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PHOTOINDUCED ELECTRON TRANSFER OF MICROPEROXIDASE-8

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Abstract—Microperoxidase 8 (MP8) has been prepared by sequential hydrolysis of cytochrome *c* by pepsin and trypsin. This five-coordinated heme-octapeptide fragment provides a uniques structure to evaluate the electronic coupling efficiency to the iron through axial position and porphyrin edge. At alkali pH, $Ru(bpy)_2(im)_2^{2+}$ was bound to iron through imidazolate coordination. The luminescence of $Ru(bpy)_2(im)_2^{2+}$ is completely quenched in AcMP8Ru complex. Transient kinetics measurement showed the decay rate to be ~ 1×10^{12} s^{-1}. Ruthenium bipyridine complex with a carboxyl group substituted bipyridine has been prepared and reacted with MP 8 to yield N-terminus bound RuMP8 complex. The luminescence decay rate has been measured as 1×10^9 s^{-1}. By using semiclassical electron transfer theory, we found the electron transfer efficiency through axial position of iron porphyrin is as good as through porphyrin edge.

INTRODUCTION

Microperoxidases are hydrolysis products of cytochrome c by proteolytic enzymes. Cytochrome *c* contains 104 amino acids and an iron protoporphyrin to carry out its natural responsibility to transport electron. This protoporphyrin is covalently attached to protein backbone through two thiother linkages, *i.e.* cysteine 14 and 17. It has been shown that treated with pepsin results a fragment from residue 11-21 (MP11), with trypsin results residues 14-22 (MP9), and with both pepsin and trypsin yidles the a fragment of residue 14-21 (MP8) heme-containing peptides.[1] These heme-containing peptide fragments all have the histidine-18 intact as the fifth ligand and leave the sixth coordination site open. This heme environvement resembles peroxidase structure and exhibits peroxidase activity. The peroxidase activity gives the name, microperoxidase, for these heme-containing peptide fragments.

A lysine residue has remained in MP9 and MP11 fragments and may act as the sixth ligand to iron. The peroxidase activity is dramatically reduced if iron has no open coordination site. Even in MP8, the N-terminus can coordinate and from inactive dimer at high concentration. To preserve peroxidase activity, the acetyl protected on N-terminus AcMP8 is often used and is the main microperoxidase discussed in this paper.

The open coordination on iron also provides a unique structure to evaluate electron transfer (ET) efficiency. Cytochrome *c* is an important biological electron transport protein.

Extensive studies on ruthenium modified cytochrome c have indicated that electron transfer through both heme edges and axial ligands ard highly efficient.[2,3] Many porphyrin based synthetic donor/acceptor complexes have demonstrated extremely fast ET reaction coupled through porphyrin edges.[4,5] However, the ET through axial site has not been thoroughly investigated.[6,7]

Transition metal complexes are effective sensitizer for many photoinduced electron transfer. Ruthenium-2,2′-bipyridine(Ru-bpy)complexes, in particular, possess many attractive features for this sort of chemistry. The metal-to-ligand charge-transfer excited states of Ru-bpy($(Ru(bpy)_3^{2+}$, $Ru(bpy)_2LL^{2+}$)complexes have 0.1-1.0 μs lifetimes and are potent and efficient one-electron reductants ($E_{1/2}$ ~ -0.8 V *vs.* NHE). One electron oxidized ruthenium complexes are stable for seconds and are power oxidants. Furthermore, the vast body of research into the photophysics and photochemistry of these complexes provides nearly limitless information.[8]

Ruthenium complexes are perfect candidates for photoinduced reaction with AcMP8. In this paper we report the photoinduced ET reactions of ruthenium complexes with AcMP8.

MATERIALS AND METHODS

Material

General. $Ru(bpy)_2(im)_2Cl_2$[9] and 4′-methyl-2,2′-bipyridinecarboxylic acid ($CH_3bpyCOOH$)[10] were prepared according to the published methods.

