

Headline Articles

Intramolecular Electron Transfer of Cytochrome *c* Modified with *N*,*N*,*N*′,*N*″,*N*″-Diethylenetriaminepentaacetatocobaltate(Ⅲ)

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Horse heart cytochrome *c* (cyt*c*) modified with an *N*,*N*,*N*′,*N*″,*N*″-diethylenetriaminepentaacetatocobaltate(Ⅲ) ion, [cyt*c*^{Ⅲ}{Co^{Ⅲ}(dtpa)}], was prepared and characterized. The major component of singly modified [cyt*c*^{Ⅲ}{Co^{Ⅲ}(dtpa)}] contains a [Co^{Ⅲ}(dtpa)] ion at Lys13, of which the ε-amino group is linked with a carboxylate ion of dtpa. A one-electron reduced protein, [cyt*c*^{Ⅱ}{Co^{Ⅲ}(dtpa)}], was prepared by reduction with a methylviologen-radical cation, which was produced in situ by a photoreduction using a tris(2,2′-bipyridine)ruthenium(Ⅱ) ion in the presence of disodium dihydrogen ethylenediaminetetraacetate at 25 °C, pH 7.5 (0.010 mol dm^{-3} tris(hydroxymethyl)aminomethane–HCl buffer), and an ionic strength of 0.50 mol dm^{-3} (NaCl). The reaction of [cyt*c*^{Ⅱ}{Co^{Ⅲ}(dtpa)}] to form [cyt*c*^{Ⅲ}{Co^{Ⅱ}-(dtpa)}] was very slow, and was followed by conventional spectrophotometry. The reaction was of first order in the protein and the first-order rate constant ($(3.1 \pm 0.5) \times 10^{-4}$ s^{-1}) was independent of the concentration of the protein, indicating that the intramolecular electron-transfer (ET) process is predominant. The reaction mechanism is discussed on a through-bond or through-space pathway. The slow intramolecular ET rate might arise not only from the long distance, but also from the large reorganization energy for the Co(Ⅲ)/Co(Ⅱ) couple.

Intramolecular electron-transfer (ET) reactions of metalloproteins have received considerable attention both in chemistry and biology, because the ET in biological systems consists of consecutive long-range ET reactions containing metalloproteins and/or substrates.[1–11] In this matrix, electron-donor(s) and -acceptor(s) are located in a fixed distance and orientation like in a macromolecule. The dependence of the distance on the intramolecular ET rate had been proposed as the electronic coupling of spacers by Larsson;[12,13] thereafter, the following equation was presented for the intramolecular ET rate constant:[1,14]

$$k_{et} = 10^{13} \cdot \exp[-\beta(r - r_0)] \cdot \exp[-(\Delta G^0 + \lambda)^2/4\lambda RT]. \quad (1)$$

Here, r is the distance between the donor and acceptor centers, r_0 is the contact distance at which the reaction becomes adiabatic, β is the decay constant which describes the sensitivity of the coupling to changes in distance, ΔG^0 is the diving force, λ is the reorganization energy, which equals the mean value of the contribution from donor and acceptor, $(\lambda_D + \lambda_A)/2$, R is the gas constant, and T is the absolute temperature.

It is one of important problems in biological long-range ET reactions whether an electron is transferred from the donor to the acceptor by a through-space or a through-bond mechanism. A chemical modification of metalloproteins is a very useful technique for elucidating the ET pathways in such systems. A number of studies of ET reactions of chemically modified metalloproteins have been reported.[4,6,8] A pathway analysis for hemoproteins succeeded to explain the distance dependence on the ET reaction rate, where through-bond, van der Waals, and hydrogen-bonding interactions are important.[15–19] Recently, Gray et al. proposed a tunneling timetable for the distance dependence on the ET rate in ruthenium-modified proteins,[10] where the β values are 1.0 Å^{-1} (1 Å = 1×10^{-10} m) along α helices and 1.3 Å^{-1} along β strands, respectively. An empirical tunneling expression with the packing density between the redox cofactors has also been reported by Dutton et al.[11] Scott et al. reported that the intramolecular ET reduction of the oxidized form of cytochrome *c* (cyt*c*^{Ⅲ}) attached to a Co(Ⅱ) cage complex is independent of the separate distance between the heme and Co(Ⅱ), and that the calculated rate constants were much faster than the observed ones.[8,20,21] They assumed that the ET is controlled by a conformational gating of cyt*c*.

We recently reported on artificial metmyoglobin, of which Lys residues are modified with one of the strong chelating reagents, *N*,*N*,*N*′,*N*″,*N*″-diethylenetriaminepentaacetic acid (metMb(dtpa)$_n$, n = 1, 2, 4, and 5; see Chart 1), and their cobalt(Ⅲ) complexes ([metMb{Co^{Ⅲ}(dtpa)}$_n$], n = 1, 2, 4, and 5).[22–26] In this work, we applied a chemical modification by dtpa to cyt*c*, where the ε-amino group of Lys13 is linked with

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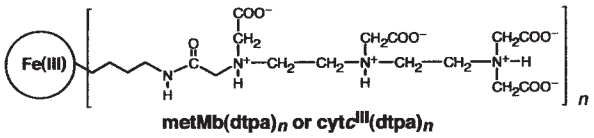

Chart 1. Structure.

