Ruthenium-modified Proteins. Reactions of *cis*-$[Ru(NH_3)_4(OH_2)_2]^{2+}$ and *cis*-$[Ru(en)_2(OH_2)_2]^{2+}$ with Azurin, Myoglobin and Cytochrome c

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Abstract

Reactions of *cis*-$[Ru(en)_2(OH_2)_2]^{2+}$ (or *cis*-$[Ru(NH_3)_4(OH_2)_2]^{2+}$) with *Pseudomonas aeruginosa* azurin (Az), horse heart myoglobin (Mb^h), and horse heart cytochrome c (cyt c) give Ru-labelled proteins. The ruthenium binding sites in the singly modified derivatives are His-83 (Az), His-81 (Mb^h), and His-33 (cyt c). Spectroscopic and electrochemical measurements indicate that the structures of the proteins are not perturbed by the surface-bound ruthenium complexes. The E_f° values of the Ru(III)/(II) couple in these Ru-modified proteins fall between −0.07 and −0.13 V *vs.* NHE.

Recent experiments have demonstrated that the surface histidines of proteins react rapidly with $[a_5Ru(OH_2)]^{2+}$ (a = NH_3), and derivatives of horse heart cytochrome c (cyt c), *Pseudomonas aeruginosa* azurin (Az), and sperm whale myoglobin (Mb^{sw}) have been characterized [1–3]. We have begun an investigation of the binding of other ruthenium–amine complexes with proteins, because of the need for electron-transfer experiments with variations in the reaction driving force. Complexes of the types *cis*- and *trans*-$[Ru^{II}L_4(OH_2)_2]^{2+}$ (L_4 = 4 monodentate amines, or 2 bidentate amines, or a tetradentate amine) are particularly attractive in this context, because their chemistry is well worked out [4, 5]. In this communication, the products of the reactions of horse heart myoglobin (Mb^h), Az, and cyt c with *cis*-$[Ru(NH_3)_4(OH_2)_2]^{2+}$ (**1**) and *cis*-$[Ru(en)_2(OH_2)_2]^{2+}$ (**2**) are described.

A solution of **1** (or **2**) was generated by Zn(Hg) reduction of *cis*-$[Ru(NH_3)_4Cl_2]^+$ (or *cis*-$[Ru(en)_2Cl_2]^+$) in tris·HCl or phosphate buffer (pH 6.5) under an inert atmosphere for 1 h [5]. The modification reactions and the properties of the Ru-labelled proteins are summarized in Table I. In most preparations, the reaction was quenched by applying the solution to a Sephadex G-25 column and the Ru-labelled protein was then oxidized by $Co(dipic)_2^-$ and purified by standard procedures [1–3]. For Mb^h, both 1:1 (**A**) and 2:1 (**B**) derivatives were isolated. Tryptic peptide analyses showed conclusively that the sites of modification in the 1:1 Ru-labelled proteins are His-81 of Mb^h and His-83 of azurin[§]. In

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[§]The peptide-mapping experiments were done in collaboration with J. B. Shelton, J. R. Shelton and W. A. Schroeder. Procedures for tryptic hydrolyses, separation of the resulting peptides by reversed-phase HPLC, and amino acid analyses have been described previously [1–3]. Absorbances were recorded at 220 (peptide bonds) (**A**,**B**,**D**,**E**), ~450 (*cis*-$[Ru(en)_2(OH)(His)]$) (**A**,**B**,**D**), and 370 nm (*cis*-$[Ru(NH_3)_4(OH(His)]$ (**E**).

TABLE I. Preparation and Characterization of Ruthenium-modified Proteins

Reaction[a]	Product	pI	E_f° (V *vs.* NHE)[b]
1 and 50 mg Mb^h, 4–5 h	*cis*-$[Ru(en)_2(OH)](His\text{-}81)Mb^h$ (**A**)	8.2	−0.12
1 and 50 mg Mb^h, 24 h	*cis*-$[Ru(en)_2(OH)]_2Mb^h$ (**B**)	9.2	−0.13
1 and 50 mg cyt c, 24 h	*cis*-$[Ru(en)_2(OH)](His\text{-}33)$cyt c (**C**)	[c]	0.265[d]
1 and 12 mg Az, 4 h	*cis*-$[Ru(en)_2(OH)](His\text{-}83)$Az (**D**)	7.2	−0.09
2 and 10 mg Az, 4 h	*cis*-$[Ru(NH_3)_4(OH)](His\text{-}83)$Az (**E**)	7.2	−0.10 0.30[e]

