Preparation, Characterization, and Intramolecular Electron Transfer in Pentaammineruthenium Histidine-26 Cytochrome b_5 Derivatives: Role of the Intervening Medium in Long-Range Donor-Acceptor Electronic Coupling

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Abstract: Wild-type, mutant, and deuteroporphyrin-substituted bovine cytochromes b_5 have been modified with pentaammineruthenium (a_5Ru) for intramolecular electron-transfer (ET) studies. The reactivity of the three surface histidines of the wild-type trypsin-solubilized protein (Tb_5) with $[a_5Ru(OH_2)]^{2+}$ increases in the order His-15 < His-80 < His-26. Intramolecular ET rates from Fe(II) to Ru(III) have been measured by flash photolysis for a_5Ru(His-26)-modified Tb_5, Ru(H26)Tb_5; mutant (Asn-57 to Asp, Gln-13 to Glu, Glu-11 to Gln, His-15 to Asn, His-80 to Asn) lipase-solubilized cyt b_5, Ru(H26)LMb_5; and deuteroporphyrin-substituted Tb_5, Ru(H26)DPb_5 (0.5-3 μM protein, μ = 0.5 M sodium phosphate, pH 7.0, 25 °C). k_{ET}: 1.4 (1) s^{-1}, Ru(H26)Tb_5; 5.9 (5) s^{-1}, Ru(H26)LMb_5; 0.2 (1) s^{-1}, Ru(H26)DPb_5. The rates do not directly correspond to differences in driving force (-0.08, -0.10, -0.13 eV) or edge-to-edge donor-acceptor separation (12.1, 12, 12.9 Å). Evaluation of the donor-acceptor electronic coupling (H_{AB}) in terms of specific through-bond and through-space interactions in the intervening medium for Ru(H26)Tb_5 and Ru(H26)LMb_5 revealed a probable ET pathway consisting of eight covalent bonds from C_γ of His-26 to the end of the Leu-25 side chain and then a through-space jump (3.8, 3.7 Å) to the heme 2-vinyl. Ru(H26)DPb_5 lacks the 2-vinyl, requiring a longer jump (4.5 Å) to the heme 3-methyl. Because H_{AB} is predicted to decay rapidly with distance, the calculated rate constants (assuming λ = 1.2 eV) reflect the differences in the through-space jumps for the three derivatives: Ru(H26)Tb_5, 1.9 s^{-1}; Ru(H26)LMb_5, 4.4 s^{-1}; Ru(H26)DPb_5, 0.16 s^{-1}. The close agreement of calculated and observed rate constants indicates that the electronic coupling in this system is reasonably described by the His-26 → Leu-25 → through-space jump to heme pathway.

Recent investigations involving ruthenium-modified derivatives of myoglobin[1] and horse heart cytochrome *c*,[2,3] as well as zinc-substituted hybrids of hemoglobin,[4] have demonstrated that intramolecular electron-transfer (ET) rates in these proteins decrease exponentially with increasing donor-acceptor separation distance. However, rates greater than predicted by simple exponential decay relationships[5] have been reported for intramolecular ET in ruthenium-modified *Candida krusei* cytochrome *c*,[6] and anomalously low rates have been observed in ruthenium-modified derivatives of cytochrome c_{551},[7] plastocyanin,[8] azurin,[9,10] and the high-potential iron-sulfur protein (HiPIP) from *Chromatium vinosum*.[11] These observations suggest that the structure and composition of the protein medium between the electron-donor and -acceptor centers are critical factors in determining the rate of intramolecular ET. Initial attempts to analyze the nature of this dependence on the intervening medium have produced promising results.[12]

Ruthenium modification of bovine liver cytochrome b_5 provides an attractive system for further investigation of structural regulation of intramolecular ET for several reasons. First, the three-dimensional structures of reduced and oxidized lipase-solubilized cytochrome b_5 have been determined to 2.0-[13] and 1.53-Å[14] resolution, respectively. Second, the electron-transfer properties of the trypsin-solubilized cytochrome have been studied through electrochemical[15] and kinetics[16] analyses. Third, the lipase-[17] and trypsin-solubilized[18] derivatives of the bovine protein have been expressed in *Escherichia coli* from synthetic genes, and the structure of a triple mutant of the protein produced in this manner has been determined to 1.9-Å resolution.[17] Finally, the heme prosthetic group of cytochrome b_5 is bound noncovalently, so heme-substituted derivatives of the protein can be prepared readily.[19]