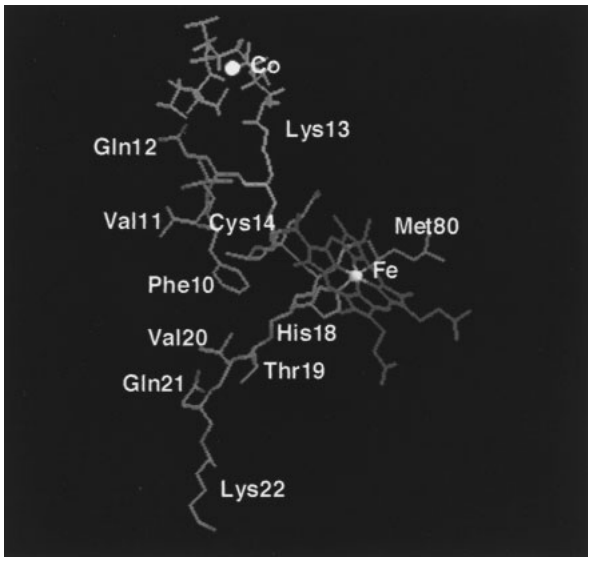

Fig. 1. Display of heme linked with Cys14, Lys13, and a [Co^{III}(dtpa)] moiety in horse heart cytc^{III}.

a [Co^{III}(dtpa)] ion (see Fig. 1), and found a slow intramolecular ET reaction with a rate constant of $(3.1 \pm 0.5) \times 10^{-4}$ s^{-1} at 25 °C in the one-electron reduced [cytc^{II}{Co^{III}(dtpa)}]. A time scale of hours has never been reported for the ET of cyt*c*. This slow ET reaction is almost certainly not controlled by a conformational change of the protein, in contrast to Scott's results and, thus, the mechanism is expected to be ET controlled. We discuss a slow intramolecular ET reaction based on the ET pathway and the reorganization energy, especially the Co(III) center. It is important to elucidate whether the ET proceeds by a through-space or a through-bond mechanism. Lys residues are flexible, and an estimation of the distance between the donor and the acceptor remains uncertain; that is, the through-space edge to edge distance between the heme and the Lys13 residue lies in a range of 6–10 Å. This estimated through-space distance is, however, completely different from the through-bond distance of 18.6 Å via Cys14 and Lys13 residues (see Fig. 1). It is, therefore, expected to distinguish between the through-space and the through-bond mechanisms in the case of [cytc^{II}{Co^{III}(dtpa)}].

Experimental

Materials. Horse heart cyt*c* (Sigma) was purified as previously described.[27] *N*,*N*-Bis[2-(2,6-dioxomorpholino)ethyl]glycine was purchased from Dojindo Laboratories and was used without further purification. Cobalt(II) chloride hexahydrate was the purest product from Wako Pure Chemical Industries, Ltd. All other chemicals used were of reagent grade. All of the aqueous solutions were prepared from redistilled water. The ionic strength (*I*) of the solution was adjusted with NaCl.

Preparation of dtpa-modified cyt*c*. Horse heart cyt*c* was treated with a 1.5-fold excess of *N*,*N*-bis[2-(2,6-dioxomorpholino)ethyl]glycine in a 0.10 mol dm^{-3} tris(hydroxymethyl)aminomethane (Tris)–HCl buffer (pH 8.6 ± 0.1) at room temperature, and the solution was treated with a 3-fold excess of potassium hexacyanoferrate(III) ($K_3[Fe(CN)_6]$). The following procedures were carried out at 4 °C. After the solution was dialyzed with water and then with a 0.010 mol dm^{-3} sodium phosphate buffer at pH 6.0, it was passed through a Whatman DM-52 cellulose column at pH 6.0. The effluent was then loaded on a Whatman CM-52 cellulose column at pH 6.0 and all of the cyt*c* species were eluted with a 1.0 mol dm^{-3} NaCl solution containing a 0.010 mol dm^{-3} sodium phosphate buffer at pH 6.0. After the effluent was dialyzed with a 0.010 mol dm^{-3} sodium phosphate buffer at pH 6.0, the mixture of the cyt*c* species was separated with a Tosoh CM-5PW column (7.5 mm × 7.5 cm) in a Shimadzu LC-10 HPLC system. The elution conditions were: a linear gradient from a 0.010 mol dm^{-3} sodium phosphate buffer at pH 6.0 to a 0.10 mol dm^{-3} sodium phosphate buffer at pH 6.0, a flow rate of 1.0 mL/min, and wavelength monitoring at 280 nm.

