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**Mechanism of Electron Transfer from
Cytochrome *c* to Cytochrome *c* Oxidase Regulated by
Hydrophobic Interaction**

(疎水性相互作用で制御されるシトクロム *c* –
シトクロム *c* 酸化酵素間の電子伝達機構)

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CONTENTS

ACKNOWLEDGMENTS.....	I
LIST OF PUBLICATIONS.....	II
LIST OF PRESENTATIONS.....	III

CHAPTER I: General Introduction.....	1
1.1. Intermolecular Electron Transfer in the Mitochondrial Respiratory Chain.....	2
1.2. Electron Transfer Reaction from Cytochrome <i>c</i> to Cytochrome <i>c</i> Oxidase.....	4
1.3. Specific Interprotein Interactions upon the Cyt <i>c</i> -CcO Complexation (Chapter II).....	6
1.4. Contribution of Dehydration near the Heme in Cyt <i>c</i> to ET to CcO (Chapter III).....	6
1.5. Exploring the Electron Transfer Pathway from Cyt <i>c</i> to CcO (Chapter IV).....	7
References.....	8

CHAPTER II: Thermodynamic Study on the Cytochrome <i>c</i>-Cytochrome <i>c</i> Oxidase Complex Using Osmotic Pressure.....	11
Abstract.....	12
2.1. Introduction.....	13
2.2. Experimental Procedures.....	16
Protein Expression and Purification.....	16
Site-directed Mutagenesis.....	17
Steady-state Kinetics for Cyt <i>c</i> Oxidation by CcO.....	17
2.3. Results.....	19
Examination of Dehydration upon the Cyt <i>c</i> -CcO Complex Formation.....	19
Dehydration Sites on Cyt <i>c</i>	23
Contribution of Dehydration to the Cyt <i>c</i> -CcO Complex Stability.....	26
2.4. Discussion.....	29
Slight and Specific Dehydration upon Cyt <i>c</i> -CcO Complex Formation.....	29
Functional Significance of Dehydration from Ile81 of Cyt <i>c</i>	30
References.....	31

CHAPTER III: Contribution of Dehydration near the Heme in Cytochrome <i>c</i> to Electron Transfer Activity to Cytochrome <i>c</i> Oxidase.....	36
Abstract.....	37
3.1. Introduction.....	38
3.2. Experimental Procedures.....	39
Ferrous Cyt <i>c</i> Autoxidation Kinetics.....	39

Absorption Spectroscopy.....	39
Circular Dichroism Spectroscopy.....	40
Resonance Raman Spectroscopy.....	40
Fluorescence Spectroscopy.....	41
Near-infrared Spectroscopy.....	41
¹ H- ¹⁵ N HSQC NMR Spectroscopy.....	42
Potentiometric Titration.....	43
3.3. Results.....	44
Electron Transfer from Cyt <i>c</i> in the Presence of PEG.....	44
Structural Properties of Cyt <i>c</i> in the Presence of PEG.....	45
Observation of Dehydration Induced by PEG.....	50
Dehydration Sites Causing the Change of Heme Position.....	52
Positional Change of Heme Triggering the Autoxidation.....	54
Conformational Change of Cyt <i>c</i> Associated with Change of the Heme Position.....	55
Influence of Positional Change of Heme on Its Redox Potential.....	59
3.4 Discussion.....	60
Functional Significance of Dehydration to Electron Transfer.....	60
References.....	62

CHAPTER IV: Exploring the Electron Transfer Pathway from Cytochrome *c* to

Cytochrome <i>c</i> Oxidase.....	65
Abstract.....	66
4.1. Introduction.....	67
4.2. Experimental Procedures.....	69
<i>Pathways</i> Model.....	69
Preparation of the Sample Solution for Flow-Flash Measurement.....	69
Flow-flash Measurement.....	70
4.3. Results.....	71
Prediction of the Structure of Cyt <i>c</i> -CcO Complex.....	71
Identification of the Residues Forming the ET Pathway from Cyt <i>c</i> to CcO.....	73
Determination of <i>T</i> _{DA} in the Cyt <i>c</i> -CcO Complex.....	75
Contribution of Hydrophobic Residues near the Heme of Cyt <i>c</i> to ET.....	79
4.4. Discussion.....	80
Importance of the Hydrophobic Interactions near the Redox Centers for ET.....	80
Possibility of Conformational Rearrangement to an Optimal Orientation for ET.....	83
References.....	85

CHAPTER V: CONCLUSIONS.....87

CHAPTER I

General Introduction

1.1. Intermolecular Electron Transfer in the Mitochondrial Respiratory Chain

All eukaryotes, including yeast, plant and human, use an organic molecule, ATP, which participates in many chemical reactions in cells and is often called as the “molecular unit of currency” for intracellular energy transfer. Actually, on any given day we turn over our body weight equivalent in ATP [1]. Such abundant ATP is mainly generated in one of the intracellular organelles, mitochondria.

In the mitochondria, ATP is generated primarily through the oxidative phosphorylation of ADP, a process in which electrons are transferred along a series of carrier molecules called a mitochondrial respiratory chain (Figure 1). These electrons are generated from NADH or succinate, which are produced by oxidation of glycolytic products or fatty acid, and are ultimately transferred to molecular oxygen [2]. The mitochondrial respiratory chain consists of four respiratory enzyme complexes in the mitochondrial inner membrane; NADH:ubiquinone oxidoreductase (Complex I), succinate:ubiquinone oxidoreductase (Complex II), ubiquinone:cytochrome *c* oxidoreductase (Complex III, Cyt *bc*₁ complex), and cytochrome *c* oxidase (Complex IV, CcO).

Complex I functions as the entry point for electrons into the respiratory chain from NADH and these electrons are used to reduce lipid-soluble electron carrier, ubiquinone (CoQ) [3–5]. Complex II also participates in the electron transport chain by transferring electrons from succinate to CoQ [6]. Then, CoQ is subsequently oxidized by Complex III, which catalyzes the transfer of electrons from CoQ to Cytochrome *c* (Cyt *c*) [7–9]. Cyt *c* functions as an electron shuttle from Complex III to Complex IV. In the terminal step of respiration, Complex IV catalyzes the transfer of electrons from Cyt *c* to molecular oxygen, yielding two water molecules [2,10]. The electron transfer (ET) reactions through Complex I, Complex III, or Complex IV, induce the proton pump from the mitochondrial matrix to the cytosolic side of the inner membrane [11–14]. Then, the proton gradient produced by the ET through these complexes is used as an electrochemical gradient that drives F₀F₁-ATP synthase (Complex V)

to generate ATP from ADP and inorganic phosphate [15–17]. Therefore, the series of unidirectional intermolecular ET reactions in the mitochondrial respiratory chain significantly contribute to sustain cellular life by ATP synthesis, and many scientists have been interested in the physiologically crucial molecular mechanism of these intermolecular ET reactions.

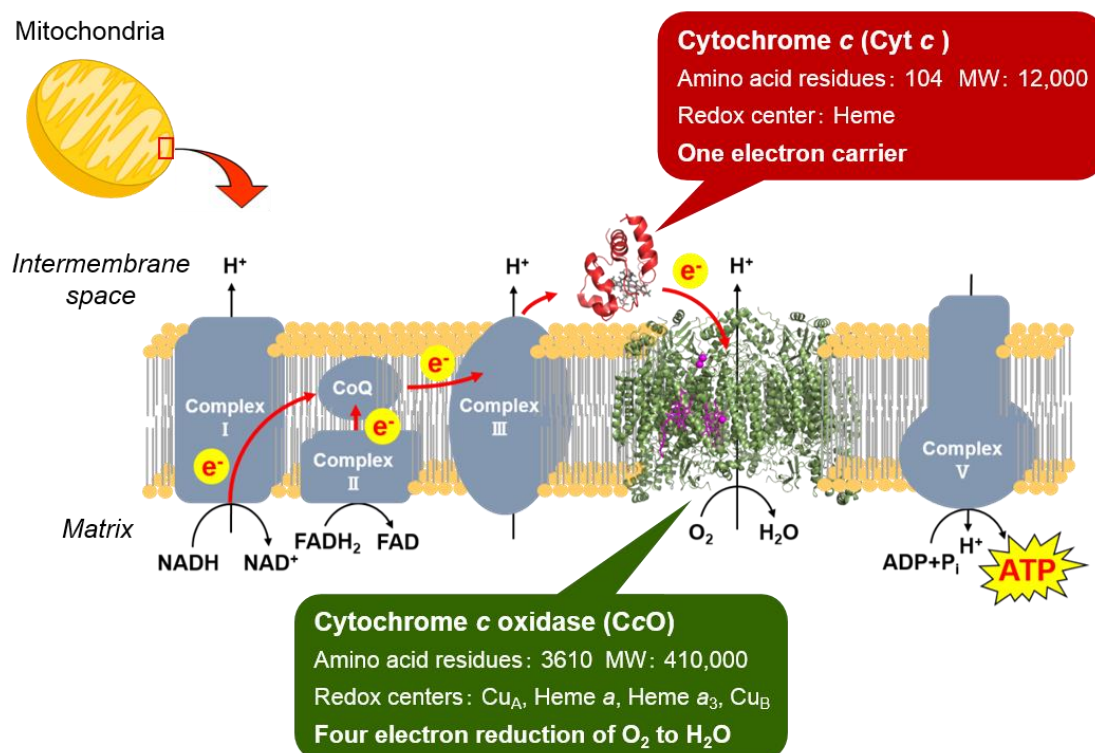


Figure 1. The enzymes of the mitochondrial inner membrane involved in oxidative phosphorylation. Complex I, II, III, and IV (Cytochrome c oxidase, CcO) form the electron transfer chain from NADH or FADH₂ to molecular oxygen. With the exception of Complex II, these enzymes translocate proton across the mitochondrial membrane. The proton gradient is used by ATP synthase to generate ATP. Cytochrome c (Cyt c) functions as an electron mediator between Complex III and IV.

1.2. Electron Transfer Reaction from Cytochrome *c* to Cytochrome *c* Oxidase

As described above, a series of electron transfer (ET) reactions in the mitochondrial respiratory chain terminated at Complex IV (CcO) (Figure 1), where molecular oxygen is reduced to two water molecules. Electrons that reduce the molecular oxygen are donated from an electron carrier protein, Cyt *c* [18]. As shown in Figure 2, Cyt *c* is a 12 kDa globular protein, containing a heme group covalently attached to two cysteine residues by thioether linkage [19], which readily accepts and releases an electron. Although the reduction of molecular oxygen to water molecules requires four electrons, Cyt *c* can carry only one electron, meaning that Cyt *c* is required to repetitively associate with and dissociate from CcO to consecutively donate an electron. Therefore, the interprotein ET from Cyt *c* to CcO was supposed to be controlled by the specific interactions between two proteins, but not accidentally occur by random collision.

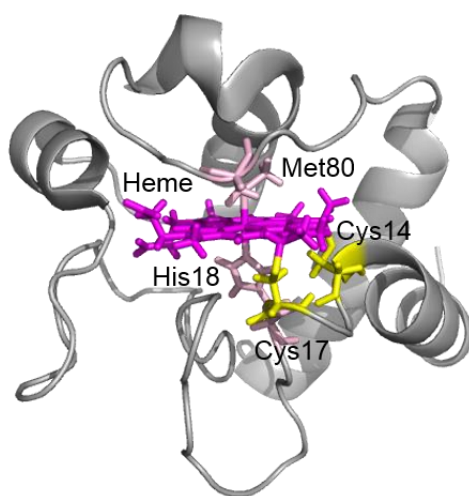


Figure 2. Tertiary structure of Cyt *c*.

The heme, axial ligands and Cys residues by thioether linkage are shown as magenta, pink and yellow stick, respectively.

For elucidating the molecular mechanism of the ET reaction from Cyt *c* to CcO, many researchers have focused on the interactions between Cyt *c* and CcO. The detailed tertiary structures of Cyt *c* and CcO, which were determined in the mid- and late-twenty century,

respectively, provided detailed structural information for understanding the interprotein interactions. The X-ray crystal structure of Cyt *c* showed that many lysine residues, positively charged residues, are localized on the surface of Cyt *c* and surround the redox center, heme [19]. In contrast, CcO contains several negatively charged residues, such as glutamate and aspartate, around the Cu_A site, which is known as an electron entry site [20]. Based on these information, Cyt *c* has been proposed to dock specifically with CcO by these electrostatic interactions for the ET to CcO (Figure 3). In contrast, recent NMR analysis of the Cyt *c*-CcO complex identified several hydrophobic residues that contribute to the formation of the ET complex [21]. However, the role and contribution of hydrophobic interactions for the ET reaction from Cyt *c* to CcO has not been fully understood.

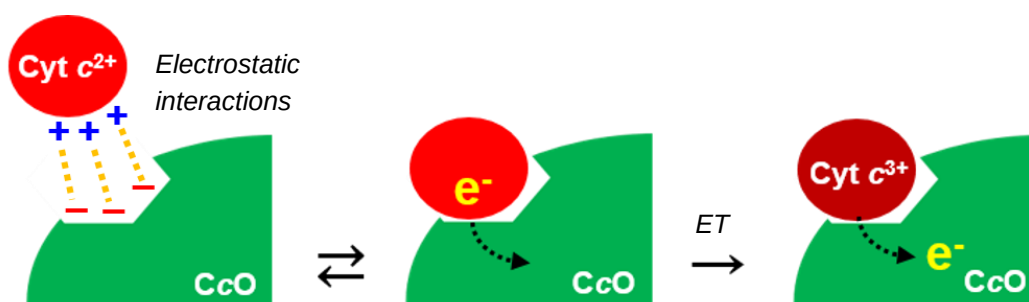


Figure 3. Proposed mechanism of ET reaction from Cyt *c* to CcO.

In this mechanism, the ET-active Cyt *c*-CcO complex is formed by electrostatic interactions between positively-charged residues in Cyt *c* and negatively-charged residues in CcO.

1.3. Specific Interprotein Interactions upon the Cyt *c*-CcO Complexation (Chapter II)

While many researchers have demonstrated the involvement of electrostatic interactions mediated by lysine residues in Cyt *c* and acidic residues in CcO for the Cyt *c*-CcO complex formation, several recent studies on protein-protein interactions suggested that hydrophobic interactions were the primary thermodynamic factor that stabilized protein-protein complex formation and electrostatic interactions were almost negligible [22]. However, the quantitative characterization of the hydrophobic interactions essential for the complexation between Cyt *c* and CcO has not yet been fully investigated. Thus, the interactions primarily dominating the specific complex formation between Cyt *c* and CcO and the detailed stabilization mechanism for the complex formation by the hydrophobic interactions are still key open questions.

One of the experimental approaches to evaluate the thermodynamic contribution of hydrophobic interactions in the Cyt *c*-CcO complex is to examine the “dehydration” of hydrophobic residues in the interaction site [23,24], because the dehydration at protein surface is based on the increase in entropy upon the complexation [25,26]. Here, in Chapter II, I experimentally identified the residues of Cyt *c* forming the hydrophobic interactions with CcO and evaluated the contribution of such hydrophobic interactions to the stability of the Cyt *c*-CcO complex on the basis of the entropy change from the dehydration around the interface of the complex. Based on these experimental analyses, I discussed the thermodynamic significance of the hydrophobic interactions for the Cyt *c*-CcO complexation.

1.4. Contribution of Dehydration near the Heme in Cyt *c* to ET to CcO (Chapter III)

As revealed in Chapter II, formation of the Cyt *c*-CcO complex are driven by specific hydrophobic interaction near the heme of Cyt *c*, accompanied by an increase in the entropy from the “dehydration” around the hydrophobic residues. On the other hand, recent studies have proposed that dehydration of protein surface would affect the conformations and functions of proteins [27,28]. In the case of Cyt *c* and CcO, however, the influence of the dehydration near

the heme periphery of Cyt *c* on its ET function was still unknown.

In this chapter, to examine the functional and structural significance of the dehydration from Cyt *c* upon the Cyt *c*-CcO complexation, I characterized the structure and ET function of Cyt *c* in the presence of polyethylene glycol (PEG), which causes dehydration from the surfaces of proteins [29,30]. Based on various spectroscopic measurements and combined with the results of Chapter II, I discussed the contribution of the specific dehydration from the hydrophobic region near the heme periphery to the ET reaction from Cyt *c* to CcO in terms of heme redox potential.

1.5. Exploring the Electron Transfer Pathway from Cyt *c* to CcO (Chapter IV)

In Chapters II and III, I suggested that the dehydration from the hydrophobic residues near the heme periphery of Cyt *c* contributes to not only the stabilization of the Cyt *c*-CcO complex but also the change of heme position to negatively shift its reduction potential, which promotes the ET reaction. However, the mechanism how an electron is transferred from Cyt *c* to CcO after the complex formation is a still open question because of the lack of structural information about the Cyt *c*-CcO complex.

In this chapter, to examine the ET pathway from heme to Cu_A in the Cyt *c*-CcO complex, I predicted the complex between Cyt *c* and CcO by protein docking simulation and identified the residues involved in the ET pathway from Cyt *c* to CcO. I further experimentally determined the electronic donor-to-acceptor tunneling coupling mediated by the intervening (T_{DA}) for the ET reaction between heme and Cu_A in the Cyt *c*-CcO complex, to assess the contribution of the hydrophobic residue near the heme periphery to form the ET pathway. By these combined results from experimental and theoretical analysis of the ET reaction in the Cyt *c*-CcO complex, I deduced the molecular mechanism of the ET reaction from Cyt *c* to CcO after the complexation.

For elucidating the molecular mechanism of the ET reaction from Cyt *c* to CcO, in this thesis, I focused on the hydrophobic interactions which has not been fully investigated compared to electrostatic interactions and aimed to reveal the functional significance of the hydrophobic interactions for the Cyt *c*-CcO ET reaction based on the thermodynamic, structural and kinetic analyses using the various spectroscopic measurements and theoretical analysis.

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CHAPTER II

Thermodynamic Study on the Cytochrome *c*-Cytochrome *c*

Oxidase Complex Using Osmotic Pressure

Abstract

To examine the hydrophobic interaction dominating the formation of Cyt *c*-CcO complex, I determined the number of dehydrated water molecules accompanying the formation of the hydrophobic interaction and the dehydration sites upon the Cyt *c*-CcO complexation by the osmotic pressure analysis. Based on the osmotic pressure dependence of the ET rates from Cyt *c* to CcO, about 17 water molecules were estimated to be dehydrated upon the complex formation. Mutations at the interaction site of Cyt *c* with CcO revealed that nearly half of the dehydrated water molecules were located around Ile81, one of the hydrophobic residues near the exposed heme periphery of Cyt *c*. The entropy change by such a specific dehydration from the hydrophobic residue was estimated to be about 60% of the total entropy change upon the Cyt *c*-CcO complexation, indicating compensation for the entropy decrease by the association of Cyt *c* with CcO. These results suggest that Cyt *c* associates with CcO for the ET reaction by forming specific hydrophobic interactions near the heme periphery as the driving force for the Cyt *c*-CcO complexation.

2.1. Introduction

For elucidating the detailed molecular mechanism of the ET reaction from Cyt *c* to CcO, many studies have demonstrated the involvement of electrostatic interactions mediated by lysine residues surrounding the heme edge in Cyt *c* and acidic residues around Cu_A in CcO [1–5]. In contrast, recent NMR analysis of the Cyt *c*-CcO complex identified several hydrophobic residues, including Ile9, Ile11, Met12, Cys17, and Ile81, as well as many lysine residues, which participate in the formation of the complex [6]. Especially, recent studies on protein-protein interactions suggested that hydrophobic interactions were the primary factor to thermodynamically stabilize the protein complex and electrostatic interactions were almost negligible [7]. However, the experimental evaluation of the hydrophobic interactions to the stabilization of the Cyt *c*-CcO complex has not yet been examined. Therefore, the interactions primarily dominating the complex formation between Cyt *c* and CcO remain unclear.

To examine the formation of hydrophobic interactions, I focused on the hydrating water molecules around the side chain of hydrophobic residues at the interaction sites with CcO on Cyt *c*. When the hydrophobic residues are involved in the interaction site with the partner protein, hydrating water molecules around the side chain of the residues would be expelled to bulk solvent, followed by formation of the direct hydrophobic interaction with the residues of the partner protein. Thus, the observation of the dehydration of hydrophobic residues at the interaction site is crucial experimental evidence for the formation of hydrophobic interactions in protein-protein complexes [8–10]. Although the dehydration is also observed for hydrophilic residues [11,12], the energetic contribution of hydrophilic interactions to the formation of the protein-protein complex is proposed to be much smaller than that of hydrophobic interactions, because the energetically favorable hydrophilic interactions, such as electrostatic interactions, are nearly cancelled out by dehydration due to its large unfavorable enthalpy change [7]. In contrast, the dehydration of hydrophobic residues induces the favorable entropy change [7,13], suggesting that formation of hydrophobic interactions accompanying dehydration is a key

process in the formation of the Cyt *c*-CcO complex.

