Genetic Engineering of Redox Donor Sites: Measurement of Intracomplex Electron Transfer between Ruthenium-65–Cytochrome b_5 and Cytochrome c[†]

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ABSTRACT: The de novo design and synthesis of ruthenium-labeled cytochrome b_5 that is optimized for the measurement of intracomplex electron transfer to cytochrome c are described. A single cysteine was substituted for Thr-65 of rat liver cytochrome b_5 by recombinant DNA techniques [Stayton, P. S., Fisher, M. T., & Sligar, S. G. (1988) *J. Biol. Chem. 263*, 13544–13548]. The single sulfhydryl group on T65C cytochrome b_5 was then labeled with [4-(bromomethyl)-4′-methylbipyridine](bisbipyridine)ruthenium^{2+} to form Ru-65–cyt b_5. The ruthenium group at Cys-65 is only 12 Å from the heme group of cytochrome b_5 but is not located at the binding site for cytochrome c. Laser excitation of the complex between Ru-65–cyt b_5 and cytochrome c results in electron transfer from the excited state Ru(II*) to the heme group of Ru-65–cyt b_5 with a rate constant greater than 10^6 s^{-1}. Subsequent electron transfer from the heme group of Ru-65–cyt b_5 to the heme group of cytochrome c is biphasic, with a fast-phase rate constant of $(4 \pm 1) \times 10^5$ s^{-1} and a slow-phase rate constant of $(3 \pm 1) \times 10^4$ s^{-1}. This suggests that the complex can assume two different conformations with different electron-transfer properties. The reaction becomes monophasic and the rate constant decreases as the ionic strength is increased, indicating dissociation of the complex. The ionic strength dependence of the second-order rate constant is nearly the same as for the reaction between native cytochrome b_5 and cytochrome c [Eltis, L. D., Herbert, R. G., Barker, P. D., Mauk, A. G., & Northrup, S. H. (1991) *Biochemistry 30*, 3663–3674], indicating that the same electrostatic interactions are involved in both reactions.

Interprotein electron-transfer reactions often involve the transient formation of a complex between two redox proteins. However, only a limited number of techniques are available to measure the actual rate of electron transfer within such complexes. These include pulse radiolysis (McLendon & Miller, 1985) and flash photolysis utilizing flavins (Hazzard et al., 1987, 1988a,b; Qin et al., 1991) or zinc-substituted heme proteins (McLendon & Miller, 1985; Liang et al., 1988; Wallin et al., 1991; Everest et al., 1991). We have recently introduced a new method to study electron transfer that utilizes a covalently attached tris(bipyridine)ruthenium complex (Pan et al., 1988, 1990; Durham et al., 1989; Geren et al., 1991; Hahm et al., 1992). The photoexcited metal-to-ligand charge-transfer state, Ru(II*), is a strong reducing agent that can transfer an electron to a protein redox center on a nanosecond time scale (Durham et al., 1989). The present paper describes a general method for the de novo design and synthesis of a ruthenium-labeled redox protein that is optimized for the measurement of interprotein electron transfer. A variant of cytochrome b_5 has been prepared with a single cysteine at residue 65 (Stayton et al., 1988). A new sulfhydryl-selective reagent (Geren et al., 1991) was used to attach ruthenium to this cysteine residue, forming Ru-65–cyt b_5 (Figure 1). The position of the ruthenium group was designed to promote rapid electron transfer from Ru(II*) to the heme group of cytochrome b_5 but not interfere with cytochrome c binding (Figure 1). This has allowed measurement of intracomplex electron transfer from the heme group of Ru-65–cyt b_5 to the heme group of cytochrome c.