Experimental Section

Materials and General Methods. Distilled water passed through a Barnstead Nanopure purification system (specific resistance > 18 MΩ cm) was used to prepare all aqueous solutions. Sodium phosphate (NaP_i), 2,2',2''-nitrilotriethanol (TEA), and 4-(2-hydroxyethyl)-1-

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piperazineethanesulfonic acid (HEPES) buffers were prepared with analytical grade reagents. Argon was passed through a manganese oxide scrubbing column to remove residual dioxygen.

Protein samples were deoxygenated by at least five vacuum/purge cycles on a dual-manifold vacuum/argon line. Non-protein reagents were degassed with at least five freeze, pump, thaw cycles and were handled by standard Schlenk techniques. Other manipulations of air-sensitive materials were performed under Ar in a Vacuum Atmospheres Co. glovebox equipped with a Dri-Train (Model HE-493).

Proteins were concentrated and buffers were exchanged by ultrafiltration in stirred cells (YM-5 membranes, Amicon) or by centrifugation (Centricon-10, Amicon).

Preparation of Cytochromes b_5. Several forms of bovine liver microsomal cytochrome b_5 were used in this study. Reference to these various forms is abbreviated as follows: Tb_5 refers to trypsin-solubilized cytochrome b_5 isolated from bovine liver or from *E. coli* transformed with a plasmid bearing a gene coding for this protein.[18] Lb_5 refers to a triple mutant[17] of lipase-solubilized cytochrome b_5 that possesses Gln for Glu-11, Glu for Gln-13, and Asp for Asn-57.[17] LMb_5 refers to a mutant of Lb_5 involving replacement of histidyl residues (Asn for His-15 and for His-80) constructed by using oligodeoxyribonucleotide-directed site-specific mutagenesis as described previously[17] and expressed with the same system as the wild-type protein.[17] Final purification of each of these cytochrome forms was achieved by ion-exchange chromatography with an FPLC system.[17] DPb_5 refers to Tb_5 in which the protoheme IX prosthetic group has been replaced with deuteroheme IX as described previously.[16c]

Preparation of Ruthenium-Modified Cytochromes b_5. Tb_5 and DPb_5 (20–50 mg; 2 mg/mL; Tb_5, $\epsilon_{412.5} = 117$ mM^{-1} cm^{-1};[19] DPb_5, $\epsilon_{403} = 122$ mM^{-1} cm^{-1}[19]) were each modified by reaction with a 25-fold molar excess of $[a_5Ru(OH_2)](PF_6)_2$ (a = NH_3)[20] in 325 mM HEPES (pH 7.5) at room temperature. After 2 h, reaction was quenched by passing the product mixture over a gel filtration column (Sephadex G-25; 1.5 × 47 cm) equilibrated and eluted with 20 mM TEA, pH 7.3. Oxidation in air was sufficient to produce the substitutionally inert Ru(III) derivative.[21] LMb_5 was modified by a similar procedure, except that the protein concentration was 1.4 mg/mL, a 30-fold excess of $[a_5Ru(OH_2)](PF_6)_2$ was used, and the reaction was allowed to proceed for 12 h. In each case, the products were separated with a Pharmacia FPLC system fitted with a Mono Q (HR 5/5 or 10/10) anion-exchange column eluted with a gradient formed by mixing buffer A (20 mM TEA, pH 7.3) with an increasing proportion of buffer B (A + 1.0 M NaCl). For Mono Q 5/5, the following gradient was used: 0–4 mL (0% B), 4–8 mL (ramp to 15% B), 8–60 mL (ramp to 24% B), followed by a high-salt wash. The gradient used for the larger Mono Q 10/10 column was analogous, but with gradient elution volumes multiplied by a factor of four.

NMR Spectra. Spectra were recorded with a Bruker AM500 spectrometer at 298 K and referenced to DSS. Samples were prepared by exchanging the fully oxidized protein into D_2O and then into 100 mM NaP_i in D_2O (uncorrected pH 7.0) by centrifugal ultrafiltration. Resonance from residual water was not suppressed. The sharp histidine C_2H resonances were enhanced by Gaussian multiplication, and their assignments[16e] were verified by pH titration with DCl and NaOD.

