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Chemistry

Preparation and characterization of a pentaammineruthenium(III) derivative of horse heart ferricytochrome *c*

(ruthenium-histidine binding/modified cytochromes/electrochemistry)

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ABSTRACT **A stable complex between pentaammineruthenium(III) and histidine-33 in horse heart ferricytochrome *c* is formed in the reaction between aquopentaammineruthenium(II) and the protein at pH 7. HPLC of the tryptic hydrolysate of the modified protein was employed to identify the pentaammineruthenium binding site. Spectroscopic measurements show that the integrity of the native structure in the vicinity of the heme *c* group is maintained in the ruthenium-modified protein. The reduction potentials are: heme *c* ($Fe^{3+/2+}$), 0.26 V; $Ru(NH_3)_5(His\text{-}33)^{3+/2+}$, 0.15 V (vs. normal hydrogen electrode).**

A central theme of our research in bioinorganic chemistry has been the elucidation of the factors that control the rates of metalloprotein electron transfer reactions. One factor that we know to be important is the distance that separates the redox centers of the protein and the substrate in the activated complex undergoing electron transfer (1). To explore rate–distance relationships in detail, we have begun to prepare protein derivatives in which one or more redox-active metal complexes are attached covalently to ligands supplied by the polypeptide chains. The idea is to produce synthetic multisite metalloproteins, whose redox centers are fixed at several different separation distances, and then to measure the intramolecular electron transfer rates at those known distances.

Matthews has shown (2–5) that $Ru(NH_3)_5H_2O^{2+}$ is a good reagent for modification of histidine residues in proteins, and we have found that the reaction of $Ru(NH_3)_5H_2O^{2+}$ with horse heart cytochrome *c*, followed by oxidation, produces several stable $Ru(NH_3)_5^{3+}$-ferricytochrome *c* derivatives. Characterization of one of these derivatives, $Ru(NH_3)_5(His\text{-}33)^{3+}$-ferricytochrome *c*, is reported here.

MATERIALS AND METHODS

Horse heart cytochrome *c* (type VI) was obtained from Sigma. The protein was purified by cation exchange chromatography on carboxymethylcellulose (6). Trypsin treated with *N*-tosylphenylalanine chloromethyl ketone was from Worthington. Baker HPLC reagent-grade acetonitrile was used for HPLC. Ruthenium atomic absorption standard solutions were obtained from Alfa-Ventron and Aldrich. 4,4-Bipyridyl·$2H_2O$ was obtained from Aldrich and was recrystallized from water. Materials for column chromatography were AG50W-X2, 200 mesh (Bio-Rad); CM52 (Whatman); Sephadex G-25-150 (Sigma).

Protein Modification (at California Institute of Technology). Cytochrome *c* was reacted with $Ru(NH_3)_5H_2O^{2+}$ under argon at room temperature in 85 mM sodium phosphate buffer (pH 7.0) for 24–72 hr. Each preparation generally employed 100 mg of purified cytochrome *c* at a concentration of *ca.* 0.2 mM, and a 50-fold excess of ruthenium reagent ([Ru] ≈ 10 mM). $Ru(NH_3)_5H_2O^{2+}$ was generated by the reduction of $[Ru(NH_3)_5Cl]Cl_2$ (7, 8) over zinc/mercury amalgam or $[Ru(NH_3)_5H_2O](PF_6)_2$ (9) was used directly. The reaction was terminated by applying the solution to a Sephadex G-25 column. The pooled fractions from the Sephadex column were oxidized and concentrated in a stirred ultrafiltration cell. Prior to rechromatography or other experiments, materials were equilibrated into the buffer of choice (or water) by several cycles of dilution and ultrafiltration. The components of the mixture from the Sephadex column were separated by cation exchange chromatography on a 2 × 50–60 cm column of Whatman CM-cellulose (CM52) that had been equilibrated with 85 mM phosphate buffer (pH 7.0). All ion exchange chromatography was performed at 4°C. The chromatogram was developed by a linear gradient between 1.0 liter each of 85 mM and 150 mM sodium phosphate buffer (pH 7.0) at a flow rate of *ca.* 30 ml/hr. Five-milliliter fractions were collected, and the absorbance of every other fraction was read at 410 nm.