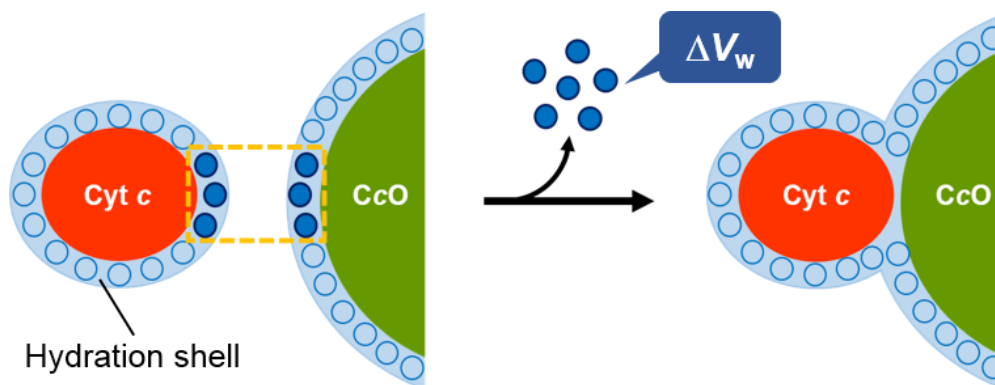


Figure 1. A schematic presentation of dehydration around protein interface upon the complex formation.

Although spectroscopic detection of dehydration is quite difficult, dehydration can be monitored as the change in the partial molar volume of proteins (ΔV_w) [14–16], which is altered during the dehydration (Figure 1). Assuming that the molecular volume of each protein is unchanged after the complex formation, the change in ΔV_w corresponds to the volume of the water molecules involved in the dehydration (negative volume change) or hydration (positive volume change) [14,17]. ΔV_w can be determined by the dependence of the dissociation constant (K_d) on osmotic pressure (see Results). Then, to examine the dehydration associated with the complex formation, I examined the effect of osmotic pressure on K_d for the formation of the Cyt *c*-CcO complex. In this case, however, the determination of K_d for the “ET-active” complex between “reduced Cyt *c*” and “oxidized CcO” is difficult due to the rapid ET reaction between the complex. Thus, I applied Michaelis-Menten constant (K_M), representing the stability of the ET-active complex, instead of K_d , to estimate ΔV_w for ET from Cyt *c* to CcO, assuming that Cyt *c* is in instantaneous chemical equilibrium with the Cyt *c*-CcO complex.

In this chapter, to characterize the hydrophobic interaction upon the Cyt *c*-CcO complex

formation, I experimentally identified the residues in Cyt *c* forming the hydrophobic interactions with CcO by determining the number of dehydrated water molecules by the osmotic pressure analysis. Furthermore, the contribution of the entropy change from such hydrophobic interactions accompanying the dehydration to stabilize the Cyt *c*-CcO complex was quantified in terms of the thermodynamic parameters upon the complexation. Based on these experimental analyses, I discussed a functional significance of the hydrophobic interactions for the Cyt *c*-CcO complexation.

2.2. Experimental Procedures

Protein Expression and Purification

Cyt *c* was expressed in *Escherichia coli* and purified as previously described [6,18]. Briefly, Rosetta2(DE3)pLysS (Novagen, Madison, WI, USA) cells transformed with the plasmids containing DNA of human Cyt *c* were inoculated in 5 mL of 2xYT medium and grown overnight. This pre-cultured medium was added to 1 L of 2xYT and the bacteria were further incubated at 37 °C. The expression of Cyt *c* was initiated by adding 0.8 mM isopropyl 1-thio- β -D-galactopyranoside to the culture when the optical density at 600 nm reached 0.6. Then, 0.1 mM δ -aminolevulinic acid was also added to promote heme biosynthesis. After incubation for additional 24 h, the cells were harvested by centrifugation.

The cell pellet was resuspended in 50 mM Tris-HCl (pH 7.5) containing 1 g/L lysozyme, 50 mg/L DNase I, and 50 mg/L RNase A and suspended for 3 h to lyse the cell pellet completely. The supernatant of the crude extract was obtained by centrifugation at 18,000 rpm for 5 min and 40,000 rpm for 1 h. This supernatant was purified by a HiPrep 16/10 SP XL column (GE Healthcare, Uppsala, Sweden) with a linear gradient of 1–300 mM NaCl. The elution sample was concentrated by Amicon ultrafiltration using 5-kDa cutoff membranes. Because the concentrated Cyt *c* was a mixture of ferric and ferrous forms, Cyt *c* was oxidized by 10-fold potassium ferricyanide with stirring for 1 h. After being dissolved in 50 mM sodium phosphate buffer (pH 7.0), Cyt *c* was further purified by a Mono S 10/100 GL column (GE Healthcare) with a linear salt gradient of 1–300 mM NaCl. The purified Cyt *c* fractions were pooled, concentrated, and applied to a HiLoad 16/60 Superdex 75 gel filtration column (GE Healthcare).

Site-directed Mutagenesis

Mutagenesis was conducted utilizing a PrimeSTAR mutagenesis basal kit from Takara Bio (Otsu, Japan). DNA oligonucleotides were purchased from Eurofins Genomics (Tokyo, Japan). The mutated genes were sequenced to ensure that only the desired mutations were introduced (Eurofins Genomics).

Steady-state Kinetics for Cyt *c* Oxidation by CcO

The electron transfer reaction from Cyt *c* to CcO was measured with a Hitachi U-3310 UV-visible spectrophotometer in 50 mM sodium phosphate and 0.1% *n*-decyl β -D-maltoside (DM) (pH 6.8). Ferrous Cyt *c* was prepared by the reduction of ferric Cyt *c* with dithionite and excess reductant was removed by a PD MiniTrap G-25 column. The concentrations of Cyt *c* were varied between 0.5 and 40 μ M, and oxidation of Cyt *c* was followed by the absorbance at 550 nm after adding CcO to the reaction solution at the final concentration of 1 nM. The absorption at 550 nm was recorded with an interval of 1 s for 3 min. To determine the end point of the electron transfer reaction, a small amount of potassium ferricyanide (III) was added to the reaction solution. A first-order kinetics was observed for oxidation of reduced Cyt *c* as reported in the previous reports [19,20]:

$$V = -\frac{d[\text{Cyt } c^{2+}]}{dt} = k_{\text{obs}}[\text{Cyt } c^{2+}] \quad (1)$$

where V is the oxidation rate of reduced Cyt *c*, and k_{obs} is the apparent rate constant at the various concentrations of Cyt *c*, which was estimated by Equation 2:

$$\ln[\text{Cyt } c^{2+}] = -k_{\text{obs}}t + \ln[\text{Cyt } c^{2+}]_0 \quad (2)$$

where $[\text{Cyt } c^{2+}]_0$ denotes the initial concentration of reduced Cyt *c*. Consistent to the previous reports [19,20], a single rectangular relationship between V_0 (initial oxidation rate) and $[\text{Cyt } c^{2+}]_0$ was obtained under the present experimental conditions, as given in Equation 3.

$$V_0 = \frac{V_{\max} [\text{Cyt } c^{2+}]_0}{K_M + [\text{Cyt } c^{2+}]_0} \quad (3)$$

The Michaelis-Menten parameters were estimated using $[\text{Cyt } c^{2+}]_0$ and V_0 that was determined by Equation 1 ($V_0 = k_{\text{obs}}[\text{Cyt } c^{2+}]_0$). The catalytic constant, k_{cat} , was calculated by Equation 4:

$$k_{\text{cat}} = \frac{V_{\max}}{[\text{CcO}]} \quad (4)$$

where $[\text{CcO}]$ denotes the total concentration of CcO (1 nM).

2.3. Results

Examination of Dehydration upon the Cyt *c*-CcO Complex Formation

To examine the formation of hydrophobic interactions between Cyt *c* and CcO, I investigated dehydration around the interaction sites of Cyt *c* with CcO upon the complex formation by determining the Michaelis constants (K_M) under various osmotic pressure. The K_M values were obtained by the steady-state kinetics for the ET reaction from Cyt *c* to CcO. The osmotic pressure of the sample solution was increased by the addition of sugars or polyalcohols such as sucrose, D-glucose, glycerol and ethylene glycol, which do not significantly affect the interactions between the native states of proteins [21–23]. To avoid decrease in association rate between Cyt *c* and CcO due to increase in viscosity by addition of osmolytes, the oxidation reaction of Cyt *c* by CcO was measured at constant viscosity (1.6 mPa·s). Figure 2 shows the dependence of the initial rate on the concentrations of reduced Cyt *c* under various osmotic pressures. The Michaelis-Menten parameters (K_M and k_{cat}) for the ET reaction from Cyt *c* to CcO were determined by fitting the initial rates to Equation 3 (Table 1).

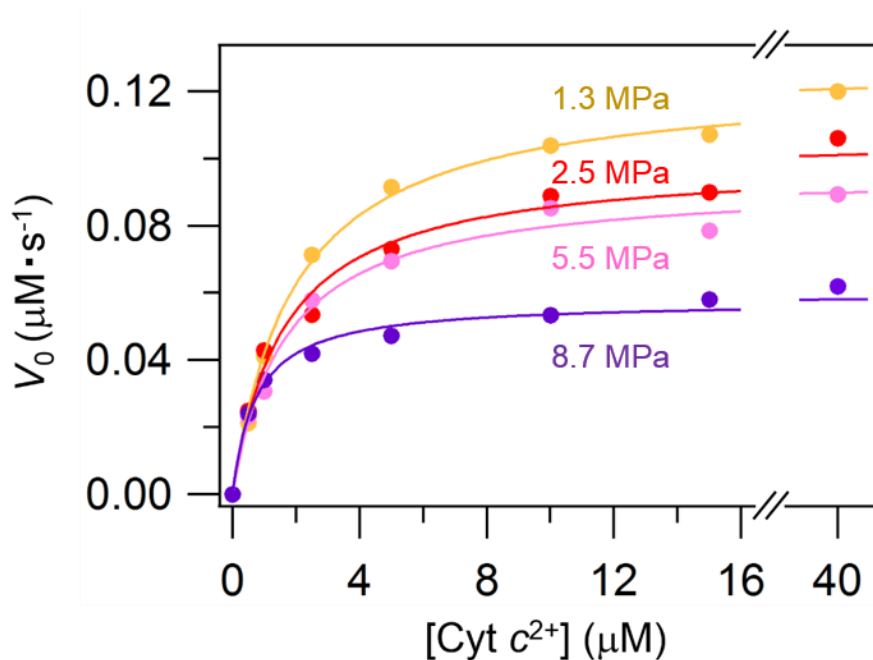


Figure 2. The dependence of the initial rate constants on the concentrations of Cyt *c* in the presence of various kinds of osmolytes.

Sucrose, orange; D-Glucose, red; Glycerol, pink, and Ethylene glycol, purple, respectively. The solid curves are best fits to the Equation 3.

Table 1. Dependence of the Michaelis constants on osmotic pressure for formation of the Cyt *c*-CcO complex.

Osmolyte and Concentration	Osmotic Pressure (MPa)	K_M (μM)	k_{cat} (s ⁻¹)	k_{cat}/K_M (μM ⁻¹ s ⁻¹)
15.2% Sucrose	1.3	2.05 ± 0.14	126 ± 2	61
15.6% D-Glucose	2.5	1.94 ± 0.27	105 ± 3	54
17.5% Glycerol	5.5	1.38 ± 0.13	99 ± 2	72
18.6% Ethylene Glycol	8.7	0.80 ± 0.11	59 ± 2	74

As described in introduction, assuming that the molecular volume of each protein is unchanged after the complexation, the change in the partial molecular volume corresponds to the volume of the dehydrated or hydrated water molecules (ΔV_w). ΔV_w is simply correlated with the osmotic pressure (π) dependence of the dissociation constant (K_d) [14,15,24]:

$$\left(\frac{\partial \ln K_d}{\partial \pi}\right)_T = \frac{\Delta V_w}{RT} \quad (5)$$

where R is the gas constant and T is temperature. As described above, in this study, I used K_M instead of the dissociation constant K_d , assuming that Cyt *c* is in instantaneous chemical equilibrium with the Cyt *c*-CcO complex. As Table 1 shows, K_M decreased with increasing osmotic pressure, corresponding to the negative volume change (Equation 5). The $\ln K_M$ value was plotted against the osmotic pressure (Figure 3) and fitted to Equation 5. Based on the slope of the fitting line (red) in Figure 3, ΔV_w was estimated to be $-314 \pm 50 \text{ mL mol}^{-1}$ (Table 2). The negative value implies that the Cyt *c*-CcO complex formation induces dehydration. Given the assumption that the volume change for dehydration from one hydrophobic amino acid residue is $-17.5 \text{ mL mol}^{-1}$ [25], the volume change obtained here corresponded to 17 ± 3 water molecules (Table 2).

Table 2. Volume changes (ΔV_w) and numbers of the dehydrated water molecule (N_{H_2O}) associated with formation of the Cyt *c*-CcO complex.

Cyt <i>c</i>	$\Delta V_w \text{ (mL mol}^{-1}\text{)}$	N_{H_2O}
WT	-314 ± 50	17 ± 3
I9A	-317 ± 48	18 ± 3
I11A	-246 ± 72	14 ± 4
K13A	-241 ± 20	13 ± 1
K79A	-278 ± 44	15 ± 2
I81A	-139 ± 38	8 ± 2

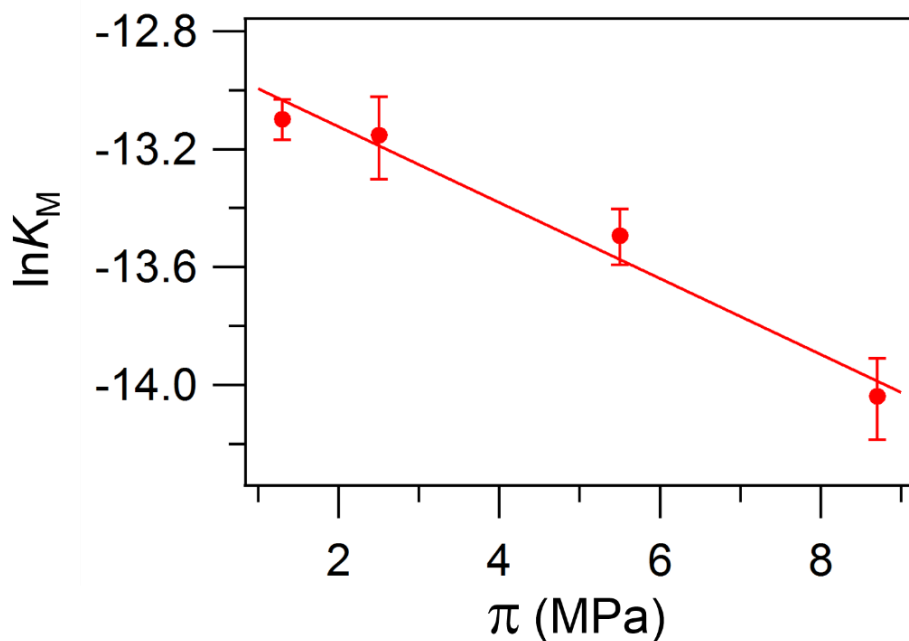


Figure 3. Dependence of K_M on osmotic pressure for the complex formation between Cyt *c* and CcO. Values of K_M were determined by Equation 3. The solid line represents the fit of these data to Equation 5.

The osmotic pressure experiment was also conducted using Cyt *c* complexed with porphyrin (demetallized heme) [26]. In this case, a positive volume change was reported. However, the structure and stability of porphyrin Cyt *c* significantly differ from those of native Cyt *c* owing to the lack of the two axial heme ligands. Although porphyrin Cyt *c* has native-like α -helix, its tertiary structure is substantially disordered, and its stability against heat or denaturant is much lower than that of native Cyt *c*, rather similar to that of the acidic molten globule state [27]. Thus, the positive volume change upon the association of CcO with porphyrin Cyt *c* appeared to involve the conformational change of porphyrin Cyt *c* upon the complexation, and the observed negative volume change upon the association of CcO with native Cyt *c*, which retains the rigid tertiary structure, (Figure 2 and Table 2) would be attributed to the dehydration.

Dehydration Sites on Cyt *c*

To identify the dehydration sites on Cyt *c*, I mutated the hydrophobic residues identified as the interaction site with CcO [6] to examine the osmotic pressure effects on K_M for the Cyt *c*-CcO complexation. As previously reported [6], three isoleucine residues, Ile9, Ile11, and Ile81, are located at the interaction site (Figure 4). Fractions of solvent accessible surface area (ASA) on Ile9, Ile11 and Ile81 were estimated to be 15, 42 and 85%, respectively, using the web server “VADAR” [28] (used PDB code; 1J3S). Considering that the number of the hydrated water molecules of the isolated isoleucine is 8.6 [29], the number of hydrated water molecules around side chains of Ile9, Ile11, and Ile81 were estimated to be 1, 4, and 7, respectively (summarized in Table 3). Each Ile residue was mutated to alanine, which is a less hydrophobic residue with fewer hydrating water molecules, and the oxidation rates of Cyt *c* by CcO at various osmotic pressures were measured to obtain ΔV_w . As recently reported, these mutations induced highly localized structural changes and the structures of the mutant Cyt *c*-CcO complexes were almost identical to that of the WT protein. Thus, I assumed that the mutations affect only the hydration at the mutation site.

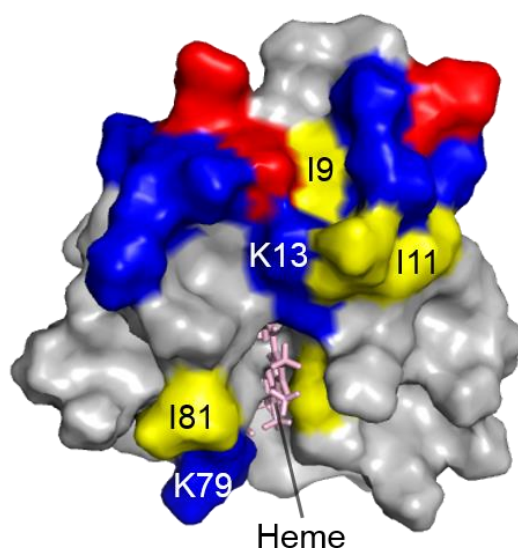


Figure 4. Interaction site for CcO on Cyt *c* [6].

Positively charged, negatively charged and hydrophobic residues represent blue, red and yellow, respectively.

Table 3. Hydrated water molecules around some residues in the interaction site of Cyt *c* for CcO.

Amino acid residue	ASA ^a (%)	^{hydr} N ^b	$\Delta^{\text{hydr}}N^c$
Ile	100	$\sim 8.6^{10)}$	—
Lys	100	~ 10	—
Ala	100	$\sim 1.8^{10)}$	—
Ile9	15 ± 6	1.3 ± 0.5	1.0 ± 0.4
Ile11	42 ± 6	3.6 ± 0.5	2.9 ± 0.4
Lys13	30 ± 7	3.0 ± 0.7	2.5 ± 0.6
Lys79	89 ± 7	8.9 ± 0.7	7.3 ± 0.6
Ile81	85 ± 13	7.3 ± 1.1	5.8 ± 0.9

a : Accessible surface area to solvent. These values were calculated by using the web server “VADAR”. I used structural data from Protein Data Bank (PDB) (PDB code; 1J3S).

b : The number of hydrated water molecules. This number was calculated by the following equation:

$$^{\text{hydr}}N = \text{ASA}(\%) / 100 \times ^{\text{hydr}}N(\text{amino acid residue})$$

where ASA is the accessible surface area to solvent and ^{hydr}N (amino acid residue) is the number of the hydrated water molecules of the isolated amino acid residue. We use 8.60 and 1.75 as ^{hydr}N (amino acid residue) for Ile and Lys residues, respectively.

c : The estimated number of decrease of hydrated water molecules by mutation

As illustrated in Figure 5, the slope of the osmotic pressure dependence of K_M for the Ile11 and Ile81 mutants was less steep than that for the wild-type protein. The estimated volume changes upon I11A or I81A mutant Cyt *c* upon the complex formation by Equation 5 were -246 ± 72 and $-139 \pm 38 \text{ mL mol}^{-1}$, respectively, which were smaller than that upon WT Cyt *c*-CcO complexation (Table 2). The decreased volume changes in the I11A and I81A mutants represented the small number of dehydrated water molecules of 14 ± 4 and 8 ± 2 , respectively. Assuming that the mutations to alanine affect only the hydration at the mutation site, the reduced number of dehydrated water molecules for these Ile mutants indicates that the hydrating water molecules were expelled from Ile11 and Ile81 to the solvent upon the formation of the ET complex. Particularly, the number of water molecules released from Ile81 was approximately 9, corresponding to about half of the total number of dehydrated water molecules.

Therefore, it can be concluded that Ile81 of Cyt *c* is one of the primary dehydration sites for the complex formation between Cyt *c* and CcO, and nearly 9 water molecules are released from Ile81 at the interaction site.

I also mutated two lysine residues located near the heme of Cyt *c*, Lys13 and Lys79, at the interaction sites (Figure 4), with alanine, and measured the oxidation rates of Cyt *c* by CcO at various osmotic pressures. The lysine mutations were less effective for changing the number of the dehydrated water molecules (Figure 5 and Table 2). Although Lys13 is a key residue for the complex formation with CcO [6], the number of the dehydrated water molecule of the K13A mutant was decreased by 4, which was much smaller than that of the I81A mutant. The mutation of Lys79, which is located adjacent to Ile81, also caused only minor perturbation of the dehydration. The numbers of dehydrated water molecules in the lysine mutants were similar to that in WT Cyt *c*, suggesting the preferential and specific dehydration from Ile81 occurred upon the formation of the Cyt *c*-CcO complex.