Flash Photolysis. Kinetics measurements were made with a microsecond flash-photolysis system described previously[22] that was modified as follows. The transient recorder was replaced with a microcomputer (80286 CPU) equipped with a 12-bit A/D card (Microway A2D-160). A variable amplifier was added, and fiber optics were used to direct the probe light to the sample. Data collection was controlled by commercial software (UnkelScope, version 2.25, Unkel Software, Inc.), and data were analyzed with SI-FIT, version 1.0 (On-Line-Instrument Systems, Inc.), and with fitting routines described elsewhere.[23]

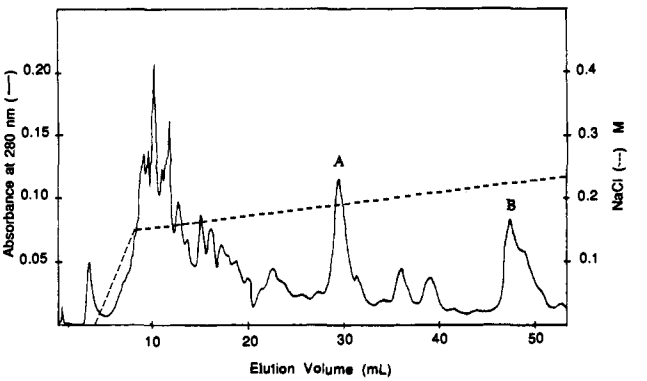

Figure 1. FPLC elution profile of the reaction mixture from a_5Ru modification of Tb_5 quenched after 2 h (Mono Q 5/5; 20 mM TEA, pH 7.3; NaCl gradient as shown). Peak A is singly modified Ru(H26)Tb_5; peak B is unreacted native Tb_5.

Flash-photolysis experiments were performed with the sample placed in a water-jacketed (25 °C) cell (15-cm path length). The protein solution (ca. 11 mL) contained the following components: 7.25 mM Na_2EDTA, 65 μM $[Ru(bpy)_3]Cl_2$ (bpy = 2,2′-bipyridine),[24] and 0.5–3 μM protein in μ = 500 mM NaP_i (pH 7.0). The solutions used to prepare the sample were degassed separately and mixed under an argon atmosphere in the glovebox. To prevent premature $[Ru(bpy)_3]^{2+}$ excitation, the reaction mixture was shielded from light prior to photolysis, and the probe light was filtered (cutoff < 523 nm). Electron-transfer reactions were followed by monitoring the protein oxidation state by visible absorption at 555 nm (Tb_5 and LMb_5) or at 545 nm (DPb_5), and only data from the first flash were used.

Molecular Modeling. Measurements of intramolecular distances were made by using BIOGRAF, version 2.1 (Biodesign, Inc.). The refined coordinates for bovine cytochrome b_5 with the corrected major heme orientation[13e] were provided by F. S. Mathews and R. Durley.[14] A model for DPb_5 was made by deleting the 2- and 4-vinyl groups from this structure. The coordinates for Lb_5[17] were provided by G. D. Brayer and T. L. Lo and were used for measurements pertaining to LMb_5. The two additional mutations in LMb_5 (His-15 to Asn and His-80 to Asn) are conservative surface substitutions and are not expected to perturb the structure of the protein.[25]

Results and Discussion

Modification and Characterization. Cytochrome b_5 possesses three histidyl residues (positions 15, 26, and 80) that are not coordinated to heme iron and that are available for modification with ruthenium. A representative elution profile, obtained during separation of a reaction mixture by ion-exchange chromatography, is shown in Figure 1. In this separation, the cytochrome elutes earlier in the gradients, as it is more extensively modified. On the basis of ^{1}H NMR and peptide-mapping analysis of reaction products resolved in this manner (vide infra), it was established that the susceptibility of histidyl residues to modification with ruthenium correlates with the extent of solvent exposure in the order His-26 > His-80 > His-15. This result is somewhat different from the reported reactivity of these residues with diethyl pyrocarbonate (DEP), which exhibits the order His-26 > His-15 > His-80.[26]