Five distinct peaks (A–E) from four separate $Ru(NH_3)_5Cl^{2+}$/Zn (amalgam) preparations were pooled and purified as follows: Peak A (*ca.* 32 mg of cytochrome *c*) was rechromatographed on a column (2.3 × 32 cm) of CM52 with 85 mM sodium phosphate buffer (pH 7.0); peak B (*ca.* 42 mg) was rechromatographed on CM52 with 85 mM sodium phosphate buffer (column bed, 2.3 × 26 cm); peak C (*ca.* 48 mg) also was eluted from a CM52 column (2.3 × 19 cm) with 85 mM sodium phosphate buffer; peaks D (*ca.* 24 mg) and E (*ca.* 43 mg) were rechromatographed on CM52 with 100 mM sodium phosphate buffer (pH 7.0). Column beds were 2.3 × 19 cm (peak D) and 2.3 × 17 cm (peak E). In all cases, a flow rate of 30 ml/hr was maintained and 5-ml fractions were collected. The absorbance of every other fraction was read at 410 nm.

The fractions corresponding to each of the major species that eluted upon rechromatography were pooled (A′–E′). All experiments reported here are for E′ (concentrated solutions that were to be used within a few days were kept at 4°C; other samples were shell-frozen in liquid nitrogen and stored at −10°C) or its analogue in the Rutgers' preparation. E′ was analyzed for Ru by flame atomic absorption. The Ru/heme *c* ratio was found to be 1:1 (±10%).

Protein Modification (at Rutgers). A 20-ml solution of $Ru(NH_3)_5H_2O^{2+}$ (≈0.2 M) was prepared by zinc/mercury amalgam reduction of $Ru(NH_3)_5Cl^{2+}$. The pH was adjusted to 7 with 100 mM phosphate buffer and cytochrome *c* (125 mg)

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Abbreviations: AUFS, absorbance unit full scale; SSCE, saturated NaCl calomel electrode; NHE, normal hydrogen electrode.

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(0.01 mmol in 3 ml of 100 mM phosphate buffer contained in a dialysis bag) was added to the ruthenium solution. After 48 hr, the dialysis bag was removed and washed (four washes of 50 ml each) with ascorbic acid solution in an ultrafiltration cell by using a UM05 membrane. The solution then was concentrated to 10 ml and potassium ferricyanide (2 equivalents) was added with stirring. The solution then was charged onto a CM52 cellulose column (45 × 1.5 cm) (equilibrated with 100 mM phosphate buffer at pH 7). The first band eluted from this column with 100 mM phosphate. The second band eluted when a KCl/phosphate buffer salt gradient was used. A third band remained at the top of the column and did not elute with 1 M KCl. The second band (analyzed as the ruthenium-modified cytochrome *c*) was collected, washed with water, and lyophilized. The first band was native protein (no detectable ruthenium). The second band had a Ru/Fe ratio of 1:1 (±10%) by flame atomic absorption and chemical analyses.

Tryptic Hydrolyses. The protein (native or E′) was digested by trypsin and the resulting peptides were separated by HPLC (10). A 4-mg sample of the cytochrome *c* species in 2 ml of water was adjusted to pH 8.5 with 0.1 M NaOH. NH_4HCO_3 was added to give a concentration of 0.025 M. At 0 and 6 hr, 25 μl of trypsin solution (2 mg/ml in 0.001 M HCl) was added. The solution was stirred at 37°C for a total of 24 hr. The pH of the solution then was lowered to 2.1. After filtration through a 0.5-μm cellulosic membrane filter, the solution was lyophilized to dryness. The lyophilized material was dissolved in chromatography pH 2.85 phosphate buffer (49 mM KH_2PO_4/5.4 mM H_3PO_4) and refiltered. A 2-mg sample of the filtered cytochrome *c* dissolved in 0.1 ml of pH 2.85 phosphate buffer was injected onto the HPLC column (Altex Ultrasphere, Berkeley, CA; 4.6 × 250 mm). The peptides were eluted in 120 min with a linear gradient from 0 to 45% in acetonitrile at a flow rate of 1 ml/min. Absorbance at 220 nm was recorded at 1.0 absorbance unit full scale (AUFS); absorbance at 300 nm was recorded at 0.1 AUFS. Fraction size was 0.5 ml. Fractions corresponding to those peaks that were later subjected to amino acid analysis or examined spectrally were pooled and blown dry with filtered air in a 40°C water bath.

