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Enantioselectivity in Electron-Transfer Reactions between Cytochrome *c* and (*S*)-/(*R*)-Ruthenium Complexes. Non-Polar Interaction in a Molecular Recognition Process

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Redox reactions between horse heart cytochrome *c* and optically active Ru complexes were kinetically studied. The kinetic parameters for the oxidation of ferrocytochrome *c* with Ru^{III} complexes indicated an apparent enantioselectivity, which clearly shows that exact molecular recognition is achieved through non-polar interactions rather than electrostatic ones.

Cytochrome *c*, which is an electron-transport protein that has been studied by many biochemical and chemical researchers, is involved in the electron-transfer reactions in most respiratory chains and photosynthesis systems.[1] The redox center of cytochrome *c* is an iron–protoporphyrin IX (heme) covalently connected to two cysteine residues of a polypeptide chain.[2] The heme is buried in the crevasse of the protein surface, so that only 6–10% of the total heme surface is exposed to solvent.[3] There are four lysine residues (Lys13, Lys27, Lys72, and Lys79) around the exposed heme edge. Acetylation of these lysines causes a decrease in the electron-transfer rate of cytochrome *c* with its physiological redox partner.[4] This fact suggests that the cationic lysine residues contribute to the dynamic recognition between cytochrome *c* and its electron transport partner. Recently, X-ray structures of the physiological redox complexes, that is, cytochrome *c*–cytochrome bc_1 complex, cytochrome c_2–reaction center, and plastocyanin–cytochrome *f*, have been analyzed, and the local structures of molecular recognition site in the static state has been examined in detail.[5] Interestingly, it has been shown that exact recognition is achieved by weak non-polar interactions, such as hydrophobic and van der Waals interactions as well as electrostatic ones.[6] At this stage, the understanding of dynamic molecular recognition process is desired.

To clarify the structural information of the molecular recognition site of electron-transport proteins in solution, redox reactions between the electron-transport proteins and inorganic complexes have actively been investigated still.[7] We have also developed a new molecular recognition probe, optically active Ru complexes, $[Ru^{II/III}(bcmaa)(bpy)]$ (bcmaa = *N*,*N*-bis(carboxymethyl)-(*S*)-/(*R*)-amino acid; bpy = 2,2′-bipyridine) (Fig. 1). Electron-transfer reactions with electron-transport proteins, such as azurins and cytochrome c_{553} (cyt c_{553}), were investigated kinetically using these probes.[8] We have demonstrated that these small ruthenium complexes are useful as a probe for the molecular recognition site structures of the electron-transport proteins. In this paper, we describe a kinetic study on electron-transfer reactions between horse heart cytochrome *c* (cyt *c*)[2] and [Ru(bcmaa)(bpy)] complexes (bcmaa = bcmpa (*N*,*N*-bis(carboxymethyl)-(*S*)-/(*R*)-phenylalanine) and bcmal (*N*,*N*-bis(carboxymethyl)-(*S*)-/(*R*)-alanine)) and show that hydrophobic interactions, not electrostatic ones, are essential in the dynamic molecular recognition process of cyt *c*.

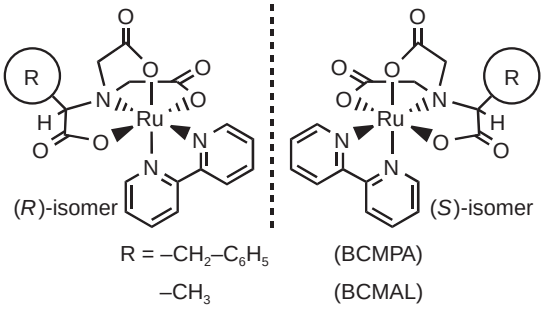

Fig. 1. Structures of optically active $[Ru^{II/III}(bcmaa)(bpy)]$ complexes.

The kinetic measurements for the oxidation of ferrocytochrome *c* (Fe^{II}cyt *c*) with $[Ru^{III}(bcmaa)(bpy)]^0$ and the reduction of ferricytochrome *c* (Fe^{III}cyt *c*) with $K[Ru^{II}(bcmaa)(bpy)]$ were carried out by using stopped-flow spectrometry, in which the decrease/increase in the absorbance of Soret band of heme of cyt *c* under pseudo-first-order conditions was monitored.[9] A linear relationship was obtained for the pseudo-first-order rate constants with respect to the concentration of the complexes (Fig. S1), and saturation kinetics were not observed, indicating that the interaction between cyt *c* and Ru complexes is too weak to kinetically separate the equilibrium constants for the formation of the precursor and electron-transfer rate constants. The temperature dependence of these reactions (Fig. S2) was also investigated.[10] The evaluated rate constants (k), and the activation parameters, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$, are summarized in Table 1, together with differences in the activation parameters between enantiomers, $\Delta\Delta H^{\ddagger}_{S-R}$ ($=\Delta H^{\ddagger}_{S} - \Delta H^{\ddagger}_{R}$) and $T\Delta\Delta S^{\ddagger}_{S-R}$ ($=T(\Delta S^{\ddagger}_{S} - \Delta S^{\ddagger}_{R})$).