With the availability of three surface histidyl residues for modification with ruthenium, there are four possible derivatives that could be produced by reaction at more than one site. However, the multiplicity of products that elute earlier in the gradient (and therefore are more extensively modified) suggests the occurrence of nonspecific modification of or binding to the highly negative cytochrome b_5 surface. Production of these multiply modified products was minimized by conducting the

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Table I. Rate Constants for Fe(II) to Ru(III) ET in Ru(H26)(cyt b_5) Derivatives

sample	$\Delta G°$, eV[a]	k_{ET}, s^{-1}	pathway (jump, Å)	$(\chi_{AB})^2$	k_{calc}, s^{-1} λ_{b_5} = 1.2 eV[b]	k_{calc}, s^{-1} λ_{b_5} = 1.4 eV[b]	k_{calc}, s^{-1} Ru(H26)Tb_5
Ru(H26)Tb_5	−0.08	1.4 (4)	8C, 1S (3.8)	2.8×10^{-8}	1.9 [4.5]	0.27 [0.65]	
Ru(H26)LMb_5	−0.10	6 (1)	8C, 1S (3.7)	4.7×10^{-8}	4.4 [5.7]	0.66 [0.85]	3.3 [1.8]
Ru(H26)DPb_5	−0.13	0.2 (1)	9C, 1S (4.5)	9.9×10^{-10}	0.16 [4.4]	0.02 [0.63]	0.12 [1.4]

[a] $E°(Fe^{3+/2+})$: 5.1 mV, Tb_5;[15] −14 mV, LMb_5;[17] −44 mV, DPb_5[16c] (vs NHE, 25 °C, μ = 0.1 M (NaP_i)). $E°(Ru^{3+/2+})$ = 80 mV.[1,2] [b] Referenced to Ru(H33)(cyt c): k_{ET} = 30 s^{-1}, $(\chi_{AB})^2 = 8.0 \times 10^{-8}$. Rates in brackets were calculated by using an exponential decay expression: $k_{calc} = k_{ref} \exp[-\beta(d - d_{ref})](FC/FC_{ref})$, where β = 0.9 Å^{-1}.[36]

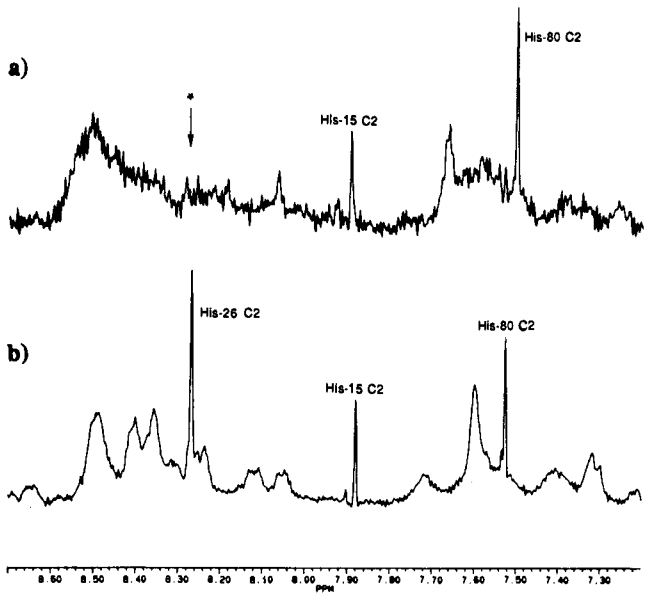

Figure 2. Aromatic region of the ^1H NMR spectra (50 mM D_2O NaP_i, pH 7.1) of (a) peak A (Figure 1) and (b) unmodified Tb_5. The His-26 C_2H resonance is absent in the modified sample.

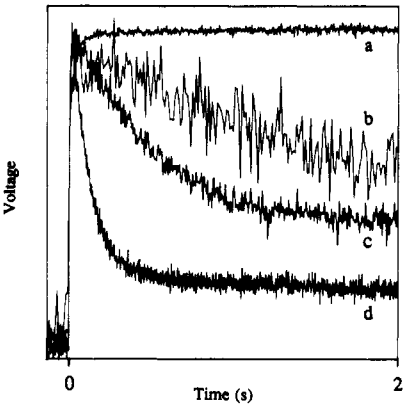

Figure 3. Kinetics traces for (a) unmodified Tb_5, (b) Ru(H26)DPb_5, (c) Ru(H26)Tb_5, and (d) Ru(H26)LMb_5. Voltages were converted to absorbances prior to fitting.[10] Flash photolysis was performed on the fully oxidized proteins (1.0 μM) with 65 μM $Ru(bpy)_3^{2+}$ and 7.25 mM EDTA in μ = 0.5 M NaP_i, pH 7.0.