Amino Acid Analyses. These experiments employed a modified Beckman 120 amino acid analyzer. The sensitivity of the instrument was such that 5 nmol of amino acids was adequate for calculation.

Spectra. Spectra of HPLC fractions were recorded on a Hewlett–Packard 8450 UV-Vis spectrophotometer with a 7225A Graphics Plotter. The dried sample corresponding to a given HPLC peak was rediluted with 1 ml of water and the spectrum was recorded in a small volume, 1-cm cell. The pH of the solution was ≈3. Other optical absorption spectral measurements were made on Cary 219 and Cary 118C spectrophotometers. Circular dichroism spectra were measured on a Cary 60. NMR spectra were obtained on a Bruker 360 MHz instrument.

Cyclic Voltammetry. For these experiments a PAR 174A potentiostat was used in conjunction with a Hewlett–Packard 7004B-X-Y recorder. A Keithley 177 Microvolt DMM voltmeter was used to confirm the starting potential prior to a scan. The all-glass cell consisted of two compartments separated by a sintered glass disk. It also was equipped with a side arm for degassing the sample. The working electrode was a gold disk (3 mm in diameter), and the auxiliary electrode was a coil of platinum wire. The reference electrode was a saturated NaCl calomel electrode (SSCE). Solutions of cytochrome *c* (1.5–2.0 ml of 0.1–0.2 mM) also were 0.1 M in 4,4′-bipyridyl (11). The supporting electrolyte was 0.1 M $NaClO_4$ in sodium phosphate buffer (pH 7, μ = 0.05 M). All solutions were deoxygenated by bubbling with argon for several minutes prior to use.

RESULTS AND DISCUSSION

The site of attachment of $Ru(NH_3)_5^{3+}$ to the protein in E′ was identified by locating and isolating the ruthenium-containing

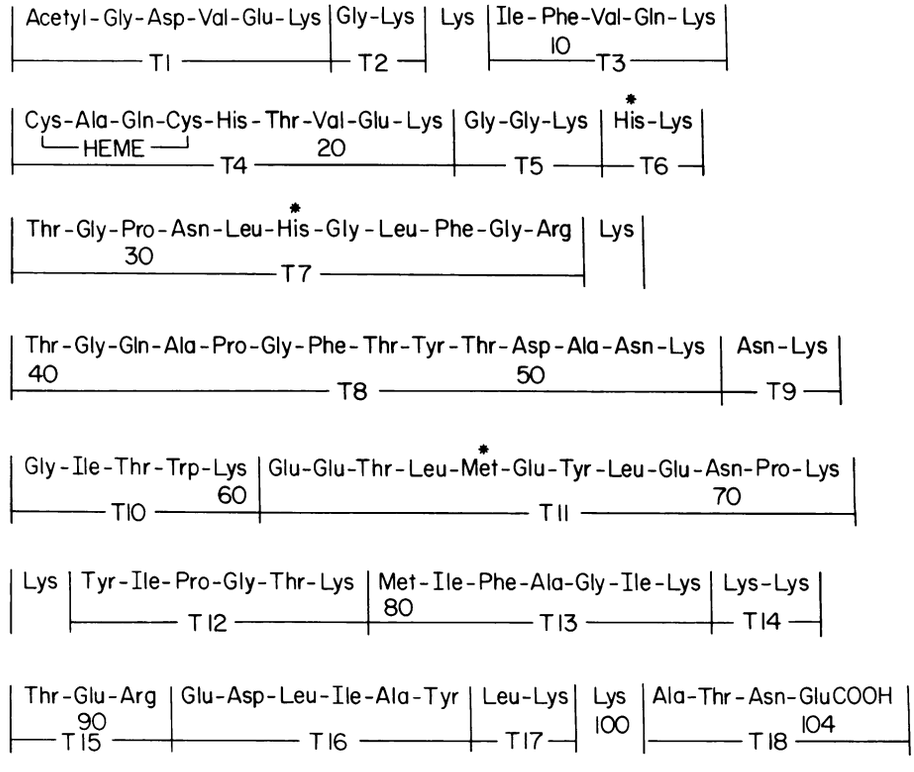

FIG. 1. The tryptic peptides (T1–T18) of horse heart cytochrome *c* (vertical lines indicate the points of hydrolysis by trypsin). Potential $Ru(NH_3)_5^{3+}$ ligands are His-26 (T6), His-33 (T7), and Met-65 (T11).

