Preparation, Characterization, and Intramolecular Rate Constant for Ru(II) → Fe(III) Electron Transfer in the Pentaammineruthenium Histidine Modified Cytochrome c_{551} from *Pseudomonas stutzeri*

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Abstract: The acidic (pI ~ 4.7) mono-heme protein cytochrome c_{551} (M_r 9250; 82 amino acids) from bacterial *Pseudomonas stutzeri* has a single uncoordinated surface histidine at position 47. Reaction with $[Ru(NH_3)_5H_2O]^{2+}$ followed by oxidation with $[Fe(CN)_6]^{3-}$ yields singly modified ferricytochrome c_{551} Ru(III). The product, purified by mono-Q column FPLC chromatography, gives on analysis by ICP atomic emission spectroscopy a 1:1 ratio of Fe:Ru. The His47 residue no longer reacts with diethyl pyrocarbonate (DEPC), and the sharp ^{1}H NMR His47 C_2H resonance at 8.2 ppm is lost due to paramagnetic line broadening by the adjacent Ru(III). On pulse radiolysis, using the formate radical $CO_2^{\bullet-}$ to reduce the fully oxidized protein (pH 7, ~20 °C), rapid reduction is observed (1.9×10^9 M^{-1} s^{-1}), which can be partitioned between the Fe(III) (~90%) and Ru(III) (~10%) centers, yielding stable $Fe^{II}Ru^{III}$ and metastable $Fe^{III}Ru^{II}$ products, respectively. The latter with a donor–acceptor (C^γ of His47 to S of the axial Met61) separation of 7.9 Å and driving force (difference in reduction potentials) of ~200 mV decays with an intramolecular rate constant of 13 s^{-1} to yield the stable $Fe^{II}Ru^{III}$ product. This result is discussed alongside other values for fixed-distance electron transfer in Ru-modified metalloproteins.

Cytochrome c_{551} is an electron-transport protein that can be isolated from, e.g., various pseudomonads, where its role in the bacterial respiratory chain is analogous to that of cytochrome c in eukaryotes.[1] The X-ray crystal structures of both oxidation states of cytochrome c_{551} from *Pseudomonas aeruginosa* have been determined and refined to 1.6 Å resolution.[2] Bond lengths from the axially coordinated His16 (N-atom) and Met61 (S-atom) to the heme iron and other properties are consistent with the presence of low-spin iron. Although smaller (82 amino acids) than cytochrome c (normally 104 amino acids), there are some striking similarities in the tertiary structures. The main difference is the deletion of cytochrome c residues 41–55,[2] present toward the bottom of the heme crevice in the view with the exposed heme edge vertical and the axially coordinated methionine to the left. These same features are expected to be retained in cytochrome c_{551} from *Pseudomonas stutzeri*, which is the subject of the present study. In general there are fewer lysine residues around the exposed heme edge than in cytochrome c, and the protein is acidic (pI = 4.7 for reduced cytochrome c_{551} from *Pseudomonas aeruginosa*).[3] The reduction potential of 277 mV is similar to that of other cytochrome c_{551}'s (280 mV)[4,5] and cytochrome c (260 mV),[6] although a somewhat lower value of 240 mV has also been reported.[7] Attached to the heme ring are two propionic acid substituents, one exposed to solvent and readily ionized at pH 4 and the other buried and stabilized by H-bonding.[8]

Amino-acid sequences of cytochrome c_{551}'s, including that from *P. stutzeri*, have been reported.[5,9] The occurrence of an additional histidine at position 47, the imidazole ring of which is ~10 Å from the heme Fe, is the focal point of the present studies. The complex $[Ru(NH_3)_5H_2O]^{2+}$ is a good reagent for the modification of metalloproteins because of its affinity for accessible surface histidine residues. It has, for example, been attached previously to specific histidine residues on two plastocyanins,[10] high-potential iron–sulfur protein,[11] cytochrome c,[12] azurin,[13] and myoglobin.[14] Because of their kinetic inertness in the Ru(II) and Ru(III) states the modified proteins lend themselves to the study of intramolecular electron-transfer processes.[15,16]

Experimental Section

Protein Isolation. The bacterium *Pseudomonas stutzeri* (strain 224) was grown by a modification of the procedure of Ambler[17] in 15-L batches in an LH fermenter at 30 °C, pH 7.6, with aeration (9–12 L/min) over 20 h. The cell paste was removed by centrifugation and stored at 4 °C as an acetone dried powder. The cytochrome c_{551} was extracted by a modification of the method of Liu.[18] All steps were