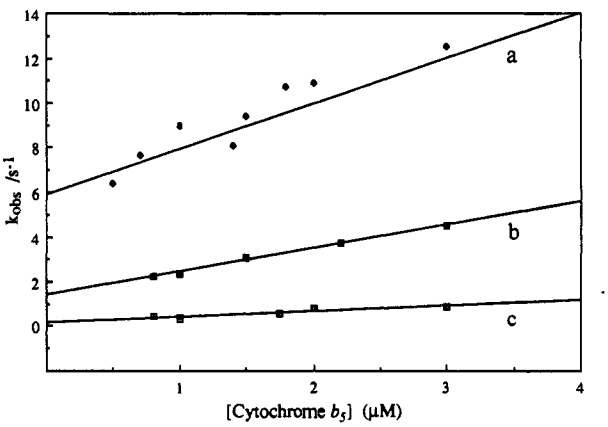

Figure 4. Dependence of observed rate constants for flash photolysis of (a) Ru(H26)LMb_5, (b) Ru(H26)Tb_5, and (c) Ru(H26)DPb_5 on protein concentration. The lines are least-squares fits to the data. See Figure 3 and Experimental Section for conditions.

reaction at high ionic strength (the highly modified material could be converted to the unmodified cytochrome by reduction). Nevertheless, the occurrence of these more highly modified products restricted the range of reaction times that could be employed in this work and reduced the yield of singly modified products.

Identification of the derivative that is modified only at His-26 (Ru(H26)Tb_5)[27] was achieved by HPLC tryptic peptide mapping[28] and by ^1H NMR spectroscopy. Figure 2 shows the aromatic region of the NMR spectra obtained for Tb_5 and Ru(H26)Tb_5. The sharp His-26 C_2H resonance at 8.23 ppm is absent in the spectrum obtained for the modified protein as the result of paramagnetic shifting and broadening by the bound Ru(III).

As expected, DPb_5 exhibited reactivity in the ruthenium-modification reaction that was comparable to that of Tb_5; however, Lb_5 was much less reactive. This difference in reactivity is probably attributable to interference from the nine additional amino acid residues at the C-terminus of this form of the cytochrome, since this peptide extension is in close spatial proximity to His-26. The modification reaction for the LMb_5 mutant (possessing a single His at position 26) could be allowed to proceed for longer reaction times to increase the yield of Ru(H26)LMb_5 with less formation of multiply modified material. The identity of this product was established by the absence of the one remaining C_2H resonance in the ^1H NMR spectrum of LMb_5 (8.15 ppm; pH 7.2) in this singly modified sample (data not shown).

Kinetics. In the flash-photolysis experiment,[2,24] the a_5Ru(His) and heme sites are initially oxidized, and ET is initiated by photogeneration of the highly reducing excited state of $[Ru(bpy)_3]^{2+}$. The low reduction potential of the cytochrome b_5 heme center [$E_{m,7}(Fe^{3+/2+})$ = 5 mV[15]] results in a thermodynamic driving force that favors ET from the heme to the pendant a_5Ru center [$E_{m,7}(Ru^{3+/2+})$ = 80 mV[1,2]]. With photolysis, the reducing equivalents partition between Fe(III) and Ru(III). Direct heme reduction is probably facilitated by the extensive exposure of the heme edge to the solvent (the fraction of the protein surface that is heme is 5 times greater in cytochrome b_5 than in cytochrome c[16f]) and by the binding of $[Ru(bpy)_3]^{2+*}$ to the negative electrostatic potential surface of the protein where the heme becomes exposed to the solvent.[29]

Unfortunately, the same factors that lead to efficient heme reduction probably also contribute to bimolecular ET by attracting the positively charged a_5Ru^{III} from another protein in a stacking

(27) Atomic absorption analysis performed by the analytical services at Brookhaven National Laboratory indicated Ru:Fe = 1.2 ± 0.2.