Microperoxidase-8 (MP8). MP8 was prepared from horse heart cytochrome c (Sigma) by a modification of literature procedures.[11,12] 500 mg of cytochrome *c* and 13 mg of pepsin (Sigma) were dissolved in 20 mL of water. The pH was adjusted to 2.1 by addition of 1 M HCl. After 30 min, another portion of pepsin (13 mg) was added. After standing overnight, the solution was brought to pH 8.1 by addition of ammonium, hydroxide (Fischer) and trypsin(18 mg)(Sigma) was added. The resulting red solution was allowed to stand at 30°C overnight. Crude MP8

was precipitated by addition of solid ammonium sulfate (29g) and collected by centrifugation. The supernatant was discarded and the remaining red solid was dissloved in water. Residual ammonium sulfate was removed by washing with water in an Amicon ultrafiltration cell (YM1 membrance). The crude MP8 was purified by FPLC chromatography using a Pharmacia PepRPC 10/10 column running a 12-36% acetonitrile/water gradient. FPLC solvents contain 0.1% trifluoroacetic acid. Purified MP8 was lyophilized and stored in a refrigerator. N-acetyl MP8 was prepared by slow addition of a 500-fold excess of acetic anhydride (Aldrich) to a solution of purified MP8 in 0.1 M potassium bicarbonate solution.[12] Excess bicarbonate was removed by repeated washing with water in an Amicon cell and purified by reverse phase FPLC. The concentrations of stock solution of MP8 were determined spectrophotometrically (ε_{396} = 1.57 mM^{-1} cm^{-1}; pH 8).[12]

Ru(bpy)$_2$(CH$_3$bpyCO)MP8(RuMP8). Ru(bpy)$_2$(CH$_3$bpy-COOH)Cl$_2$ was prepared from reflux Ru(bpy)$_2$CO$_3$ with carboxyl substituted bipyridine ligand.[10] The carboxyl group was further activated by N-hydroxysuccinimide to succinamide.[10] In large excess, this ruthenium succinamide reacts with the N-terminus amine of MP8 and yieds ruthenium covalently bounded to MP8. Final purification is done by reverse phase column chromatography similar to the procedure of MP8.

Methods

General. Absorption spectra were measured on a HP 8453 photodiode array spectrometer. Emission spectra were obtained by using an Aminco Bowman Series 2 Luminescence Spectrometer.

Lifetime measurement. Lifetimes in ns-region were measured by home constructed time resolved Laser spectrometer and was described elsewhere.[13] Picosecond laser spectroscopic measurements were performed at the Laser Resource Center at Beckman Institute of California Institute of Technology. Transient absorption was measured by exciting the AcMP8Ru complex at the third harmonic of an active-mode-locked ND:YAG laser (FWHM = 10 ps). A continuum white light, generated from irradiating D_2O/H_2O mixture, was used as the probe light for transient absorption. The luminescence decay was measured by using Time-Correlated Single-Photon-Counting. Decay traces were transferred to a personal computer loaded with the commercial software Origin 3.5.

Electrochemical Measurements. The redox potentials of AcMP8Ru and RuMP8 complexes were determined by cyclic voltammetry by using a EG G Potentiostate/Galvan (Model 273). Electrochemical measurements in aqueous solutions (buffers, μ = 0.1M) were perfomed at room temperatured in a standard three-electrode configuration consisting of a pyrolytic graphite working electrode (6.2 mm dia.), a Pt wire as auxiliary electrode, and a saturated calomel reference electrode(SCE).

RESULTS

Complex formation between AcMP8 and Ru(bpy)$_2$(im)$_2^{2+}$ (AcMP8Ru). The absorption spectrum of AcMP8 exhibits a soret band at 396 nm which is a typical high spin five coordination Fe porphyrin electronic transition.[14] The soret band is very sensitive to the iron's coordination environment and is utilized to monitor the sixth ligand binding reaction. Upon binding the sixth strong field ligand, it changes to low spin iron and the soret band shifts to longer wavelength. At neutral pH, Ru(bpy)$_2$(im)$_2^{2+}$ has no free coordinate site and, therefore, no reaction occurred with AcMp8, but at pH 10, imidazole is deprotonated and is a good ligand to iron. The complex formation is evident by the soret band shift to 406 nm as shown in Fig 1a. After gel filtration, a 1:1 complex of AcMP8 is isolated (Fig.1b).