The reaction between cytochrome b_5 and cytochrome c is a particularly attractive system for studies of biological electron transfer. In the first application of computer graphics docking to electron-transfer proteins, Salemme (1976) proposed a model for the 1:1 complex between cytochrome b_5 and cytochrome c based on the X-ray crystal structures of the individual proteins (Takano & Dickerson, 1981; Argos & Mathews, 1975). This complex is stabilized by charge interactions between cytochrome c amine cations on Lys-13, -27, -72, and -79 and cytochrome b_5 carboxylate anions on Glu-48, Glu-44, Asp-60, and the exposed heme propionate, respectively (Figure 1). The heme groups of the two proteins are nearly coplanar in the complex, with their edges separated by 8.4 Å. The interaction between these two proteins has been studied using a number of different experimental approaches. Chemical modification of the cytochrome c amino groups on Lys-13, -25, -27, -72, and -79 decreases the rate of reaction with cytochrome b_5, supporting their involvement in binding (Ng et al., 1977; Stonehuerner et al., 1979; Smith et al., 1980). Mauk et al. (1982) obtained direct spectroscopic evidence for the formation of a 1:1 complex at low ionic strength and found that methyl esterification of the cytochrome b_5 heme propionate alters the orientation of the complex (Reid et al., 1984; Mauk et al., 1986; Eltis et al., 1988). Rodgers et al. (1988) and Rodgers and Sligar (1991) used site-directed mutagenesis to change specific cytochrome b_5 carboxylate groups to the corresponding amides, resulting in decreases in binding strength and specific volume changes which map the interaction domain to that proposed by Salemme (1976). NMR and molecular dynamics studies have indicated that the complex is in dynamic equilibrium between several different conformations (Ely & Moore, 1983; Burch et al., 1990; Whitford et al., 1990; Wendoloski et al., 1987). McLendon and Miller (1985) used pulse radiolysis to measure a rate constant

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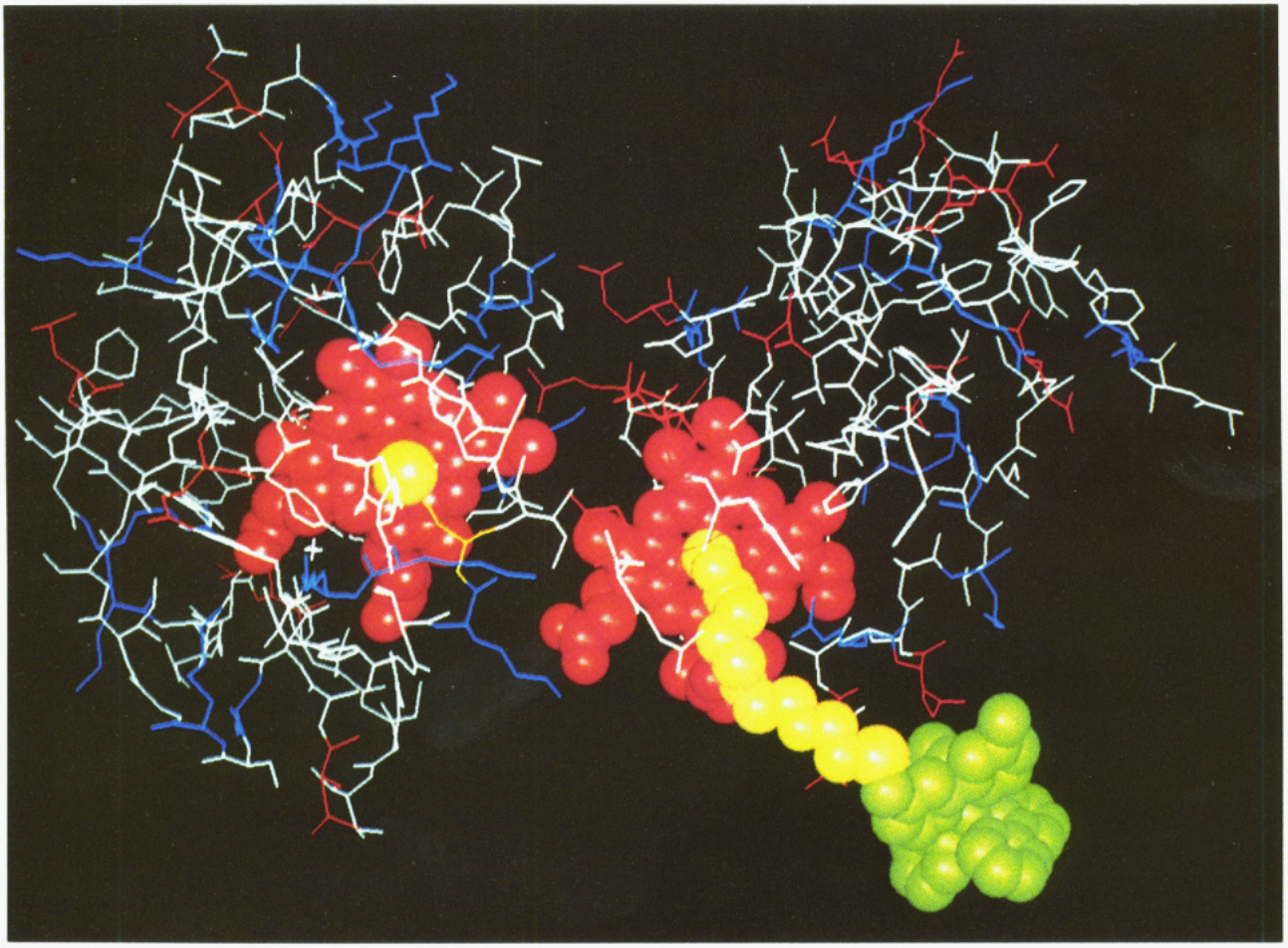