In the oxidation of Fe^{II}cyt *c* with $[Ru^{III}(bcmpa)(bpy)]^0$ bearing a bulky benzyl group, $\Delta\Delta H^{\ddagger}_{S-R}$ and $T\Delta\Delta S^{\ddagger}_{S-R}$ were significantly large. In contrast, $\Delta\Delta H^{\ddagger}_{S-R}$ and $T\Delta\Delta S^{\ddagger}_{S-R}$ for the case of $[Ru^{III}(bcmal)(bpy)]$ which has a smaller methyl group, were almost equal to zero. These results were drastically different from those for the cases of azurin-1, azurin-2, and cyt c_{553} reported previously.[8] Considering that these are originated from the molecular recognition site structure of cyt *c*, it is clear that [Ru(bcmaa)(bpy)] can detect the difference in the molecular recognition processes with Fe^{II}cyt *c*.

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Table 1. k and Activation Parameters for the Electron-Transfer Reactions of Cyt c with [Ru(bcmaa)(bpy)][a)]

bcmaa	Oxidation of Fe^{II}cyt c with $[Ru^{III}(bcmaa)(bpy)]^0$					Reduction of Fe^{III}cyt c with $[Ru^{II}(bcmaa)(bpy)]^-$				
	k[b)] /10^5 M^{-1} s^{-1}	$\Delta H^{\ddagger}$ /kJ mol^{-1}	$\Delta S^{\ddagger}$ /J mol^{-1} K^{-1}	$\Delta\Delta H^{\ddagger}_{S-R}$ /kJ mol^{-1}	$T\Delta\Delta S^{\ddagger}_{S-R}$[b)] /kJ mol^{-1}	k[b)] /10^4 M^{-1} s^{-1}	$\Delta H^{\ddagger}$ /kJ mol^{-1}	$\Delta S^{\ddagger}$ /J mol^{-1} K^{-1}	$\Delta\Delta H^{\ddagger}_{S-R}$ /kJ mol^{-1}	$T\Delta\Delta S^{\ddagger}_{S-R}$[b)] /kJ mol^{-1}
(*S*)-BCMPA	5.33 ± 0.04	13.3 ± 0.4	-90 ± 2	-16.6	-15.1	7.68 ± 0.05	38.1 ± 0.2	-25 ± 1	-1.3	-0.9
(*R*)-BCMPA	3.15 ± 0.02	29.9 ± 0.4	-39 ± 2			6.39 ± 0.01	39.4 ± 0.2	-22 ± 1		
(*S*)-BCMAL	2.48 ± 0.02	22.3 ± 0.5	-67 ± 2	0.3	0.4	7.86 ± 0.03	35.8 ± 0.1	-31 ± 1	-1.0	-0.9
(*R*)-BCMAL	2.49 ± 0.03	22.0 ± 0.5	-68 ± 2			7.34 ± 0.02	36.8 ± 0.1	-28 ± 1		

a) pH 6.0 (1 mM phosphate buffer), $I = 0.05$ M (KCl). b) 298 K.