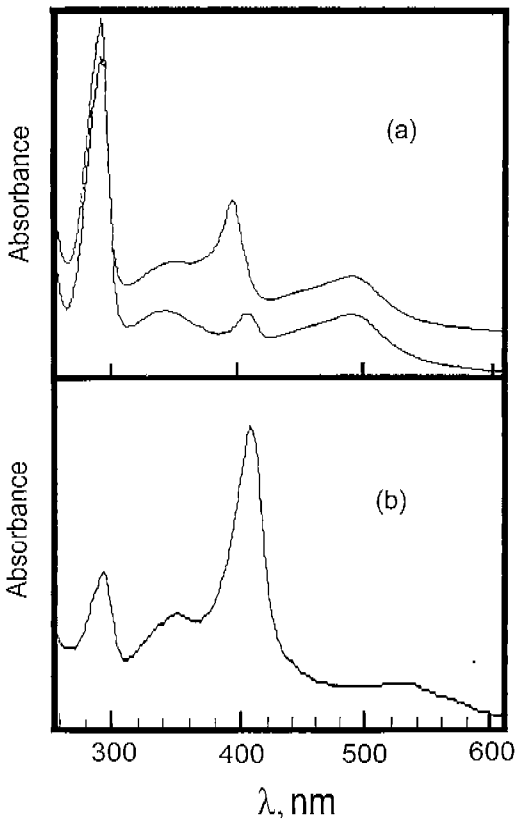


Figure 1. (a) Absorption spectra of AcMP8 with excess Ru(bpy)$_2$(im)$_2^{2+}$. Upper trace: pH = 7, lower trace: pH = 10. (b) Spectrum of 1:1 AcMP8Ru complex.

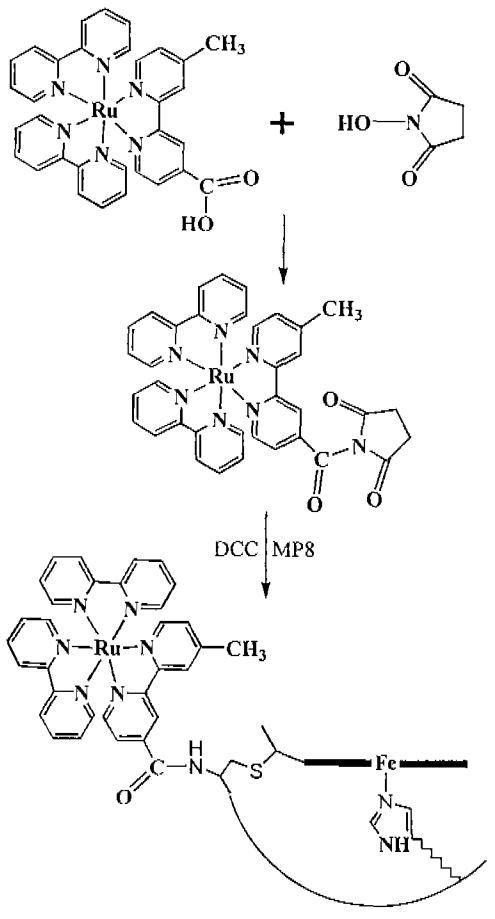
Photoinduced electron transfer in AcMP8Ru complex. The intense absorption of Ru(bpy)$_2$(im)$_2^{2+}$ around 450 nm has been assigned to metel-to-ligand charge-transfer (MLCT) band (Table 1). Excitation into the MLCT band of Ru(bpy)$_2$(im)$_2^{2+}$ results in a broad emission centered around 670 nm. However, there is no observable emission of AcMP8Ru complex indicating the excited-state of Ru(bpy)$_2$(im)$_2^{2+}$ is completely quenched. As one would expect the ET rate would be very fast for donor-acceptor at such a short distance, the emission result is not surprising. Picosecond transient absorption exhibits intense bleach in the soret region. The decay kinetics monitored at 400 nm, as shown in Fig. 2, is very close to the instrument response function. Deconvoluting the sig-

Table 1. Absorption, emission, lifetime, and redox potential of $Ru(bpy)_2LL^{2+}$ complexes.

