Site-Dependent Stereoselective Binding of Ruthenium Aquobipyridine Complexes to Histidine Side Chains in Horse Heart Cytochrome *c*

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Stereoselective recognition of substrate molecules by enzymes, proteins, and DNA is an active area of investigation.[1] Enantiomeric preference for substrates is observed in the rates and equilibria of redox reactions between proteins and transition-metal complexes[2] and also in the electrostatic and interchalative interactions between small molecules and DNA.[3] The stereoselective covalent binding of metal complexes to proteins and DNA has the potential for being useful in carrying out stereoselective transformations for a variety of organic compounds.[4]

We have been studying the covalent binding of ruthenium ammine and bipyridine complexes to cytochrome *c* and the dependence of their intraprotein electron-transfer rates on distance and protein structure for a number of years.[5] We report here the first example of stereoselective binding of two racemic ruthenium(II) aquobipyridine complexes to histidine residues (His **26** and His **33**) of horse heart cytochrome *c* (Hh cyt *c*). The stereoselectivity of ruthenium binding varies with the surface exposure of the modification site, and it is further enhanced when steric interactions are increased by the addition of two methyl groups per bipyridine ligand. Furthermore, the diastereomeric complexes formed when one or the other enantiomer of a given ruthenium complex binds at a given site possess distinct spectroscopic, thermodynamic, and kinetic properties. While evidence for stereospecific interactions of transition-metal complexes with proteins is known, isolable stereospecific protein−metal complexes that exhibit measurable isomeric differences in the spectroscopy, thermodynamics, and kinetics of their reactions are rare.

The three potential histidine binding sites for ruthenium on Hh cyt *c* (His **33**, His **26**, and His **18**) are located in different regions of the protein, with His **33** a somewhat exposed surface residue, His **26** an interior residue within the protein, and His **18** a residue bound to the heme iron and thus unavailable for modification in neutral media. Hydrophilic complexes such as $[Ru(NH_3)_5(OH)_2]^{2+}$ and $[Ru(NH_3)_4L(OH_2)]^{2+}$ (L is a nitrogen heterocycle) specifically bind to surface histidine residues such as His **33**,[5,6] whereas hydrophobic ruthenium(II) aquobipyridine complexes bind both to surface (His **33**) and to protein-buried (His **26**) His residues. For example, the hydrophobic *cis*-$[Ru(bpy)_2(H_2O)_2]^{2+}$ (bpy = 2,2′-bipyridine) covalently binds to the His **26** and His **33** residues of Hh cyt *c*, with only a small preference for the more surface-exposed His **33** site.[7,8] The chiral discrimination exhibited by Hh cyt *c* toward these ruthenium aquobipyridine complexes bound in different locations results in different spectroscopic, electrochemical, and kinetic properties for each stereoisomer.

Circular dichroism (CD) was used to determine the stereoselective enrichment ratios (enantiomeric excess, ee) by comparison of the CD spectrum of the chromatographically isolated His **26** and His **33** ruthenium-derivatized cytochrome *c* with that of the Λ- and Δ-*cis*-$[Ru(bpy)_2(H_2O)Pyridine]^{2+}$, the small molecule reference.[9] For modifications at the His **33** site, a large stereoselectivity for the Λ- over the Δ-isomer (ee ∼34%) (Figure 1A) is observed for both ruthenium derivatives, $[Ru(bpy)_2(H_2O)]$-His **33**-cyt *c* and $[Ru(dmbpy)_2(H_2O)]$-His **33**-cyt *c* (dmbpy = 4,4′-dimethyl-2,2′-bipyridine).[10]

At the His **26** site (Figure 1B), binding favors the Δ isomer over the Λ isomer; however, the enantiomeric excess is only 6% in favor of Δ-$[Ru(bpy)_2(H_2O)]$-His **26**-cyt *c*. In contrast, when the bipyridines have 4,4′-methyl substituents, the ee in favor of Δ-$[Ru(dmbpy)_2(H_2O)]$-His **26**-cyt *c* increases to ∼38%. Our experiments, therefore, are clear examples of the stereochemical control exerted by the environment of the histidine side chains and how the bipyridine substituents can amplify the stereochemical preference.