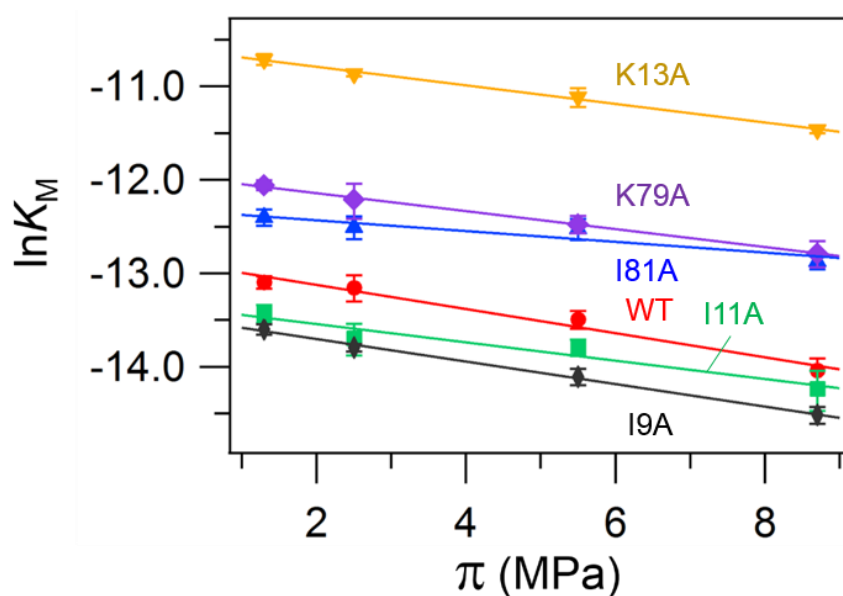


Figure 5. Dependence of K_M on osmotic pressure for the complex formation between various Cyt *c* mutants and CcO.

Values of K_M were determined from Equation 3. The solid line represents the fit of these data to Equation 5. WT, red; I9A, black; I11A, green; K13A, yellow; K79A, purple, and I81A, blue.

Contribution of Dehydration to the Cyt *c*-CcO Complex Stability

To evaluate the contribution of the specific dehydration from the interaction site accompanied by formation of hydrophobic interactions, I estimated the entropy change for the dehydration upon the Cyt *c*-CcO complexation. Assuming that the entropy change for dehydration of one water molecule from a hydrophobic residue is estimated to be $5.5 \pm 1.3 \text{ J K}^{-1} \text{ mol}^{-1}$ [30], the dehydration of 17 ± 3 water molecules in the WT Cyt *c*-CcO binding system corresponds to $93.5 \pm 27.6 \text{ J K}^{-1} \text{ mol}^{-1}$ and $-27.4 \pm 8.1 \text{ kJ mol}^{-1}$ in the entropy change and free energy change at 293 K, respectively (Table 4). To evaluate the contribution of this entropy change accompanied with dehydration ($^{\text{dehyd}}\Delta S$) to the Cyt *c*-CcO complexation, I experimentally obtained the enthalpy (ΔH) and total entropy change (ΔS) for the complex formation between Cyt *c* and CcO by measuring K_d under various temperature (Equation 6).

$$\ln K_d = \frac{\Delta H}{R} \frac{1}{T} - \frac{\Delta S}{R} \quad (6)$$

Table 4. Thermodynamic parameters for the complex formation between Cyt *c* and CcO.

Thermodynamic parameter (kJ mol ⁻¹)	WT	I81A
ΔG	-34.1 ± 5.2	-33.6 ± 4.2
ΔH	13.0 ± 3.6	-3.5 ± 3.0
$-T\Delta S$	-47.1 ± 3.7	-30.1 ± 3.0
$-T^{\text{dehyd}}\Delta S$	-27.4 ± 8.1	-12.9 ± 4.4

Figure 6 shows the $\ln K_M$ values for the complex formation between Cyt *c* and CcO plotted against reciprocal of temperature. By fitting the data to Equation 6 (red line, Figure 5), ΔH and $-T\Delta S$ at 293 K for formation of the Cyt *c*-CcO complex were estimated to be $13.0 \pm 3.6 \text{ kJ mol}^{-1}$ and $-47.1 \pm 3.7 \text{ kJ mol}^{-1}$, respectively (Table 4). Both ΔH and ΔS were positive values, indicating that the Cyt *c*-CcO complexation was driven by increase in the entropy. As described above, the entropy change due to the dehydration ($-T^{\text{dehyd}}\Delta S$) was calculated to be $-27.4 \pm 8.1 \text{ kJ mol}^{-1}$, which represents about 60% of total entropy change of $-47.1 \pm 3.7 \text{ kJ mol}^{-1}$. It is noted

that when one protein associates with another protein, the entropy change from the overall rotational and translational degrees of freedom ($-T^{\text{trans}}\Delta S$) is proposed to decrease by approximately $+9.8 \text{ kJ mol}^{-1}$ at 293 K [31–33]. The positive entropy change by dehydration of the hydrophobic residues compensates for the reduced entropy upon the complex formation. The dehydration upon the formation of the hydrophobic interaction is, therefore, one of the primary factors that drive the Cyt *c*-CcO complexation by increasing the entropy.

To investigate that the increase in the entropy upon the Cyt *c*-CcO complexation is suppressed by decrease in the number of dehydrated water molecules, I further determined the thermodynamic parameters for the complexation between I81A Cyt *c* and CcO, in which the less number of water molecules was dehydrated upon the complexation (Table 3). As shown in Figure 5, unlike WT Cyt *c*, the K_M value slightly increased with increasing temperature up to 308 K. The fitting data to Equation 6 (blue line, Figure 5) provided ΔH and $-T\Delta S$ at 293 K for the I81A Cyt *c*-CcO complexation of $-3.5 \pm 3.0 \text{ kJ mol}^{-1}$ and $-30.1 \pm 3.0 \text{ kJ mol}^{-1}$, respectively (Table 4). Although the association of I81A mutant Cyt *c* with CcO was also primarily driven by increasing the entropy, the value of $-T\Delta S$ decreased by approximately 17 kJ mol^{-1} at 293 K by the mutation of Ile81 to alanine, representing the change of ΔS in approximately $-58 \text{ J K}^{-1} \text{ mol}^{-1}$. Since $^{\text{dehyd}}\Delta S$ of one water molecule from a hydrophobic residue is estimated to be $5.5 \pm 1.3 \text{ J K}^{-1} \text{ mol}^{-1}$ [30], the entropy change of $58 \text{ J K}^{-1} \text{ mol}^{-1}$ corresponds to the dehydration of approximately 10 water molecules. As described in Table 2, the number of water molecules released upon the dehydration of Ile81 was approximately 9, which was almost identical to the decreased number of dehydrated water molecules by the mutation of Ile81 to alanine. Thus, the decrease in the contribution of ΔS by mutation of Ile81 to alanine was attributed to the decreased number of dehydrated water molecules. The comparable ΔS in the I81A mutant to that in WT Cyt *c* also supports the proposal that Cyt *c* uses the increase in the entropy from the preferential and specific dehydration from Ile81 as the driving force for the formation of the Cyt *c*-CcO complex (Figure 7).

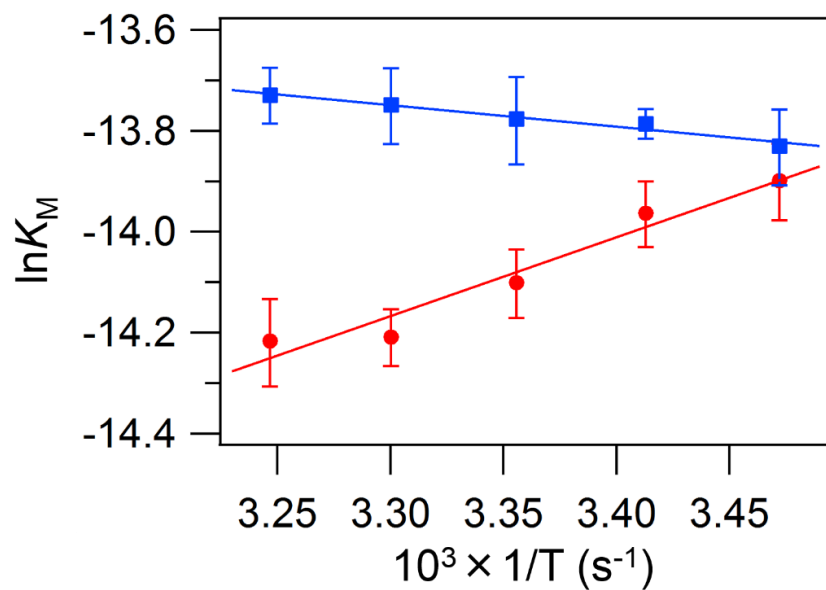


Figure 6. Dependence of K_M on temperature for the complex formation between WT (red) or I81A mutant (blue) Cyt *c* and CcO.

Values of K_M were determined from Equation 3. The solid line represents the fit of these data to Equation 6.

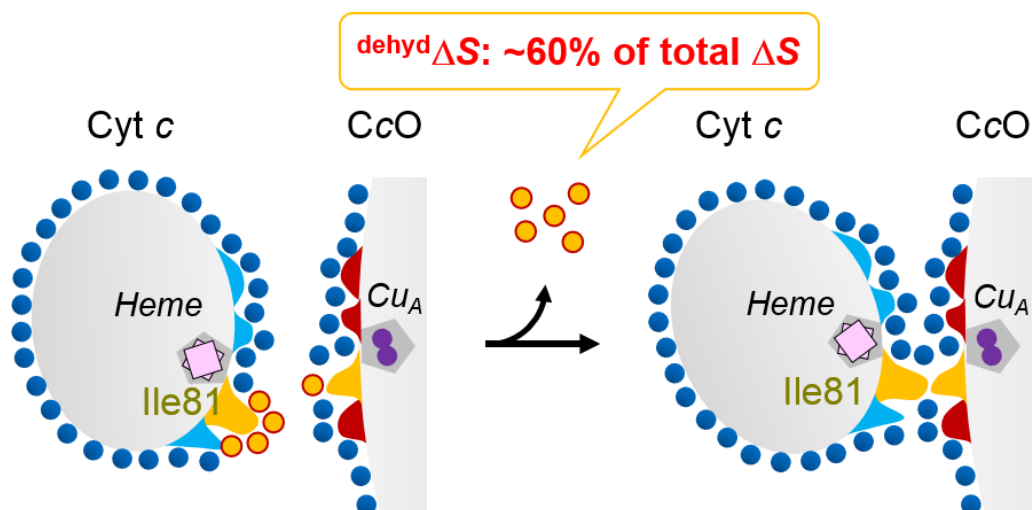


Figure 7. Proposed mechanism of complex formation between Cyt *c* and CcO by specific dehydration around heme periphery.

2.4. Discussion

Slight and Specific Dehydration upon Cyt *c*-CcO Complex Formation

In this chapter, I found that approximately 17 hydrated water molecules are expelled to the bulk solvent when Cyt *c* associates with CcO (Table 2). The primary dehydration sites on Cyt *c* were estimated to be Ile81, Ile11 and Lys13 (Table 3), all of which are located near the heme, and a sum of ASA of these residues corresponds to approximately 260 Å² (calculated by using “VADAR” [28]). Assuming that the number of the dehydrated water molecules depends on only the area of the interaction site, the estimated number of dehydrated water molecules in this study appeared to be quite small, because previous NMR experiments [6] estimated that the interaction area for CcO on Cyt *c* (Figure 4) is about 1800 Å², from which nearly 100 water molecules can be expelled. Furthermore, mutational analyses indicated that the number of dehydrated water molecules around Ile81 account for about half of the total number of dehydrated water molecules (Table 2). These data suggest that the interaction sites that induce dehydration are remarkably small and localized near the heme such as around Ile81. In other words, most of hydrating water molecules at the Cyt *c*-CcO interface except the dehydration sites would remain after the formation of the complex. In the Cyt *c*-CcO complex, most of hydrating water molecules at the interface remain, implying that the distances between the interacting residues are relatively long enough for the hydrated water molecules to be kept there. Thus, most of the interactions between Cyt *c* and CcO would be rather weak. In fact, the affinity for Cyt *c* to CcO (K_M) was determined to be approximately 1 μM (Table 1), reflecting a weak interaction between two proteins. These specific but not strong interactions between Cyt *c* and CcO mediated by hydrophobic residues would be suitable for the rapid dissociation and association of Cyt *c* to facilitate the donation of the electron to CcO.

The highly hydrated and small dehydrated interface have also been proposed in several other redox protein complexes such as the structure of Cytochrome *bc*₁ with Cyt *c* [34–39], and the affinity for the protein complexes represent approximately within 10 μM [40–44], which are

similar to that for the Cyt *c*-CcO complex. Thus, the specific but not strong interactions would not be typical for the Cyt *c*-CcO complex, but would be one of the general properties in interprotein complexes in biological systems.

Functional Significance of Dehydration from Ile81 of Cyt *c*

One of the notable finding in this chapter is that the primary dehydration site upon the formation of the Cyt *c*-CcO complex, such as Ile81, was localized near the heme periphery of Cyt *c*. Then, I discussed the contribution of the specific dehydration near the redox center to the ET reaction. As described above, replacement of Ile81 with alanine decreased the number of dehydrated water molecules (Table 3, 4). However, the total free energy change (ΔG) at 293 K for the association of CcO with I81A mutant Cyt *c* was estimated to be -33.6 ± 4.2 kJ mol⁻¹, comparable to that with WT (-34.1 ± 5.2 kJ mol⁻¹). This is because the decrease in the contribution of entropy change of -30.1 ± 3.0 kJ mol⁻¹ by the mutation was compensated by the favorable enthalpy change of -3.5 ± 3.0 kJ mol⁻¹, implying that functional significance of the dehydration from Ile81 is not to ensure the affinity for the Cyt *c*-CcO complex.

To explore the thermodynamic significance of the specific dehydration around the hydrophobic region near the heme periphery as the driving force for the Cyt *c*-CcO complexation, I focused on the interface of the Cyt *c*-CcO complex. As described above, Ile81 is located near the hydrophobic area that includes a hydrophobic thioether group (Figure 3), whereas the Cu_A site of CcO, which is the electron entry site for CcO, is also in the hydrophobic region constructed by several aromatic amino acid residues on the protein surface of CcO [45]. The combined information of the X-ray crystal structure of each protein and the osmotic pressure experiments suggest that the dehydration at the interface between the hydrophobic area around the exposed heme periphery of Cyt *c* and the hydrophobic region of CcO would occur upon the complexation. Such interprotein hydrophobic interactions around the redox centers are also encountered in other ET complexes. Like the Cyt *c*-CcO complex, many of other ET

complex formation were found to be entropy-driven [41–43,46,47], supporting the primary role of interprotein hydrophobic interactions in the complex formation. These information suggest that several hydrophobic interactions at Cyt *c* (Ile81 and Cys17) near the redox center would fix the relative positions of redox centers close enough to ensure the ET.

In summary, I found that the several hydrophobic residues such as Ile81 near the heme of Cyt *c* specifically form the hydrophobic interaction with CcO, and the dehydration upon the formation of the hydrophobic interaction is one of the primary factors that facilitate the Cyt *c*-CcO complexation by increasing the entropy. I, therefore, proposing that Cyt *c* associates with CcO for ET reaction by forming specific hydrophobic interactions near the heme periphery as the driving force for the Cyt *c*-CcO complexation.

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CHAPTER III

Contribution of Dehydration near the Heme in Cytochrome *c* to Electron Transfer Activity to Cytochrome *c* Oxidase

ABSTRACT

The Cyt *c*-CcO ET complex was found to be formed by increase in entropy from the dehydration at hydrophobic region near the heme periphery as shown in Chapter II. On the other hand, recent studies have proposed that the alteration of hydration environment around protein surface such as dehydration would affect the conformation and functions of proteins. In this chapter, I investigated the influence of dehydration near the heme periphery of Cyt *c* on its ET function by addition of PEG as the cosolute causing dehydration from the surfaces of proteins. In the presence of PEG, reduced Cyt *c* was rapidly oxidized even in the absence of CcO. The underlying the rapid oxidation was explored by examining the structural changes in Cyt *c* in the presence of PEG using various spectroscopic analyses and mutational experiments, suggesting that the dehydration around the heme periphery, including Ile81 and Val83, caused a modest tertiary structural change accompanied by a slight shift in the heme position (<1.0 Å). Such change of heme position would alter its accessible surface area to solvent, which induces the downshift of redox potential of heme and, in turn, contributes to unidirectional ET to CcO.

3.1. Introduction

In Chapter II, I found that the dehydration at several hydrophobic residues of Cyt *c* such as Ile81 was a key factor for thermodynamically promoting the formation of Cyt *c*-CcO complex. Recently, alteration of hydration environment around protein surface such as dehydration has been suggested to affect the conformation and functions of proteins, as well as the protein binding [1–3]. Especially, Petra and co-workers have proposed that cytochrome *c*₅₅₂ (Cyt *c*₅₅₂) negatively shifted its redox potential of approximately 90 mV upon the association with the Cu_A fragment from *ba*₃ oxidase, which was derived from the change in the solvent environment on the interface of Cyt *c*₅₅₂ accompanying the conformational changes [4]. In the case of Cyt *c* and CcO, however, the influence of the dehydration near the heme periphery of Cyt *c* on its electron transfer (ET) activity was almost unknown.

One of the useful approaches to forming an environment promoting the dehydration involves the use of synthetic cosolutes, polyethylene glycol (PEG). High concentrations of polyethylene glycol (PEG) are known to cause dehydration from the surfaces of proteins [5–7].

In this chapter, to gain the insight into the contribution of the specific dehydration near the heme periphery of Cyt *c* to ET activity to CcO, I examined the structure and ET function of Cyt *c* in the presence of PEG. Based on the results of various spectroscopic measurements, I discussed the functional significance of the dehydration from the hydrophobic region around the surface-exposed heme periphery in terms of the heme redox potential.

3.2. Experimental Procedures

Autoxidation Kinetics of Ferrous Cyt *c*

The ferrous Cyt *c* autoxidation kinetics were studied using a Hitachi U-3310 UV-visible spectrophotometer (Tokyo, Japan) at 293 K in a 50 mM sodium phosphate buffer (pH 7.0). Ferrous Cyt *c* was prepared by reducing ferric Cyt *c* with sodium dithionite, and excess reductant was removed using a PD MiniTrap G-25 column (GE Healthcare). The concentration of PEG 4000 was varied between 0 and 20%, and oxidation of Cyt *c* was monitored by measuring the absorbance at 550 nm after adding ferrous Cyt *c* to the reaction solution at a final concentration of 3 μ M. The absorption at 550 nm was recorded in intervals of 0.5 s over 5 min. The end point of Cyt *c* autoxidation was determined by adding a small amount of potassium ferricyanide to the reaction solution.

Absorption Spectroscopy

The absorption spectra were collected using a Hitachi U-3310 UV-visible spectrophotometer from solutions prepared at 293 K in 50 mM sodium phosphate buffer (pH 7.0). Spectra were recorded between 250 and 700 nm or 600 and 800 nm in intervals of 1 nm or 0.2 nm, respectively. The PEG 4000 concentration was varied between 0 and 20%, and the Cyt *c* concentration was 8 μ M (for the 250–700 nm measurement) or 0.3 mM (for the 600–800 nm measurement).

Circular Dichroism Spectroscopy

Circular dichroism (CD) spectra were recorded using a JASCO J-1500 CD spectrometer (Tokyo, Japan) with 10 mm path length cuvettes at room temperature in 50 mM sodium phosphate buffer (pH 7.0). Each spectrum represented the integration of three consecutive scans from 390 to 440 nm or from 190 to 250 nm at 0.2 nm intervals, with a scan speed of 20 nm/min. The spectrum bandwidth was fixed at 1.0 nm. The PEG 4000 concentration was varied between 0 and 20%, and the Cyt *c* concentration was 10 μ M for measurement of the Soret region or 2 μ M for measurement of the far-UV region. The ellipticity was expressed as the mean residue molar ellipticity ($\text{deg cm}^2 \text{dmol}^{-1}$), calculated using the JASCO software.

Resonance Raman Spectroscopy

Resonance Raman spectra were recorded using a single monochromator (SPEX500M, Jobin Yvon, Edison, NJ, USA) equipped with a liquid nitrogen-cooled CCD detector (Spec-10:400B/LN, Roper Scientific, Princeton, NJ). Samples were excited at a wavelength of 441.6 nm delivered by a helium-cadmium laser (IK5651R, Kimmon Koha, Tokyo, Japan). The laser power at the sample point was adjusted to 5 mW. Raman shifts were calibrated using indene and CCl_4 . The accuracy of the well-defined Raman band peak positions was $\pm 1 \text{ cm}^{-1}$. The PEG 4000 concentration was varied between 0 and 20%, and the Cyt *c* concentration was 50 μ M.

