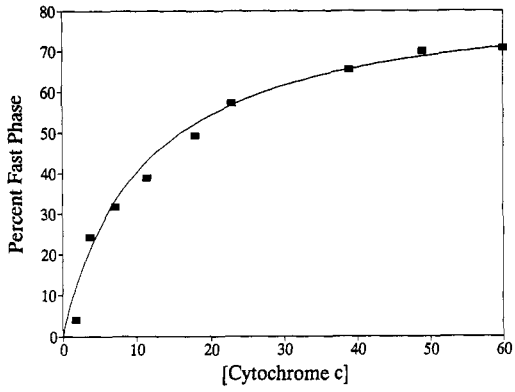

FIGURE 4: Concentration dependence of the reaction between Ru-65–cyt b_5 and horse cytochrome *c* at 10 mM ionic strength. The buffer contained 2 mM sodium phosphate, pH 7, and 6 mM *N*-phenylglycine. The percent amplitude of the fast phase, f_{obs}, was fit to the equation $f_{obs} = f[\text{cyt } c]/(K_d + [\text{cyt } c])$, where K_d is the dissociation constant of the complex and f is the percent of the fast phase at infinite cytochrome *c* concentrations. The smooth curve is the best fit with $f = 80 \pm 10\%$, and $K_d = (11 \pm 1)$ μM. The rate constant of the fast phase remained constant at $(3 \pm 1) \times 10^5$ s^{-1}.

The reactions of Ru-65–cyt b_5 with the F82Y;C102T and F82G;C102T variants have the same overall kinetic behavior as the reaction with wild-type iso-1-cytochrome *c*, but with different kinetic constants. The rate constants k_a and k_b for the F82Y;C102T variant at low ionic strength are both smaller than those of wild-type iso-1-cytochrome *c* (Table I). When the ionic strength was increased to 50 mM, the concentration dependence of k_{obs} obeyed eq 1 with $k_c = (3.1 \pm 0.6) \times 10^4$ s^{-1}, which is significantly faster than the slow-phase k_b observed at low ionic strength (Tables I and II). The fast phase of the reaction of the F82G;C102T variant at low ionic strength is nearly 2-fold faster than that of wild-type yeast cytochrome *c*, while the slow phase is 5-fold slower (Table I). As the ionic strength was increased, the rate constant of the slow phase increased and that of the fast phase decreased until at 50 mM ionic strength a single phase was observed with $k_c = 1.9 \times 10^4$ s^{-1} (Table II). The kinetics of the F82G;C102T variant are similar at pH 6 and pH 7 (Tables I and II).

The kinetics of the F82L;C102T and F82I;C102T variants are similar to each other but significantly different from those of wild-type iso-1-cytochrome *c* at pH 7. At low ionic strength the fast phase of F82L;C102T accounted for only 24% of the reaction, with two slow phases making up the remainder (Table I). Increasing the ionic strength increased the rate of the slow phases dramatically, and at 50 mM a single phase was observed with $k_c = (1.2 \pm 0.3) \times 10^4$ s^{-1} (Table II). Since the pK for the alkaline transition of both the F82L;C102T and the F82I;C102T variant is 7.2, it was thought that one of the slow phases could have arisen from the alkaline form. Therefore, the kinetic studies were repeated at pH 6 where very little alkaline form should be present. The fast phase accounted for over 50% of the reactions of the F82L;C102T and F82I;C102T variants at low ionic strength and pH 6 (Table I). The slow phases of both variants increased with increasing ionic strength, and at 50 mM ionic strength a single phase with $k_c = (5 \pm 1) \times 10^4$ s^{-1} was observed (Table II). All of the variants obey second-order kinetics at ionic strengths above 100 mM, and the second-order rate constants are compared in Table III.

DISCUSSION

The position of the ruthenium complex on the surface of Ru-65–cyt b_5 was designed to promote rapid photoinduced electron transfer from ruthenium to the heme group but not interfere with the binding of redox partners such as cytochrome *c* (Willie et al., 1992). There is a direct 12-covalent-bond pathway for electron transfer between the bipyridine ligand of the ruthenium complex and the imidazole group of His-63 which ligands the heme group iron. We have recently measured the rate constant for photoinduced electron transfer from Ru(II*) to the heme group Fe(III) to be $(1.4 \pm 0.3) \times 10^7$ s^{-1} and the rate constant for the thermal back reaction from Fe(II) to Ru(III) to be $(6.0 \pm 1) \times 10^6$ s^{-1} (Scott et al., 1993). These values are consistent with theoretical predictions based on the effect of distance, pathway, and driving force on electron transfer in ruthenium-labeled proteins (Jacobs et al., 1991; Meade et al., 1989; Therien et al., 1990; Beratan et al., 1990; Durham et al., 1989; Chang et al., 1991).