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Table I. Effect of Redox Cycling (RC) on Product Distribution for the *P. stutzeri* Cytochrome c_{551} Ru-Modification Reaction[a]

	%	additional % yield after RC of band 1
band 1 (multiply modified)	61	45
band 2 (singly modified)	24	13
band 3 (native)	15	3

[a] Conditions used for modification were: ferricytochrome c_{551} (0.4 mM; 55 mg), $[Ru(NH_3)_5H_2O]^{2+}$ (6 mM; 44 mg) pH 7.0, and reaction time 180 mins. Redox cycling was carried out as described in the text.

carried out at 4 °C. The acetone dried powder (80 g) was mixed with 0.04 M Tris/HCl buffer, pH 8.3 (1 L), containing DNAase (1 mg/L) and magnesium chloride (0.15 g/L), and sonicated in an ice bath in 100-mL batches for 10 min. After centrifuging at 20000 g for 30 min, the pellet was sonicated with further buffer, and the combined centrifugated aqueous extracts were dialyzed overnight against 5 mM Tris/HCl buffer (pH 7.6). The solution was loaded onto a DE-52 column (20 × 7 cm) washed with buffer and then eluted with 0.1 M NaCl in the same buffer. The protein-containing material was Amicon ultrafiltered (YM5 membrane) into 5 mM Tris/HCl buffer (pH 7.6) and loaded onto a DE52 column (60 × 2.5 cm) to which a continuous gradient of NaCl (0–0.15 M, total volume 800 mL) was applied. The sample of cytochrome c_{551} was fully reduced (dithionite), ultrafiltered (Amicon YM5 membrane) into 20 mM Tris/HCl buffer (pH 7.6), and applied to a Sephadex G50-80 gel column (80 × 2.5 cm) to give protein with an absorbance (A) purity ratio of A_{551}/A_{280} = 1.35 for reduced protein (literature 1.39).[9] A final purification step with an FPLC (Pharmacia) Mono-Q column, with NaCl gradient 0 to 1.0 M in 20 mM Tris/HCl buffer (pH 8.08), gave a single sharp symmetrical band at 0.09M NaCl, corresponding to the pure protein.

Ru Complex. The aquapentammineruthenium(III) complex $[Ru(NH_3)_5H_2O](PF_6)_2$ was prepared by a modification of the literature method.[19] In this, the more water soluble trifluoromethanesulfonate complex $[Ru(NH_3)_5(CF_3SO_2)](CF_3SO_3)_2$,[20] was used in place of $[Ru(NH_3)_5Cl]Cl_2$. The composition was confirmed by analysis. The complex was stored at 4 °C under argon and used within 2 weeks of preparation.

Modification Procedure. To purified ferrocytochrome c_{551} (55 mg) in 5 mL of a Hepes buffer (50 mM, pH 7.26), where Hepes is *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Sigma Chemicals), was added $[Ru(NH_3)_5H_2O](PF_6)_2$ (44 mg) in 10 mL of the same buffer. After 3 h the reaction was quenched by passage down a Sephadex G25 (3 × 25 cm) column, previously equilibrated with the same buffer, thereby removing excess $[Ru(NH_3)_5H_2O]^{2+}$. The reactant solution and gel column were saturated with argon gas to ensure the complete exclusion of oxygen. The protein band was immediately oxidized with a slight excess of $[Fe(CN)_6]^{3-}$, and the solution was ultrafiltered into 20 mM Tris/HCl buffer (pH 8.1). Total protein recovery based on the known absorption coefficient, and assuming no significant change in the absorbance spectrum at 525 nm upon modification, was ~85%.

The mixture of products was readily separated by FPLC with a Mono-Q anion-exchange column (at pH 8.1). A typical elution profile is shown in Figure 1. The relative yields of various fractions are given (Table I). The multiply modified fraction (which did not bind to the Mono-Q column) was subjected to redox cycling.[10] Thus it was ultrafiltered into Hepes buffer (50 mM, pH 7.26), and the solution (15 mL) was then reduced with excess dithionite (under argon). After 2–3 h the solution was ultrafiltered into Tris/HCl buffer (20 mM, pH 8.1) oxidized with $[Fe(CN)_6]^{3-}$ and then subjected to FPLC chromatography. Approximately 20% of the multiply modified protein was converted to the desired product, and ~5% to native cytochrome c_{551}. Combined multiply modified material from several syntheses was combined and subjected to several redox-cycling experiments. The desired, singly modified protein was subjected to a final purification on the Mono-Q FPLC column with the same buffer system and appeared as a symmetrical sharp peak at 0.07 M NaCl concentration.