Fluorescence Spectroscopy

Fluorescence measurements were collected using a JASCO FP-8500 spectrofluorometer from solutions prepared at 293 K in 50 mM sodium phosphate buffer (pH 7.0). Unpolarized emission spectra were recorded between 300 and 450 nm using a 280 nm excitation wavelength and a scan speed of 200 nm/min. The excitation and emission slit widths were 5 and 10 nm, respectively. Each spectrum represents the integration of three consecutive scans. The measurements were carried out in the absence or presence of 6 μ M Cyt *c* with 0, 10, or 20% PEG, and the spectra of the PEG solution were subtracted from the spectra of the Cyt *c*-containing solution.

Near-infrared Spectroscopy

Near-infrared spectra were recorded using a JASCO V-570 spectrometer with a 1 mm path length cuvette, using solutions prepared at room temperature in 50 mM sodium phosphate buffer (pH 7.0). Each spectrum represents the integration of three consecutive scans from 1300 to 1600 nm in 0.2 nm intervals, with a scan speed of 200 nm/min. The spectral bandwidth was fixed at 2.0 nm. The measurements were carried out in the absence or presence of 50 μ M Cyt *c* and 0, 5, or 10% PEG, and the spectra of the PEG solutions were subtracted from the spectra of the Cyt *c*-containing solutions.

NMR Spectroscopy

^1H - ^{15}N HSQC NMR measurements were performed at 293 K using a Bruker AVANCE III HD 600 MHz spectrometer. All spectrum were processed by NMRpipe [8], and the data were analyzed using SPARKY. In the ^1H - ^{15}N HSQC experiments, each sample contained 0.15 mM oxidized Cyt *c* and 5% D_2O in a 50 mM sodium phosphate buffer (pH 7.0). ^{15}N -labeled Cyt *c* was measured in the presence of 20% PEG. ^1H - ^{15}N HSQC spectra were corrected using the spectral widths 16 ppm and 38 ppm for the ^1H and ^{15}N dimensions, respectively, and 2048 and 300 complex points were set for the ^1H and ^{15}N dimensions, respectively [9].

After identifying the amide signals based on sequential assignments and peak fits using SPARKY, the PEG-induced changes in the backbone amide proton and nitrogen chemical shifts in Cyt *c* were determined. ^1H and ^{15}N chemical shifts were combined using the following equation [10] to calculate the composite chemical shifts.

$$\Delta\delta_{\text{NH}} = \{\Delta\delta_{\text{H}}^2 + (\Delta\delta_{\text{N}}/6.5)^2\}^{1/2} \quad (1)$$

All NMR samples used for the ^1H measurements contained 0.15 mM oxidized Cyt *c*, 10 or 20% PEG and 5% D_2O in 50 mM sodium phosphate buffer (pH 7.0). Paramagnetic ^1H NMR measurements were performed using an Agilent Unity INOVA 600 spectrometer. Chemical shifts were referenced to the signals obtained from 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS). A series of ^1H NMR experiments was performed using a presaturation pulse program with a spectral width of 80 ppm. Significant signals from PEG were suppressed by adding a WURST-2 pulse 2 ppm wide and centered at 4.23 ppm.

Potentiometric Titration

Potentiometric titrations were performed in an anaerobic cuvette at 293 K with continuous flow of humidified nitrogen gas. A three-electrode configuration consisted of a platinum wire meshes as a working electrode, a platinum wire meshes as a counter electrode, and saturated Ag/AgCl electrode as a reference electrode. The potentials of these electrodes were controlled by a Hokuto Denko HSV-110 potentiostat/galvanostat (Tokyo, Japan). Samples (0.4 mL) containing 40 μ M Cyt *c* in 50 mM sodium phosphate buffer (pH 7.0) were used. Electrode-solution mediation was facilitated by the following mediators: 15 μ M *N,N*-dimethyl-*p*-phenylendiamine ($E_m = +340$ mV), 20 μ M *N,N,N',N'*-tetramethyl-*p*-phenylendiamine ($E_m = +260$ mV), 15 μ M 2,6-dichlorophenolindophenol ($E_m = +217$ mV), 50 μ M 1,2-Napthoquinone ($E_m = +145$ mV), phenazine methosulfate ($E_m = +80$ mV), 15 μ M gallocyanine ($E_m = +20$ mV), 15 μ M indigo trisulfonate ($E_m = -80$ mV), 15 μ M 2-hydroxy-1,4-napthoquinone ($E_m = -120$ mV), and 10 μ M anthraquinone-2-sulfonic acid ($E_m = -230$ mV). The absorption spectra were recorded from 350 to 700 nm with a Hitachi U-3310 UV-visible spectrophotometer equipped with a Peltier-type temperature control system. The potential of electrode was decrease in 50 mV steps until the protein was fully reduced and increased stepwise until reoxidation was complete. Heme reduction was monitored by the increase in the α -band absorption at 550 nm, where the absorption of the mediators was negligible compared to that of the protein. Potential values were described relative to the normal hydrogen electrode (NHE) by adding 197 mV to the imposed potentials. The titration data were analyzed by fitting with the Nernst equation for a single-electron process.

3.3. Results

Electron Transfer from Cyt *c* in the Presence of PEG

To examine the ET activity of Cyt *c* under the environment with low dielectric constant in the aqueous solvent, I tried to obtain the ET rate to CcO in a solution containing PEG 4000 by measuring the apparent oxidation rate of Cyt *c* in the presence of PEG. However, the absorbance at 550 nm was decreased immediately after adding PEG, even in the absence of CcO (Figure 1). This observation was somewhat surprising because Cyt *c* is stable to reduction due to its high redox potential of +235 mV [11]. Under anaerobic conditions, Cyt *c* was not oxidized in the absence and presence of PEG, indicating that the heme of Cyt *c* was oxidized by molecular oxygen (O₂) only in the presence of PEG. To estimate the apparent oxidation rate of reduced Cyt *c* by O₂, k_{app} , the absorbance change at 550 nm measured at 1 s intervals over 300 s was fit to a following equation:

$$Abs_{550}(t) = A \exp(-k_{app}t) + B \quad (2)$$

where $Abs_{550}(t)$ is the absorbance at 550 nm as a function of time, A is a constant related to the initial absorbance, and B is the final absorbance, respectively. The k_{app} in the presence of 10% PEG was estimated to be $0.0031 \pm 0.0001 \text{ s}^{-1}$ using equation 2, indicating that Cyt *c* was oxidized completely within approximately 20 min. By contrast, the absorbance at 550 nm in the absence of PEG remained constant over 1 week in dark. These results suggest that PEG significantly promoted electron flow from Cyt *c* to molecular oxygen, leading to oxidation of the heme.

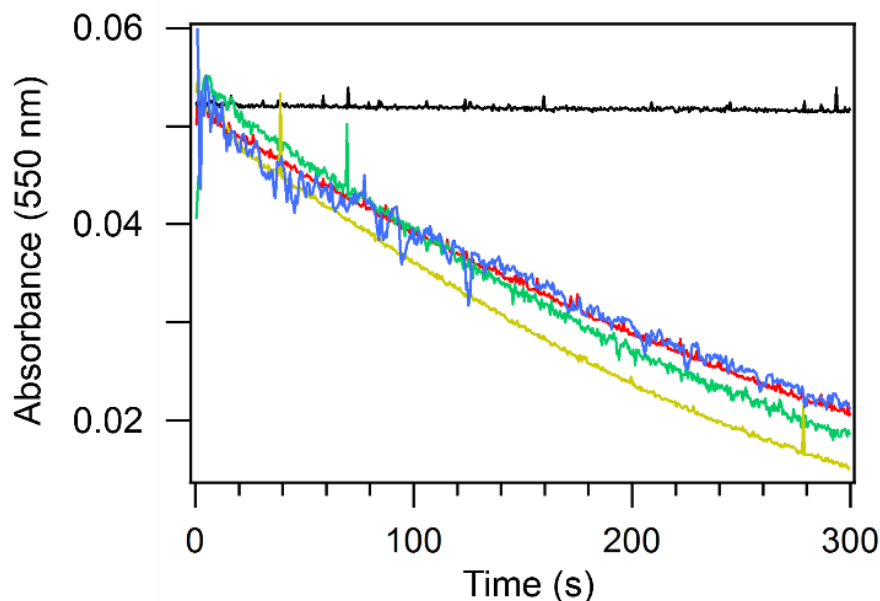


Figure 1. Absorbance changes at 550 nm after addition of PEG 4000.

The measurements were performed by mixing of 3 μ M ferrous Cyt *c* and 0 (black), 5 (red), 10 (yellow), 15 (green), and 20 (blue)% solutions of PEG, dissolved in 50 mM sodium phosphate buffer (pH 7.0) at 293 K.

Structural Properties of Cyt *c* in the Presence of PEG

One of the driving forces underlying the rapid oxidation of Cyt *c* in the presence of PEG was PEG-induced partially denaturation of Cyt *c*. Figure 2A plots the absorption spectra of ferric Cyt *c* in the region between 250 and 700 nm. In the presence of 5–20% PEG, almost no shift in the Soret peak at 409 nm was observed, suggesting that a dramatic conformational change around the heme center was not induced. I also examined the dissociation of Met80 from the heme iron by the absorbance at 695 nm, which was derived from the S(Met80) $\rightarrow$ Fe charge-transfer transition. As shown in Figure 2B, the peak at 695 nm was not significantly affected by the addition of PEG up to 20%, indicating that Met80 did not dissociate from the heme. These results clearly indicate that PEG does not partially denature the heme periphery of Cyt *c*.

To reveal the structural factor for the autoxidation of heme, the structure of Cyt *c* in the presence of PEG was further characterized by using a variety of spectroscopic techniques. As shown in Figure 2C, the CD spectra in the far-UV region were insensitive to the PEG concentration, indicating that the secondary structure of Cyt *c* was not remarkably altered by the addition of PEG. In the absence of PEG, I observed a slightly asymmetric, negatively biased couplet with a minimum at 417 nm and a maximum at 406 nm reflecting the conformation near the heme periphery in the Soret region of the CD spectrum (Figure 2D), in agreement with results obtained from horse heart Cyt *c* [12]. These signals did not change significantly by the addition of PEG, consistent with the results observed in the absorption spectra (Figure 2A). I moreover measured the resonance Raman spectra. In the low-frequency region, some heme peripheral modes such as methyl, propionates, and thioether bonds, are observed [13]. Because some amino acid residues in Cyt *c* interact with the heme peripheral groups, we examined whether the PEG induced a structural change that alters such interactions. Figure 2E shows the resonance Raman spectra of ferric Cyt *c* in the absence and presence of PEG. The sharp bands at 396 and 691 cm^{-1} correspond to the vibrations of the thioether linkages [$\delta(\text{C}_\beta\text{C}_\alpha\text{S})$] and C-S stretching $\nu(\text{C}_\alpha\text{S})$, respectively [13]. The intensity and frequency of these bands in a 20% PEG solution were very similar to those observed in the absence of PEG (Figure 2E). The spectroscopic data shown in Figures 2C, 2D, and 2E suggest that the geometry around the heme did not undergo a dramatic change upon the addition of PEG.

Figure 2

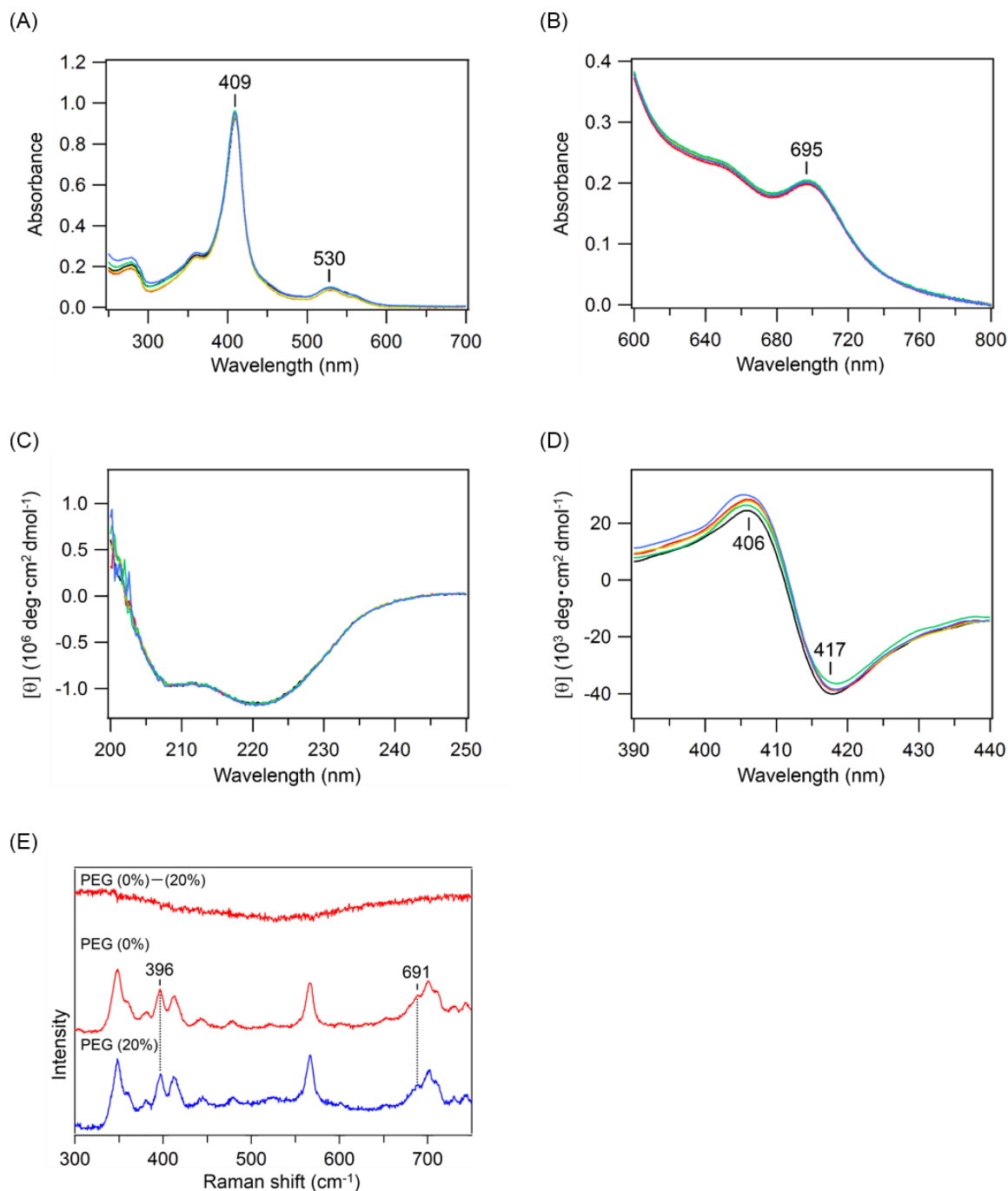


Figure 2. UV-visible absorption, CD, and resonance Raman spectra of Cyt c in the presence of PEG.

Absorption spectra in the region between 250 and 700 nm of 8 μM oxidized Cyt c (A) and between 600 and 800 nm of 0.3 mM oxidized Cyt c (B). CD spectra in the far-UV region of 2 μM Cyt c (C) and in the Soret region of 10 μM Cyt c (D). These measurements were carried out in the presence of 0% (black), 5% (red), 10% (yellow), 15% (green), and 20% (blue) PEG. (E) Resonance Raman spectra in the low frequency region of 50 μM Cyt c. These measurements were carried out in the presence of 0% (red) and 20% (blue) PEG. For all measurements, sample were dissolved in 50 mM sodium phosphate buffer (pH 7.0) at 293 K.

To further investigate the factors that induce the autoxidation of heme, I focused on the tertiary structure of Cyt *c*. I examined the tertiary structural changes for the rapid autoxidation of heme in the presence of PEG by estimating the distance between the heme and any point in Cyt *c* using fluorescence resonance energy transfer (FRET) measurements. The FRET efficiency (E) is determined by the fluorescence intensity transferred from a donor molecule to an acceptor molecule, and can be described as:

$$E = \frac{1}{1 + (r / R_0)^6} \quad (3)$$

where R_0 and r are the fluorophore pair Förster distance and donor-to-acceptor separation distance, respectively. Assuming that the orientations of the dipole moments of the donor and heme are not altered during conformational changes, the fluorescence intensity from the donor would depend only on the distance between the donor and the heme (Equation 3).

Although Cyt *c* includes one tryptophan at position 59, fluorescence from Trp59 is almost quenched by heme because it is located about 8 Å from the heme. Considering that the Förster distance between tryptophan and the heme is 29 Å [14], the FRET efficiency from Trp59 to heme is estimated to be nearly 100% (Equation 3). To improve the sensitivity of the distance between the heme and the tryptophan estimated by FRET, I introduced tryptophan at a position close to the R_0 value. I mutated Asp2 to Trp (D2W) because this residue is located about 20 Å from the heme. Prior to measuring the fluorescence spectra, the ^1H - ^{15}N HSQC spectrum of the D2W mutant was measured to confirm whether the heme structural environment in the D2W mutant remained intact. Except around the mutated position, the HSQC spectrum of the D2W mutant was essentially superimposable with that of the WT including heme periphery, indicating that this mutant possessed the similar tertiary structure and the same heme environmental structure as WT Cyt *c*.

The fluorescence spectra of the D2W mutant in the presence of PEG are shown in Figure 3. The fluorescence signal at 350 nm was mainly derived from Trp2, because fluorescence from

Trp59 was quenched by heme due to the Trp59-heme close distance. As shown in Figure 3, the fluorescence intensity at 350 nm significantly decreased from 1080 to 890, by 23%, as the PEG concentration increased to 20%. Based on equation 3, a decrease in the fluorescence intensity was interpreted as a shortening of the distance between Trp2 and heme by approximately 1.0 Å. Since the fluorescence maximum of the D2W mutant was not significantly shifted, I assumed that the environment around Trp2 was not affected by the addition of PEG. Thus, these fluorescence spectra suggest that PEG induces tertiary conformational changes including the change of relative position of heme to Trp2 in Cyt *c*.

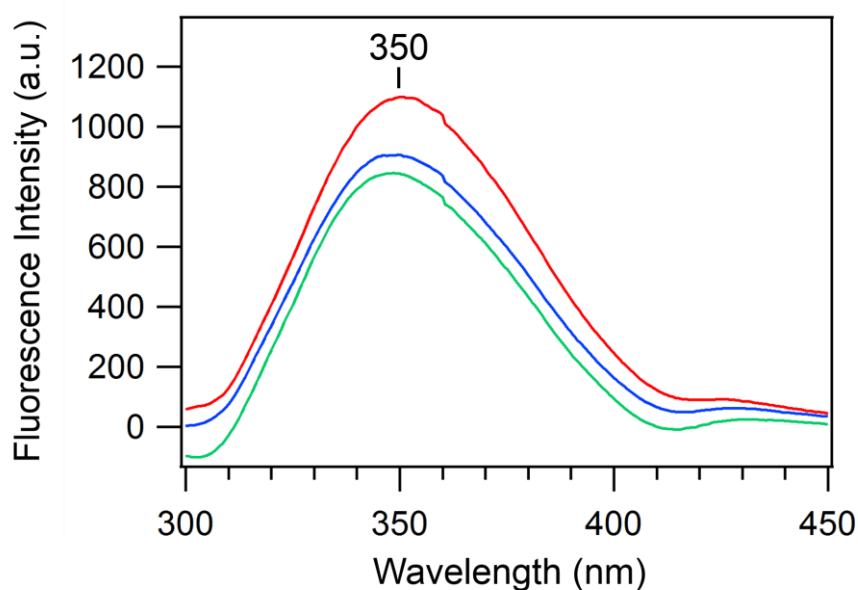


Figure 3. Fluorescence spectra for the D2W mutant Cyt *c*.

The measurements were carried out in the presence of 6 μ M Cyt *c* with 0 (red), 10 (blue), and 20 (green)% solutions of PEG, dissolved in 50 mM sodium phosphate buffer (pH 7.0) at 293 K.

Dehydration Induced by PEG

As described above, high concentrations of PEG induce reduction in the dielectric constant in an aqueous solvent, causing dehydration from the surfaces of proteins [5–7,15]. Such dehydration has been proposed to induce conformational changes in proteins [2,5]. In fact, a recent study of the structures of several polypeptides in a cellular environment suggested that a decrease in the dielectric constant alters the polypeptide conformation and exposes its hydrophobic components to solvent [3]. Because heme is a strongly hydrophobic molecule, it prefers more hydrophobic environment. Based on the results from these studies, it is plausible that PEG induces conformational changes accompanied by the change of heme position in Cyt *c* and perturbs the hydration environment around the protein.

Then, I examined PEG-induced dehydration from Cyt *c* using near-infrared (NIR) spectroscopy, which can be monitored hydrated water molecules around a protein. Figure 4A shows the NIR spectra of 50 μM Cyt *c* in a 50 mM sodium phosphate buffer. The broad band at 1450 nm corresponds to a combination of symmetric and asymmetric stretching vibrations in the water molecules [16,17]. The structural properties of the water molecules that hydrated Cyt *c* were investigated by subtracting the NIR spectrum of the buffer from that of Cyt *c* (Figure 4B). The difference spectrum revealed a positive peak at 1412 nm and a negative peak at 1490 nm, which were assigned to vibrations from the weakly and strongly hydrogen-bonded water, respectively [16,17]. The positive peak at 1412 and negative peak at 1490 nm in the difference spectrum between 10 μM Cyt *c* and the buffer were smaller than those in the difference spectrum between 50 μM Cyt *c* and the buffer. In other words, these absorption peaks depend on the Cyt *c* concentration. Thus, the peaks at 1412 and 1490 nm were postulated to be derived from water molecules that hydrated Cyt *c*. The difference spectra shown in Figure 4B reflect a structural change in the water molecules surrounding Cyt *c*: an increase in the number of the weakly hydrogen-bonded water molecules and a decrease in the number of the strongly hydrogen-bonded water molecules, upon the addition of Cyt *c*.