The ruthenium complex on Ru-65–cyt b_5 is located on a surface remote from the proposed binding domain for cytochrome *c* and therefore should not sterically interfere with cytochrome *c* binding (Willie et al., 1992). Willie et al. (1992) found that the dissociation constant of the 1:1 complex between Ru-65–cyt b_5 and horse cytochrome *c* is less than 5 μM at 2 mM ionic strength, increasing to greater than 20 μM at 20 mM ionic strength. The concentration-dependence data shown in Figure 4 indicate that the dissociation constant is 11 ± 2 μM at 10 mM ionic strength. These values are comparable to the dissociation constant of the complex between unmodified bovine liver cytochrome b_5 and horse cytochrome *c*, which is 0.25 μM at 1 mM ionic strength and 12.5 μM at 10 mM ionic strength (Mauk et al., 1982). Willie et al. (1992) have also shown that the ionic strength dependence of the second-order rate constant of the reaction of Ru-65–cyt b_5 with horse cytochrome *c* is nearly the same as for bovine cytochrome b_5 measured by stopped-flow (Eltis et al., 1991). Taken together, these data indicate that there are no significant conformational differences between Ru-65–cyt b_5 and unmodified cytochrome b_5 that affect either the dissociation constant of the complex at low ionic strength or the second-order rate constant at high ionic strength. The 2+ charge on the ruthenium complex is evidently too far away from the interaction domain to affect these parameters. The visible spectrum of the photoreduced heme in Ru-65–cyt b_5 is the same as that of chemically reduced cytochrome b_5, indicating that the electronic state is the same (Willie et al., 1992; Scott et al., 1993).

The biphasic kinetics observed for the reaction of Ru-65–cyt b_5 with all of the variants of cytochrome *c* at low ionic strength indicates that there are at least two different conformational forms of the complex. This is consistent with previous studies indicating that the cytochrome b_5–cytochrome *c* complex may exist in dynamic equilibrium between several different forms (Mauk et al., 1986; Ely & Moore, 1983; Burch et al., 1990; Whitford et al., 1990; Wendoloski et al., 1987). One possible mechanism for the photoinduced electron-transfer reaction is shown in Scheme I, where f and s stand for the "fast" and "slow" forms of the complex. Since a closed algebraic solution of Scheme I does not exist, numerical integration methods (Strickland et al., 1975) were used to obtain the complete time course of the reaction without making any assumptions (e.g., steady-state or rapid equilibrium). A wide range of rate constants were then explored to obtain the best fits to the experimental transients. The concentration independence of the rate constants at low ionic strength could only be fit by assuming that the dissociation constant of the complex was small ($k_2/k_1 \approx k_6/k_5 < 2$ μM) and that the rates of interconversion between the fast and slow forms of the complex were slow compared to k_{etf} and k_{ets}. Under these

Scheme I

$$\begin{array}{ccccc} b_5^{o}\text{-}c_f^{o} & \xrightarrow{h\nu} & b_5^{r}\text{-}c_f^{o} & \xrightarrow{k_{etf}} & b_5^{o}\text{-}c_f^{r} \\ k_3 \uparrow\downarrow k_4 & & k_7 \uparrow\downarrow k_8 & & \uparrow\downarrow \\ b_5^{o}\text{-}c_s^{o} & \xrightarrow{h\nu} & b_5^{r}\text{-}c_s^{o} & \xrightarrow{k_{ets}} & b_5^{o}\text{-}c_s^{r} \\ k_1 \uparrow\downarrow k_2 & & k_5 \uparrow\downarrow k_6 & & \uparrow\downarrow \\ b_5^{o} + c^{o} & \xrightarrow{h\nu} & b_5^{r} + c^{o} & & b_5^{o} + c^{r} \end{array}$$

conditions, k_{etf} would be equal to the experimental rate constant k_a, and k_{ets} would be equal to k_b. An alternative explanation of the slow phase is that it involves a form of the complex that is inactive in electron transfer ($k_{ets} = 0$), in which case the observed rate constant would represent the rate of conversion from the inactive complex to the active complex in a conformational gating mechanism ($k_b = k_7$; Hoffman & Ratner, 1987).

