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SITE–DEPENDENT CHIRAL DISCRIMINATION OF HISTIDINE SIDE CHAINS IN HORSE HEART CYTOCHROME C FOR RUTHENIUM AQUO BIPYRIDINE COMPLEXES

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ABSTRACT

A much higher stereoselectivity was observed in the reaction between Hhcyt c and cis–$[Ru(L–L)_2(H_2O)_2]^{2+}$ (L–L = 2,2′–bipyridine (bpy), and 4,4′–dimethyl–2,2′–bipyridine (dmbpy)). It was shown that the stereoselectivity is dependent of the binding sites on the cytochrome c surface as well as the substituent groups on the bipyridine ligand. The $[Ru(L–L)_2(H_2O)]$–His26–cyt c and $[Ru(L–L)_2(H_2O)]$–His33–cyt c products contain aquo ions in the protein interior and at the protein–water interface respectively. Interactions of these aquo ions with the protein matrix result in unusual spectroscopic, electrochemical, and kinetic properties.

The substitution reaction of racemic ruthenium complexes cis–$[Ru(L–L)_2(H_2O)_2]^{2+}$ (L–L = 2,2′–bipyridine (bpy) or 4,4′–dimethyl–2,2′–bipyridine (dmbpy) on the surface His 33 residue and the more buried His 26 residues of Horse heart cytochrome c (cyt c) takes place in a stereoslective manner. Whereas large enantiomeric excess (ee) for Δ–$[Ru(dmbpy)_2(H_2O)]$–His26–cyt c (38%), only small ee for Δ–$[Ru(bpy)_2(H_2O)]$–His26–cyt c (6%) are observed. Similar ee (~30%) for both bpy and dmbpy ruthenium complexes modified proteins, ∧–$[Ru(L–L)_2(H_2O)]$–His33–cyt c; were observed. Different redox potentials were determined for the different isomers with current ratios consistent with the circular dichroism of the ruthenated proteins. The rates of substitution of imidazole on ruthenium aquo proteins are shown to proceeds with retention of configuration at the ruthenium site. For different ruthenium protein isomers, substitution rates of isomers determined were one to two orders of magnitude slower than those with the corresponding small molecule racemic complexes.

INTRODUCTION

Stereoselective recognition of substrate molecules by enzymes, proteins and DNA is a subject of continuing interst[1–4] for fundamental and pharmacological applications. Stereoselective electron transfer reactions between optical active reagents and metalloproteins including cytochrome c (cyt c), Zn–myoglobin,[7] plastocyanin,[8,9] plant ferredoxin[3], cytochrome c peroxidase,[10] and superoxide dismutase,[11] have already been reported. Chiral recognition was also observed in a proton transfer reaction[12] and luminescence quenching of lanthanide complexes by cytochrome c,[13] as well as mediated electron transfer on chiral–monolayer–electrodes.[14,15]Stereoselective intercalation and covalent binding of metal complexes to DNA is another subject under extensive investigations.[16–24] The reported high stereoselectivity in ccovalent binding of ruthenium aquo complexes to DNA showed different enantiomericselectivities from that in noncovalent binding (intercalation). These types of interactions hold promise for carrying out many stereoselective oxidation reactions.[24,25] Evidence of stereoselectivity of covalent binding[26] and noncovalent binding to cytochrome c[6] were also observed, though the chiral discrimination obtained was extremely small (less than 6%). The electron transfer studies showed different optical isomeric preference of cytochrome c for different cobalt complexes.

A much higher stereoselectivity was observed in the reaction between Hhcyt c and cis–$[Ru(L–L)_2(H_2O)_2]^{2+}$ (L–L = 2,2′–bipyridine (bpy), and 4,4′–dimethyl–2,2′–bipyridine (dmbpy)). It was shown that the stereoselectivity is dependent of the binding sites on the cytochrome c surface as well as the substituent groups on the bipyridine ligand. The $[Ru(L–L)_2(H_2O)]$–His26–cyt c and $[Ru(L–L)_2(H_2O)]$–His33–cyt c products contain aquo ions in the protein interior and at the protein–water interface respectively. Interactions

of these aquo ions with the protein matrix result in unusual spectroscopic, electrochemical, and kinetic properties.