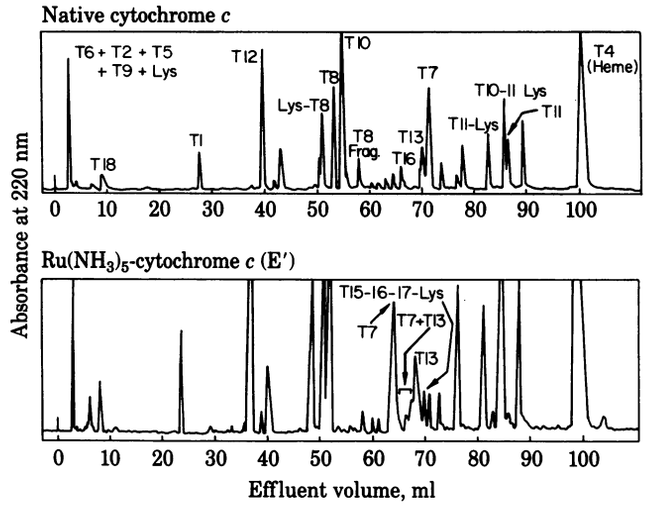

FIG. 2. Reversed-phase HPLC of tryptic digests of native cytochrome *c* and $Ru(NH_3)_5^{3+}$-cytochrome *c* (E′) at A_{220} (1.0 AUFS). A 2-mg sample of hydrolyzed protein was chromatographed with a linear gradient between 0% and 45% acetonitrile in pH 2.85 phosphate buffer in 120 min. The labeled peaks were identified by amino acid analyses.

tryptic peptide (Fig. 1). The separations of the tryptic digests of both native cytochrome *c* and E′ on a reversed-phase HPLC column are shown in Fig. 2. The major difference between the chromatograms of the native and ruthenium-modified proteins is in the position of peptide T7. This (His-33)-containing peptide has shifted to a considerably lower elution volume in the HPLC of the ruthenium-modified cytochrome *c*. Analytical data (numbers of amino acid residues) confirm that the modified peptide is T7: Lys, 0.16; His, 0.87; Arg, 0.94; Asp, 1.27; Thr, 0.90; Glu, 0.38; Pro, 0.93; Gly, 2.90; Ala, 0.34; Ile, 0.34; Leu, 2.24; Tyr, 0.31; Phe, 0.94.

The HPLC of a native cytochrome *c* tryptic digest was rerun, this time monitoring the absorbance of the eluting peptides at 300 nm (Fig. 3). In the 300-nm chromatogram of native protein, only the peaks corresponding to the T10 (tryptophan containing) and T4 (heme containing) peptides are evident but, in addition, a new peak at an elution volume of ≈60 ml is observed in the $Ru(NH_3)_5^{3+}$ derivative. This peak is identified as the T7 peptide

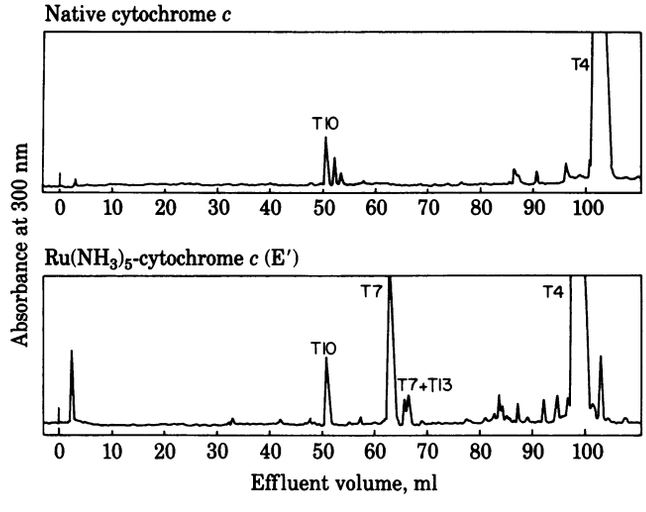

FIG. 3. Reversed-phase HPLC of tryptic digests of native cytochrome *c* and $Ru(NH_3)_5^{3+}$-cytochrome *c* (E′) at 300 nm (0.1 AUFS) (same conditions as in Fig. 2). The labeled peaks were identified by analogy to the 220-nm chromatograms.