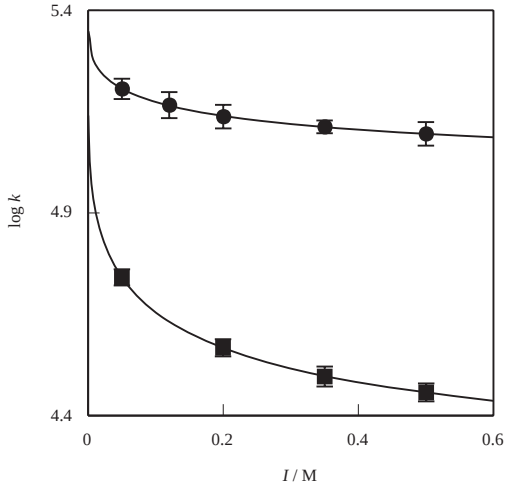

Fig. 2. Ionic strength dependence of the second-order rate constant for the reduction of Fe^{III}cyt c with [Ru^{II}((*S*)-bcmpa)(bpy)]. Experimental conditions: pH 6.0 (1 mM phosphate buffer), 298 K, KCl was used to adjust the ionic strength. Concentration: [Fe^{III}cyt c] $= 1 \times 10^{-6}$ M, [Ru^{II}] $= 1.5 \times 10^{-4}$ M. Fe^{III}cyt c (filled square) and Fe^{III}cyt c_{553} (filled circle). The best fit for the equation $\log k = a + bI^{0.5}/(1 + cI^{0.5})$ results in $a = 5.1 \pm 0.1$, $b = -2.9 \pm 0.9$, and $c = 2.9 \pm 0.9$ for Fe^{III}cyt c and $a = 5.3 \pm 0.1$, $b = -1.0 \pm 0.9$, and $c = 2.5 \pm 2.5$ for Fe^{III}cyt c_{553}, respectively.

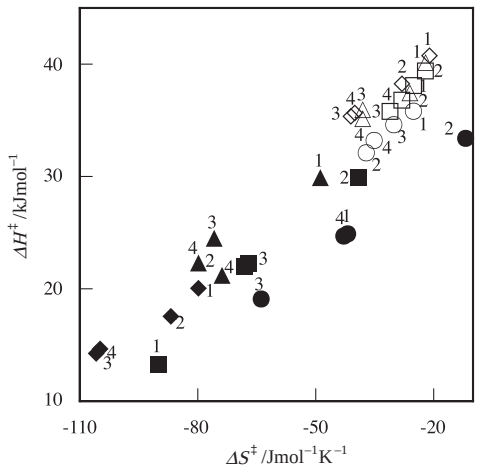

Fig. 3. $\Delta H^{\ddagger}/\Delta S^{\ddagger}$ compensation plots for the oxidation of reduced protein with $[Ru^{III}(bcmaa)(bpy)]^0$ (filled symbol) and the reduction of the oxidized protein with K[Ru^{II}-(bcmaa)(bpy)] (open symbol); protein = cyt c (square), cyt c_{553} (circle), azurin-1 (triangle), and azurin-2 (diamond); BCMAA = (*S*)-BCMPA (**1**), (*R*)-BCMPA (**2**), (*S*)-BCMAL (**3**), and (*R*)-BCMAL (**4**). Experimental condition: pH 6.0 (1 mM phosphate buffer), $I = 0.05$ M (KCl).

On the other hand, $\Delta\Delta H^{\ddagger}_{S-R}$ and $T\Delta\Delta S^{\ddagger}_{S-R}$ for the reduction of Fe^{III}cyt c with [Ru^{II}(bcmaa)(bpy)] were almost the same in spite of differences in the bulkiness of amino acid residues BCMAL and BCMPA. Such a difference involving the reaction directions, oxidation or reduction, was also observed in the cases of azurin-1, azurin-2, and cyt c_{553}.[8] These behaviors can be explained in terms of the charges of Ru complexes and electron-transport proteins, that is, in the oxidation of Fe^{II}cyt c, [Ru^{III}(bcmaa)(bpy)] is neutral, and in the case of the reduction of Fe^{III}cyt c, that of K[Ru^{II}(bcmaa)(bpy)] is -1. Therefore, the former will not be affected by the electrostatic interaction, whereas the latter will be influenced by the electrostatic attraction/repulsion according to the charges of the molecular recognition site of Fe^{III}cyt c. To examine the influence of the electrostatic interaction in the reduction of Fe^{III}cyt c by K[Ru^{II}(bcmaa)(bpy)], the ion strength dependence of rate constants was investigated (Fig. 2).[11] The ion strength dependence was fitted according to the extended Debye–Hückel equation,[12,13] and the effective charges were estimated as follows: that of Fe^{III}cyt c (+3) is larger that those (+1) of Fe^{III}cyt c_{553} and azurin-1Cu^{II}. Therefore, the electrostatic attraction between Fe^{III}cyt c and K[Ru^{II}(bcmaa)(bpy)] will contribute more strongly to the electron-transfer reaction than that of the interaction with Fe^{III}cyt c_{553} or azurin-1Cu^{II}. In the case of cyt c, the decrease in the absolute values of $\Delta\Delta H^{\ddagger}_{S-R}$ and $T\Delta\Delta S^{\ddagger}_{S-R}$ ($|\Delta\Delta H^{\ddagger}_{S-R,\mathrm{ox}}| - |\Delta\Delta H^{\ddagger}_{S-R,\mathrm{red}}|$ and $|T\Delta\Delta S^{\ddagger}_{S-R,\mathrm{ox}}| - |T\Delta\Delta S^{\ddagger}_{S-R,\mathrm{red}}|$) was larger than those of cyt c_{553} and azurin-1. These findings indicate that electrostatic attraction is unfavorable for the recognition of the chiralities of [Ru(bcmaa)(bpy)].