METHODS AND MATERIALS

All the chemicals were reagent grade and were used as received unless otherwise indicated. The 2,2|–bipyridine and 4,4|–dimethyl–2,2|–bipyridine were purchased from Aldrich and ruthenium trichloride hydrate was obtained from Matthey Bishop. Imidazole (Im) was recrystallized from water before used. The $Ru(bpy)_2Cl_2$[27], $Ru(bpy)_2CO_3$,[28] and $Ru(dmbpy)2CO_3$[29] were prepared according to literature procedures.

Horse–heart (Hh) cytochrome c (cyt c) (Type VI) was obtained from Sigma and was purified using literature procedures.[30] Protein solution was concentrated using ultrafiltration units containing YM3 or YM10 membranes (Amicon). G–25 Sephadex (Pharmacia) was used for gel filtration chromatography. Cation exchange chromatography was carried out using CM–52 Cellulose (Whatman) for protein purification and Bio–Rex 70 resin (BioRad) (minus 400 mesh) for Ru–protein separations.

INSTRUMENTS AND TECHNIQUES

UV–Visible spectra were obtained on a Hewlett–Packard 8452A diode array spectrometer. Fluorescenece spectra were measured in 1 cm quartz cells using a FluoroMaxspectrofluorometer. Circular dichroism (CD) spectra were obtained on a Model 60DS Aviv CD spectrometer with a programmable, thermoelectrically controlled cell holder. 10 μM protein in 50 mM potassium phosphate (KPi) buffer, pH 7.0 was used. Spectra in the wavelength range from 230 to 600 nm were obtained using quartz cells with a 1 cm pathlength. Cells with a pathlength of 0.1 cm were used to obtain spectra in the UV range from 200 nm to 300 nm. The obtained ellipticity was converted to the difference of molar extinction coefficients for left–handled and right–handed circularly polarized light using the relation (1):

$$\Delta\varepsilon\ (\varepsilon_L - \varepsilon_R) = [\theta]_M / 3298.2 = 100\theta deg/cl/3298.2 \quad (1)$$

whereθdeg is the measured ellipticity in degrees, c is the molar concentration in M, and 1 is the path length in cm.

Cyclic voltammograms (CV) and Osteryoung square wave voltammograms (OSWV) were recorded using a BAS 100A electrochemical analyzer (Bioanalytical Systems, West Lafayette, IN). Electrochemical experiments were carried out in a conventional three–electrode glass cell containing a working electrode, a platinum wire auxiliary electrode and a saturated sodium chloride calomel reference electrode (SSCE) and all potentials reported in this dissertation are vs. SSCE. Cyt c electrochemistry (SSCE) and all potentials reported in this dissertation are vs. SSCE, Cyt c electrochemistry was normally carried out I n a 100 mMKPi buffer solution (pH 7.0) with a pyrolytic carbon electrode as the working electrode at room temperature. Before each scan, the carbon working electrode was freshly polished with a 0.03 mm alumina slurry. The typical conditions for an OSWV are as follows: step height: 4 mV, square–wave amplitude: 25 mV, frequency, 5 Hz.

The digitized voltammograms were fitted to accurately measure the peak separations when closely spaced OSWV waves were observed for the ruthenated cytochromes using Igor 3.00 on a Macinotosh computer.[32] A version of the "Custom Peak Measurement and Fitting" demonstration experiment (Igor Technical Note NO. 020–A available by anonymous FTP from d31rz0.stanford.edu) was adapted for OSWV fitting using the relation:

$$i_{dif} = F[0]\ \{P/(1+P)^2\} \quad (2)$$

where P = exp (E – F[1]) RT/nF. F[0] and F[1], which stand for peak amplitude and formal potential (E°) respectively, are the parameters for peak fitting, n is assumed to be 0.8.