LL	Absorption (MLCT), nm	Emission, nm	Lifetime, ns	Redox potential, V *vs.* NHE
$(Im)_2$	490	670	70	0.98
$CH_3bpyCOO^-$	456	625	576	1.28

nal results a decay rate constant of ~ 1×10^{12} s^{-1}.

Covalently bound RuMP8 complex. Ruthenium complex with one carboxyl group substituted bipyridine ligand can be activated and convalently bound to the N-terminus amine of the MP8. The syntheses are summarized in Scheme 1.



Scheme 1.

Mass spectrum measured by electrospray ionization technique is shown in Fig. 3, where the M^{2+}, M^{3+}, and M^{4+} are at 1057.9, 705.6, 529.4, respectively.

Electron transfer through porphyrin edge. Ruthenium complex, $Ru(bpy)_2(CH_3bpyCOO)^+$, exhibits a strong emission at 625 nm in pH 7, sodium phosphate solution, but the emission intensity is dramatically reduced in RuMP8 complex. Emission decay measured by time-correlated single-photon-counting gives a lifetime of 1 ns.

Redox potential of AcMP8Ru and RuMP8 complexes.

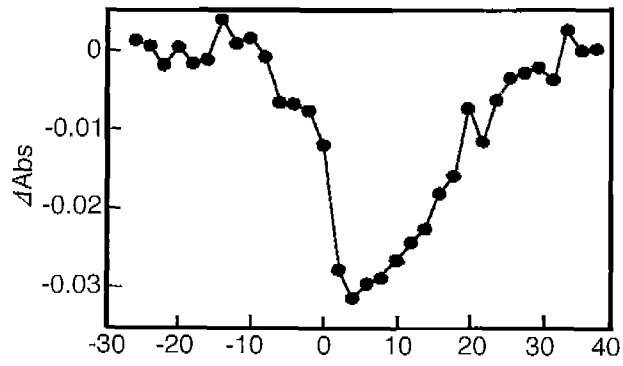


Figure 2. Transient kinetics of AcMP8Ru, monitored at 400 nm.

Direct cyclic voltammetry measurement of the AcMP8 Ru complex at pH 10 exhibits two reversible redox couples at -0.140 and 1.01 V *vs.* NHE. There is only one redox couple at 0.98 V *vs.* NHE for $Ru(bpy)_2(im)_2^{2+}$ complex. Redox potential of $Ru(bpy)_2(CH_3bpyCOO)^+$ was measured at pH 7 and has one quasi-reversible peak at 1.28V *vs.* NHE.

DISCUSSION

Since AcMP8 does not absorb strongly in the emission region of $Ru(bpy)_2(im)_2^{2+}$, energy transfer is less likely to be responsible for the quenching. While the decay rate of $Ru(bpy)_2(im)_2^{2+}$ itself is 1.4×10^7 s^{-1}, the ET rate form Ru complex to AcMP8 through axial ligation is ~1×10^{12} s^{-1}.