FIGURE 1: Molecular model of the complex between Ru-65–cyt b_5 and cytochrome *c*. The geometry of the complex is the same as that of the complex involving native cytochrome b_5 proposed by Salemme (1976). The heme groups (red), heme group ligands (yellow), and ruthenium complex (green) are represented by CPK models. The atoms forming an electron-transfer pathway between the ruthenium complex and the heme group of Ru-65–cyt b_5 are represented by yellow CPK models. The lysine and arginine residues are blue, while the aspartate and glutamate residues are red.

of $1600 \pm 700\ s^{-1}$ for electron transfer within the cytochrome b_5–cytochrome *c* complex at low ionic strength. The second-order reaction between cytochrome b_5 and cytochrome *c* has recently been subjected to a detailed electrostatic analysis (Eltis et al., 1991).

EXPERIMENTAL PROCEDURES

Preparation and Characterization of Ru-65–cyt b_5. T65C cytochrome b_5 was prepared as described by Stayton et al. (1988). Ru-65–cyt b_5 was prepared by treating 0.2 mM T65C cytochrome b_5 with 3 mM [4-(bromomethyl)-4′-methylbipyridine][bis(bipyridine)]ruthenium^{2+} for 18 h at 25 °C in 1 mL of 50 mM Tris-HCl (pH 8) as described by Geren et al. (1991). The reaction mixture was passed through a Bio-Gel column (1 × 10 cm) equilibrated with 5 mM sodium phosphate (pH 7) to remove excess reagent. The labeled protein was purified using a Waters 625 LC system equipped with a DEAE 8HR column eluted with a linear gradient from 25 to 500 mM sodium phosphate, pH 7 (Figure 2). The major peak was concentrated and washed twice with 5 mM sodium phosphate, pH 7, using an Amicon concentrator. The location of the ruthenium label was determined by digesting Ru-65–cyt b_5 with 10% *Staphylococcus aureus* protease in 50 mM sodium phosphate (pH 7.8), which specifically cleaves peptide bonds following Asp and Glu residues. Reverse-phase HPLC of the digest was carried out as described by Geren et al. (1991) using two detectors in series set at 210 and 450 nm.

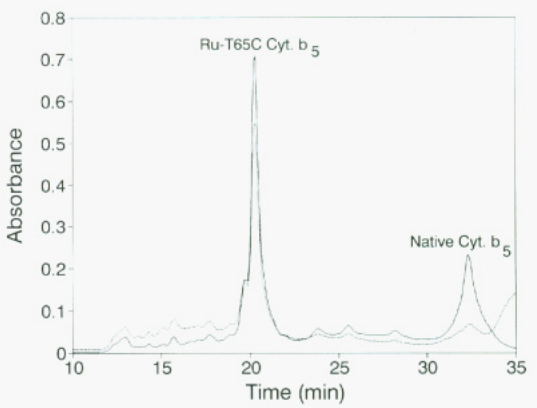

FIGURE 2: Purification of Ru-65–cyt b_5. Crude Ru-65–cyt b_5 (1.5 mg) was loaded onto a DEAE 8HR Waters ion-exchange column and eluted with a linear gradient from 25 to 500 mM sodium phosphate, pH 7.0, at rate of 1.0 mL/min using a Waters 625 LC. (—) 412-nm absorbance; (- - -) 286-nm absorbance.