Modification of Cytochrome c with cis–[RuII(L–L)$_2$(H$_2$O)$_2$] (L–L = bpy and dmbpy)

Protein modification was carried out following a published procedure with minor modifications. A KPi solution (pH 7.0) containing 50 mM of ferrocytochrome c (2 mM, 4 mL) was incubated with 5 mg (~2 mM)

racemic $Ru(L–L)_2CO_3$ for 18–24 hr at room temperature in the dark under anaerobic conditions. The solution was oxidized with potassium ferricyanide and passed through a G–25 gel column (1 × 40cm) to remove the excess reagent. The protein solution was then loaded on a BIo–Rex 70 cation exchange column (1.5 × 4 cm) and eluted with a linear gradient from 0 to 100% 250 mMKCl in 100 mM potassium phosphate buffer. The Ru–modified species collected were concentrated and equilibrated with 50 mM potassium phosphate buffer (pH 7.0) by ultrafiltration. To insure purity, the Ru–modified cyt c species was rechromatographed once (even twice based on whether baseline separation is achieved) on a BIo–Rex 70 cation exchange column under the same conditions as above. The total yield of this modified protein after repurification was ~15%. The presence of a single ruthenium bis–bypyridine species per cyt c was identified from the UV–Visible spectra of the modified proteins, OD_{292}/OD_{410}= 0.65 for ferricytochrome c or OD_{292}/OD_{416} = 0.55 for ferrocytochrome c. The purified Ru–modified cyt c was stored at –5°C.

DETERMINATION OF THE SITE OF MODIFICATION

The aquo group of the ruthenium complex attached on the protein was substituted by imidazole before tryptic digestion by incubating the reduced ruthenated protein in 1 M imidazole phosphate buffers (pH 7.0) for ~ 5 days under dark, anaerobic conditions. The reduced protein was then oxidized by ferricyanide and the excess reagents (imidazole and ferricyanide) were then removed by ultrafiltration.

Tryptic digestion. Native and ruthenium modified ferricytochrome c, ~2 mg, were equilibrated with digestion buffer containing 0.1 M NH_4HCO_3 and 0.1 mM $CaCl_2$ (pH 8.3), and concentrated to a volume of ~ 1 mL by ultrafiltration. To the protein solution, TPCK–treated trypsin (stored at 0°C in 0.1 mMHCl with a concentration of 1 mg/mL) was added (0.01 mg trypsin per mg cytochrome c, or 1 % by mass) and incubated for 60 min at 37°C. A second 1% aliquot of trypsin was added and the digestion was continued for another 30 min. The final solution was lyophilized and the dried digested protein was stored below 0°C until the HPLC analysis.

PEPTIDE MAPPING

Tryptic peptide mapping was conducted by reversed–phase high–performance liquid chromatography (HPLC). The HPLC system consisted of a waters model M45 solvent delivery system operated in isocratic mode, a Rheodyne model 7125 manual injector equipped with a 20 μL loop, a Waters 994 Programable Photodiode Array Detector, and the Autochrom M320 HPLC Gradient Workstation. All buffers were filtered through 0.2μ Nylon 66 membrane filters (Rainin) and degassed before use. The following conditions were used to separate trypticdigestied peptides:

Column: Vydec 218TP54 C_{18} reversed phase (4.6 × 250 mm).
Detection: 210 nm and 290 nm.
Flow: 1 mL/min.
Injection volume: 10 – 20 μL.
Mobile phase: A. 0.05% (v/v) trifluoroacetic acid in water.
B. 0.05% (v/v) trifluoroacetic acid in 80% (v/v) acetonitrile in water.

The tryptic digested peptides were dissolved in 100–200 μL water before HPLC analysis. In all experiments, a linear gradient from 0 to 60% mobile phase B in 50 minutes was used. The spectrum of each peptide fragment was taken on–line using Waters 994 Programmable Photodiode Array Detector.

RESULTS

Preparation of cis–[Ru(L–L)$_2$(H$_2$O)]–HisX–cyt c (X=33, 26)

cis–$[Ru(L–L)_2(H_2O)]$–HisX–cyt c was prepared using a literature procedure.[33] Separation was achieved by cation exchange. Two different ruthenium modified proteins with one Ru atom per cytochrome c are obtained (Figure 2.1). The presence of a mononuclear species was determined from the UV–Visible spectra

of the modified proteins, OD_{292}/OD_{410} = 0.65 for ferricytochrome c or OD_{292}/OD_{416} = 0.55 for ferrocytochrome c (Figure 2.2). The first species that eluted from the cation–exchange column was assigned to the His26 modified species, and the second assigned the His33 modified species based on Durham et al'stryptic digest analysis[33] and confirmed by our own tryptic digest analysis as described next.