To examine the effect of PEG on the hydration state of Cyt *c*, I measured the NIR spectra of 50 μ M Cyt *c* in the presence of 5% PEG. Figure 4C shows the difference spectra obtained by subtracting the spectrum of the buffer from that of Cyt *c* in the PEG solution. As depicted in Figure 4C, the positive peak at 1412 nm decreased significantly in the presence of 5 or 10% PEG, supporting the hypothesis that most of the weakly hydrogen-bonded water molecules around Cyt *c* were dehydrated from the surface by addition of PEG.

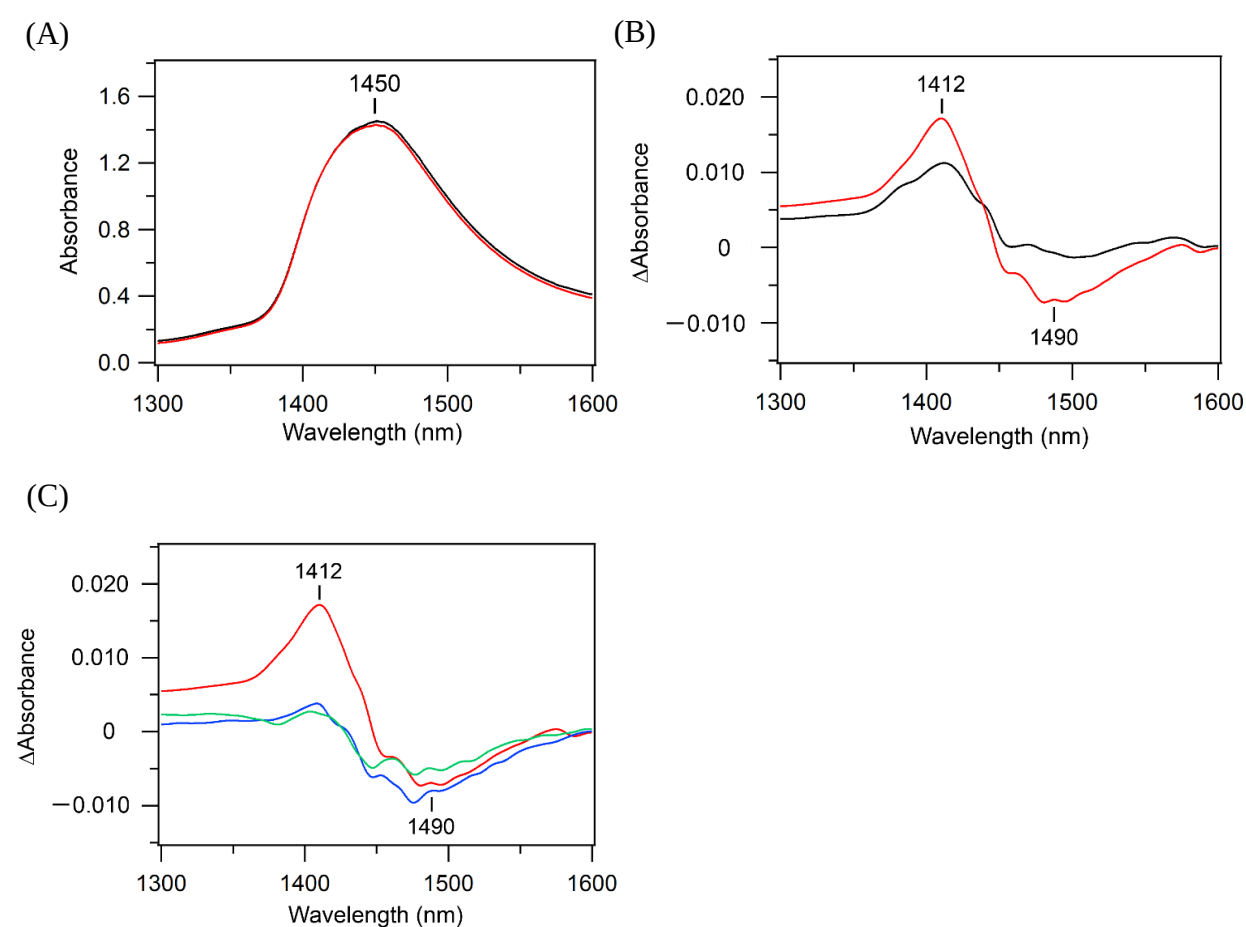


Figure 4. NIR spectra of Cyt *c* in the region between 1300 and 1600 nm.

(A) NIR spectra in the absence (black) and presence of 50 μ M Cyt *c* (red) dissolved in 50 mM sodium phosphate buffer (pH 7.0) at 293 K. (B) Difference NIR spectra subtracting the spectrum of buffer from that of 50 μ M Cyt *c* (red) and that of 10 μ M Cyt *c* (black). (C) Difference NIR spectra subtracting the spectra of buffer including 0 (red), 5 (blue), and 10% (green) PEG from that of 50 μ M Cyt *c* in 0 (red), 5 (blue), and 10% (green) PEG solution.

Dehydration Sites to Induce Positional Change of Heme

To get insights into the relationship between the dehydration at the surface of Cyt *c* from the NIR spectra (Figure 4C) and the change in the heme position in the presence of PEG (Figure 3), the dehydration sites on Cyt *c* were investigated. Most residues on the surface were hydrophilic, including lysine and arginine, and only a few hydrophobic residues such as Ile81 and Val83 were present near the heme (Figure 5). Especially, Ile81 significantly contributed to the stabilization of the Cyt *c*-CcO complex by forming hydrophobic interactions upon dehydration as discussed in Chapter II. I examine functional significance of this hydrophobic residue near the heme by site-directed mutagenesis experiments. Ile81 was replaced with serine to alter the environment of hydration around position 81.

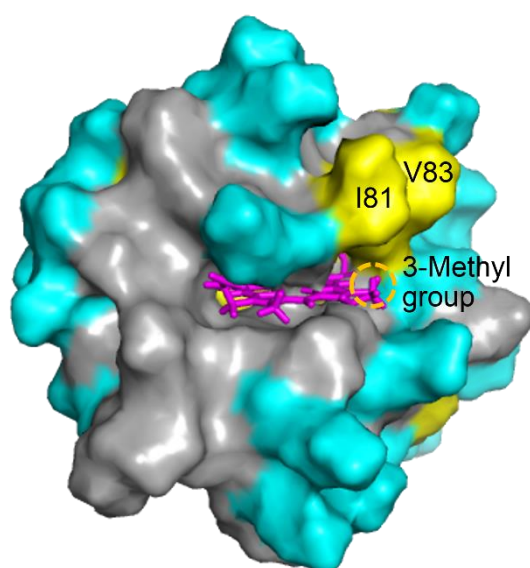


Figure 5. Surface residues on Cyt *c*.

Hydrophilic residues (Arg, Lys, Asp, Gln, Asn, and Glu) and hydrophobic residues (Ile, Phe, Val, and Leu) are colored in cyan and yellow, respectively. The dashed orange circle indicates the 3-methyl group of heme.

I measured the NIR spectra of the I81S mutant in the absence of PEG. The difference spectrum, obtained by subtracting the spectrum of the buffer from that of the I81S mutant, did not show a positive peak at 1412 nm (Figure 6A), unlike the WT Cyt *c* in the absence of PEG (Figure 4B). The decrease in the positive peak at 1412 nm in the difference spectrum of the I81S mutant indicates that the replacement of hydrophobic isoleucine with hydrophilic serine reduced the number of weakly hydrogen-bonded water molecules around the mutation site. Thus, the weakly hydrogen-bonded water molecules would exist around the hydrophobic region near the heme periphery, such as Ile81. Considering that the NIR difference spectra of WT Cyt *c* showed a decrease in the 1412 nm peak in the presence of PEG (Figure 4C), the hydrophobic region near the heme periphery, Ile81, appeared to be a primary dehydration sites.

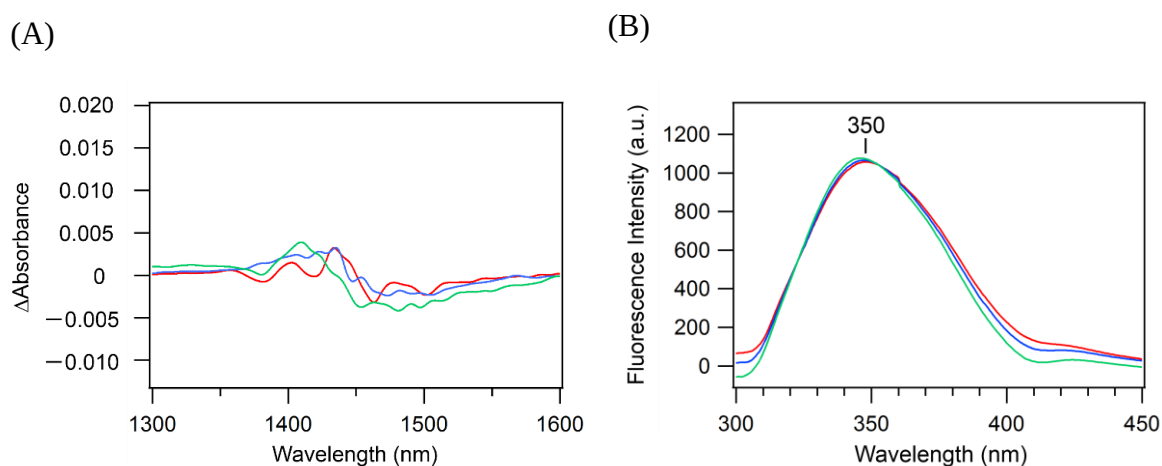


Figure 6. Difference NIR spectra and fluorescence spectra of mutants Cyt *c* in the presence of PEG.

(A) Difference NIR spectra subtracting the spectra of buffer including 0 (red), 5 (blue), and 10% (green) PEG from that of 50 μ M I81S mutant Cyt *c* in 0 (red), 5 (blue), and 10% (green) PEG solution. The experimental conditions were the same as Figure 4C. (B) Fluorescence spectra for D2W/V83S mutant Cyt *c* in the presence of 0 (red), 10 (blue), and 20% (green) PEG. The experimental conditions were the same as Figure 3.

Then, I examined the correlation between the dehydration around the heme and a change of the heme position by replacing Asp2 of the I81S mutant with Trp and measuring the FRET efficiency in the presence of PEG. The I81S/D2W mutant was not well expressed in *E. coli*. Considering that Val83 was also located near the heme (Figure 5), I constructed the V83S/D2W double mutant. Figure 6B shows the fluorescence spectra of the V83S/D2W mutant in the presence of PEG. The fluorescence maximum at 350 nm, derived from Trp2, did not change significantly by the addition of PEG up to 20%, in contrast with the D2W mutant (Figure 3), indicating that the mutation of Val83 to serine suppressed the positional change of heme, suggesting that, in WT Cyt *c*, the positional change of heme in the presence of PEG was induced by dehydration around hydrophobic residues located near the heme periphery.

Positional Change of Heme Triggering the Autoxidation

The contributions of the positional change of heme caused by the dehydration from the hydrophobic region near the heme including Ile81 and Val83 to the rapid autoxidation of heme were assessed by the oxidation of the I81S and V83S mutants, in the 5% PEG solution. In Figure 7, the absorbance changes at 550 nm for these mutants were plotted and compared to that obtained from WT Cyt *c* after the addition of PEG. The observed oxidation rates of these mutants significantly decreased compared to that of WT Cyt *c* (Figure 7). Based on the NIR spectra of the I81S mutant (Figure 6A) and FRET measurements of the V83S/D2W mutant (Figure 6B), I propose that the reduced oxidation rates of these mutants resulted from the suppressed heme displacement upon introduction of a hydrophilic serine moiety at the protein surface.

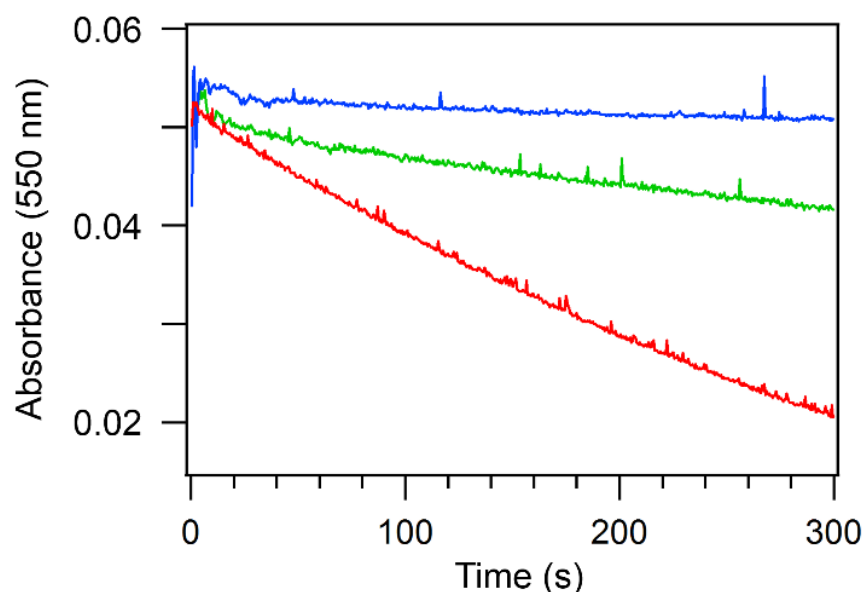


Figure 7. Absorbance change at 550 nm of mutants Cyt *c* in the presence of PEG.

Absorbance changes at 550 nm of WT (red), I81S (blue), and V83S (green) Cyt *c* after addition of 5% PEG. The experimental conditions were the same as Figure 1.

Conformational Changes in Cyt *c* Associated with the Positional Change of Heme

To evaluate the conformational changes in Cyt *c* induced by PEG, I measured the two-dimensional ^1H - ^{15}N HSQC spectra of ^{15}N -labeled Cyt *c* in the absence or presence of 20% PEG. Figure 8A shows ^1H - ^{15}N HSQC spectra of the oxidized Cyt *c* in the presence and absence of PEG. Some peaks displayed chemical shift perturbations upon the addition of PEG, reflecting environmental changes in Cyt *c*. The chemical shift perturbations of the backbone amides upon the addition of PEG were illustrated in Figure 8B. The N-H cross-peaks from Met12, Gln16, Cys17, His18, Arg38, Gly56, Glu61, Gly77, Phe82, Val83, and Lys86 were substantially shifted. These chemical shift perturbations indicate that structural changes in local tertiary conformations alter the magnetic shielding and/or interactions between the residues by the addition of PEG [10]. In fact, several reports have reported that longer chain PEG (e.g. PEG

5,000) can nonspecifically interact with hydrophobic proteins due to the presence of a hydrophobic methylene group in PEG [18–20]. In my study, however, the residues perturbed by PEG included several surface-exposed hydrophilic residues (Gln16, Glu61, and Lys86) as well as hydrophobic residues, suggesting that PEG did not specifically perturb hydrophobic residues. Moreover, the residues exhibiting the chemical shift perturbations upon the addition of PEG were relatively gathered in the heme periphery region (Figure 8C). The residues that exhibited a decrease in the peak intensity also included hydrophilic residues. Thus, these chemical shift changes appeared to be attributed to local perturbations in the surface-exposed residues rather than from nonspecific direct contact with PEG.

As described above, the distributions of chemical shift perturbations induced by PEG were mapped onto the structure of Cyt *c*, as shown in Figure 8C. Most of the perturbed residues were located near the exposed heme edge, in agreement with results reported from *Saccharomyces cerevisiae* Cyt *c* [21]. Considering that PEG induced the positional change of heme position as revealed in FRET measurement (Figure 3), the chemical shift perturbations near the heme of Cyt *c* observed upon the addition of 20% PEG (Figure 8B and 8C) can be attributed to tertiary structural changes that accompanied the heme displacement. These ^1H - ^{15}N HSQC spectra also support the proposal that the change of heme position was induced by addition of PEG, which facilitates dehydration from the surface of Cyt *c*.

The ^1H - ^{15}N HSQC spectra of the I81S mutant were also measured in the absence and presence of PEG. The residues that displayed chemical shift perturbations upon the addition of PEG are displayed in Figures 8D and 8E. The PEG-induced chemical shift perturbations observed in the I81S mutant were similar to those observed in WT Cyt *c* (Figure 8B or 8C). These results of the HSQC spectra in the I81S mutant implied that the perturbation of heme also occurred in the I81S mutant. In this mutant, however, the chemical shift changes observed in Gly77, Val83, and Lys86, located near heme, substantially decreased compared to those in WT Cyt *c*. The spectroscopic data obtained from the I81S mutant suggest that the PEG-induced

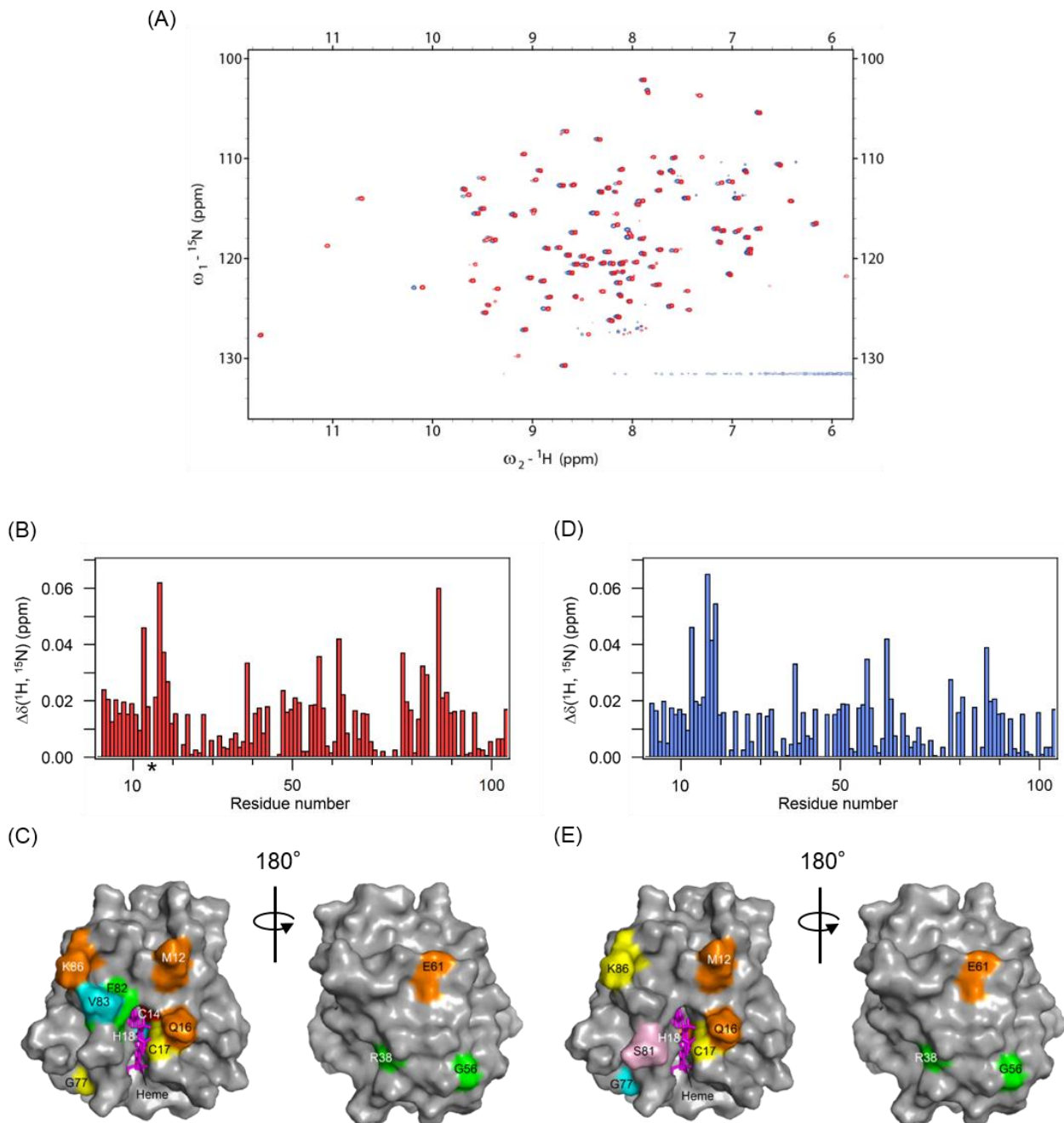


Figure 8. Chemical shift perturbation in Cyt *c* in the presence of PEG.