DEPC Modification. The reaction of both native and Ru-modified protein with diethyl pyrocarbonate (DEPC, Sigma Chemicals), was monitored between 220 and 300 nm. To solutions of protein (~30 μM) in 100 mM phosphate buffer (pH 7.0) was added 20 equiv of DEPC (~0.1 M, standardized with imidazole before use) in absolute alcohol.[21] With native protein, a peak developed at ~240 nm over 40 min, corresponding to ~88% of that expected for a 1:1 adduct (based on a molar absorption coefficient of 3200 M^{-1} cm^{-1} for *N*-ethoxyimidazole).[21] However, a value of ϵ = 2900 M^{-1} cm^{-1} has been reported for *N*-ethoxyhistidine,[22] and a value of 2750 ± 100 M^{-1} cm^{-1} has been indicated in other previous studies.[23] The absorbance change observed is consistent therefore with 1:1 modification as in (1). In a similar experiment the Ru-modified protein gave no significant absorbance change between 230 and 300 nm. This is consistent with attachment of $Ru(NH_3)_5$ to His47 and the blocking of the same site to reaction with DEPC.

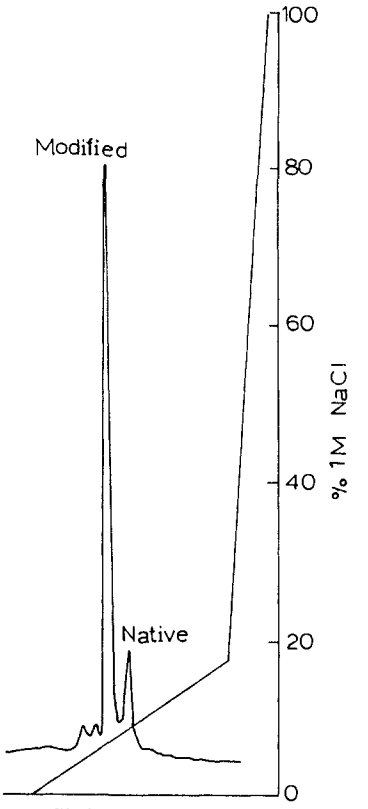
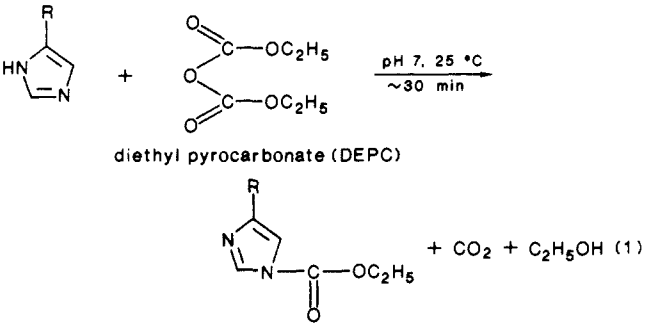


Figure 1. Elution profile by FPLC (absorbance monitored at 280 nm) of reaction mixture from Ru modification of *P. stutzeri* cytochrome c_{551}. A mono-Q column in 20 mM Tris/HCl buffer, pH 8.1, was used with a 0–17% linear gradient in 1 M NaCl. The peak labeled I is for Ru-modified and peak II for native protein. Multiply modified protein was not held by the column.



NMR Spectra. Proton NMR spectra at 300 MHz were acquired with solutions (5–10 mM) of fully oxidized native and Ru-modified proteins in D_2O-containing phosphate buffer (100 mM at pH 7; pH's were not adjusted to p²H). The ^1H NMR spectra of native and Ru-modified proteins in the region 6.5 to 10.0 ppm are compared in Figure 2. The sharp singlet at 8.2 ppm in the native spectrum, which corresponds to the C_2H proton of the imidazole ring of His47,[24] is absent in the ^1H NMR spectrum of the Ru-modified protein, as expected for attachment of a paramagnetic Ru species to the imidazole ring of His47. The C_4H proton