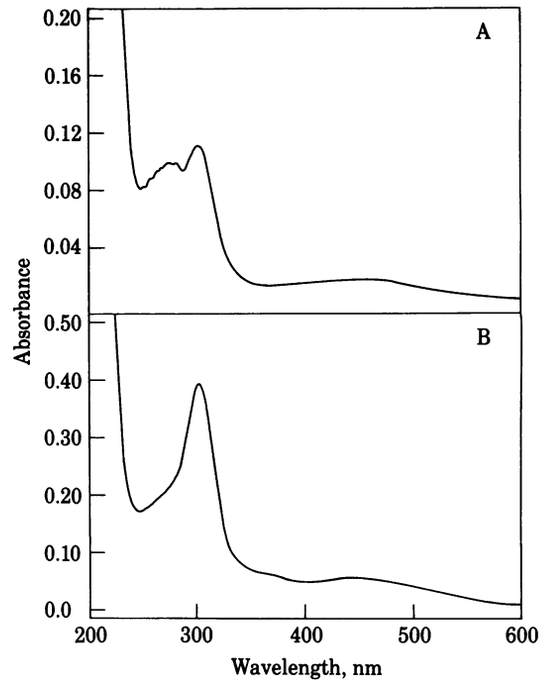

FIG. 4. (*A*) Absorption spectrum of the (His-33)-containing peptide, T7, of $Ru(NH_3)_5^{3+}$-cytochrome *c* (E′), isolated by tryptic hydrolysis and HPLC (Fig. 3); pH 3. (*B*) Absorption spectrum of a 0.2 mM solution of $[Ru(NH_3)_5His]Cl_3$; pH 7.

by comparison with the 220-nm chromatogram of the $Ru(NH_3)_5^{3+}$-cytochrome *c* (Fig. 2). The attachment of $Ru(NH_3)_5^{3+}$ to His-33 in the T7 peptide makes it possible to detect T7 at 300 nm in

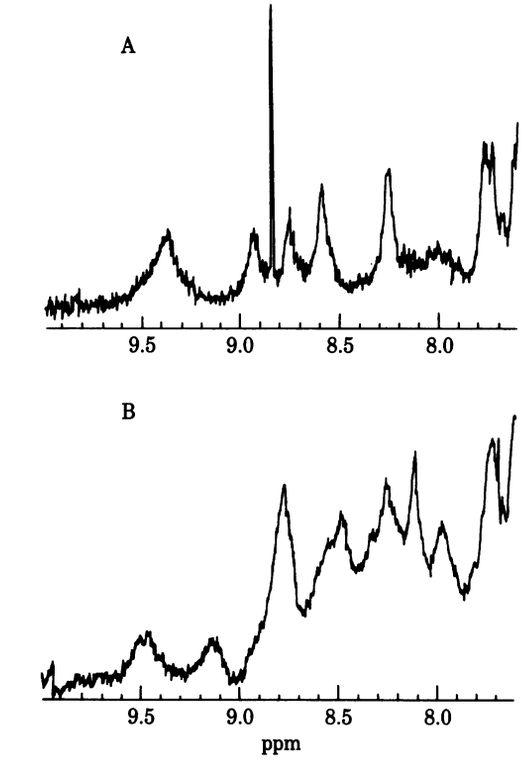

FIG. 5. High-field (360 MHz) NMR spectra of native (*A*) and $Ru(NH_3)_5^{3+}$-modified (*B*) ferricytochromes *c* (pH 5, 25°C, ^2H_2O) in the region of imidazole C-2 proton resonances [internal 2,2-dimethyl-2-silapentane-5-sulfonate (DSS)].