Peptide Mapping of Native and Ru–modified Cytochrome c

Tryptic digestion of horse heart cytochrome c resulted in a series of peptides. Detailed amino acid analysis of these tryptic peptides has been done elsewhere.The sequence and sizes of the obtained peptides are summarized in Figure 2.3. The peptide maps for the native horse heart cytochrome c obtained at 210 nm and 290 nm are shown in Figure 2.4. Only two peaks observed at 290 nm, corresponding to T10 and T4 respectively.[34–36] The T4 and T10 peptides were therefore taken as internal standard peaks for, characterization of other tryptic peptides (see Figure 2.5 for the absorption spectra of T4 and T10). The ruthenated peptide fragments were located using the characteristic absorption of the ruthenium chromophore. From the retention times of the ruthenium peptides, the site of modifications (His26 and His33) was confirmed by comparison with tryptic fragments of the native proteins. Peptide maps for all the four ruthenated proteins. The results are consistent for the assignment of the His26 modified peptide (T6) which eluted ten to fifteen minutes earlier than the corresponding His33 modified peptide (T7). The bipyridine ruthenium modified peptides eluted four to eight minutes earlier than the corresponding dmbpy ruthenium modified peptides. For His33 modified proteins, the T7 peaks shifted to higher retention times than the native protein because of the additional hydrophobicity of the ruthenium complexes. The UV–visible spectrum of T7 fragment of $[Ru(bpy)_2(Im)]$–His33–cyt c is shown in Figure 2.10. For His26 modified proteins, no similar comparison can be made because T6 in native protein elutes with the column void volume together with other small peptides such as T2, T5, and T9. In the tryptic maps of His26 modified proteins, more than one peak was observed containing ruthenium complexes the UV–visible spectra of the His26 fragments at retention time 27.5 min and 41.2 min. The fragment eluted after T4 has a strong absorption peak around 400 nm which indicates the presence of the heme peptide in addition to the ruthenium peptide. These results imply incomplete tryptic digestion and the presence of a much larger peptide fragements than the normal tryptic peptides. Similar observations were made for the tryptic map of $[Ru(dmbpy)_2(Im)]$–His26–cyt c.

CD Spectra

The CD spectra in near UV and visible range of cytochrome c and the ruthenated proteins are shown in Figure 2.12. The CD spectra of all the ruthenated proteins in the range of 200–250 nm are almost identical to that of native cytochrome c. The CD spectra in this far–UV range of cis–$[Ru^{II}(bpy)_2(H_2O)]$– His33–cyt c and cis–$[Ru^{II}(bpy)_2(H_2O)]$– His26–cyt c are presented in Figure 2.13 together with that of native cyt c for comparison.

A solution of cis–$[Ru^{II}(bpy)_2(H_2O)]$– His33–cyt c in 50 mMKPi buffer (reduced by ascorbic acid) was incubated in 1M imidazole for 5days, oxidized by ferricyanide, passed through G–25 column, and then adjusted to a concentration of 10 μM.The Difference CD spectra between the modified and unmodified proteins are shown in Figure 2.14 (for modification at His33) and Figure 2.15 (for modification at His26).

ELECTROCHEMISTRY

The voltammetry (OSWV) of cis–$[Ru(bpy)_2(H_2O)]$– His33–cyt c at pH 7 showed a band that correspond to the redox potential of cyt c(II/III) at 32 mV and two other bands at 427mV and 534mV. The band at 427mV changes to ~510mV or 311mV as the pH changes to 5.6 or 8.5 respectively. The electrochemistry of the ruthenium aquo proteins and cis–$[Ru^{II}(bpy)_2(H_2O)(Im)]$ are compared at pH 5.6, 7.0 and 8.5. Similar behavior was also observed for the closely related ruthenium protein derivative cis–$[Ru(dmbpy)_2(H_2O)]$–His33–cyt c .