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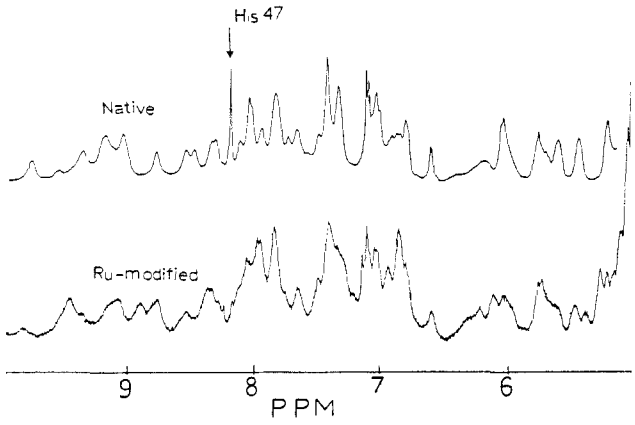

Figure 2. 300-MHz ^{1}H NMR spectra in the aromatic region for native *P. stutzeri* cytochrome c_{551} (upper spectrum) and the Ru(III)-modified protein (lower spectrum).

could not be unequivocally identified in the native ^{1}H NMR spectrum, so it was not possible to observe the effect of Ru attachment on this resonance.

Other Products of Ruthenation. The presence of more positively charged products in the FPLC elution profile has already been discussed in the context of redox cycling. Attachment of two Ru species per protein was confirmed by ICP Atomic Emission Spectroscopy (see below). A small amount of another singly Ru modified species was also present. This is most probably a product of a ligand substitution reaction in the $Ru(NH_3)_5$ core, as solutions of the chromatographically pure major singly modified product, when left at room temperature over several days showed the appearance of more of this minor product upon rechromatography. A species that eluted at higher NaCl concentration than native protein was also obtained, but as this corresponds to a more negatively charged species than native protein, it is clearly not simply the product of Ru attachment and was not subjected to further investigation.

Analysis for Metals. The Fe:Ru ratios of the various products were determined on an Inductively Coupled Plasma Atomic Emission Spectrometer (Bausch and Lomb ARL-2350). The major and minor bands that eluted before the native protein from the Mono-Q anion-exchange column showed Fe:Ru ratios of 1:0.94 and 1:1.04, respectively, and the material that did not bind to the column had a ratio ~1:1.5.

Pulse Radiolysis Studies. These were carried out at the Cookridge Radiation Research Centre, using a 2.5M eV electron beam, with pulse length in the range 0.2–1.2 μS. The reducing radical yield [R] was calculated from (2), where V(SEC), the voltage of the secondary emission

$$[R] = V(SEC)S(SEC)G_R \quad (2)$$

chamber, was measured directly and S(SEC) (the sensitivity) was determined by thiocyanate[25] or iodide dosimetry,[26] and G_R is the yield of radicals per joule of energy absorbed by the system.

Pulse radiolysis experiments were performed on solutions of fully oxidized protein (5–30 μM) in a 1, 2.5, or 5 cm cell, at room temperature (19 ± 1.5 °C). The reduction reactions were in general monitored at 551 nm, the absorbance maximum of the so-called α-band of the reduced protein. The $\Delta\epsilon$ at this wavelength was determined as 22 000 (Figure 3). Absorbance changes were calculated from the change in the photomultiplier voltage, eq 3, where V_0 and V_t are signals from the photomultiplier

$$\Delta A = \log (V_t/V_0) \quad (3)$$

before the pulse and at a time t after the pulse, respectively. An OY6 Chance filter was used to exclude light of wavelength below 500 nm. All solutions were prepared with triply distilled water and buffered with phosphate. The solutions were 100 mM in phosphate at pH 7.0 and additionally contained 100 mM sodium formate (giving I = 0.31 M). They were N_2O saturated prior to use and kept under a positive pressure of argon throughout the experiment. The concentration of protein (and the absence of any reduced material) in solutions was determined by visible-range spectrophotometry after bubbling with N_2O and prior to use. The protein solutions were reoxidized with $[Fe(CN)_6]^{3-}$ immediately after use and frozen as soon as possible to minimize any decomposition reaction. This enabled samples to be reused several times. Protein samples were oxidized with $[Fe(CN)_6]^{3-}$, purified by FPLC chromatography, and exhaustively ultrafiltered into the buffer solution on the day prior to pulse radiolysis experiments.