(28) Ruthenium-modified peptides of cytochrome b_5 fail to elute in the normal gradient previously reported for HPLC peptide mapping of this protein (Mauk, M. R.; Mauk, A. G. *Eur. J. Biochem.* **1989**, *186*, 473–486) and, therefore, were identified by their absence (data not shown).

(29) Cyt b_5 is known to bind small, positively charged metal complexes with a 3–/4– binding site.[16e] Also, $[Ru(bpy)_3]^{2+*}$ emission (610 nm) quenched by cyt b_5 exhibits saturation kinetics, indicating that binding does occur.[10]

arrangement. As a result, it was necessary to conduct experiments at relatively high ionic strength to obtain reproducible intramolecular kinetics results. Representative kinetics traces are shown in Figure 3. A weak dependence of the observed rate constants on protein concentration was observed even at high ionic strength, so the intramolecular rate constants were obtained by extrapolation of the apparent rate constants to zero protein concentration (Figure 4). The rate constants determined for ET from Fe(II) to Ru(III) for Ru(H26)Tb_5, Ru(H26)LMb_5, and Ru(H26)DPb_5 are given in Table I.

Analysis of ET Rates. The ET rate constant in the weak-overlap (nonadiabatic) limit is given by the following expression:[5]

$$k_{ET} = (4\pi^2/h)(H_{AB})^2(FC) \quad (1)$$

In eq 1, H_{AB} is the electronic coupling between the donor and acceptor, and the Franck–Condon term (FC) contains details of the nuclear motion coupled to the electron transfer. In the one-mode, high-temperature, harmonic limit

$$FC = (4\pi k\lambda T)^{-1/2} \exp[-(\lambda + \Delta G°)^2/4\lambda kT] \quad (2)$$

Thus, k_{ET} is expected to depend on the electronic coupling (H_{AB}), the driving force of the reaction ($-\Delta G°$), and the reorganization energy (λ) of solvent dipoles and redox center ligands.

In view of their structural similarities, it is somewhat surprising that the ET rates in the modified proteins (Ru(H26)Tb_5, Ru(H26)LMb_5, and Ru(H26)DPb_5) differ by over an order of magnitude. It is also striking that Ru(H26)DPb_5, which has the largest driving force for Fe(II) to Ru(III) ET, exhibits by far the slowest rate. Since the electronic and structural properties of the electron donors in these systems are virtually identical, and the acceptors are the same, it is likely that the ET reorganization energies are not significantly different in the three modified proteins.[10] Therefore, we conclude that the ET rates reflect changes in the long-range donor–acceptor electronic coupling.

It is commonly assumed that H_{AB} decays exponentially with the donor–acceptor separation (d):[1-5]

$$H_{AB} = H_{AB}°\chi_{AB} \quad (3)$$

$$\chi_{AB} = \exp[-\beta(d - d_0)/2] \quad (4)$$

with values of β in the range 0.8–0.9 Å^{-1}. $H_{AB}°$ is the matrix element at donor–acceptor contact ($d_0 \approx 3$ Å). Hoffman and co-workers compared ET from electronically excited ZnPP and ZnDP to Fe^{III}(H$_2$O)PP (PP = protoporphyrin; DP = deuteroporphyrin) in hemoglobin hybrids and reported k_{ET}(ZnPP)/k_{ET}(ZnDP) = 2.8.[30] They credited a vinyl group in the ZnPP-substituted protein with extending the conjugation of the porphyrin and thus reducing d by 1 Å. Their ratio of k_{ET} values agrees with $\exp(-\beta\Delta d) \approx 2.5$ ($\beta = 0.9$ Å^{-1}). In the present work, comparison of Ru(H26)Tb_5 and Ru(H26)DPb_5 also gives $\Delta d \approx 1$ Å (Figure 5). However, a value of $\beta = 1.9$ Å^{-1} is required to reproduce the observed ratio of ET rate constants with a simple exponential distance decay model.