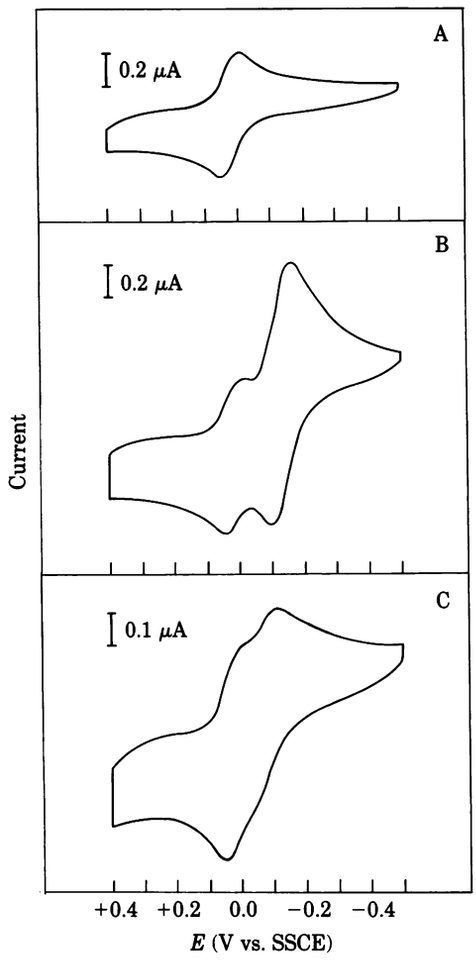

FIG. 6. Direct current cyclic voltammetry of E′ and related species. All species are in pH 7 phosphate buffer (μ = 0.05 M) (gold electrode) in the presence of 0.01 M 4,4′-bipyridyl. Scan rate = 5 mV/s. (*A*) Native cytochrome *c*, 0.21 mM: $E_{1/2}$ = 27 ± 3 mV (263 ± 3 mV vs. NHE). (*B*) Native cytochrome *c*, 0.21 mM, with $[Ru(NH_3)_5His]Cl_3$, 0.21 mM: $E_{1/2}$ (heme *c*) = 19 ± 1 mV (255 ± 1 mV vs. NHE); $E_{1/2}$ $[Ru(NH_3)_5His^{3+/2+}]$ = − 128 ± 2 mV (108 ± 2 mV vs. NHE). (*C*) E′, 0.12 mM: $E_{1/2}$ (heme *c*) = 21 ± 3 mV (257 ± 3 mV vs. NHE); $E_{1/2}$ $[Ru(NH_3)_5(His\text{-}33)^{3+/2+}]$ = 85 ± 3 mV (151 ± 3 mV vs. NHE).

the modified protein [the spectrum of $Ru(NH_3)_5His^{3+}$ exhibits a peak at 303 nm (molar extinction coefficient ε = 2,100 $M^{-1}\cdot cm^{-1}$) (12)]. The fractions corresponding to the T7 peptide in the 300-nm chromatogram of $Ru(NH_3)_5^{3+}$-cytochrome *c* were collected, dried, and redissolved in water. The spectrum of the modified T7 peptide is exactly that of $Ru(NH_3)_5His^{3+}$ with an additional peak at ≈275 nm attributable to the presence of Phe in the T7 peptide (Fig. 4).

The $Ru(NH_3)_5^{3+}$ binding site also was confirmed by high-field NMR measurements (Fig. 5). The general features in the spectrum of the ruthenium derivative are broader than those observed for the native protein, and it is apparent that the His-33 C-2 proton resonance (13) at 8.84 ppm (Fig. 5*A*) is absent in Fig. 5*B*.

The visible and ultraviolet spectra of E′ in both the oxidized and dithionite-reduced forms are indistinguishable from those of native cytochrome *c*. In the fully oxidized state, features attributable to $Ru(NH_3)_5His^{3+}$ are obscured by heme absorption in the spectrum. The presence of the conformation-sensitive 695-nm band (14) in the spectrum of the oxidized ruthenium-modified protein indicates that the heme *c* site is not perturbed significantly. A similar conclusion follows from the observation that the ultraviolet circular dichroism spectra of native and modified cytochromes are virtually identical.

The ruthenium derivative is ideally suited for the electron transfer kinetic studies mentioned earlier. The attached $Ru(NH_3)_5^{3+}$ group is inert to substitution, and by cyclic voltammetry we have demonstrated that both the heme *c* [0.26 V vs. normal hydrogen electrode (NHE)] and $Ru(NH_3)_5(His\text{-}33)^{3+}$ (0.15 V vs. NHE) centers are redox-active (Fig. 6). One important result that emerges is that the heme *c* potential is unperturbed by the presence of the $Ru(NH_3)_5^{3+}$ group on the surface of the protein. Elucidation of the factors that tune the potential of $Ru(NH_3)_5(His\text{-}33)^{3+}$ 40 mV higher than that of isolated $Ru(NH_3)_5His^{3+}$ is an important subject that remains to be explored.

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