The His **33** and the His **26** sites experience different interactions with the protein matrix as evidenced by the crystal structures.[11]

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(9) Although *cis*-Λ (or Δ)-$[Ru(bpy)_2(H_2O)His]^{2+}$ has not been assigned, a related study showed that the sign of the CD at 300 nm for Δ (Λ)-$[Ru(L-L)_2L_1L_2]^{2+}$, where L−L = bpy and phenanthroline, L_1,L_2 = pyridine, imidazole, or Cl^-, is not sensitive to the type of ligand L_1 and L_2 ((a) Hua, X. Ph.D. thesis, 1993, Institute of Inorganic Chemistry, University of Fribourg, Switzerland. (b) Bosnich, B. *Inorg. Chem.* **1968**, *7*, 178). Throughout this paper, we used the CD at $\Delta\epsilon_{294nm} = -170$ for *cis*-Δ-$[Ru(bpy)_2(py)(H_2O)]^{2+}$ in aqueous solution (Hua, X.; Lappin, A. G. *Inorg. Chem.* **1995**, *34*, 992), as the small molecule model to calculate the enantiomeric excess (ee) of the ruthenium complexes in the proteins.

(10) A slight preference (less than 1% of the values observed in this work) for the Λ isomer of the $[Ru(bpy)_2(imidazole)(HisX)]^{2+}$ (X = 66, 58, 55) of mutant yeast cytochrome *c* was reported earlier (Casimiro, D. R., Richards, J. H. Winkler, J. R., Gray, H. B. *J. Phys. Chem.* **1993**, *97*, 7, 13073).

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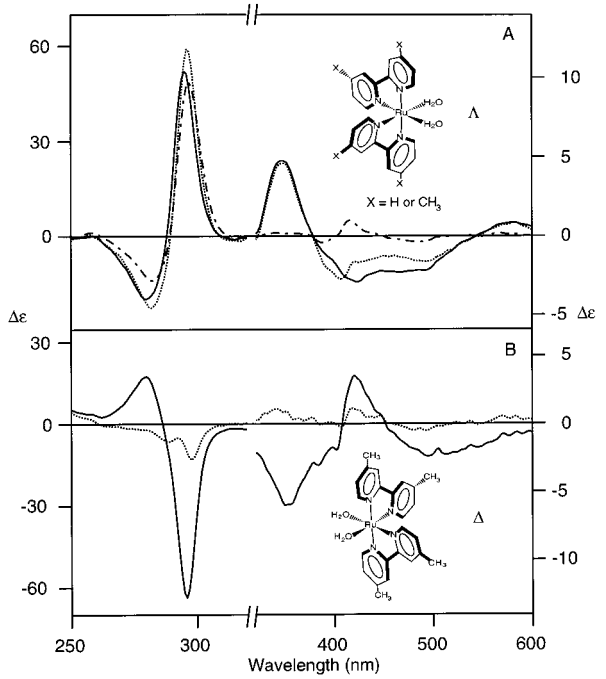

Figure 1. (A) The difference CD spectra between unmodified and ruthenium-modified Hh cyt *c* for the reaction of Hh cyt *c* with the racemic *cis*-[Ru^{II}(bpy)$_2$(H_2O)]-His **33**-cyt *c* (•••) and *cis*-[Ru(dmbpy)$_2$(H_2O)]-His **33**-cyt *c* (—). The stereochemistries of the bound ruthenium complexes are assigned by comparison with the model compounds. Both of the modified proteins show enrichment of the Λ isomer (Insert). The ruthenium configuration at the His **33** site is retained upon substitution of imidazole in *cis*-[Ru^{II}(bpy)$_2$(H_2O)]-His **33**-cyt *c* (-•-). (B) The difference CD spectra between unmodified and ruthenium-modified Hh cyt *c* for the reaction of Hh cyt *c* with the racemic *cis*-[Ru^{II}(bpy)$_2$(H_2O)]-His **26**-cyt *c* (•••) and *cis*-[Ru(dmbpy)$_2$(H_2O)]-His **26**-cyt *c* (—). The stereochemistries of the bound ruthenium complexes are assigned by comparison with the model compounds. The CD of *cis*-[Ru(dmbpy)$_2$(H_2O)]-His **26**-cyt *c* shows the large enrichment in the Δ isomer (Insert).

The His **33** site is surrounded by flexible, charged amino acid side chains (Lys 22, Glu 104, see Figure 2A) and has a single hydrogen bond to the carbonyl oxygen of Val **20** (Figure 2A). Such weak interactions are expected to be broken upon binding of the ruthenium complex, *cis*-[Ru(bpy)$_2$(H_2O)$_2$]$^{2+}$ to the His side chain. The selectivity of the **His 33** site for the Λ isomer is presumably due to the preferential binding of the ruthenium complex to these charged residues and the hydrogen bonding between the water ligand and the protein donor groups.[12] This stereoselective recognition of **His 33** for both the [Ru(bpy)$_2$(H_2O)$_2$]$^{2+}$ and [Ru(dmbpy)$_2$(H_2O)$_2$]$^{2+}$ is independent of their size since [Ru(dmbpy)$_2$(H_2O)$_2$]$^{2+}$ has four more methyl groups oriented into solution than the [Ru(bpy)$_2$(H_2O)$_2$]$^{2+}$.