Beratan and Onuchic have developed a model of long-range interactions in proteins in which χ_{AB} is a function of the specific composition of the medium between the donor and the acceptor.[31] This electron-tunneling-pathway model has been successful in predicting ET rate constants for several ruthenium-modified cytochrome c mutants.[12] In the calculation of ET pathways,[31] the peptide network that bridges the electron donor and acceptor is partitioned into three types of interactions: covalent bonds (C), hydrogen bonds (H), and through-space jumps (S). The coupling is estimated to decay by a fixed amount over each covalent bond in the pathway; hydrogen bonds and through-space jumps are expected to result in more rapid decay. The electronic coupling

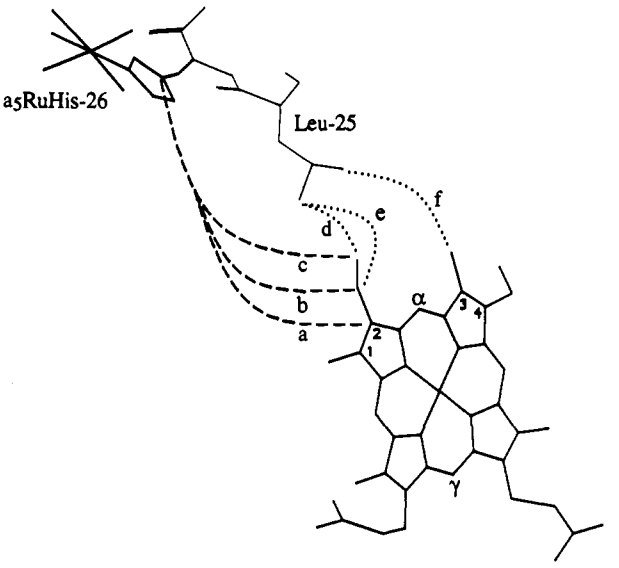

Figure 5. ET distances in Ru(H26)(cyt b_5) derivatives. Edge-to-edge distances (---) and best pathway through-space jumps (···) for Ru(H26)Tb_5 (b, 11.9 Å; e, 3.8 Å), Ru(H26)LMb_5 (c, 12 Å; d, 3.7 Å), and Ru(H26)DPb_5 (a, 12.9 Å; f, 4.5 Å) are indicated. Because the 2-vinyl group is conjugated with the porphyrin π-system, it is counted as part of the heme in the pathway analysis (d, e). The nonconjugated 3-methyl adds one covalent bond to pathway f.

factor χ_{AB} associated with each ET pathway is calculated as follows (d_H is a hydrogen-bond distance; d_S is a through-space distance in Å):[32]

$$\chi_{AB} = \prod_{i=1}^{N_C}\epsilon_C(i)\prod_{j=1}^{N_H}\epsilon_H(j)\prod_{k=1}^{N_S}\epsilon_S(k) \quad (5)$$

$$\epsilon_C = 0.60 \quad (6)$$

$$\epsilon_H = 0.36 \exp[-1.7(d_H - 2.9)] \quad (7)$$

$$\epsilon_S = 0.60 \exp[-1.7(d_S - 1.4)] \quad (8)$$

A computer program has been written employing this formalism to search crystallographic coordinates for the optimum ET pathways through proteins.[32]

A search of the medium between His-26 and Fe in Tb_5 with this program revealed that the pathway with the largest χ_{AB} consists of eight covalent bonds from C_γ of His-26 to the end of the Leu-25 side chain; the pathway is completed by a 3.8-Å through-space jump to the closest heme atom, C_α of the 2-vinyl (Figure 5). In LMb_5, the Leu-25 to heme jump is only 3.7 Å to C_β of the 2-vinyl (Figure 5), because in this mutant the vinyl has pivoted relative to the Tb_5 structure.[33,34] A much larger perturbation of the through-space jump in the pathway is provided by DPb_5, which has a proton in place of the 2-vinyl group. Since χ_{AB} decays rapidly with distance in through-space jumps, removing the 2-vinyl effectively shuts down the shortest pathway. The next best option in DPb_5 involves nine covalent bonds and a 4.5-Å jump from Leu-25 to the heme 3-methyl (Figure 5); consequently, the