The rate constant k_a of the fast phase of the reaction of wild-type iso-1-cytochrome c at low ionic strength is about 4-fold less than that of horse cytochrome c. This presumably reflects subtle differences in the orientation of the two complexes. Although the charged residues surrounding the heme crevice of these two cytochromes c are very similar, there are differences in other residues in this region. As the ionic strength increases, the rate constant of the fast phase for wild-type iso-1-cytochrome c decreases until a single phase is observed at 50 mM ionic strength. The hyperbolic dependence of k_{obs} on concentration (Figure 3) can be fit by Scheme I if a rapid-equilibrium assumption is made, that is, if the rate constants for complex dissociation (k_6) and for interconversion between the slow and fast forms (k_7, k_8) are large compared to k_{etf} and k_{ets}. Increasing ionic strength is expected to weaken the electrostatic interactions that stabilize each form of the complex, increasing the values of k_6, k_7, and k_8. Under this rapid equilibrium assumption Scheme I obeys eq 1, with K representing a dissociation constant, and k_c an effective electron-transfer rate constant. The value of k_c, 3.8×10^4 s^{-1}, is intermediate between k_{etf} and k_{ets}, suggesting that electron transfer proceeds through a complex involving rapid equilibrium between the two conformations. Of course, the slow and fast conformations themselves could change with increasing ionic strength, changing k_{etf} and k_{ets} as well as k_7 and k_8. Because of this complication, we do not report specific fits of Scheme I to the data at 50 mM ionic strength. The K value for the reaction between Ru-65–cyt b_5 and wild-type iso-1-cytochrome c is 14 μM at 50 mM ionic strength and pH 7. By comparison, the equilibrium dissociation constant, K_d, is 14 μM for the complex between unmodified bovine liver cytochrome b_5 and iso-1-cytochrome c at 25 mM ionic strength and pH 6.7 (Mauk et al., 1991). The agreement between the two values is reasonable considering the differences in pH and ionic strength as well as the fact that K is a kinetic constant representing the interaction between reduced Ru-65–cyt b_5 and oxidized cytochrome c, while K_d is a dissociation constant for the fully oxidized complex. Much higher ionic strength is needed to dissociate the complex between Ru-65–cyt b_5 and iso-1-cytochrome c than that involving horse cytochrome c. Mauk et al. (1991) have also observed this behavior in their equilibrium binding studies of unmodified cytochrome b_5 and suggest that hydrophobic interactions are more important for the yeast cytochrome c. Similar observations have been made for the interaction of iso-1-cytochrome c with cytochrome c peroxidase (Geren et al., 1991; Hahm et al., 1992).

The rate constant k_a for the fast phase of the F82G;C102T variant is 2-fold greater than that of wild-type iso-1-cytochrome c at low ionic strength, while the slow-phase rate constant k_b is 5-fold smaller (Table I). As the ionic strength increases, the fast-phase rate constant decreases and the slow-phase rate constant increases until a single phase is present at 50 mM ionic strength (Table II). This behavior is consistent with Scheme I if both the dissociation and the interconversion rate constant increase with increasing ionic strength to satisfy the rapid equilibrium assumption at 50 mM ionic strength. The very dramatic increase in the rate constant of the slow phase with increasing ionic strength suggests that this phase might be controlled by conformational gating, with $k_b = k_7$. The rate constant k_a for the F82Y;C102T variant is over 2-fold less than that of wild-type iso-1-cytochrome c, while the rate constant k_c of the single phase at 50 mM ionic strength is nearly the same (Tables I and II). It is interesting that at 50 mM ionic strength, k_c of wild-type iso-1-cytochrome c is closer to that of k_b, and k_c of F82G-cyt c is intermediate between k_a and k_b, while k_c of F82Y-cyt c is closer to that of k_a. It appears that the side chain at residue 82 affects not only the rate constants at low ionic strength but also the dynamic equilibrium between the different forms of the complex at 50 mM ionic strength.