(A) Superposition of the ^1H - ^{15}N HSQC spectra of ^{15}N -labeled Cyt *c* in the absence (red) and presence of PEG (blue). Overview of the combined backbone chemical shift changes (bar graph) and line broadening (asterisk) of WT (B) and I81S mutant (D) Cyt *c* induced by addition of 20% PEG. Mapping of the affected residues on the surface of WT (C) and I81S mutant (E) Cyt *c*. The residues with chemical shift changes of $\Delta\delta_{\text{NH}} \geq 0.042$, $0.042 > \Delta\delta_{\text{NH}} \geq 0.037$, $0.037 > \Delta\delta_{\text{NH}} \geq 0.032$, and $0.032 > \Delta\delta_{\text{NH}} \geq 0.027$ are colored in orange, yellow, green, and cyan, respectively. In addition, red color represents the residues showing the specific line broadening. The measurements were carried out in the presence of 0.15 mM WT (B) or I81S (D) Cyt *c* with 0 and 20% solutions of PEG, dissolved in 50 mM sodium phosphate buffer (pH 7.0) at 293 K.

changes in the local protein conformation around the mutation site were specifically suppressed by the introduction of serine, which is consistent with the result of the FRET measurements of the V83S/D2W mutant (Figure 6B).

To investigate the PEG-induced environmental changes around the heme in Cyt *c*, I further measured paramagnetic ^1H NMR spectra of Cyt *c* in the presence of PEG. As shown in Figure 9, the signals from the porphyrin ring (3-methyl, 8-methyl, and 7-propionate) and axial ligands (His18 and Met80) [22] shifted as the PEG concentration increased. The signal from the 3-methyl group on the heme, in particular, displayed a subtle but more prominent shift by approximately 0.16 ppm than was observed from 8-methyl and 7-propionate by 0.06 and 0.08 ppm, respectively. Considering that the surface-exposed hydrophobic residues (Ile81 and Val83) were located near 3-methyl (Figure 5) group and the signal from the 3-methyl group was slightly shifted, also supporting the suggestion that the addition of PEG induced subtle tertiary conformational changes around Ile81 and Val83 near the 3-methyl group of heme.

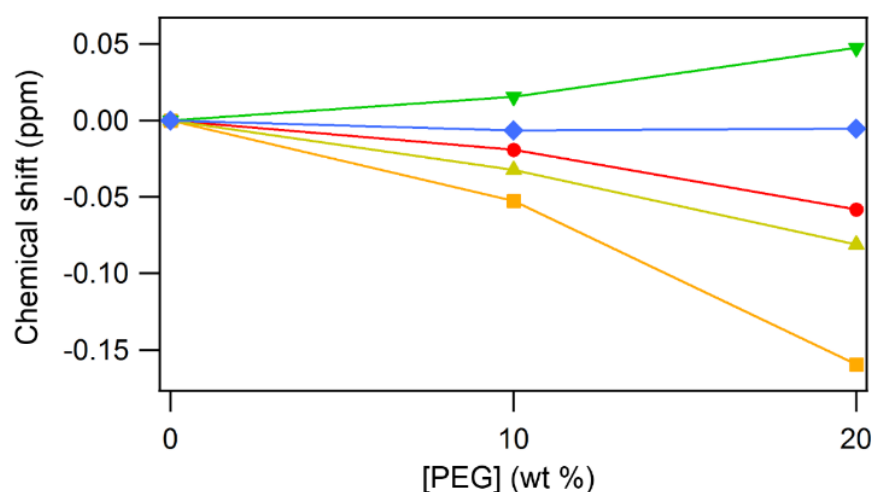


Figure 9. ^1H -NMR spectra of oxidized Cyt *c* in the absence and presence of 10 and 20% PEG at 293 K. The titration curves for Cyt *c* represent the chemical shifts from 8-methyl (red), 3-methyl (orange), 7-propionate group of heme (yellow), β -proton in His18 (green), and in Met80, respectively. For all measurements, sample were dissolved in 50 mM sodium phosphate buffer (pH 7.0).

Influence of Positional Change of Heme on Its Redox Potential

To assess the effect of the tertiary structural change of Cyt *c* by addition of PEG on the rapid oxidation, we conducted the spectroelectrochemical titration of Cyt *c* in the absence and presence of PEG. The plots of the relative ratio of the reduced heme against the measured potential in the absence or presence of PEG are shown in Figure 10A and 10B, respectively. For the reductive titration, the fit of the data obtained at 550 nm to the Nernst equation yielded midpoint reduction potential, E_m . As shown in Figure 10A and 10B, the reductive and oxidative titration curves are superimposed, which indicates that the redox process is reversible. In the absence of PEG, the E_m potential of Cyt *c* was estimated to be $+236 \pm 3$ mV vs NHE (Figure 10A), in agreement with results obtained from horse heart Cyt *c* of +235 mV [11]. On the other hand, the E_m potential of Cyt *c* in the presence of 20% PEG was found to be $+200 \pm 4$ mV vs NHE (Figure 10B). This corresponded to a downshift of approximately 40 mV of the Cyt *c* due to the addition of PEG.

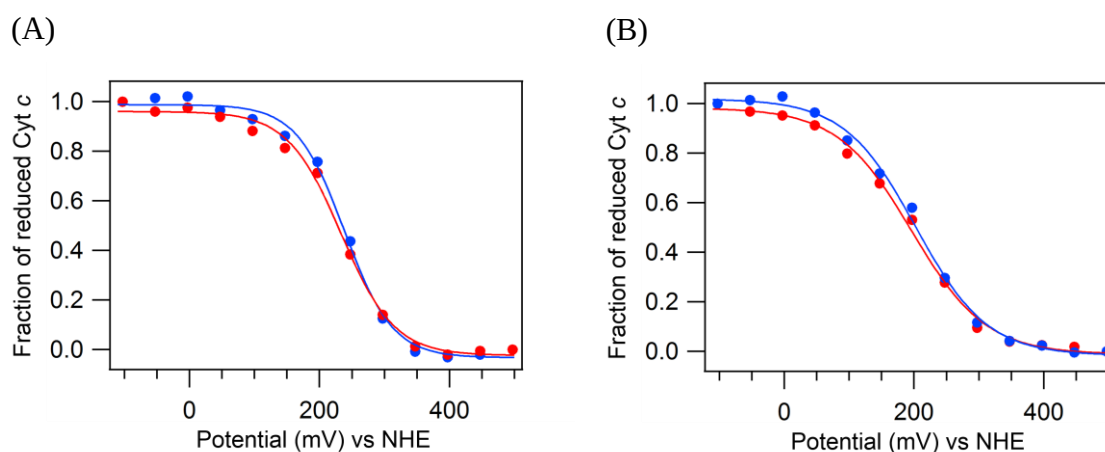


Figure 10. Spectroelectrochemical titration of Cyt *c* in the (A) absence and (B) presence of 20% PEG. The potential of electrode was decrease in 50 mV step from +500 to -100 mV vs NHE (red) and then increase from -100 to +500 mV vs NHE (blue). The solid lines represent the curves obtained by fitting of the data to the Nernst equation. The measurements were carried out in the presence of $40 \mu\text{M}$ Cyt *c* with 0 and 20% solutions of PEG, dissolved in 50 mM sodium phosphate buffer (pH 7.0) at 293 K.

3.4. Discussion

Contribution of Dehydration to ET to CcO

Based on various spectroscopic analyses and mutational experiments, I found that the dehydration around the hydrophobic residues located near the heme periphery of Cyt *c*, including Ile81 and Val83, caused a subtle tertiary structural change accompanied by a slight shift in the heme position of 1.0 Å, and such change of heme position resulted in the rapid autoxidation of Cyt *c* (ET to molecular oxygen) (Figure 11A). I deduced the contribution of the heme displacement induced by dehydration from Ile81 and Val83 to the increase in the oxidation rate of Cyt *c*. As described above, the oxidation/reduction rates of Cyt *c* are dominated by its redox potential [23]. Gray and co-workers analyzed the redox potentials of eight structurally characterized *c*-type cytochromes and proposed that the redox potentials of Cyt *c* could be tuned by roughly 500 mV by varying the heme exposure to solvent due to the increase of the polarity of the heme environment [24]. Fantuzzi et al. investigated the relationship between the reduction potential and the accessible surface area (ASA) of the Cyt *c*₅₅₃ heme [25]. They mutated Met23 or Gly51, which are located near heme and exposed to solvent, to Cys residues and constructed M23C-M23C or G51C-G51C homodimers through specific disulfide bridge formation to decrease the ASA of heme. Potentiometric titration of the Cyt *c*₅₅₃ mutants showed that the reduction potential shifted toward positive values, from 20 to 105 mV, as the ASA decreased from 73 to 50 Å². These results indicate that the less solvent-exposed heme is more stable in the reduced state. In the case of human Cyt *c*, the redox titration showed the decrease in the E_m by approximately 40 mV by the addition of PEG (Figure 10). Considering the slight shift in the heme position by approximately 1.0 Å (Figure 3), PEG would induce a subtle change of heme position to a solvent-exposed region, thereby moderately increasing the ASA of heme and decreasing the reduction potential of the Cyt *c*, although the detailed correlation between the redox potential and ASA remains to be elucidated. The observed negative shift in the redox potential of heme was expected to accelerate its oxidation.

As described above, change of heme position of Cyt *c* in the presence of PEG was thought to be induced by dehydration of the surface-exposed hydrophobic residues, Ile81 and Val83. It is notable that the hydrated water molecules around Ile81 were expelled when Cyt *c* forms a complex with CcO through hydrophobic interactions, as revealed in Chapter II. The tertiary structural changes that accompanied the heme displacement upon the addition of PEG were estimated based on the ^1H - ^{15}N HSQC spectra (Figures 8B and 8C). Chemical shift changes or line broadening upon the addition of PEG were observed on Met12, Gln16, Cys17, His18, Lys79, Met80, Ile81, Phe82, Val83, and Lys86. A previous NMR spectroscopy study revealed the interaction sites between Cyt *c* and CcO [9]. The chemical shift changes or line broadening observed upon the addition of CcO were observed at the residues located near the heme, Met12, Cys17, His18, Lys79, Met80, Ile81, Val83, and Lys86, as well as several positively or negatively charged residues. These results provide the possibility that the tertiary conformational changes at the heme periphery in the presence of PEG are similar to those involved in Cyt *c*-CcO complex formation. These tertiary conformational changes would support the suggestion for the heme displacement of Cyt *c* during the Cyt *c*-CcO complexation. Such PEG-induced dehydration from the surface of Cyt *c* would partially mimicked dehydration at the interaction sites near the heme periphery of Cyt *c* with CcO (Figure 11B). Thus, when Cyt *c* associated with CcO, heme was more exposed to solvent upon dehydration of Ile81 and Val83, leading to a negative shift in the heme redox potential.

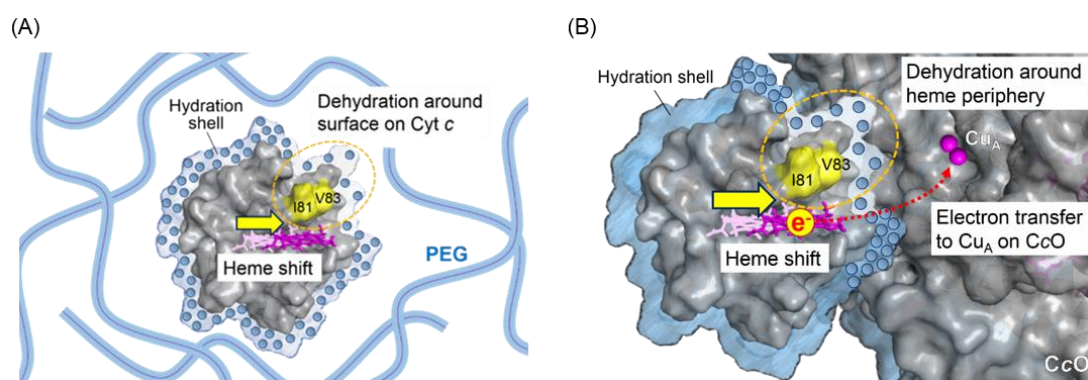


Figure 11. Proposed regulation mechanism of electron donation on Cyt *c* by specific dehydration around heme periphery.

In summary, I found that the dehydration around the hydrophobic residues located near the heme periphery of Cyt *c* caused a subtle tertiary structural change accompanied by a slight shift in the heme position, and such change of heme position resulted in the downshift of its redox potential and rapid autoxidation of Cyt *c* (ET to molecular oxygen). Considering that these hydrophobic residues are the primary sites dehydrated upon formation of the complex between Cyt *c* and CcO as shown in Chapter II, tertiary conformational changes in Cyt *c* with heme displacement are expected to occur upon the Cyt *c*-CcO complexation. Such change of heme position would negatively shift the reduction potential by increasing the ASA of heme, contributing the electron donation to CcO. This would be one of the reasons for using the specific dehydration around the hydrophobic region near the heme periphery as the driving force for the Cyt *c*-CcO complexation.

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CHAPTER IV

Exploring the Electron Transfer Pathway from Cytochrome *c* to Cytochrome *c* Oxidase

Abstract

Although the results from Chapters II and III showed that the dehydration from the hydrophobic residues near the heme of Cyt *c* contributes not only to stabilize the Cyt *c*-CcO complex but also to negatively shift the reduction potential, the mechanism how an electron is transferred from Cyt *c* to CcO after the complexation remains unclear because of the lack of the structural information about the Cyt *c*-CcO complex. In this chapter, to examine the mechanism of the ET reaction from Cyt *c* to CcO, I predicted the structure of the Cyt *c*-CcO complex by protein docking simulation and identified the residues involved in the ET pathway. The theoretical analysis showed that the electron is transferred from heme of Cyt *c* to Cu_A of CcO through the hydrophobic region at the interface between Cyt *c* and CcO, which was formed by the several hydrophobic residues near the redox centers, such as Ile81 and Val83 of Cyt *c*. To investigate the contribution of the hydrophobic residue to the formation of the ET pathway, I further experimentally determined the electronic coupling constant (T_{DA}) for the ET reaction in the Cyt *c*-CcO complex, representing the electronic coupling of the reactants-electronic state with the products. T_{DA} was estimated by the temperature dependence of the ET rate from Cyt *c* to CcO (k_{ET}), which was determined by the transient absorption spectroscopy. When the environment around the interface near the heme in the Cyt *c*-CcO complex was altered to more hydrophilic by the mutation of Ile81 to serine, T_{DA} for the Cyt *c*-CcO complex significantly decreased, indicating that Ile81, located near the heme, was one of the significant hydrophobic residues to form the ET pathway to CcO. Based on these theoretical and experimental analyses, I discuss the molecular mechanism of the ET reaction in the Cyt *c*-CcO complex.

4.1. Introduction

In Chapter II, I investigated the osmotic pressure experiments for the Cyt *c* oxidation by CcO, and found that formation of the Cyt *c*-CcO complex is promoted by dehydration from the hydrophobic residue near the heme of Cyt *c*, Ile81, which is a primary factor increasing the entropy upon the complexation. The results from Chapter III further suggested that the dehydration from the heme periphery would negatively shift the reduction potential of heme owing to the tertiary structural change accompanied by the change of heme position, leading to the efficient ET to CcO. However, the mechanism how an electron is transferred from Cyt *c* to CcO after the complexation was still unknown, because the structural information in Chapters II and III was limited in Cyt *c*.

In this chapter, to examine the ET pathway from Cyt *c* to CcO, I predicted the structure of the Cyt *c*-CcO complex using protein docking simulation. One of the most useful methods to determine the ET pathway in a protein complex such as the Cyt *c*-CcO complex is to evaluate its electronic donor-to-acceptor coupling constant (T_{DA}), representing the electronic coupling of the reactants-electronic state with the products. T_{DA} is known to depend on the structure of the medium between donor and acceptor [1,2], which was addressed by the *Pathways* model developed by Beratan et al. (See Material and Methods) [3,4]. This *pathways* model-based calculation provides an approach for identifying the residues involved in the ET pathway in large and complicated biomolecular systems [5–7]. In this chapter, to determine the ET pathway from heme of Cyt *c* to Cu_A of CcO, I identified the residues forming the ET pathway reflecting the maximum T_{DA} value by using the theoretical method, *Pathways* plugin [3,4] on the basis of the structural information of the Cyt *c*-CcO complex predicted by the docking simulation.

I also experimentally determined T_{DA} in the Cyt *c*-CcO complex to investigate the contribution of the hydrophobic interaction near the redox centers to the formation of the ET pathway. T_{DA} can be determined by the dependence of the ET rate from Cyt *c* to CcO (k_{ET}) on temperature [8]. Since the ET reaction from Cyt *c* to CcO was reported to occur within a sub-

millisecond time scale ($k_{\text{ET}} > 10^4 \text{ s}^{-1}$) [9], I utilized so-called “flow-flash” technique to estimate k_{ET} [10–13]. As shown in Figure 1, in this system, the sample solution containing reduced Cyt *c* and fully reduced CcO with CO was rapidly mixed with an O₂-containing solution, and CO was flashed off by a laser pulse. O₂ binding to reduced CcO was followed, and then the ET reaction from reduced Cyt *c* to O₂-bound CcO was initiated to reduce O₂ [11]. On the basis of these theoretical and experimental analyses in terms of the ET pathway, I discussed the molecular mechanism of the ET reaction after the Cyt *c*-CcO complexation.

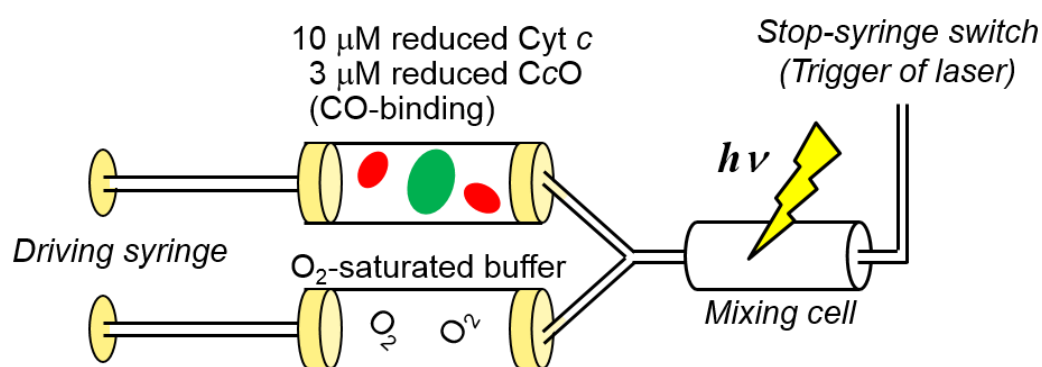


Figure 1. A schematic presentation of a flow-flash system.

One driving syringe contains the sample solution and the other syringe are filled with O₂-saturated buffer. These solutions are forced into a mixing cell. In the case of CO-bound CcO, about 30 milliseconds after mixing two solutions, CO was flashed off by a laser pulse.

4.2. Experimental Procedures

Pathways Model

The possible ET pathways from the heme iron of Cyt *c* to Cu_A of CcO were explored by the *Pathways* plugin for visual molecular dynamics (VMD) [3–5], which evaluates the donor-to-acceptor tunneling coupling constant (T_{DA}) for each pathway. The structure of WT Cyt *c*-CcO complex and its mutant were taken from the predicted structures by docking simulation. Hydrogen atoms were added using VMD. The *Pathways* model treats the tunneling propagation as a sequence of steps, each of which is mediated by a covalent bond, hydrogen bond, or vacuum, and the electronic coupling, T_{DA} , is proportional to a product of penalties for each step:

$$T_{DA} \propto \varepsilon_{\text{tot}} = \prod_i \varepsilon_i^C \prod_j \varepsilon_j^H \prod_k \varepsilon_k^S \quad (1)$$

where Π represents a product. The penalty for tunneling through a covalent bond is $\varepsilon^C = 0.6$ [14], the penalty for a through-space jump is $\varepsilon^S = \varepsilon^C \exp[-1.7(R^S-1.4)]$, where R^S is the through-space distance in Å, and the penalty for a hydrogen bond-mediated step is $\varepsilon^H = (\varepsilon^C)^2 \exp[-1.7(R^H-2.8)]$, where R^H is the hydrogen bond length (heavy atom to heavy atom) in Å, respectively.

Preparation of the Sample Solution for Flow-Flash Measurement

A sample containing 10 μM Cyt *c* and 3 μM CcO in 50 mM sodium phosphate buffer (pH 6.8) and 0.1% decyl- β -D-maltoside (DM) was placed in a Thunberg cuvette, where the atmosphere was exchanged for N₂ on a vacuum line. To reduce Cyt *c* and CcO, 4 mM sodium ascorbate and 1 μM phenazine methosulfate (PMS) were added, and the sample was incubated until reduction of these sample was complete. The atmosphere was then exchanged for CO. Reduction of Cyt *c* and CcO, and CO ligand binding to CcO were confirmed by the optical absorption spectra.

Flow-Flash Measurements

Flow-flash measurements were carried out using a locally modified stopped-flow apparatus (Applied Photophysics, Surrey, UK) [10]. The sample solution was anaerobically transferred to one of the driving syringes, which was rapidly mixed in a 1:1 ratio with the oxygen-saturated buffer containing 50 mM sodium phosphate buffer (pH 6.8) and 0.1% DM equilibrated with O₂ at $\sim 10^5$ Pa. About 30 ms after mixing, the reaction was initiated by dissociating CO ligand of CcO with a 10-ns laser pulse at 532 nm from a Nd:YAG laser (200 mJ, Quantel, France). Oxidation of Cyt *c* was monitored by measuring the absorbance at 550 nm on a digital oscilloscope (Nicolet, model 490).