(30) Gingrich, D. J.; Nocek, J. M.; Natan, M. J.; Hoffman, B. M. *J. Am. Chem. Soc.* **1987**, *109*, 7533–7534.
(31) Beratan, D. N.; Onuchic, J. N. *Photosynth. Res.* **1989**, *22*, 173–186.
(32) Beratan, D. N.; Onuchic, J. N.; Betts, J. N.; Bowler, B. E.; Gray, H. B. *J. Am. Chem. Soc.* **1990**, *112*, 7915–7921.
(33) There is some uncertainty in the vinyl orientations in Tb_5 and in LMb_5. The dominant heme orientation in lipase-solubilized wild-type cyt b_5, as determined by NMR spectroscopy (Keller, R. M.; Wuthrich, K. *Biochim. Biophys. Acta* **1980**, *621*, 204–217) and by X-ray diffraction,[13f] has the 2-vinyl in proximity to Leu-25. A minor form, with the heme rotated 180° about the α,γ meso axis (see Figure 5), is in slow equilibrium with the major form (major:minor = 90:10).[34] Lb_5 has the same major heme orientation but with an estimated 60:40 relative abundance. There is some ambiguity in the exact positions of the 2- and 4-vinyl groups in the crystal structure.[17]
(34) (a) La Mar, G. N.; Burns, P. D.; Jackson, J. T.; Smith, K. M.; Langry, K. C.; Strittmatter, P. *J. Biol. Chem.* **1981**, *256*, 6075. (b) Walker, F. A.; Emrick, D.; Rivera, J. E.; Hanquet, B. J.; Buttlaire, D. H. *J. Am. Chem. Soc.* **1988**, *110*, 6234–6240. (c) McLachlan, S. J.; La Mar, G. N.; Burns, P. D.; Smith, K. M.; Langry, K. C. *Biochim. Biophys. Acta* **1986**, *874*, 274–284.

value of $(\chi_{AB})^2$ in Ru(H26)DPb_5 is predicted to be over an order of magnitude lower than the others. From the microscopic perspective of the pathway model, a 0.7-Å change in d_S has a dramatic effect on the ET rate.

To calculate ET rate constants from χ_{AB} values (eqs 1 and 2), an estimate of the reorganization energy accompanying ET in ruthenium-modified cyt b_5 (λ_{b_5}) is required. A reasonable approximation of λ_{b_5} (1.2 eV) can be obtained from an analysis of ET rates in Ru(H33)(cyt c).[35] Because there is some evidence[16f] that cyt b_5 may have a heme reorganization energy that is roughly 0.2 eV greater than that of cyt c, calculations using λ_{b_5} = 1.4 eV were performed for comparison.

One final approximation was made in calculating ET rate constants. Since $H_{AB}°$ is not known, χ_{AB} was scaled to a reference value, $\chi_{AB_{ref}}$, and a corresponding experimental rate constant, k_{ref}, to give k_{calc}:

$$k_{calc} = k_{ref}[(\chi_{AB})^2/(\chi_{AB_{ref}})^2](FC/FC_{ref}) \quad (9)$$

The rate constants calculated by using this approach, referenced both to the well-studied His-33 cyt c system[35] and to Ru(H26)Tb_5 itself, are given in Table I. The ratio of Franck–Condon factors corrects for differences in $\Delta G°$ and/or λ.[36] Rate constants calculated in the same way but with an exponential decay model for χ_{AB} (β = 0.9 Å^{-1}) are included for comparison.

It is clear from the calculated and observed ET rates in Table I that the pathway model is superior to an exponential distance decay relationship in predicting both the trends in rates and their orders of magnitude. The good fit for predictions referenced to Ru(H33)cyt c (λ_{b_5} = 1.2 eV) and the internally consistent values referenced to Ru(H26)Tb_5 suggest that similar ET mechanisms operate in the two modified proteins. Of particular note is the relatively weak electronic coupling in Ru(H26)DPb_5, which strongly indicates that through-space interactions can play a key role in controlling long-range ET rates in proteins.

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Registry No. His, 71-00-1; leu, 61-90-5; heme, 14875-96-8; deuteroheme IX, 18922-88-8.

(35) Meade, T. J.; Gray, H. B.; Winkler, J. R. *J. Am. Chem. Soc.* **1989**, *111*, 4353–4356.

(36) FC and FC_{ref} (λ = 1.2 eV, $\Delta G°$ = –0.19 eV) were calculated by using eq 2.