The kinetics of the F82L;C102T and F82I;C102T variants at pH 7 are complicated by the fact that the pK_a for the alkaline transition of both these variants is 7.2 (Pearce et al., 1989). Barker et al. (1992) have recently found that the redox potential of the alkaline form of the F82I;C102T variant is −160 mV. Thus, the alkaline form present in solution at pH 7 will not be able to accept electrons from Ru-65–cyt b_5. This accounts for the fact that the relative amplitude of the fast phase of F82I;C102T and F82L;C102T at low ionic strength is only 25%, and two slow phases are present. The amplitude of the fast phase was not affected by adding an excess of either variant, indicating that the alkaline form binds strongly to Ru-65–cyt b_5 but does not react. One of the slow phases probably represents a process involving dissociation of the alkaline form from photoreduced Ru-65–cyt b_5 followed by the binding and reaction of the native form. Increasing the ionic strength increased the rates of the slow phases until a single phase was observed at 50 mM ionic strength (Table II). This is probably due in part to an increase in the rate of dissociation of the alkaline form from the complex until rapid equilibrium conditions are satisfied for all forms of the complex. At pH 6 and low ionic strength the fraction of the fast phase of the F82L;C102T variant increased to 67%, consistent with the absence of the alkaline form. Increasing the ionic strength to 50 mM at pH 6 leads to a single phase with $k_c = 5 \times 10^4$ s^{-1}, consistent with rapid equilibrium conditions for Scheme I. The kinetics of the F82L;C102T, F82I;C102T, and F82G;C102T variants are quite similar to one another at pH 6 (Tables I and II). The kinetics of wild-type iso-1-cytochrome c and the F82G;C102T variant are nearly the same at pH 6 and 7 under low ionic strength conditions, indicating that there are no significant differences in the forms of these complexes at the two pH values.

The position-82 variants of iso-1-cytochrome c have been extensively characterized by a number of different techniques, as reviewed by Mauk (1991). The large negative Soret–Cotton effect in the circular dichroism spectrum of wild-type iso-1-cytochrome c is retained in the F82Y;C102T variant but is greatly decreased in the nonaromatic position-82 variants

(Rafferty et al., 1990). Nuclear magnetic resonance studies have indicated that Tyr-82 occupies the same position in the heme crevice of the F82Y;C102T variant as Phe-82 in wild-type iso-1-cytochrome *c*, but Leu-85 is rotated away from Tyr-82 to make room for the hydroxyl group (Pielak et al., 1988). This subtle structural change could lead to the decreased fast-phase rate constant k_a of the F82Y;C102T variant relative to wild-type iso-1-cytochrome *c*. The X-ray crystal structure of the F82G;C102T variant revealed that the peptide segment which contains Gly-82 collapses toward the heme group to fill in the space occupied by Phe-82 in wild-type iso-1-cytochrome *c* (Louie & Brayer, 1989). This change in the heme crevice structure could account for the larger k_a rate constant of the F82G;C102T variant relative to wild type. The fact that the F82G;C102T, F82L;C102T, and F82I;C102T variants have similar kinetics at pH 6 is consistent with a number of studies showing that the spectroscopic and electrochemical properties of these variants are similar to one another (Mauk, 1991).

The rate constant for the fast-phase intracomplex reaction of Ru-65–cyt b_5 with horse cytochrome *c* is more than 2 orders of magnitude larger than the value of 1600 ± 700 s^{-1} measured for the reaction between bovine cytochrome b_5 and horse cytochrome *c* by pulse radiolysis (McLendon & Miller, 1985) or by acridine flash photolysis (Qin et al., 1991). Similarly, the fast-phase rate constant for the reaction of Ru-65–cyt b_5 with wild-type iso-1-cytochrome *c*, 1×10^5 s^{-1}, is much larger than the value of 900 s^{-1} for the reaction between bovine cytochrome b_5 and iso-1-cytochrome *c* measured by pulse radiolysis (McLendon, 1988). It is possible that these differences could arise from changes in the conformation of the complex due to the ruthenium group at Cys-65. However, this appears unlikely because both the dissociation constant at low ionic strength and the second-order rate constant at high ionic strength of the Ru-65–cyt b_5–horse cytochrome *c* pair are the same as the corresponding parameters for the unmodified bovine cytochrome b_5–horse cytochrome *c* pair (Mauk et al., 1982; Eltis et al., 1991; Willie et al., 1992). Another possibility is that the soluble reductants used in the pulse radiolysis and flash photolysis experiments are not able to reduce cytochrome b_5 in the active fast-reacting form of the complex, but only in a less active or inactive form of the complex where the heme is accessible to the solution. Thus, the observed rate constant for electron transfer could be that of the less active form of the complex or could even represent the rate of conversion from the inactive complex to the active complex in a conformational gating mechanism (Hoffman & Ratner, 1987; Kostić, 1991). Willie et al. (1992) did not observe such a slow phase in the reaction between Ru-65–cyt b_5 and horse cytochrome *c* at low ionic strength, but because of signal-to-noise limitations, it would not have been possible to detect this phase if its amplitude was less than 10% of the total. The rate constant of the fast phase of the reaction between Ru-65–cyt b_5 and horse cytochrome *c*, 4×10^5 s^{-1}, is essentially the same as the rate constant for the reaction between native cytochrome b_5 and ^{3}*Zn(porph)-cyt *c*, 5×10^5 s^{-1}, even though the driving force of the former reaction is much lower (0.25 V vs 0.76 V) (McLendon & Miller, 1985). This behavior would be expected if the reorganization energy was about 0.5 V rather than 0.8 V as proposed by McLendon and Miller (1985). However, differences in conformation between the two systems cannot be ruled out, so it is not possible to evaluate the reorganization energy.