Figures show the electrochemistry of cis–[Ru(bpy)$_2$(H$_2$O)]– His26–cyt c and cis–[Ru(dmbpy)$_2$(H$_2$O)]– His26–cyt c respectively. Two redox bands at 510 mV and 550 mV were observed in the OSWV of cis–[Ru(bpy)$_2$(H$_2$O)]– His26–cyt c and only one for cis–[Ru(dmbpy)$_2$(H$_2$O)]– His26–cyt c. Unlike other complexes, no change in the OSWV between pH 5.6–8.5 was observed for these complexes. Figures show the results of fitting the OSWV waves of all four ruthenated proteins. The results are summarized in Table.

Substitution and Aquation of Imidazole in [Ru(bpy)$_2$(H$_2$O)]–HisX–cyt c (X=33, 26)

[Ru(bpy)$_2$(H$_2$O)]– His33–cyt c and its model compound [Ru(bpy)$_2$(Im) (H$_2$O)] are not luminescent. However when this aquo group is replaced by imidazole, the product [Ru(bpy)$_2$(Im)]– His33–cyt c is luminescent with an emission centered around 650 nm (when excited at 436 nm).[33] This different fluorescence behavior between the aquo and imidazole complexes (excitation at λ=436 nm and λ_{max} at 665 nm) was used to monitor the substitution and aquation of imidazole (Figure 2.24). The quantum yield of fluorescence for [Ru(bpy)$_2$(Im)]–His33–cyt c was found to be similar to that of a mixture made from a 1:1 molar ratio [Ru(bpy)$_2$(Im)]$^{2+}$ and cyt c (Figure 2.24). The substitution process was also monitored by OSWV since both complexes have different OSWV waves. The kinetics of substitution of imidazole on the aquo protein was monitored as product formation and is shown in Figure 2.26. The substitution of Im on [Ru(bpy)$_2$(H$_2$O)]–His26–cyt c shows a double exponential kinetics plot while that on [Ru(dmbpy)$_2$(H$_2$O)]–His–26–cyt c shows only single exponential kinetics plot. Kinetic analysis of the substitution of Im on [Ru(bpy)$_2$(H$_2$O)]His–26 cyt c shows a double exponential kinetics plot.[34–36] Kinetic analysis of the substitution of Im on [Ru(bpy)$_2$(H$_2$O)His–33 cyt c] and [Ru(dmbpy)$_2$(H$_2$O)]His–33 cyt c showed almost single exponential in both cases; however, double exponential kinetics yielded better and more reasonable fitting and therefore was used as the basis for our modeling of the reaction. The fitting results and error (the sigma values) are included in Table.

DISCUSSION

OSWV Fitting for Ruthenated Cytochromes

Square wave voltammeter (OSWV) is a highly sensitive and rapid electrochemical technique which capitalizes on the new inventions of modern electronics. The theoretical OSWV peak function is simple in concept but unwieldy computationally. For the purpose of peak separation, however, a mathematical function that can capture the shapre of the OSWV peak is adequate, since no kinetic simulation is required. Analysis showed that the square wave voltammetry produces results similar to that produced by square wave polarography.

Theoretically a half–width of the OSWV peak is 90.5 mV for n = 1. In our measurements, a typical half–width is ~100 mV. This may reflect the quasireversibility feature of the protein system[41] and can be accurately simulated by empirical adjustment of n (the number of electrons for the redox process) from 1.0 to 0.8. The value of 0.8 was used in all subsequent calculations.

STRUCTURAL AND KINETIC ANALYSIS OF STEREOSELECTIVITY

The reaction between Hhcyt c and racemic cis–[Ru(bpy)$_2$(H$_2$O)$_2$]$^{2+}$ and cis– [Ru(dmbpy)$_2$(H$_2$O)$_2$]$^{2+}$ proceeds slowly (half lifeca 24 hours) and can be described by a preequilibrium step to form an encounter complex where the ruthenium species is associated with the cyt c protein surface, followed by an intramolecular rate limiting step (or steps), where the coordinated water is exchanged with the π nitrogen of the Histidine side chain. The nature of these intermediate steps depend on the location of the Histidine side chain in the protein and the neighboring amino acid residues which determine the protein folding in that region.