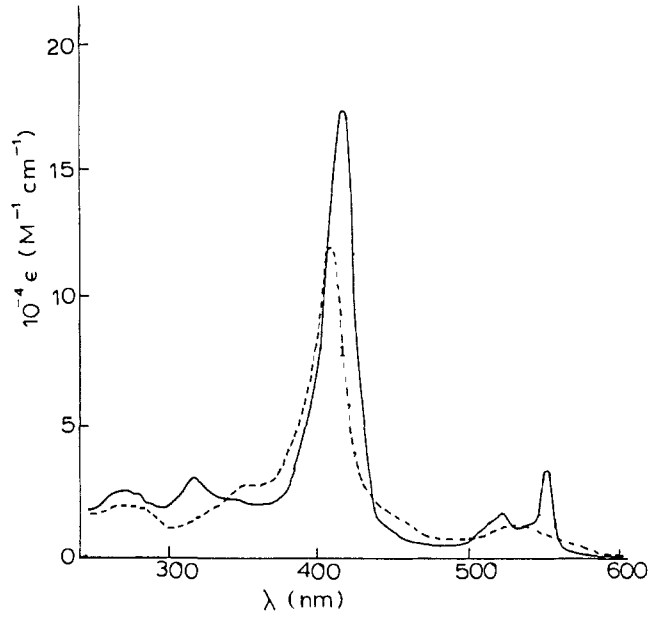

Figure 3. UV–vis spectra of reduced (—) and oxidized (---) cytochrome c_{551} from *P. stutzeri*.

Pulse radiolysis under conditions described rapidly generates the formate radical $CO_2^{\bullet-}$ (G_R = 7.38 × 10^{-7} mol J^{-1} for the formate radical[27]) as the sole significant radical species. These radicals are highly reactive (−2.0 V)[27] and preferable in protein studies to the still more reactive solvated electron, e^-_{aq} (−2.9 V).[28] The formate radical has been used in a number of other studies involving metalloprotein reduction,[29] and the rate of radical bimolecular decay ($2CO_2^{\bullet-} \rightarrow C_2O_4^{2-}$) is well-known.[30] Consequently, the radiation dose could be adjusted to ensure the concentration of reduced protein generated was <10% of the total protein concentration, and hence that pseudo-first-order conditions were retained throughout the experiment. Several experiments were performed with methyl viologen, 1,1′-dimethyl-4,4′-bipyridinium dichloride (Aldrich) present as MV^{2+} (2 mM), to generate the methyl viologen radical cation ($MV^{\bullet+}$). Pentaerithrytol, 2,2-bis(hydroxymethyl)-1,3-propanediol (Aldrich), was also investigated to generate on pulsing the potentially reducing radical species $(CH_2OH)_3CCHOH$, in this case solutions 100 mM in pentaerithrytol and 100 mM in phosphate buffer and saturated with N_2O. For all investigations on Ru-modified protein, to avoid complications resulting from the formation of the doubly reduced $Ru^{II}Fe^{II}$, which could undergo bimolecular reduction reaction as in (4), solutions

$$Ru^{III}Fe^{III} + Ru^{II}Fe^{II} \rightarrow 2Ru^{III}Fe^{II} \quad (4)$$

were pulsed only once, and the dose was such that the total concentration of reduced species was <10% of the total protein present.

The pulse-radiolysis traces was treated for first-order kinetic behavior. Plots of ln $[(A_\infty - A_t)/(A_\infty - A_0)]$ against time were linear for 3 half-lives (Figure 4). The relative rates of reaction of the ruthenium(II) and iron(III) centers with the reducing radicals could be determined from the relative magnitudes of the absorbance changes corresponding to the rapid step (Fe(III) reduced by $CO_2^{\bullet-}$ radical directly) and slower step (Fe(III) reduced by Ru(II)).

Results and Discussion

The reaction of $[Ru(NH_3)_5H_2O]^{2+}$ with *P. stutzeri* cytochrome c_{551} proceeds an order of magnitude faster than that reported for horse-heart cytochrome *c*, as expected from their relative overall charge. Small changes in the ionic strength, pH, or buffer type do not produce significant differences in the product composition, and increases in the reagent concentration or reaction time produce quantitatively the changes in product composition one would expect. Redox cycling of the multiply modified product (after