Concar et al. (1991) and Whitford et al. (1991) have used nuclear magnetic resonance saturation transfer to measure intracomplex rate constants of 1.4, 0.5, and 0.7 s^{-1} for reverse electron transfer between unmodified ferricytochrome b_5 and the ferro forms of horse cytochrome *c*, the C102T variant, and the F82G;C102T variant, respectively. A rate constant for the forward reaction, k_f, can be estimated from the formula $k_f = k_b K_{eq}$. The equilibrium constant of the reaction, K_{eq}, was calculated to have a value of 1.7×10^4 using redox potentials of 260 and 10 mV for cytochrome *c* and cytochrome b_5, respectively (Barker & Mauk, 1992; Reid et al., 1982). This results in k_f values of 2.4×10^4, 8.6×10^3, and 1.2×10^4 s^{-1} for the reactions involving horse cytochrome *c*, the C102T variant, and the F82G;C102 variant, respectively. However, saturation transfer cannot detect multiple forms of the complex. Furthermore, the fast phase of the reaction of Ru-65–cyt b_5 with horse cytochrome *c* has $k_f = 4 \times 10^5$ s^{-1}, and thus a separate resonance would not be present for the Met-80 methyl protons in oxidized cytochrome *c* under the conditions of the saturation-transfer experiment (Concar et al., 1991), since fast-exchange conditions would apply. No saturation transfer from oxidized to reduced cytochrome *c* would be observed for this form of the complex, and only the slow form of the complex would contribute to the observed saturation transfer. Similar arguments apply for the reactions involving the variant iso-1-cytochromes *c*.

Meyer et al. (1993) have recently studied the reaction between cytochrome b_5 and horse cytochrome *c* by flash photolysis in the presence of 5-deazariboflavin. They concluded that the rate of electron transfer within a 1:1 complex of the two proteins at 5 mM ionic strength was greater than the rate of cytochrome b_5 reduction by 5-deazariboflavin semiquinone, which was about 6000 s^{-1}. The reaction between ferrocytochrome b_5 and ferricytochrome *c* in a 1:1 complex with ferricytochrome b_5 was found to obey second-order kinetics, with a rate constant of 2.6×10^8 M^{-1} s^{-1} at 5 mM ionic strength. The rate constant increased to 1.3×10^9 M^{-1} s^{-1} at 35 mM ionic strength, and the first-order rate constant within the transient ternery complex was estimated to be greater than 17 000 s^{-1}. These results show that the conformational flexibility of the 1:1 complex increases with increasing ionic strength. The results of Meyer et al. (1993) are thus consistent with the present results using flash photolysis of Ru-65–cyt b_5.

CONCLUSIONS

These studies show that an aromatic side chain at residue 82 of cytochrome *c* is not needed for rapid electron transfer with cytochrome b_5. This argues against the proposal of Wendoloski et al. (1987) that the phenyl group of Phe-82 acts as an electron-transfer bridge between the two hemes in the complex. Previous studies have shown that an aromatic residue at postion 82 is not required for the reactions of cytochrome *c* with $Fe(EDTA)^{2-}$ (Rafferty et al., 1990), cytochrome *c* peroxidase (Concar et al., 1991; Pielak et al., 1985), zinc cytochrome *c* peroxidase (Everest et al., 1991), and cytochrome *c* oxidase (Pielak et al., 1985; Michel et al., 1989; Inglis et al., 1991; Hazzard et al., 1992). However, the rate constants observed in the present system are more than an order of magnitude larger than in other systems, providing a more critical test of the role of Phe-82.

It is interesting that the three iso-1-cytochrome *c* variants with a nonaromatic side chain at residue 82 actually have larger fast-phase rate constants k_a for electron transfer with Ru-65–cyt b_5 than native iso-1-cytochrome *c*. The structural changes in the heme crevice that accompany the substitution of Phe-82 with Gly, Leu, or Ile (Mauk, 1991) must actually

improve the pathway for electron transfer. These substitutions also greatly decrease the slow-phase rate constant k_b, indicating that the conformation responsible for this phase is very poorly aligned for electron transfer. The increase in the slow-phase rate constant with increasing ionic strength is the largest for these variants, giving the most dramatic evidence for conformational gating.

ACKNOWLEDGMENT

We thank Dr. Terrence Meyer for communicating results prior to publication.

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