[a]**1** was generated by Zn(Hg) reduction of 50 mg (**A**, **B**, **C**) or 7 mg (**D**) *cis*-$[Ru(en)_2Cl_2]ClO_4$ in 15–20 ml phosphate (**A**) or 15–20 ml tris·HCl (**B**,**C**) or 10 ml tris·HCl (**D**); **2** was generated by Zn(Hg) reduction of 7 mg *cis*-$[Ru(NH_3)_4Cl_2]Cl$ in 10 ml tris·HCl. [b]Ru(III)/(II) at pH 7.2 and 25 °C unless noted otherwise. [c]Not determined. [d]cyt c [Fe(III)/(II)]. [e]Az-[Cu(II)/(I)].

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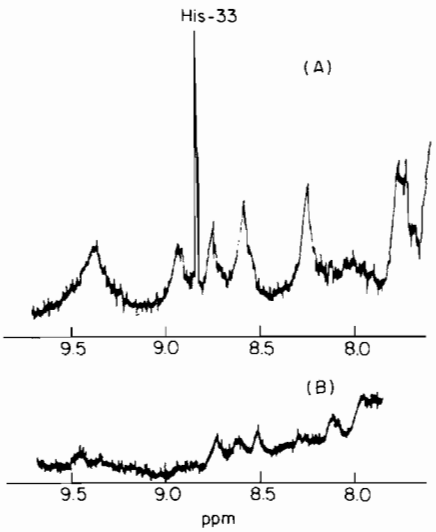

Fig. 1. Proton NMR spectra (Bruker WH500) of (a) cyt c and (b) *cis*-[Ru(en)$_2$(OH)](His-33)cyt c in the region of imidazole ^{1}H resonances (D_2O/DCl, pH 5.5; internal DSS).

product **C**, the absence of the His-33 C-2 proton peak at $\delta = 8.84$ ppm in the high-field ^{1}H NMR spectrum (Fig. 1) strongly indicates that His-33 of cyt c is labelled with **2**.

Preliminary UV–Vis circular dichroism measurements indicate that the conformations of the proteins are not perturbed significantly by the Ru complexes, a finding that is in accord with previous work on a_5Ru-proteins [1–3, 6]. Spectroelectrochemical measurements on **C** and **E** show that the structures of the redox-active metal sites (heme c and blue copper) are intact in the modified proteins.

At pH 7.2 and in tris·HCl buffer the E_f values of *cis*-[Ru(en)$_2$(OH)(His)]$^{2+/+}$ in products **A**–**D** are in the range –0.07 to –0.13 V *vs.* NHE; these E_f° values are comparable to that found for *cis*-[Ru(NH$_3$)$_4$(OH)(His)]$^{2+/+}$ (–0.10 V *vs.* NHE) in product **E**. The electrochemical results suggest that the sites of Ru–amine binding in products **A**–**E** are similar. The decrease in E_f° from *trans*-[Ru(NH$_3$)$_3$(Im)(OH$_2$)]$^{3+}$ (0.12 V) [4] to *cis*-[Ru(NH$_3$)$_4$(OH)(His)]$^{2+}$ (–0.10