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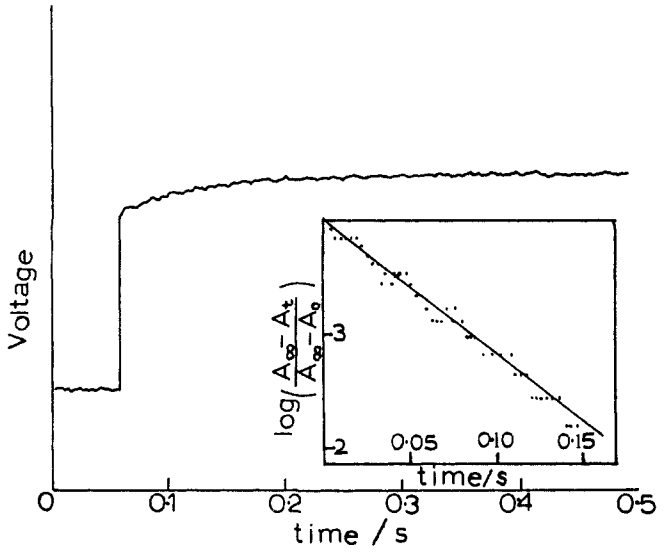

Figure 4. Pulse radiolysis trace for the reaction of the formate radical $CO_2^{\bullet-}$ (1.5 μM) with fully oxidized Ru-modified *P. stutzeri* cytochrome c_{551} (17 μM). Absorbance changes (monitored as voltage) were at 551 nm where ΔA/div = 0.0046. The rapid first stage is followed by a slower second stage corresponding to Ru(II) → Fe(III) intramolecular electron transfer. The inset shows the corresponding first-order plot over 2.8 half-lives.

Table II. Rate Constants (~20 °C) for the First Stage in the Pulse Radiolysis (Reduction with $CO_2^{\bullet-}$) of Fully Oxidized *P. stutzeri* Cytochrome c_{551}, I = 0.31 M

	[protein] (μM)	$10^{-3}k_{obs}$[a] (s^{-1})	$10^{-9}k$ (M^{-1} s^{-1})
native protein	8.8	7.1	0.81
	16.2	20	1.23
	24	27	1.13
	27	33	1.22
Ru-modified protein	7.5	13.2	1.8
	14.5	31.0	2.1

[a] Each entry is the average of at least 3 determinations.

separation from the singly modified material) significantly improves the yield of desired product. Clearly the doubly reduced Fe^{II}–Ru^{II} species did slowly undergo loss of the His47 bound ruthenium species, as shown by the elution profile in redoxcycling experiments. However, the Fe^{II}–Ru^{III} species did not apper to undergo significant loss of ruthenium over a period of hours, and the Ru-modified protein from the pulse radiolysis studies showed (by FPLC) negligible regeneration of the native protein.

The attachment of a single Ru was confirmed by ICP atomic emission spectroscopy. The blocking of the DEPC modification site on Ru modification together with the absence of the characteristic histidine C_2H resonance (as expected from paramagnetic broadening by the Ru(III)) are both consistent with attachment of the Ru complex to His47 (the only free histidine on the protein). Unlike studies on the plastocyanin[10] and azurin,[13] it was not possible to use UV–visible range spectroscopy to probe the site of attachment of the ruthenium, as the heme protein absorbs significantly in the region where $[Ru(NH_3)_5His]^{3+}$ absorbs. In fact, the spectra of native and Ru-modified proteins are not significantly different.

Pulse-radiolysis studies on the reduction of the native ferricytochrome c_{551} and the corresponding reaction in the Ru-modified protein are in the same range previously observed for horse-heart cytochrome *c*.[12] The reactions are both first order in protein, with rate constants (1.1 ± 0.2) × 10^9 and (1.9 ± 0.2) × 10^9 M^{-1} s^{-1}, respectively (Table II), indicating that the attachment of Ru has not significantly perturbed the direct reduction of the protein. The slight rate enhancement could be electrostatic in nature, as the overall charge of the Ru-modified protein is more positive as a result of attachment of the 3+ metal center. A slower reaction is also detected, corresponding to further reduction of the Fe(III)

Table III. Rate Constants (~20 °C) for the Second Stage of the Pulse Radiolysis of Fully Oxidized Ru-Modified *P. Stutzeri* Cytochrome c_{551} Corresponding to Intramolecular Electron Transfer Ru(II) → Fe(III) (pH 7.0 (100 mM phosphate), I = 0.31 M)

[protein] (μM)	k[a] (s^{-1})
5.0	13.0[b]
8.5	13.3
17.0	13.5
30.0	13.6
k = 13 (±2) s^{-1}	

[a] Each entry average of at least 3 determinations. [b] Using pentaerithrytol instead of $CO_2^{\bullet-}$ as the reducing radical.