For the more buried His **26** site, the large stereoselectivity is dependent on the rigid cavity in the protein interior where the His **26** is hydrogen bonded to two neighboring residues (Pro **44** carbonyl oxygen and Asn **31** amide hydrogen) (Figure 2B).[11] Upon binding of [Ru(dmbpy)$_2$(H_2O)$_2$]$^{2+}$ to His **26**, the nonbonding interactions with the surrounding side chains of the cavity make a better fit with the Δ isomer, whereas the smaller [Ru(bpy)$_2$(H_2O)$_2$]$^{2+}$ (without the four methyl groups) can be accommodated

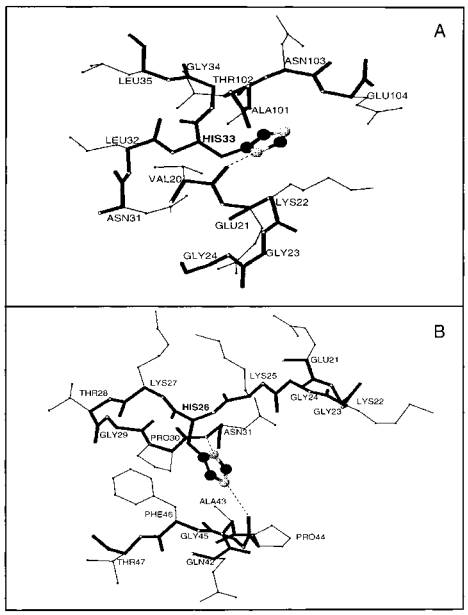

Figure 2. The peptide segments surrounding the His **33** (A) and His **26** (B) of Hh cyt *c*.[11] The main peptide chain is represented by thick lines, the side chains by thin lines, and the H-bonds by dashed lines.

with only minor isomeric preference. Thus, the stereoselectivity at the more buried His **26** is more dependent on the size of the ruthenium complex than that observed for the surface-bound His **33** residue.

Two different square wave voltammograms (0.67 V and 0.76 V)[13] are observed for the protein-buried aquo ions of D- and L-[Ru(bpy)$_2$(H_2O)]-His **26**-cyt *c* (ee 6%) and only one wave (0.64 V) for [Ru(dmbpy)$_2$(H_2O)]-His **26**-cyt *c* (ee 38%). Surprisingly, these voltammograms are pH-independent (pH 5.6−8.3), whereas the reference compound *cis*-[Ru(bpy)$_2$(H_2O)Im] (Im = imidazole) is known to have a pH-dependent voltammogram in this region.[14] Substitution of Im for the aquo ligand of ruthenium proteins proceeds with retention of configuration at slightly different rates (more details will be published separately).

In conclusion we have shown how the stereospecific binding of ruthenium complexes to the more buried His **26** and the surface-accessible His **33** residues of cyt *c* results in protein isomers with different CD spectra, redox potentials, and kinetics of substitution. The stereoselective interaction of these ruthenium complexes with histidine residues may be a useful stereospecific probe for studying the nature of the protein cavities surrounding a histidine residue at the surface or within the protein interior. Structural characterization and modeling work are in progress to unravel the molecular nature of these chiral interactions between the different ruthenium complexes and Hh cytochrome *c*.

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Supporting Information Available: CD analysis, electrochemistry, and Osteryoung square wave voltammetry (5 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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(12) The reaction between Hh cyt *c* and racemic *cis*-[Ru(bpy)$_2$(H_2O)$_2$]$^{2+}$ and *cis*-[Ru(dmbpy)$_2$(H_2O)$_2$]$^{2+}$ proceeds slowly (half-life ca. 24 h) with a preequilibrium step, followed by an intracomplex rate-limiting step (or steps). The chiral discrimination step can occur at either the preequilibrium step or the rate-limiting kinetic steps (see ref 1a for discussion of different mechanisms of chiral discrimination).

(13) All potentials were measured in 50 mM phosphate buffer at room temperature using SSCE reference electrode and are expressed vs NHE by adding 0.236 V.

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