The chromatogram of the Ru-65–cyt b_5 digest revealed three ruthenium-labeled peptides with 450-nm absorptions. An Applied Biosystems 473A protein sequencer was used to determine that these peptides were DVGHS[C]DARE, DVGHS[C]D, and VGHS[C]D. Native Cys-65 was missing from the sequence of each peptide, indicating that Cys-65 was the site labeled with ruthenium. The visible absorption spectrum of the derivative is equal to the sum of the spectra of 1 equiv of native cytochrome b_5 and 1 equiv of Ru(bpy)$_2$-

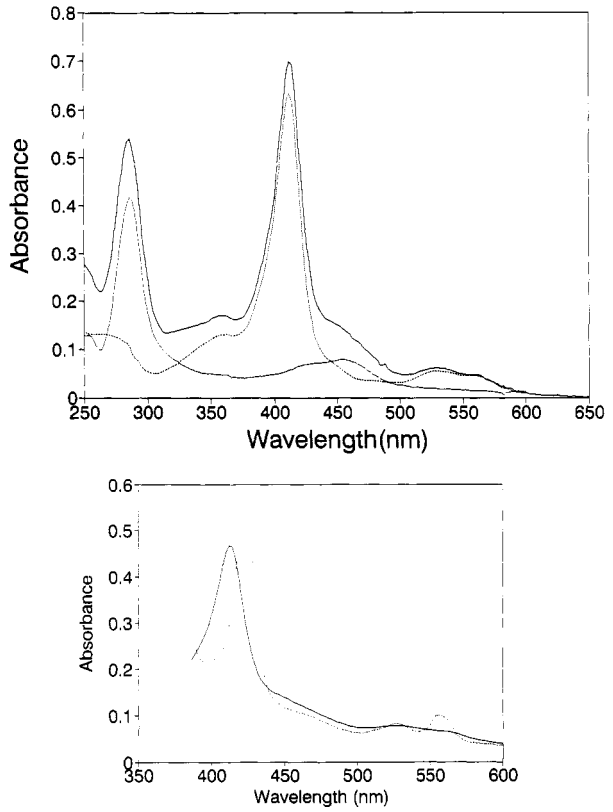

FIGURE 3: (A, top) Visible absorbance spectra of 5.4 μM Ru-65–cyt b_5 (—), 5.4 μM T65C cyt b_5 (- - -), and 5.4 μM (4,4'-dimethylbipyridine)[bis(bipyridine)]ruthenium^{2+} (···) in 50 mM sodium phosphate, pH 7. (B, bottom) Spectra of oxidized (—) and reduced (- - -) Ru-65–cyt b_5. The protein was reduced by addition of dithionite to a solution of 4 μM oxidized Ru-65–cyt b_5 in 10 mM sodium phosphate, pH 7.

(dmbpy)$^{2+}$ (Figure 3A). There are no shifts in the positions of the absorption band maxima in either redox state (Figure 3B). The redox potential of the heme group in Ru-65–cyt b_5 was determined using a 1 × 1 cm anaerobic spectroelectrochemical cell with a platinum electrode and a calomel reference electrode. Reductive titrations were carried out using 1–10 mM hexaammineruthenium(II) in 100 mM sodium phosphate, pH 7. The redox potential of the heme group in Ru-65–cyt b_5 is +15 ± 10 mV compared to +10 mV for native cytochrome b_5 (Reid et al., 1982).