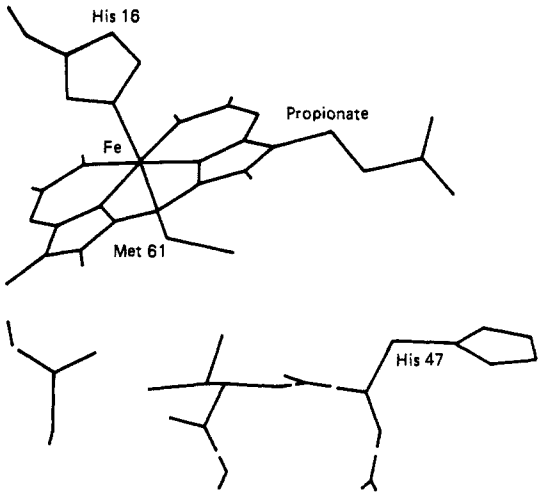

Figure 5. The relative orientations of the heme and His47 in *P. stutzeri* cytochrome c_{551}. Crystal structure information for *P. aeruginosa* cytochrome c_{551} has been used,[2] with Arg47 replaced by His47 (interactions minimized). The orientation of the buried (heme-substituent position 7) propionate is also indicated. From Evans and Sutherland computer graphics display.

center (Figure 4). Although it is not possible to monitor the fate of the Ru center in the pulse radiolysis experiments (the Ru absorbance is masked by the cytochrome), this slow reaction is consistent with electron transfer from a reduced Ru(II) center to the Fe(III). *No slow step is observed in pulse radiolysis experiments on the native protein.* With Ru-modified protein (reactant in 10-fold excess), the formate radical can reduce either the Fe(III) or the Ru(III) center, see eq 5. However, the $Fe^{III}Ru^{II}$

$$CO_2^{\bullet-} + Fe^{III}Ru^{III} \rightarrow \begin{cases} Fe^{II}Ru^{III} \\ Fe^{III}Ru^{II} \end{cases} \quad (5)$$

is thermodynamically unstable (the reduction potential for $[Ru(NH_3)_5His]^{3+/2+}$ is 80 mV),[12] and reduction of Fe(III) by the Ru(II) center can take place by either intermolecular or intramolecular electron transfer. The rate constant for the slower, first-order reaction was found to be independent of protein concentration (Table III), k = 13 (±2) s^{-1}, and hence corresponds to an intramolecular electron-transfer process.

The relative magnitudes of the absorbance changes of the fast and slow steps in Figure 5 gives an indication of the selectivity of $CO_2^{\bullet-}$ for the Fe(III) and Ru(III) centers. This selectivity amounts to only 10% in favor of the Ru, where a rate constant of 1.9 × 10^9 M^{-1} s^{-1} for $CO_2^{\bullet-}$ reduction of the Ru(III) and Fe(III) centers was obtained.

The selectivity was, unexpectedly, not significantly improved with the more hydrophobic pentaerithrytol as the reducing radical, in contrast to the results obtained for Ru-modified horse-heart cytochrome *c*.[12c] With the methyl viologen free radical, the selectivity was still further reduced, as expected for the reaction of a positively charged radical with $[Ru(NH_3)_5His]^{3+}$. The more efficient electron transfer, which is possible between the exposed

Table IV. A Comparison of Rate Constants for Intramolecular Electron Transfer in Ru-Modified Metalloproteins

protein	d (A)	$\Delta E°$ [c] (mV)	k (s^{-1})	ref
cytochrome c_{551} (P.s.)	7.9	200	13[a]	this work
HIPIP (*C.v.*)	7.9	270	18[a]	11
plastocyanin (*S.o.*)	10–12	300	<0.26	10
plastocyanin (*A.v.*)	11.9	260	<0.08	10
azurin (*P.a.*)	11.8	240	1.9[b]	13
cytochrome *c* (*h.h.*)	11.8	180	30, 53[b]	12

[a] Temperature 20 °C. [b] Temperature 25 °C. [c] Assuming an $E°$ of 80 mV for $[Ru(NH_3)_5His]^{3+/2+}$.

heme edge (with its aromatic character) and the aromatic $MV^{\bullet+}$, is also expected to increase the selectivity in favor of reduction of the Fe(III) center.