Based on the semiclassical theory,[15] the rate constant is expressed as Eq.1,

$$k_{ET} = \nu_n \kappa_E \exp\{-(\Delta G^\circ+\lambda)^2/4\lambda k_B T\} \quad (1)$$

Where ν_n is the nuclear motion frequency, κ_E is the electronic factor, and k_B is the Boltzmann constant. For adiabatic ET reactions, κ_E equlas unity and Eq.1 can be rewritten as Eq.2.

$$k_{ET} \sim 10^{13}\exp\{-(\Delta G^\circ+\lambda)^2/4\lambda k_B T\} \quad (2)$$

From the data listed in Table 1, the E_{oo} for $Ru(bpy)_2(im)_2^{2+}$ is estimated as 1.9 eV,[8] therefore, the excited-state redox redox potential of $Ru^{III/II*}$ is -0.89 V *vs.* NHE. The reduction potential for bisimidazole heme protein is about ~ 5mV *vs.* NHE in AcMP8Ru complex.[16] In AcMP8Ru complex, the negative charge from imidazolate will disfavor the reduction of the iron. By using Lever's ligand electrochemical series, it may shift the reduction potential to as much as -0.15 V.[17] The calculated value -0.145 V is in excellent agreement with the experimental result of -0.14 V *vs.* NHE. The driving force for electron transfer from excited Ru^{II} to Fe^{III} is -0.75 eV in AcMP8Ru complex. Both ruthenium and iron polypyridyl complexes are known to have small reorganization energy. From self-exchange reaction, it is estimated to be 0.57 and 0.59 eV.[15,18] The iron corrdination in AcMP8Ru complex is very similar to polypyridyl complexes, therefore, a similar reorganization energy is expected. From Marcus cross-rela-

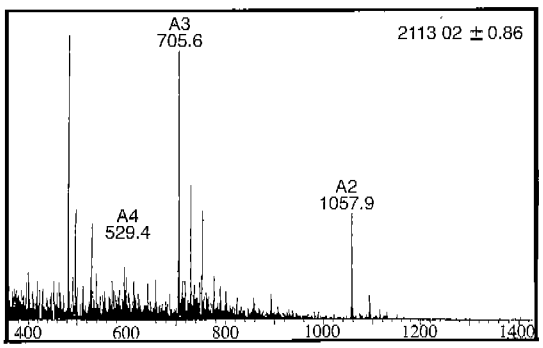


Figure 3. Electrospray mass spectrum of RuMP8 complex.

tion theory ($\lambda_{12}=1/2\lambda_{11}+1/2\lambda_{22}$), the reorganization energy of the ET reaction is 0.58 eV. The predicted adiabatic ET rate for AcMP8Ru complex, 6.2×10^{12} s^{-1}, is in the proximity of our experimental result.

For RuMP8 complexes, the excited-state redox potential of $Ru^{III/II*}$ is -0.77 V *vs.* NHE. The reduction potential for MP8 is -0.16 V *vs.* NHE at pH 7.[19] Simple calculation gives the driving force for the ET reaction in RuMPS to be -0.61 eV. While the reorganization energy for ruthenium part should be similar in both AcMP8Ru and RuMP8 complexes, it is not the same for the MP8 part. In AcMP8Ru, the iron in coordinately saturated and hence has small reorganization energy. In RuMP8 complex, the iron has one open coordination site. It is well known that five corrdinated hemoproteins favor H_2O as sixth ligand in the oxidized form while the reduced form favor five coordination under anaerobic condition. The change of the coordinated water elevates reorganization energy. From the self-exchange ET rate[20] of MP8, 1.3×10^7 M^{-1} s^{-1} and assuming precursor formation constant, K_A, of 3×10^{11}, reogranization energy is calculated to be 1.14 eV. Again from the cross-relation theory, the reorganization energy of RuMP8 is 0.855 eV. Assuming the reaction is adiabatic, we can calculate the rate from Eq. 2 to give 5.1×10^{12} s^{-1}. This calculated rate is much faster than the observed rate, therefore, the reaction cannot be adiabatic.

For nonadiabatic intramolecular ET (k_{ET}), κ_E is less than one and Eq. 1 can be rewrite as Eq. 3.