4.3. Results

Prediction of the Structure of Cyt *c*-CcO Complex

To examine the ET pathway from Cyt *c* to CcO, I predicted the structure of the Cyt *c*-CcO complex and energetically characterized the interactions essential for complex formation using protein docking simulation based on the previous studies on steady-state kinetics of the ET reaction from Cyt *c* to CcO and NMR analysis [15] of the interaction sites for CcO on Cyt *c*. Table 1 lists the calculated overall free energy change along with the energetic components from the MM-PBSA calculation [16] on the modeled Cyt *c*-CcO complex. As shown in Table 1, Coulombic energy, ΔE_{coul} , was predicted to be large negative value of approximately $-1410 \text{ kcal mol}^{-1}$, whereas desolvation energy for polar residues, ΔG_{polar} , was predicted to be large positive value of approximately $1440 \text{ kcal mol}^{-1}$. These predicted data indicated that the favorable electrostatic interactions between several charged residues of Cyt *c* and CcO (ΔE_{coul}) were nearly cancelled out by large unfavorable ΔG_{polar} , resulting in the destabilization of the Cyt *c*-CcO complex. Consequently, the electrostatic interactions, $\Delta G_{\text{electro}}$ (sum of ΔE_{coul} and ΔG_{polar}) were not the primary factor for the Cyt *c*-CcO complexation. The residual destabilizing free energy is compensated by the van der Waals interactions mediated by hydrophobic residues, ΔE_{vdw} , to give the stabilized complex. Therefore, hydrophobic interactions were the primary thermodynamic factors that promote the complex formation between Cyt *c* and CcO. This prediction was consistent with the results from Chapter II that the Cyt *c*-CcO complex formation was driven by increase in the entropy.

In the predicted structure of Cyt *c*-CcO complex, the interface between Cyt *c* and CcO contained several hydrophobic residues such as Ile81 and Val83 in Cyt *c* and Leu136 in CcO, which are localized near the redox centers (Figure 2). This structural information provided the hypothesis that an electron is transferred from heme of Cyt *c* to Cu_A of CcO through the hydrophobic region formed by these residues.

Table 1. Overall free energy change and representative energy terms upon the Cyt *c*-CcO complex formation (kcal mol⁻¹).

ΔG	ΔE_{coul}	ΔE_{vdW}	ΔG_{polar}	$\Delta G_{\text{nonpolar}}$	$\Delta G_{\text{electro}}$
-8.25	-1413.23	-139.49	1438.72	-20.19	25.49

ΔE_{coul} : Electrostatic (Coulombic) energy

ΔE_{vdW} : van der Waals energy

ΔG_{polar} : Polar contributions to solvation

$\Delta G_{\text{nonpolar}}$: Nonpolar contributions to solvation

$\Delta G_{\text{electro}}$: $\Delta G_{\text{polar}} + \Delta G_{\text{nonpolar}}$

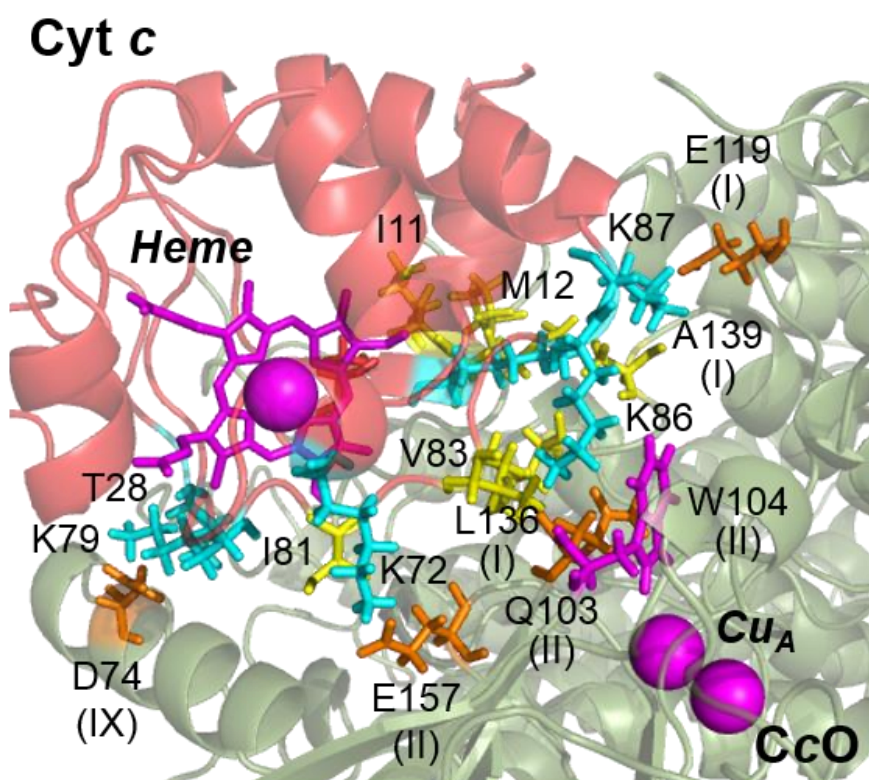


Figure 2. Predicted binding interface of the Cyt *c* and CcO complex.

Hydrophobic amino acid residues, hydrophilic residues of Cyt *c* and those of CcO are shown as yellow, cyan and orange stick, respectively. Cyt *c* and CcO are shown as red and green cartoon, respectively. The heme iron of Cyt *c* and Cu_A of CcO are represented by magenta spheres.

Identification of the Residues Forming the ET Pathway from Cyt *c* to CcO

To gain insights into the ET pathway from Cyt *c* to CcO, I identified the residues to form the ET pathway reflecting the maximum T_{DA} value by using the *Pathways* plugin for VMD [3,4]. As described in Experimental Procedure, the *Pathways* model interprets the ET reaction from donor to acceptor as a sequence of steps, and each step is mediated by a covalent bond, hydrogen bond, or vacuum, and the electronic coupling (T_{DA}) is proportional to penalties for each step (Equation 1). The complex structure of Cyt *c* with CcO used for the *Pathways* model calculation was taken from the predicted structures by the docking simulation. Figure 3A shows the most dominant ET pathway from Cyt *c* to CcO computed by the *Pathways* plugin. The calculation predicted that, in the most probable ET pathways, an electron is transferred through the iron atom of heme *c*, Met80, Ile81, Phe82, Val83, Lys86 of Cyt *c*, Gln103, Trp104, and Cu_A of CcO subunit II, most of which are hydrophobic residues in the Cyt *c*-CcO interaction surface [15]. The predicted ET pathway contains the aromatic residue near the Cu_A of CcO, Trp104, in agreement with the proposal that Trp104 is the electron entry site to Cu_A [17,18]. It is notable that the hydrophobic residues near the heme of Cyt *c* such as Ile81, which was identified as the dehydration site upon the Cyt *c*-CcO complexation in Chapter II, were also involved in the ET pathway, suggesting that the hydrophobic residues near the heme of Cyt *c* contribute to form the ET pathway by the hydrophobic interaction with CcO.

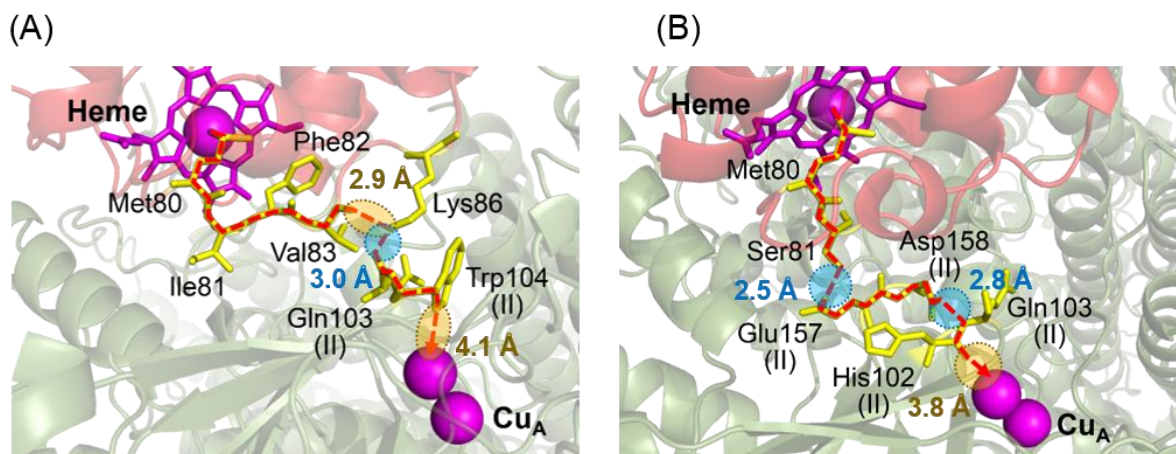


Figure 3. A dominant electron transfer-path in the WT Cyt *c*-CcO (A) and I81S mutant Cyt *c*-CcO complex (B).

ET pathway are represented by red dashed line. The amino acid residues forming the ET pathway are shown as yellow sticks. Positions of hydrogen bond-mediated step and through vacuum step are represented by blue and yellow circle, respectively. Cyt *c* and CcO are shown as red and green cartoon, respectively. The heme iron of Cyt *c* and Cu_A of CcO are represented by magenta spheres.

To investigate the relationship between the hydrophobic residues and the formation of the ET pathway, I performed a site-directed mutagenesis. Ile81 of Cyt *c* was replaced with serine to alter the hydrophobicity around position 81. Figure 3B shows the most probable ET pathway from the I81S mutant to CcO by the *Pathways* model. An electron was predicted to be transferred from the iron atom of heme *c* to Cu_A of CcO through Met80, Ser81 of Cyt *c*, Glu157, Asp158, Gln103 and His102 of CcO. Most of the residues forming the ET pathway were located on the I81S Cyt *c*-CcO interaction surface, as observed for the complex of WT Cyt *c* with CcO. However, the position of the interprotein ET process in the complex of I81S Cyt *c* with CcO was different from that of the WT Cyt *c*-CcO complex. In the complex between WT Cyt *c* and CcO, an electron was transferred through a hydrogen bond between Lys86 of Cyt *c* and Gln103 of CcO (a distance of 3.0 Å) (Figure 3A), while an electron was transferred through a hydrogen bond between Ser81 of Cyt *c* and Glu157 of CcO (a distance of 2.5 Å) in the complex of I81S Cyt *c* with CcO (Figure 3B). Thus, Ile81 of Cyt *c* was predicted to participate the formation of the ET pathway by forming the hydrophobic interaction with CcO.

Determination of T_{DA} in the Cyt *c*-CcO Complex

To demonstrate the contribution of the hydrophobic interaction at Ile81 of Cyt *c* to the formation of the ET pathway, I experimentally determined the electronic donor-to-acceptor coupling constant in the Cyt *c*-CcO complex, T_{DA} , based on the semi-classical theory developed by Marcus [8]. In this theory, the ET rate (k_{ET}) between two molecules is supposed to be controlled by the driving force ΔG , the reorganization energy λ , and T_{DA} (Equation 2):

$$k_{ET} = \frac{4\pi^2}{h} \frac{\langle T_{DA} \rangle^2}{\sqrt{4\pi\lambda k_B T}} \exp \left\{ -\frac{(\Delta G + \lambda)^2}{4\lambda k_B T} \right\} \quad (2)$$

where h is Planck's constant, k_B is Boltzmann's constant, and T is temperature. λ represents the energy barrier for ET and T_{DA} represents the degree of wave function overlap occurring between donor and acceptor. Interprotein ET reactions typically occur in the weak coupling limit owing to the relatively large donor-to-acceptor distances (5–25 Å) [19], where electronic tunneling is mediated by the virtual electronic states of the intervening medium. T_{DA} reflects the most probable ET pathway, depending on the structure of the medium between the donor and acceptor [1,2].

To obtain T_{DA} in the Cyt *c*-CcO complex, I determined the k_{ET} from Cyt *c* to CcO using transient absorption spectroscopy called “flow-flash” system [10,11]. Figure 4A shows the time course for the Cyt *c* oxidation by CcO, which was monitored by the absorbance at 550 nm. The time course data were fit by single or double exponential equations, shown along with the raw data. Residuals from each fit are shown in the top panel of Figure 4A. In the case of the Cyt *c* oxidation by CcO, fitting the absorption change to the double exponential equation (Equation 3) provided a significantly better model (red line) than the single exponential equation (blue line).

$$\text{Abs}_{550}(t) = A\exp(-k_{\text{fast}}t) + B\exp(-k_{\text{slow}}t) + C \quad (3)$$

where $\text{Abs}_{550}(t)$ is the absorbance at 550 nm as a function of time, A and B are constants related to the initial absorbance and C is the final absorbance. The k_{fast} and k_{slow} values in the presence

of 10 μM Cyt *c* at 293 K were 10700 ± 400 and $380 \pm 10 \text{ s}^{-1}$, respectively. To identify each rate constant reflect a unimolecular or bimolecular reaction, we investigated the dependence of the Cyt *c* concentration on k_{fast} and k_{slow} . As shown in Figure 4B, the k_{slow} value depended on the Cyt *c* concentration, implying that k_{slow} involves a bimolecular reaction such as the Cyt *c*-CcO complex formation. In contrast, the k_{fast} value was independent of the concentration of Cyt *c* (Figure 4C), indicating that k_{fast} reflects a unimolecular reaction. The k_{fast} value of approximately $1 \times 10^4 \text{ s}^{-1}$ was in agreement with the interprotein ET rate of a ruthenium trisbipyridine-labeled Cyt *c* to CcO measured by flash photolysis, in which an electron is transferred from the photoexcited Ru (II) to heme of Cyt *c*, followed by ET from Cyt *c* to CcO [9]. Thus, k_{fast} determined by the “flow-flash” system corresponds to the intermolecular ET rate, k_{ET} .

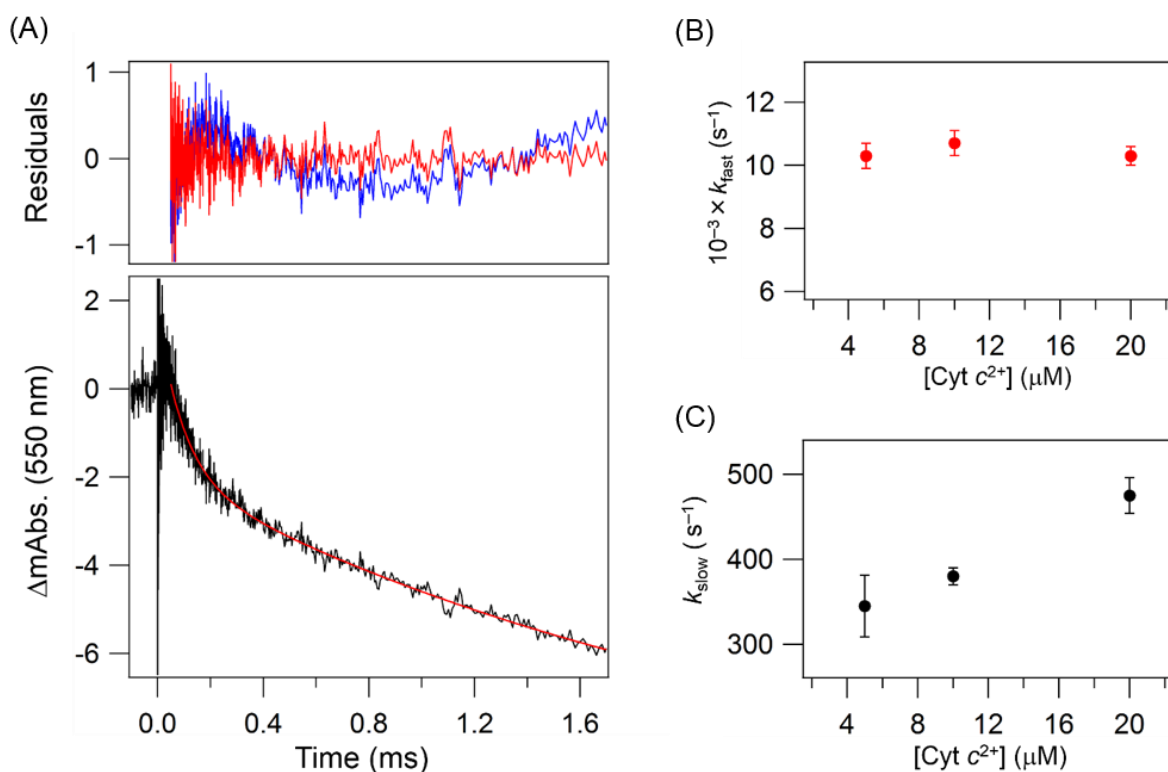


Figure 4. Kinetics of Cyt *c* oxidation by CcO monitored absorbance at 550 nm.

(A) Absorbance change at 550 nm associated with electron transfer (ET) reaction between Cyt *c* and CcO. The measurements were performed by mixing of sample solution and O_2 -saturated 50 mM sodium phosphate buffer (pH 6.8) containing 0.1% *n*-decyl- β -D-maltoside (DM). Sample solution contains 10 μM reduced Cyt *c* and 3 μM reduced CcO. The CO ligand binding to CcO was dissociated by a laser flash at time zero. Time course was fitted to single (upper panel, grey) or double exponential (middle panel, red). The solid line represents the fits of this data to Equation 3. The residuals from the single- (blue line) or double-exponential fit (red line) to the time courses are presented as the top traces. mAbs represents milli-absorbance. (B) k_{fast} and (C) k_{slow} values for Cyt *c*-CcO ET at various concentrations of CcO. Sample solution contains 5, 10, or 20 μM reduced Cyt *c* and 3 μM reduced CcO.

I obtained T_{DA} by measuring k_{ET} (k_{fast}) under various temperatures. ΔG for the ET reaction in Equation 2 corresponds to a difference in the redox potential between the redox centers (ΔE) ($\Delta G = -nF\Delta E$; n is the number of electrons transferred in the reaction, F is the Faraday's constant). Thus, in the case of ET from heme of Cyt *c* (redox potential; +235 mV) [20] to Cu_A of CcO (+245 mV) [20], ΔE was calculated to be +10 mV, corresponding to ΔG of $-0.965 \text{ kJ mol}^{-1}$. As shown in Figure 5, the k_{ET} value increased to $1.4 \times 10^4 \text{ s}^{-1}$ with temperature up to 308 K. T_{DA} was estimated to be 1.10 cm^{-1} by fitting the k_{ET} values to Equation 2 (Table 2).

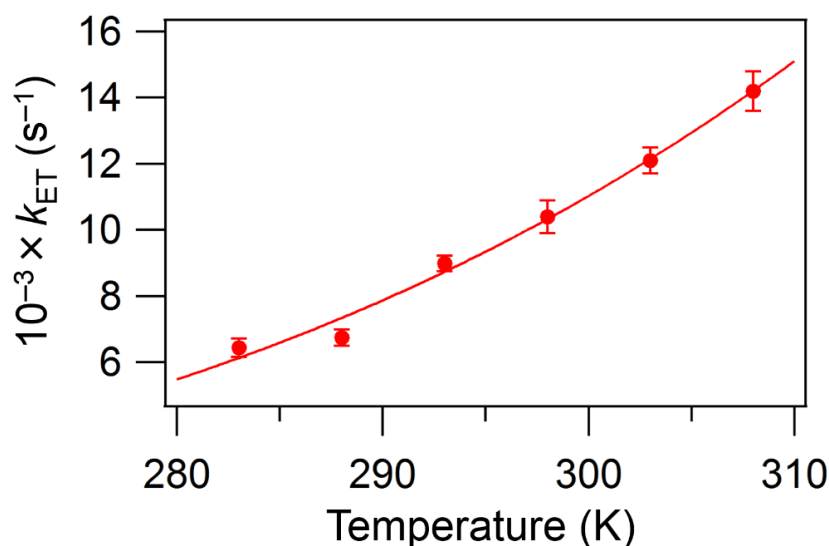


Figure 5. Dependence of k_{ET} on temperature for Cyt *c*-CcO ET.

Values of k_{ET} were determined from the data shown in Figure 4A, which were fit to Equation 3. The solid line represents the fitting of these data to Equations 2 and 4. The fitting curves to the two equations are superimposable.

Table 2. ET parameters in the Cyt *c*-CcO complex

	$T_{\text{DA}} \text{ (cm}^{-1}\text{)}$	ϵ^{tot}	$r \text{ (\AA)}$
WT	1.10	1.72×10^{-9}	10.8 – 18.6
I81S	0.104	5.45×10^{-8}	14.0 – 21.9

Contribution of Hydrophobic Residues near Heme of Cyt *c* to ET

Based on the determined T_{DA} value in the complex between WT Cyt *c* and CcO, I investigated contribution of the hydrophobic residues near heme of Cyt *c* to formation of the ET pathway between Cyt *c* and CcO by the site-directed mutagenesis. Similar to the theoretical analyses using the *Pathway* model, Ile81 was replaced with serine to alter the hydrophobicity around position 81. I measured k_{ET} for the I81S mutant Cyt *c* under various temperatures and determined T_{DA} using Equation 2. As shown in Figure 6, k_{ET} for the I81S mutant moderately increased with temperature up to 308 K compared to that of WT Cyt *c* (Figure 5). The estimated T_{DA} value for the I81S mutant was 0.104 cm⁻¹ (Table 2), about 10% of that for WT Cyt *c*. Therefore, Ile81 of Cyt *c* deeply contributes to formation of the ET pathway from Cyt *c* to CcO.