To analyze the contribution of the charges on proteins and ruthenium complexes, $\Delta H^{\ddagger}/\Delta S^{\ddagger}$ compensation plots were examined (Fig. 3). The plots for the oxidations of the reduced proteins with $[Ru^{III}(bcmaa)(bpy)]^0$ (filled symbols) were widely diverse according to the chiralities and the types of side chains of $[Ru^{III}(bcmaa)(bpy)]^0$ and the kinds of electron-transfer proteins. This distribution might be caused by the steric interactions between the electron-transfer site of proteins and the side chains of the ruthenium complexes.[8] In contrast, the plots for the reductions of the oxidized proteins with $[Ru^{II}(bcmaa)(bpy)]^-$ (open symbols) were gathered around ($\Delta S^{\ddagger}$, $\Delta H^{\ddagger}$) = (-30 J mol^{-1} K^{-1}, 35 kJ mol^{-1}). These behav-

iors indicate that electrostatic attractions lower the molecular recognition ability for the side chains of [Ru(bcmaa)(bpy)] and the proteins as well as that for the chiralities of [Ru(bcmaa)(bpy)]. Our results agree with the local structure obtained from the crystal structure of the electron-transfer complexes cytochrome *c*–cytochrome bc_1 complex and cytochrome c_2–reaction center.[5] In other words, the intermolecular interactions, which work at the vicinity of the electron-transfer site, are achieved by noncovalent interactions, such as hydrophobic, steric repulsion, and van der Waals interactions, rather than electrostatic interactions.

In conclusion, the kinetics for the redox reaction between cyt *c* and [Ru(bcmaa)(bpy)] was studied and their kinetic parameters was compared to those of the proteins azurin-1, azurin-2, and cyt c_{553}. The importance of non-polar interactions in the exact recognitions of electron-transport proteins by their redox partners rather than the polar interaction was demonstrated, and this is the first example of evidence obtained kinetically by using small probe complexes.

Experimental

Preparation of Cytochrome *c*. Fe^{III}cyt *c* (horse heart) was purchased from NACALAI TESQUE and used without further purification. Fe^{II}cyt *c*, used for kinetic measurements, was prepared by treating Fe^{III}cyt *c* with the equimolar amounts sodium dithionite.

Syntheses of $Ru^{II/III}$ Complexes. All $Ru^{II/III}$ complexes were synthesized according to previously reported procedures.[8]

Kinetic Measurements. The electron-transport reaction between cytochrome *c* and Ru complexes were investigated by using a UNISOKU stopped-flow spectrophotometer using 417 nm (increase or decrease of Soret band) under pseudo-first-order conditions ([cyt *c*] = 1 μM, [Ru]/[cyt *c*] > 50). The sample solution, containing 1 mM phosphate buffer (pH 6.0) and 0.05 M KCl, was freshly prepared. The relation of the absorbance (A) with time (t) (A/t relation) was fitted to Eq. 1 by using the non-linear least squares methods.

$$A - A_\infty = (A_0 - A_\infty)\exp(-k_{obs}t) + Bt, \quad (1)$$

where Bt is a baseline correction. All manipulations were performed under N_2 or argon.

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Supporting Information

The dependence of first-order rate constants on the concentration of Ru complexes (Fig. S1) and Eyring plots (Fig. S2). This material is available free of charge on the Web at: http://www.csj.jp/journals/bcsj/.

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9 [cyt *c*] = 1×10^{-6} M and [Ru]/[cyt *c*] = 50–300, pH 6.0 (1 mM phosphate buffer), I = 0.05 M (KCl), 298 K, under Ar.

10 [cyt *c*] = 1×10^{-6} M and [Ru] = 1.0×10^{-4} M, pH 6.0 (1 mM phosphate buffer), I = 0.05 M (KCl), 278–298 K, under Ar.

11 [cyt *c*] = 1×10^{-6} M and [Ru] = 1.5×10^{-4} M, pH 6.0 (1 mM phosphate buffer), I = 0.05–0.50 M (KCl), 298 K, under Ar.

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13 $\log k = \log k_0 + 2Az_{Ru}z_{cytc}I^{0.5}/(1 + BaI^{0.5})$, k_0: rate constant for I = 0 M, z_{Ru}: charge of Ru complex, z_{cytc}: charge of cyt *c*, A and B: the parameters depending on temperature and solvent (A = 0.51, $B = 0.33 \times 10^8$ for 25 °C, water), a: the nearest distance between Ru complex and cytochrome *c*.