Flash Photolysis Experiments. Transient absorbance measurements were carried out by flash photolysis of 300-μL solutions contained in 1-cm glass semimicrocuvettes. The excitation light flash 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 excitation beam was focused on the entire volume of the detection beam in the sample cuvette by a cylindrical lens. The absorbance detection system has been described in Durham et al. (1989) and had a response rate constant of 1 × 10^6 s^{-1}. Samples typically contained 5–20 μM Ru-65–cyt b_5, 5–40 μM cytochrome c, 10 mM aniline, and 1 mM sodium phosphate, pH 7. The sample also contained a catalytic concentration of cytochrome oxidase (20 nM) to reoxidize cytochrome c between laser pulses. Complete visible spectra were obtained for each sample before and after flash photolysis. Single transients were adequate for kinetic analysis; the transients shown in Figure 5 are the average of 10 transients. A single sample could be subjected to hundreds of laser flashes with no change in the rate constants of the transient or in the absorption spectra, indicating no significant photolysis damage.

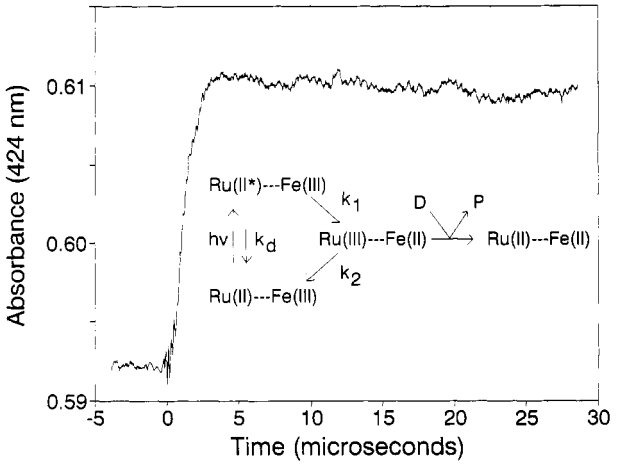

FIGURE 4: Transient kinetics at 424 nm following laser flash excitation of a solution containing 10.9 μM Ru-65–cyt b_5, 10 mM aniline, and 1 mM sodium phosphate, pH 7. The excitation flash was provided by a Phase R Model DL1400 flash-lamp-pumped dye laser to produce a 450-nm light pulse of <500-ns duration. The rate constant was (1.0 ± 0.1) × 10^6 s^{-1}, which was the same as the response rate of the detection system.

The rate constants of the transients were independent of the concentration of aniline, the sacrificial electron donor. The kinetics were similar using EDTA as a sacrificial electron donor instead of aniline, taking into account the contribution of EDTA to the total ionic strength.

RESULTS

Photoexcitation of Ru-65–cyt b_5 with a 450-nm laser flash results in electron transfer from Ru(II*) to heme Fe(III) as shown in Figure 4. 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 measured at 424 nm is greater than the response rate of the detector, which was 10^6 s^{-1}. Electron transfer from the heme Fe(II) of Ru-65–cyt b_5 to ferricytochrome c is observed upon excitation of a solution containing both proteins in 1 mM sodium phosphate, pH 7 (Figure 5). The oxidation of the ferrous heme of Ru-65–cyt b_5 was measured at 556.5 nm (a cytochrome c isosbestic) while the reduction of cytochrome c was measured at 547 nm (a cytochrome b_5 isosbestic). The transients were fitted to the appropriate convolution functions to correct for the response rate of the detection system (Demas, 1983). The transients at 556.5 and 547 nm are biphasic, each with a fast-phase rate constant of (4 ± 1) × 10^5 s^{-1} and relative amplitude of 80 ± 5% and a slow-phase rate constant of (3 ± 1) × 10^4 s^{-1} and amplitude of 20 ± 5% (Figure 5). The amount of cytochrome b_5 oxidized during the transient was the same as the amount of cytochrome c reduced, 1.2 ± 0.2 μM under the conditions of Figure 5. The two rate constants remained the same as the concentrations of Ru-65–cyt b_5 and cytochrome c were independently varied over the range 5–30 μM, while the total amplitude of the transient was proportional to the concentration of the least concentrated cytochrome. This kinetic behavior is consistent with electron transfer within a 1:1 complex between Ru-65–cyt b_5 and cytochrome c with a dissociation constant of less than 5 μM. The fact that the rate constants were independent of concentration indicates that no higher-order aggregation occurred in this concentration range. The presence of two phases suggests that the 1:1 complex can assume two different conformations.