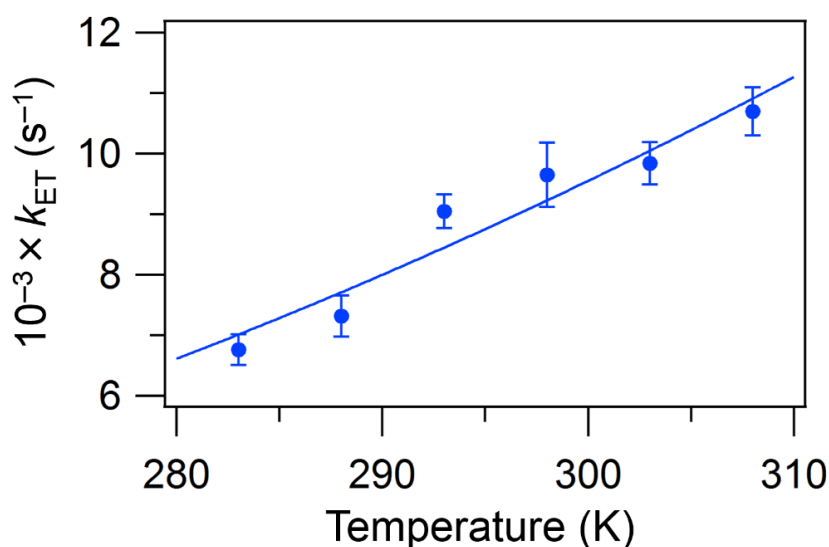


Figure 6. Dependence of k_{ET} on temperature for I81S mutant Cyt *c*-CcO ET.

Values of k_{ET} were determined from the data shown in Figure 4A, which were fit to Equation 3. The solid line represents the fitting curve of these data to Equations 2 and 4. The fitting curves to the two equations are superimposable.

4.4. Discussion

Importance of Hydrophobic Interactions near the Redox Centers for ET

As shown in Table 2, the “flow-flash” measurements indicated that the value of T_{DA} for the complex between I81S Cyt *c* and CcO was about 10 times smaller than that for WT Cyt *c*, which can be interpreted that Ile81 of Cyt *c* contributes to formation of the ET pathway from Cyt *c* to CcO. Then, to experimentally confirm the prediction *in silico* that the mutation of Ile81 to serine also induces the decrease of T_{DA} , I calculated ε_{tot} , related to T_{DA} for the Cyt *c*-CcO complex by Equation 1. The ε_{tot} value reflecting the most probable ET pathway for the complex between WT Cyt *c* and CcO (Figure 3A) was determined to be 1.72×10^{-9} , whereas that for the I81S Cyt *c* (Figure 3B) was 5.45×10^{-8} (Table 2). This observation was somewhat unexpected because the value of ε_{tot} for the complex between I81S Cyt *c* and CcO was approximately 30 times larger than that of the WT Cyt *c*, which was inconsistent results from the “flow-flash” measurements.

To explore the reason for the increase of ε_{tot} by the mutation of Ile81 to serine in the theoretical analysis and the different effect of the mutation between theoretical and experimental analyses, I focused on the character of the predicted ET pathway in each Cyt *c*-CcO complex. Table 2 shows the number of steps of electron tunneling propagation mediated by a covalent bond, hydrogen bond, or vacuum involved in the ET pathway predicted by the *Pathways* plugin for VMD. Both ET pathways in the complexes of WT and I81S Cyt *c* with CcO involved the same number of the total steps and covalent bond-mediated steps. Considering that the energy barrier for tunneling through a covalent bond, ε^C in Equation 1, represents constant value ($\varepsilon^C = 0.6$ [14]), the different ε_{tot} between the complex WT and I81S Cyt *c* with CcO was derived from the number of the hydrogen bond-mediated and/or vacuum-mediated steps. The numbers of the hydrogen bond-mediated and vacuum-mediated steps in the ET pathway from WT Cyt *c* to CcO was 1 and 2, respectively, whereas those from I81S Cyt *c* to CcO was 2 and 1, respectively (Table 3). The energy barrier for tunneling through vacuum

is known to be much higher than for tunneling through a hydrogen bond, which are addressed by the *Pathways* model (see experimental procedures) in Equation 1 [3,4]. For the WT Cyt *c*-CcO complex, by using Equation 1, the values of the energy barrier for the vacuum-mediated step (ε^S) reflecting the tunneling from Val83 to Lys86 and from Trp104 to Cu_A were estimated to be 0.047 and 0.006, respectively, and that for the hydrogen bond-mediated step (ε^H) reflecting the tunneling from Lys86 to Gln103 (Figure 3A) was 0.256. On the other hand, the values of ε^S reflecting the tunneling from His102 to Cu_A was estimated to be 0.010, and the ε^H reflecting the tunneling from Ser81 to Glu157 and from Asp158 to Gln103 in the I81S Cyt *c*-CcO complex (Figure 3B) were 0.599 and 0.360, respectively. The product of these values was 2.16×10^{-3} , which was approximately 30 times larger than that product of ε^S and ε^H in the WT Cyt *c*-CcO complex, 7.22×10^{-5} . Thus, the increase of α_{tot} (T_{DA}) by the mutation of Ile81 to serine was attributed to the decreased number of the vacuum-mediated steps and increased number of hydrogen-mediated steps by alteration of the ET pathway.

One of the primary factors to represent larger α_{tot} in the ET pathway in the complex of I81S mutant Cyt *c* with CcO is the hydrogen bond formed between Ser81 of Cyt *c* and Glu157 of CcO (Figure 3B). As shown in Figure 7, an electron is predicted to be transferred through the “solvent-exposed region”, via the hydrogen bond between these residues by the mutation of Ile81. Recent studies, however, proposed that the penetration of bulk water molecules disrupts hydrogen bonds involved in the ET pathway, leading to the decreased T_{DA} [6]. It should be noted that the *Pathways* analyses for the Cyt *c*-CcO complex performed in this chapter did not reflect the effect of bulk or hydrated water molecules, because the bulk and hydrated water molecules surrounding the Cyt *c*-CcO complex were absent in the docking simulation. It is plausible that the hydrogen bond between Ser81 to Glu157 near the solvent-exposed region was disrupted by bulk or hydrated water molecules, resulting in the decreased T_{DA} compared to the complex of WT Cyt *c* with CcO, as demonstrated from the “flow-flash” system (Table 2).

Table 3. The number of steps of electron tunneling propagation mediated by a covalent bond, hydrogen bond, or vacuum.

	Covalent bond	Hydrogen bond	Vacuum	Total
WT	21	1	2	24
I81S	21	2	1	24

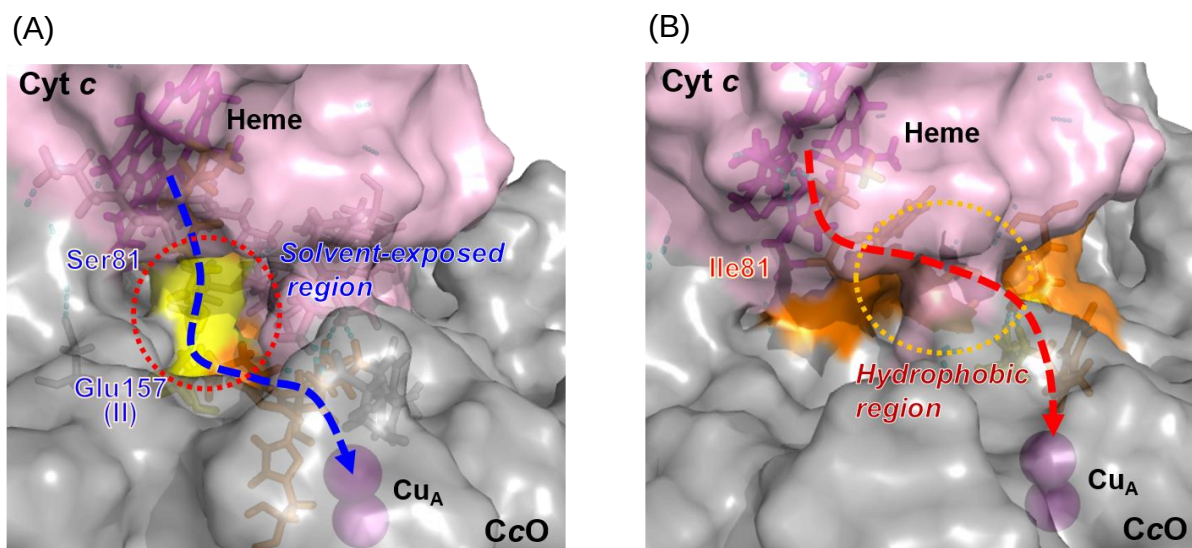


Figure 7. The predicted ET pathway in the structure of I81S Cyt *c* (A) and WT Cyt *c* with CcO (B).

ET pathways are represented by blue dashed line. (A) Ser81 of Cyt *c* and Glu157 of CcO are shown as yellow surface. Positions of solvent-exposed region are represented by dotted red circle. (B) The residues forming the ET pathways are shown as orange surface. Positions of hydrophobic region are represented by dotted orange circle.

In contrast to the I81S mutant Cyt *c*, specific hydrophobic interactions at Ile81 of WT Cyt *c* near the redox center revealed (Figure 7B) would support the formation of the ET pathway from Cyt *c* to CcO by dehydrating water molecules around the redox centers, and protect electrons from the leak to the solvent by separating the ET pathway from the bulk solvent. When the hydrophobic residues such as Ile81 were mutated to hydrophilic serine, water molecules around the redox centers were not expelled. Consequently, as described above, the remaining water molecules would disrupt the ET pathway, resulting in reduced T_{DA} . Therefore, the specific dehydration near the redox center would promote the ET reaction to CcO by constituting the hydrophobic ET pathway.

Possibility of Conformational Rearrangement to an Optimal Orientation for ET

Although the ET pathway from Cyt *c* to CcO is assumed to be formed by the specific hydrophobic interactions near the redox centers accompanied by dehydration from the hydrophobic residue, Ile81, it is difficult to completely explain the experimentally-observed fast ET from Cyt *c* to CcO ($k_{ET} > 10^4 \text{ s}^{-1}$) in Table 2. The fast ET reaction ($k_{ET} > 10^4 \text{ s}^{-1}$) is reported to occur when the distance between the redox centers is less than 18 Å [19,21], whereas the distance between the heme iron of Cyt *c* and Cu_A of CcO in the predicted complex structure of WT Cyt *c* with CcO by docking simulation is 23.0 Å.

To investigate the reason for the fast ET in the long range, I calculated the heme-Cu_A distance of the transient ET complex from the “flow-flash” ET experiment. The distance between redox centers for the ET reaction is estimated from an alternative form of the Marcus equation [8]:

$$k_{ET} = k_0 \exp\{-\beta(r - r_0)\} \exp\left\{-\frac{(\Delta G + \lambda)^2}{4\lambda k_B T}\right\} \quad (4)$$

where k_0 is the characteristic frequency of nuclei, which is set at 10^{13} s^{-1} ; r is the distance between redox centers; r_0 is the close contact distance, which is taken to be 3 Å [22]; β (Å⁻¹) is the electronic decay factor used to quantitate the nature of the intervening protein medium between donor and acceptor [22], which has been proposed to be 1.4–0.7 Å⁻¹ for analyzing ET reactions between proteins [19]. To obtain the r value between the heme iron and Cu_A for the transient ET complex, the k_{ET} values under various temperature determined by “flow-flash” system were fit to Equation 4 using β values ranging from 1.4–0.7 Å⁻¹. For the Cyt *c*-CcO complex, such an analysis using Equation 4 yielded the r values ranged from 10.8 to 18.6 Å (Table 2), which are shorter than that in the predicted complex structure of Cyt *c* with CcO by the docking simulation, 23.0 Å. Furthermore, most of experimentally-estimated heme-Cu_A distances fell within 18 Å [19,21], implying that the obtained r value appeared to be appropriate for the efficient electron tunneling to CcO.

One of the possible reasons for the different donor-to-acceptor distance between

experimental and theoretical analysis is that the optimum orientations of Cyt *c* against CcO in the ET reaction would differ from that predicted by the docking simulation. The Cyt *c*-CcO complex used for theoretical analysis was predicted as the most energetically stable complex by the docking simulation. Recent studies on the transient ET complex have proposed that redox proteins can form multiple complexes and ET occurs when the redox centers of the binding partners are close enough to ensure the efficient electron tunneling across the interface [21,23], although it might not be the most stable complex. Taking into account the possibility of the formation of the multiple Cyt *c*-CcO complexes, Cyt *c* would rearrange its orientation against CcO from the structure predicted by docking simulation to an ET-active structure that the heme-Cu_A distance is close enough to ensure the ET reaction to CcO. Such rearrangement from the most energetically stable complex to the ET active structure may contribute to protect the back electron transfer from Cu_A to heme, leading to the efficient unidirectional ET reaction to CcO.

In summary, to examine the ET pathway from Cyt *c* to CcO, I analyzed the Cyt *c*-CcO complex predicted by the docking simulation on the basis of the *Pathways* model and further measured the kinetics for the ET reaction using “flow-flash” system. Both theoretical and experimental analyses showed that the hydrophobic interaction at Ile81 of Cyt *c* with CcO in Chapter II contributes to the formation of the ET pathway to CcO. The different mutational effect on T_{DA} between theoretical and experimental methods provided a suggestion that the preferential and specific dehydration around the redox centers would protect the ET pathway from the leak of electrons to the bulk solvent, thus promoting the effective and specific ET from Cyt *c* to CcO.

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CHAPTER V

Conclusions

In the present thesis, I aimed to elucidate the mechanism of the crucial process in the mitochondrial respiratory chain, the ET reaction from Cyt *c* to CcO. For understanding the molecular mechanism of the Cyt *c*-CcO ET, many studies have demonstrated the involvement of electrostatic interactions mediated by lysine residues surrounding the heme edge in Cyt *c* and acidic residues around Cu_A in CcO on the basis of the structural information [1–5]. On the other hand, roles and contributions of hydrophobic interactions to the ET reaction have not been fully understood. Here, I focused on the hydrophobic residues near the heme periphery identified as the interaction sites for CcO on Cyt *c* [6] and examined the functional significance of the hydrophobic interaction for the Cyt *c*-CcO ET reaction based on thermodynamic, structural and kinetic analyses using the various spectroscopic techniques. The results and conclusions drawn from experimental data are highlighted below.

Thermodynamic Study on the Cytochrome *c*-Cytochrome *c* Oxidase Complex Using Osmotic Pressure (Chapter II)

In this chapter, I focused on the thermodynamic property to elucidate the interaction dominating the Cyt *c*-CcO complex formation. Measurements of the steady-state kinetics at various osmotic pressures and temperature revealed that the Cyt *c*-CcO complexation was primarily promoted by increase in the entropy from the dehydration at the hydrophobic residues near the heme periphery of Cyt *c*, which allows me to propose that Cyt *c* associates with CcO for the ET reaction by forming specific hydrophobic interactions near the heme periphery as the driving force for the Cyt *c*-CcO complexation.

Contribution of Dehydration near the Heme in Cytochrome *c* to Electron Transfer to Cytochrome *c* Oxidase (Chapter III)

To get the further insights into the functional and structural significance of the dehydration from Cyt *c* upon the Cyt *c*-CcO complexation, I focused on the hydration environment around

Cyt *c* in this chapter. The structure and ET function of Cyt *c* in the presence of PEG as an agent causing dehydration from the surface of Cyt *c* were characterized by various spectroscopic measurements and I found that the dehydration around the heme periphery induced tertiary structural changes accompanied by a slight shift in the heme position. Although I have not yet fully figured out the contribution of such positional change of heme to the ET reaction to CcO, the positional perturbation of heme would negatively shift its redox potential, facilitating the unidirectional ET to CcO. Thus, the results of various spectroscopic measurements in this chapter provided the possibility that specific dehydration upon the formation of the hydrophobic interactions contributes to the ET reaction to CcO as well as the Cyt *c*-CcO complexation.

Exploring the Electron Transfer Pathway from Cytochrome *c* to Cytochrome *c* Oxidase (Chapter IV)

In this chapter, to examine the contribution of the hydrophobic interactions to the formation of the ET pathway from heme to Cu_A in the Cyt *c*-CcO complex, I predicted the complex between Cyt *c* and CcO by protein docking simulation and identified the residues involved in the ET pathway from Cyt *c* to CcO. The theoretical analysis showed that the electron was transferred to CcO through the hydrophobic residues near the heme periphery, which were the primary dehydration sites for the Cyt *c*-CcO complexation (Chapter II) and for the change of heme position inducing the negative shift of its redox potential (Chapter III). Experimentally-determined T_{DA} for the ET in the Cyt *c*-CcO complex by transient absorption spectroscopy further provided the proposal that Ile81, located near the heme, is one of the significant hydrophobic residues to form the ET pathway to CcO. Such preferential and specific dehydration around the redox centers would protect the ET pathway from the leak of electrons to the bulk solvent, thus promoting effective and specific ET from Cyt *c* to CcO.

Concluding Remarks

As summarized above, extensive thermodynamic or kinetic analyses in Chapters II, III and IV, I found that the specific hydrophobic interactions between the hydrophobic residues near the redox center of Cyt *c* and CcO (left panel in Figure 1) contribute not only to the formation of the Cyt *c*-CcO complex to form the optimal ET pathway from heme to Cu_A (right panel in Figure 1), but also to the ET reaction to CcO by the positional change of heme causing the negative shift of redox potential (center panel in Figure 1). Considering the structural and thermodynamic similarity with interprotein ET systems, the proposed ET mechanism regulated by hydrophobic interaction in Figure 1 would not be specific for the ET reaction from Cyt *c* to CcO, but one of the general properties in interprotein ET in biological systems.

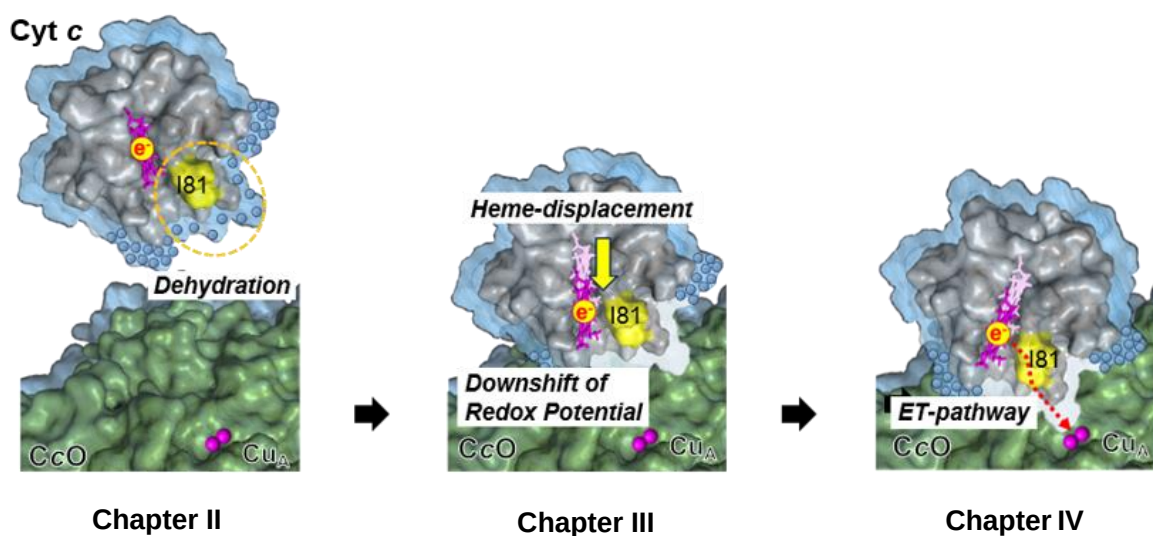


Figure 1. Proposed regulation mechanism of ET reaction from Cyt *c* to CcO.

For the ET reaction to CcO, Cyt *c* associates with CcO by forming specific hydrophobic interactions near the heme periphery as the driving force for the Cyt *c*-CcO complexation (Chapter II). Such specific dehydration upon the formation of hydrophobic interactions causes the positional change of heme, which promotes the ET reaction to CcO by negative shift of heme redox potential (Chapter III). The preferential and specific dehydration around the redox centers would protect the ET pathway from the leak of electrons to the bulk solvent, thus facilitating the effective and specific ET from Cyt *c* to CcO (Chapter IV).

On the other hand, the theoretical and experimental analyses in the Cyt *c*-CcO complex in Chapter IV also implied the concept of “conformational rearrangement”, meaning that redox proteins can form multiple complexes and ET occurs when the redox centers of the binding partners are close enough to ensure efficient electron tunneling across the interface [7,8]. Recent studies on several redox proteins have often pointed out the importance of protein dynamics for expression of ET function [7–9]. For understanding the whole picture of the molecular mechanism of unidirectional interprotein ET reaction, investigation of protein dynamics are quite essential. However, in this thesis, I would like to emphasize that the hydrophobic interaction near the redox centers is one of the important factor for regulating the effective and unidirectional ET reaction for not only the case of Cyt *c* and CcO, but also other redox proteins.

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