The three histidine residues on Hhcyt c (Hhcyt c) (His 18, His26 and His33), are located in different regions of the protein with His18 bound to heme iron, His26 buried in the protein matrix, and His33 a more exposed surface residue. The reaction of ruthenium aquo polyamines with horse heart cytochrome c has shown that these complexes specifically bind to surface histidineresidues, such as His33 in Hhcyt c, and His39 in yeast cyt c, while the ruthenium(II) aquobypyridine, due to the nature of the hydrophobic bipyridine ligands, have

been shown to bind to surface, as well as more buried His residues.[33] The cis–$[Ru(bpy)_2(H_2O)_2]^{2+}$ (bpy = 2,2|–bipyridine) covalently binds to both His26 and His33 residues, with only minor preference for the more surface exposed site (His33).

Examination of the crystal structure of Hhcyt c shows that the His 33 site which is close to the protein surface, is surrounded by flexible, charged amino acid side chains (Lys22, Glu104). The selectivity for ∧ isomer is presumably related to the preferential binding of the ruthenium complex to these charged residues and the hydrogen bonding between the coordinated water molecules and the appropriate groups on the protein surface. Thus, the flexibility of the amino acid side chains and their presence on the protein surface results in similar stereoselective recognition for both the cis–$[Ru(bpy)_2(H_2O)_2]^{2+}$ and $[Ru(dmbpy)_2(H_2O)_2]^{2+}$, independent of the size of the ruthenium complex.

The large stereoselectivity of the cis–$[Ru(dmbpy)_2(H_2O)_2]^{2+}$ over cis–$[Ru(bpy)_2(H_2O)_2]^{2+}$ to the His 26 site is achieved because the cavity around the His 26 site is in a more rigid part of the protein fold , thus upon binding of cis–$[Ru(bpy)_2(H_2O)_2]^{2+}$ to His26, the ruthenium complex is smaller than the size of the cavity. However when $[Ru(dmbpy)_2(H_2O)_2]^{2+}$ binds to His26, the additional size of the four methyl groups increases the non–bonding interaction with the surrounding side chains in the cavity such that preferential binding to the Δ isomer, makes the better fit for binding. The hydrophobic 2,2|–bipyridine ligands on ci–$[Ru(bpy)_2(H_2O)_2]^{2+}$ assist the ruthenium complex to penetrate further into the interior of the protein, resulting in displacements of H–bonding contacts of the His 26 side chain with neighboring residues as the ruthenium binds at this histidine site. Therefore the selectivity of the protein interior at His26, is more size dependent than that observed for the His 33 residue.

CONCLUSION

Different stereoselectivities of histidine residue at different sites of cyt c were observed. These reflected the wealth of recognition sites available at the protein surface. Interaction of the aquo group on these ruthenium centers results in differences in electrochemistry and kinetics of substitution of the different isomers. Such behavior is unprecedented. The in–depth understanding of the properties of these racemic ruthenium complexes lead us to a better understanding and modeling of the intramolecular ET kinetics in these complexes.

Table: A Comparison of the stereoselectivity in $[Ru^{II}(L–L)(H_2O)]$ –HisX–cyt c studied by CD, OSWV and substitution kinetic analysis

X	L–L	Isomer	pH	E°'(mV)[a]	$k_{sub}\times 10^5$ (s^{-1})[b] p(%)	(CD, OSWV, kinetics)[c]		
33	Bpy	∧	7.0 8.3	534 513	0.8±0.1 –	67, –	70, 68	79±24 –
		Δ	7.0 8.3	427 311	2.6±1.9 –	33, –	30, 32,	21±24 –
	Dmbpy	∧	7.0 8.3	420 402	1.0±0.4 –	65, –	65, 64,	45±27 –
		Δ	7.0 8.3	351 224	3.5±1.2 –	35, –	35, 36	55±27 –
26	Bpy	∧ Δ	7.0 7.0	430 517	3.1±0.8 0.46±0.08	47, 53,	45, 55,	33±7 64±7
	Dmbpy	∧ Δ	7.0 7.0	344 402	– 0.61±0.06	31, 69,	16, 84,	– –

[a]The redox potential obtained from OSWV fitting;

[b]The substitution rate of imidazole (1M) on ruthenium aquo complexes attached on cyt c;

[c]The percentage of the isomer in the mixture calculated from $\Delta\varepsilon_{294nm}$ in CD, the fitted area in OSWV fitting and double exponential kinetics analysis of imidazole substitution.

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