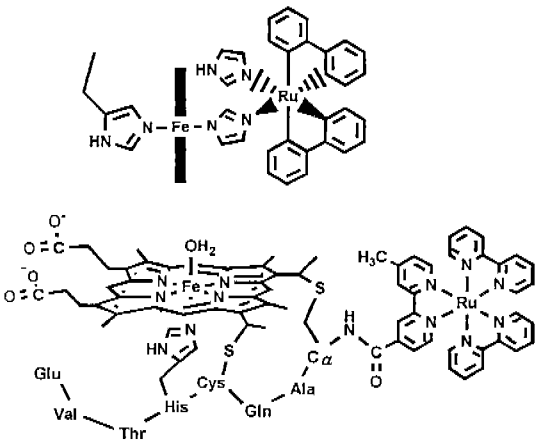
$$k_{ET} = (4\pi^3/h^2\lambda k_B T)^{1/2}(HA_B)^2\exp\{-(\Delta G^o+\lambda)^2/4\lambda k_B T\} \quad (3)$$

The H_{AB}, the electronic coupling matrix element of the reactant and the product, has been intensively investigated for long-range ET. A tunneling pathway model to evaluate H_{AB} has been proposed by Beratan and Onuchic.[21] In the model, the electromic coupling can be assessed from the product of each decay factor which from electron transfer through covalent bond, hydrogen bound, or space jump along the pathway (Eq. 4)

$$H_{AB} = \frac{\beta_A\beta_B}{E}\prod_{i=1}^{N_C}\varepsilon_C(i)\prod_{j=1}^{N_H}\varepsilon_H(j)\prod_{k=1}^{N_S}\varepsilon_S(k)$$

$$\varepsilon_C = 0.6$$
$$\varepsilon_H = 0.36\times\exp[-1.7(R-2.9)]$$
$$\varepsilon_S = 0.5\times0.6\times\exp[-1.7(R-1.4)] \quad (4)$$

As shown in Scheme 2, there are four bonds between Ru and Fe in AcMP8Ru, and 14 bonds in RuMP8. By using the tunneling pathway model, the H_{AB} value for RuMP8 is 6.0 $\times10^{-3}$ of that for AcMP8Ru and the rate is 3.3×10^8 s^{-1} for RuMP8.



Scheme 2.

It is important to notice that these two systems are on the different limit of the electron transfer reaction as we have discussed in the previous section. It is not entirely adequate to directly compare those two systems. Ruthenium modified cytochrome c is a better comparison with RuMP8 system. $Ru(bpy)_2(im)_2^{2+}$ modified on histidine-33 of cytochrome c has been thoroughly investigated, and the system demonstrates a nonadiabatic ($\kappa_E<1$) ET reaction. The direct route from Ru to Fe involves 16 covalent bonds and 1 hydrogen bond of 3.16 Å. The reported k_{max} and H_{AB} for ET reaction are 2.9 $\times10^6$ s^{-1} and 0.11, respectively.[3] From tunneling pathway model, the H_{AB} for RuMP8 is 12.0 times that for His33. Using all the data, we calculated the rate to be 2.1×10^8 s^{-1}. Both calculations give the rate in order of 10^8 s^{-1}, which is slightly slower than the observed rate, 1×10^9 s^{-1}. Since the emission spectrum of $Ru(bpy)_2(CH_3bpyCOO)^+$ complex has some overlap with the Q band of MP8 absorption, the energy transfer may also contribute to the quenching process.

CONCLUSION

The iron in the AcMP8 has a relatively small binding constant from the axial position with most of the nitrogen and sulfur ligands. However, imidazole and imidazolate do give a stable AcMP8 derivatives. Metals in AcMP8Ru share a common ligand and lead to very fast electron transfer. The rate of $\sim1\times10^{12}$ s^{-1} indicates a near adiabatic ET reaction in this case. The result demonstrates that the capability to media electron transfer through metal center in iron-porphyrin complexes is as through the porphyrin edge.

The electronic coupling matrix element (H_{AB}) of RuMP8 complex, calculated from the ET rate is in agreement with the ruthenium-modified cytochrome c data. The ET route for

His-33 in cytochrone *c* is through axial ligand His18, and in RuMP8 complex it is connected through thioether linkage to porphyrin edge. These results further illustrate that the similar electronic coupling efficiency of the axial position and porphyrin edges to the iron center.

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