The rate constants decrease and the transients become monophasic as the ionic strength is increased, indicating

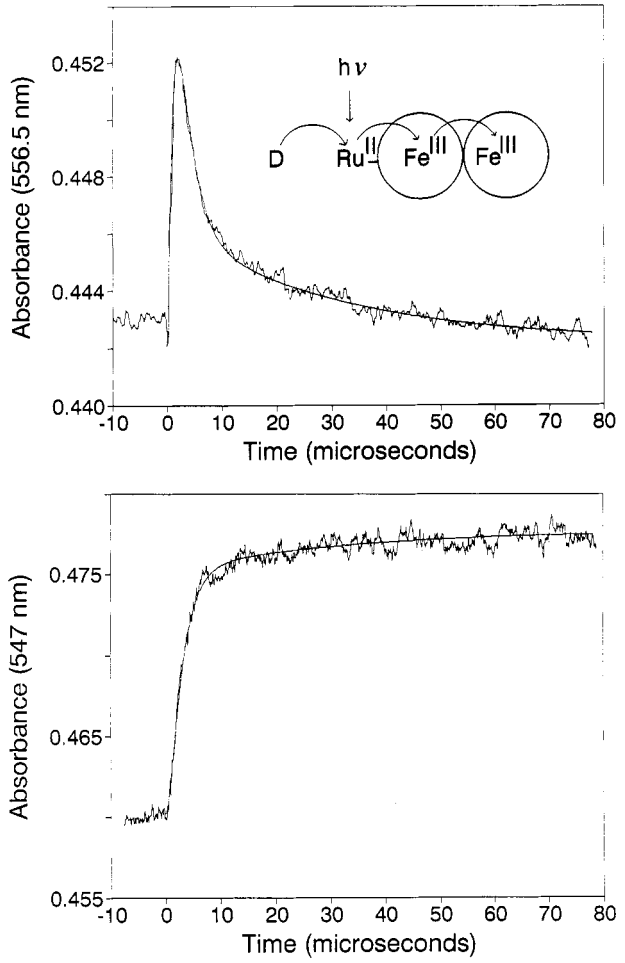

FIGURE 5: Transient kinetics for electron transfer in a solution containing 20 μM Ru-65–cyt b_5, 26 μM cytochrome *c*, 1 mM sodium phosphate, pH 7, and 10 mM aniline. (A, top) The 556.5-nm transient was fitted to the biphasic convolution function (Demas, 1983): $A(t) = A_f(e^{-k_f t} - e^{-rt})/(1 - k_f/r) + A_s(e^{-k_s t} - e^{-rt})/(1 - k_s/r)$, where A_f and A_s are the amplitudes of the fast and slow phases, k_f and k_s are their rate constants, and r is the response rate constant of the detector. $k_f = (4.0 \pm 1) \times 10^5$ s^{-1}, $k_s = (3.4 \pm 1) \times 10^4$ s^{-1}, $A_f = 0.013 \pm 0.002$, $A_s = 0.004 \pm 0.001$, and $r = (1.0 \pm 0.1) \times 10^6$ s^{-1}. (B, bottom) The 547-nm transient was fit to the biphasic convolution function (Demas, 1983): $A(t) = A_f(k_f e^{-rt}) - re^{-k_f t})/r - k_f) + A_s(k_s e^{-rt} - re^{-k_s t})/(r - k_s)$. $k_f = (4.2 \pm 1) \times 10^5$ s^{-1}, $k_s = (3.2 \pm 1) \times 10^4$ s^{-1}, $A_f = 0.015 \pm 0.002$, and $A_s = 0.003 \pm 0.001$.

dissociation of the complex (Figure 6). At 20 mM ionic strength k_{obs} increases linearly with cytochrome *c* concentration from 5 to 20 μM. A second-order rate constant k_{12} of 2×10^9 M^{-1} s^{-1} was calculated from the slope of k_{obs} vs cytochrome *c* concentration. The dissociation constant of the complex, *K*, was estimated to be greater than 20 μM because of the linear dependence of k_{obs} on cytochrome *c* concentration to greater than 20 μM (Strickland et al., 1975). The rate constant k_{12} decreases rapidly as the ionic strength is increased above 20 mM, consistent with the importance of electrostatic interactions to the reaction between the two proteins (Figure 6).