A computer graphics investigation of relative positions of the heme group and the Ru-modified His47 in *P. stutzeri* cytochrome c_{551} has been made with the *P. aeruginosa* cytochrome c_{551} crystal structure.[2] The Arg 47 residue has been replaced by histidine and its orientation adjusted to minimize interactions. The axial methionine is located on the same side of the heme ring as His47 (Figure 5). Three possible routes for electron transfer may be considered. One of these is via the propionate at position 7 on the heme ring. The propionate is known to H-bond to His47 giving rise to a high pK_a (values 8.0, 8.2, and 8.45 have been reported for His47 in the native protein.[8,9] However, unlike the unsaturate vinyl groups in hemoglobin,[31] this does not provide a facile route for electron transfer through to the heme Fe, and the route is more circuitous. The second involves transfer to the S-atom of the axial methionine, which is 7.9 Å from the C^{γ}-atom of the His47 imidazole ring. The third possibility involves transfer to the periphery of the heme ring followed by (facile) transfer through to the Fe. The relative merits of these possibilities are difficult to assess, but in view of the close similarity of the distance in each case we have used the value of 7.9 Å obtained for the C^{γ} of His47 to S of the axial Met61.

The summary of the rate constants for intramolecular transfer processes involving Ru-modified metalloproteins (Table IV) does not provide any clear pattern of behavior, or dependence on the two parameters distance (d) of separation of the donor–acceptor sites, and driving force ($\Delta E°$). Reorganization energy requirements are minimal for electron transfer between the metal centers, in this case low-spin heme-Fe^{III} (t_{2g}^5) and Ru^{II} (t_{2g}^6), as in all cases so far examined with the exception of myoglobin.[14] The through-space distance of 7.9 Å estimated from molecular graphics (Figure 5) is identical with that applying in the HIPIP study, for which a rate constant of 18 s^{-1} has been obtained. The k's are very similar, although the $\Delta E°$ values are substantially different. Electron transfer is however faster in the case of horse-heart cytochrome *c* (rate constants of 30 and 53 s^{-1}) than for cytochrome c_{551}, despite the greater donor–acceptor separation (d), and a comparable $\Delta E°$ value. In cases of weak donor–acceptor coupling it has been proposed that the electron transfer rate decreases exponentially with distance. From one such correlation[15] a rate constant of $\sim 2 \times 10^3$ s^{-1} can be predicted for the cytochrome c_{551} intramolecular rate constant based on the separation of 7.9 Å. An even bigger anomaly exists in the case of the two Ru-modified plastocyanins (rate constant <0.26 and <0.08 s^{-1})[10] and azurin.[13] Of particular interest with the plastocyanins is the very favorable electron transfer rate constant ($>5 \times 10^3$ s^{-1}) observed with unattached $[Ru(NH_3)_5(imidazole)]^{2+}$ after allowance for prior association with the protein.[11a] In this case the reductant is free to associate and react at any location on the protein surface, and clearly this approach reveals a much more favorable route for electron transfer. More information is required to establish why different electron transfer routes into the protein vary so markedly in efficiency. The relevance (and nature) of conformational changes, whether electrons can transfer in both directions or whether there are gated mechanisms,[32,33] and precisely what peptide features (aromatic residues?) are favorable for electron transfer require further careful assessment. Other relevant discussion is to be found in recent publications.[34,35]

As far as *P. stutzeri* cytochrome c_{551} is concerned there are no aromatic residues near to position 47 or in any of the three pathways indicated for electron transfer. In further studies we have explored the possibility of carrying out a similar Ru modification of *Azotobacter vinelandii* cytochrome c_{551}, which has a histidine residue at position 81. This is of interest because Phe27[5,9] is located in the path for electron transfer between His81 and the heme Fe.[2] In this instance the histidine does not undergo significant Ru modification over long reaction times. Since it does not undergo DEPC modification either it would appear that His81 is orientated and/or H-bonded in such a way as to make it unavailable for modification. The difficulty experienced in modifying some histidine residues has been noted previously for the second (uncoordinated) histidine residues in *P. aeruginosa* azurin (His35)[13] and horse-heart cytochrome *c* (His26).[12]

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Registry No. $MV^{\bullet+}$, 25239-55-8; Ru, 7440-18-8; Fe, 7439-89-6; $[Ru(NH_3)_5H_2O](PF_6)_2$, 34843-18-0; $CO_2^{\bullet-}$, 2564-86-5; $(CH_2OH)_3CCHOH$, 54954-29-9; cytochrome c_{551}, 9048-77-5.

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