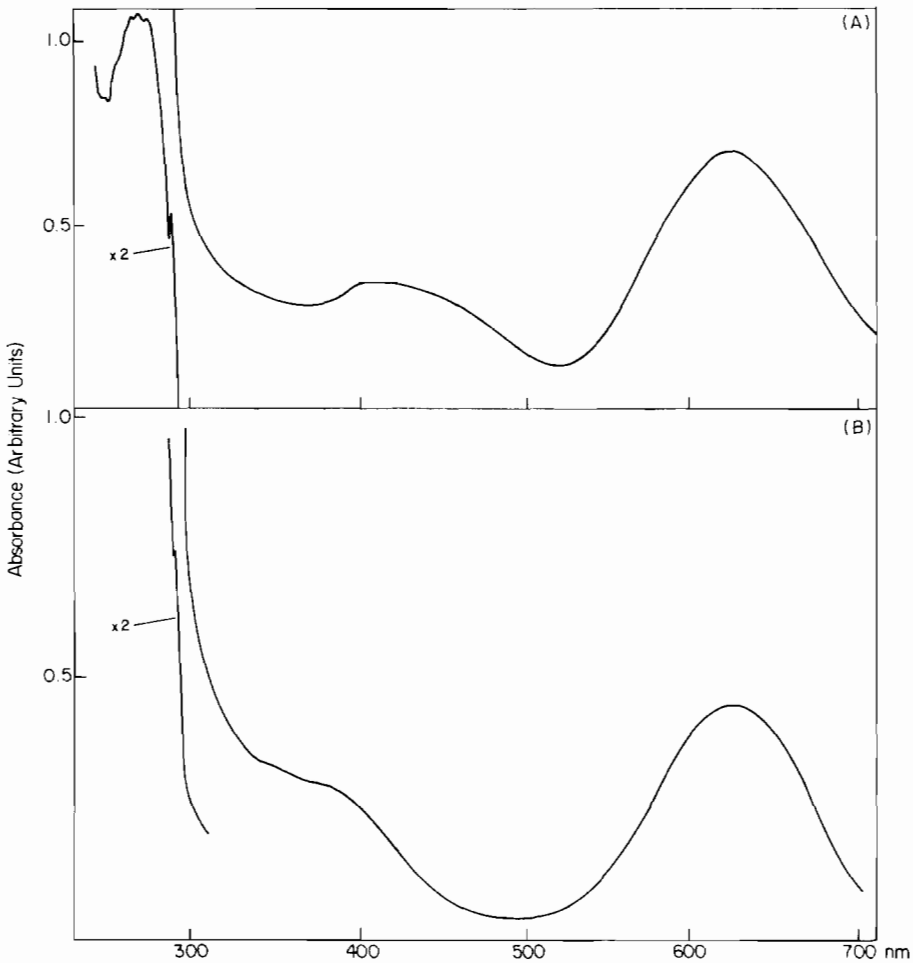

Fig. 2. UV–Vis absorption spectra of (a) *cis*-[Ru(en)$_2$(OH)](His-83)Az and (b) *cis*-[Ru(NH$_3$)$_4$(OH)](His-83)Az in tris·HCl (pH 7.2) at 25 °C.

V) to *trans*-$[Ru(NH_3)_4(Im)(SO_4)]^+$ (–0.37 V) [7] parallels decreasing charge on the Ru(III)–amine complexes. Thus the observed pH dependence of the E_f° values (e.g., **C**: pH 7.2, –0.13 V; pH 4, 0.013 V) of the Ru–amine derivatives is consistent with the formulation *cis*-$[Ru(en)_2(OH)(His)]^{2+}$ at pH 7.2.

The UV–Vis absorption spectra of **D** and **E** clearly show the blue copper signature (Fig. 2); the bands at ~390 and 430 nm are probably due to *cis*-$[Ru(NH_3)_4(OH)(His)]^{2+}$ and *cis*-$[Ru(en)_2(OH)$-$(His)]^{2+}$, respectively. A similar UV–Vis absorption spectrum has been found for *cis*-$[Ru(en)_2(OH)(His$-$81)]$ [apoMb^h] (λ_{max} ~450 nm)†.

It appears that *cis*-$[Ru(en)_2(OH_2)_2]^{2+}$ is very similar to $[a_5Ru(OH_2)]^{2+}$ as a protein modification reagent. With cyt c and Az, the same (exposed) histidines (His-33 [1] and His-83 [3], respectively) are labelled; and, with Mb^h, His-81, which is one of the major products of the reaction [2] of $[a_5Ru(OH_2)]^{2+}$ with Mb^{sw}, is readily modified.

The reduction potentials of the Ru sites in the *cis*-$[Ru(en)_2(OH)(His)]^{2+}$-proteins are roughly 0.25 V lower than those of a_5Ru^{3+} analogues. In line with this finding, neither **A** nor **B** catalyzes the aerial oxidation of ascorbate. This lack of reactivity in the low potential derivatives suggests that electron transfer from ascorbate to Ru(III) plays a key role in the observed [2a] catalytic activity of $[a_5Ru]_3Mb^{sw}$

†Virtually the same UV–Vis absorption spectrum is obtained when *cis*-$[Ru(en)_2(OH_2)_2]^{2+}$ is allowed to react with imidazole under similar conditions. In view of the electrochemical results described in the text, it is unlikely that the 450 nm band arises from a charge transfer transition of any product of a ligand oxidative dehydrogenation reaction of *cis*-$[Ru(en)_2(OH)(His)]^{2+}$.

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