DISCUSSION

The placement of the ruthenium at Cys-65 was designed to promote rapid electron transfer to the heme group of cytochrome b_5 but not interfere with electron transfer to cytochrome *c*. Molecular modeling indicates that there is a nearly direct covalent-bond pathway for electron transfer between the ruthenium complex and the heme group of Ru-65–cyt b_5 (Figure 1). This pathway involves 12 covalent bonds

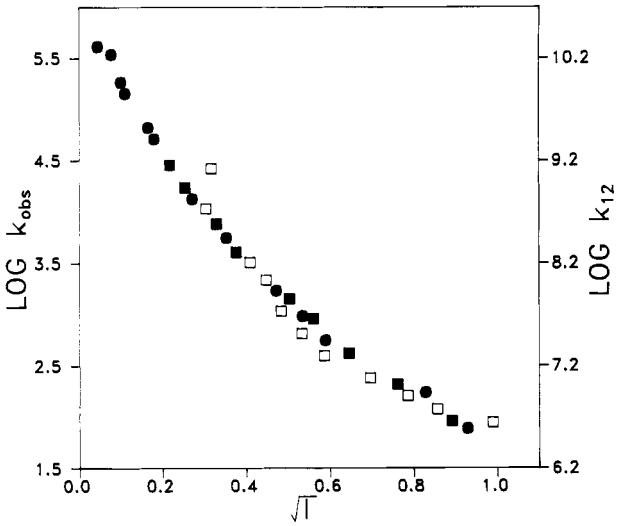

FIGURE 6: Ionic strength dependence of the reduction of cytochrome *c* by Ru-65–cyt b_5. The rate constant k_{obs} (●) (s^{-1}) measured using 20 μM Ru-65–cyt b_5 and 20 μM cytochrome is plotted against the left-hand axis. (k_{obs} is for the fast phase at low ionic strength and the single phase at ionic strengths above 20 mM.) The second-order rate constant k_{12} (■) (M^{-1} s^{-1}) is plotted against the right-hand axis. The horizontal axis is the square root of the ionic strength *I* (M). The buffer contained 1 mM sodium phosphate, pH 7, 10 mM aniline, and 0–1 M NaCl. The second-order rate constants for reduction of cytochrome *c* by native cytochrome b_5 [from Eltis et al. (1991)] are also plotted against the right-hand axis (□).

between the bipyridine ligand of the ruthenium complex at Ru–Cys-65 and the imidazole group of His-63 which ligands the heme group iron. The separation between the two ligands is 12 Å. We have recently measured the rate constants for photoinduced electron transfer between the ruthenium and heme groups to be $k_1 = (1.4 \pm 0.3) \times 10^7$ s^{-1} and $k_2 = (3.0 \pm 0.7) \times 10^6$ s^{-1} (Willie et al., 1992). These values are consistent with extensive studies of 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).

Although Ru–Cys-65 is close to the heme, it is not located at the interaction domain and should not sterically interfere with the binding of cytochrome *c* (Figure 1). The dissociation constant of the 1:1 complex between Ru-65–cyt b_5 and cytochrome *c* is less than 5 μM at 2 mM ionic strength, increasing to greater than 20 μM at 20 mM ionic strength. This is comparable to the dissociation constant of the complex between native cytochrome b_5 and 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). The ionic strength dependence of k_{12} for the reaction of Ru-65–cyt b_5 with cytochrome *c* is nearly the same as for native bovine cytochrome b_5 measured by stopped-flow analysis (Eltis et al., 1991) (Figure 6). This indicates that there are no significant conformational differences between Ru-65–cyt b_5 and native cytochrome b_5 that affect the rate constant under second-order conditions. The 2+ charge on the ruthenium complex is evidently too far away from the interaction domain to affect the second-order kinetics. The detailed electrostatic analysis of the reaction between the native proteins carried out by Eltis et al. (1991) should thus apply to the reaction between Ru-65–cyt b_5 and cytochrome *c*.

The rate constant k_{obs} increases as the ionic strength decreases until a maximal value due to intracomplex electron transfer is reached (Figure 6). The fast-phase intracomplex rate constant of 4×10^5 s^{-1} is over 2 orders of magnitude

larger than the value of 1600 ± 700 s^{-1} measured for native cytochrome b_5 by pulse radiolysis (McLendon & Miller, 1985). Eltis et al. (1991) have pointed out that the rate constant measured by pulse radiolysis at low ionic strength is much smaller than would be predicted from stopped-flow data at high ionic strength. At 0.094 M ionic strength the second-order rate constant is 1.2×10^9 M^{-1} s^{-1} (Eltis et al., 1991) and the dissociation constant is $>10^{-3}$ M (Ely & Moore, 1983), giving an estimated intracomplex rate constant of $>10^6$ s^{-1} using the analysis of Strickland et al. (1975). A number of different studies have indicated 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). The rate constant measured by pulse radiolysis at low ionic strength could thus be governed by the rate of interconversion between the different forms rather than by the actual rate of electron transfer. This "conformational gating" mechanism (Hoffman & Ratner, 1987) would occur if the soluble reductant could not reduce cytochrome b_5 in the "active complex" but only in an "inactive complex" where the heme is accessible to the solution. Electron transfer from cytochrome b_5 to cytochrome c would thus require conversion from the inactive to the active complex.

The ruthenium group attached to Cys-65 is able to rapidly reduce the heme group of Ru-65–cyt b_5 regardless of the conformational state of the complex with cytochrome c. The observation of two different phases for the reaction with cytochrome c indicates that there are at least two different conformations for the complex. The large value of the fast-phase rate constant, 4×10^5 s^{-1}, suggests that the geometry of this complex is nearly as favorable for electron transfer as the encounter complexes formed at high ionic strength. This is in contrast to several other systems where high ionic strength allows more favorable electron-transfer geometries than the thermodynamically stable complex formed at low ionic strength (Hazzard et al., 1988b; Northrup et al., 1988; Kostić, 1991). The low ionic strength 1:1 complex between Ru-65–cyt b_5 and cytochrome c thus appears to have kinetically competent electron-transfer activity. Thermodynamic and structural characterization of this complex can therefore provide an important link between structure and function.

The present studies suggest the need for a reevaluation of the dependence of electron transfer on driving force in the cytochrome b_5–cytochrome c complex. McLendon and Miller (1985) reported rate constants of 1.6×10^3 s^{-1}, 5×10^4 s^{-1}, 5×10^5 s^{-1}, and 8×10^3 s^{-1} for the reactions of cytochrome b_5 with native cytochrome c, ^{3}*Zn(porph)–cyt c, ^{3}*(porph)–cyt c, and $(porph)^{-1}$–cyt c, respectively. The driving forces for these reactions are 0.25, 0.34, 0.74, and 1.1 V, respectively. From these data, McLendon and Miller (1985) estimated that the reorganization energy in the classical Marcus theory is 0.8 V. The reaction between Ru-65–cyt b_5 and cytochrome c has the same driving force as the reaction involving the native proteins, 0.25 V. Thus, three different rate constants have been observed at a driving force of 0.25 V: 4×10^5 s^{-1} and 3×10^4 s^{-1} for the Ru-65–cyt b_5 reaction, and 1.6×10^3 s^{-1} for the reaction of the native proteins measured by pulse radiolysis. This suggests that conformational gating and/or conformational heterogeneity can have as large an effect on rate as the postulated driving force effect. It is thus difficult to analyze the effect of driving force in a system subject to as much conformational heterogeneity as the reaction between cytochrome b_5 and cytochrome c.

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