Peptide Sequence Analysis. The modified cyt*c* was digested with lysyl endopeptidase from *Achromobacter lyticus* (Wako) at 35 °C for 8 h by a standard method (cyt*c*:enzyme = 200:1).[28–30] The peptide fragments were dissolved in an aqueous solution of 0.1% trifluoroacetic acid (TFA) and separated by reversed-phase chromatography with a Tosoh ODS 120T column (4.6 mm × 25 cm) in a Shimadzu LC-10 HPLC system. The elution conditions were: a linear gradient from a 0.1% aqueous TFA to a mixed solution of aqueous TFA with MeCN (1:1), a flow rate of 1.0 mL/min, and wavelength monitoring at 215 nm. The peptide fragments different from those of native cyt*c* were analyzed with a Hitachi L-8500 automated amino-acid analyzer and a Shimadzu PPSQ-23 automated protein/peptide sequencer.

Preparation of [cytc^{III}{Co^{III}(dtpa)}]. The modified cytc^{III}dtpa, of which Lys13 was linked with dtpa, was dialyzed with a 0.010 mol dm^{-3} Tris–HCl buffer at pH 8.0. To this solution was added a 3-fold excess of $CoCl_2 \cdot 6H_2O$, and the resulting solution was left standing at 25 °C for 24 h. The solution was oxidized by a 100-fold excess of $K_3[Fe(CN)_6]$ at 25 °C for 2 h, and was dialyzed with water, then with a 0.010 mol dm^{-3} sodium phosphate buffer at pH 7.0 containing disodium dihydrogen ethylenediaminetetraacetate (Na_2H_2edta, 1.0×10^{-4} mol dm^{-3}), and finally with a 0.010 mol dm^{-3} sodium phosphate buffer at pH 7.0. After the solution was passed through a DE-52 cellulose column, the desired [cytc^{III}{Co^{III}(dtpa)}] was separated by using a CM-cellulose column with a 0.10 mol dm^{-3} sodium phosphate buffer at pH 7.0. A small amount of cytc^{III}dtpa was detected below the [cytc^{III}{Co^{III}(dtpa)}] band on the column. The [cytc^{III}{Co^{III}(dtpa)}] species was dialyzed with a 0.010 mol dm^{-3} Tris–HCl buffer at pH 7.5 for kinetic measurements.

Kinetic Measurements. A sample solution was gently purged with high-purity Ar gas, and then carefully degassed by freeze-pump-thaw cycles. The reduced [cytc^{II}{Co^{III}(dtpa)}] was prepared in situ by reduction with a methylviologen-radical cation ($MV^{\bullet+}$), which was produced by a photoreduction of MV^{2+} with the excited triplet state of tris(2,2′-bipyridine)ruthenium(II) (^{3}([Ru(bpy)$_3$]$^{2+}$)*) in the presence of Na_2H_2edta.[31] The experimental conditions were: $(4.0–5.0) \times 10^{-6}$ mol dm^{-3} [cytc^{III}{Co^{III}(dtpa)}], 5.0×10^{-6} mol dm^{-3} [Ru(bpy)$_3$]$^{2+}$, 5.00×10^{-4} mol dm^{-3} MV^{2+}, and 5.00×10^{-3} mol dm^{-3} Na_2H_2edta at 25.0 °C, pH 7.5 (0.010 mol dm^{-3} Tris–HCl buffer), and $I = 0.50$ mol dm^{-3} (NaCl) using a Photal RA-412 pulse flash apparatus with a 30 μs pulse-width Xe lamp ($\lambda > 450$ nm; a Toshiba

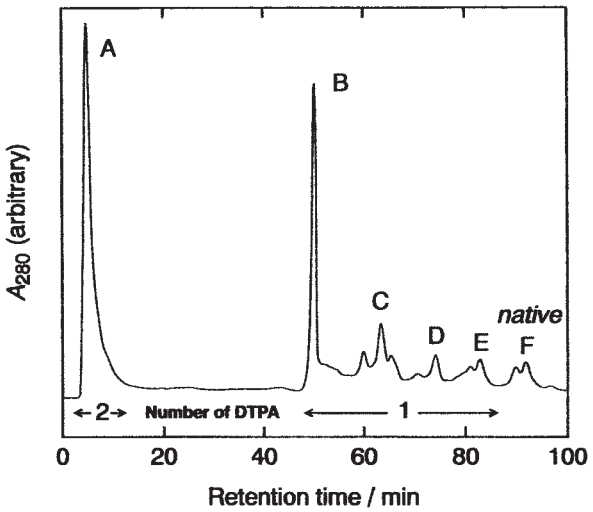

Fig. 2. HPLC profile for dtpa-modified cytc^{III}. Conditions: a linear gradient from 0.010 mol dm^{-3} to 0.10 mol dm^{-3} sodium phosphate buffer at pH 6.0, a flow rate of 1.0 mL/min, and wavelength monitoring at 280 nm.

Y-47 glass filter). The reaction of [cytc^{II}{Co^{III}(dtpa)}] was very slow and, therefore, followed by a conventional spectrophotometry with a Shimadzu UV-240 spectrophotometer.

Other Measurements. The electronic absorption spectra were recorded on Shimadzu UV-240 and MultiSpec-1500 spectrophotometers. The pHs of the solutions were measured on a Hitachi-Horiba F-14RS pH meter. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra were measured with a Finnigan MAT V2000 mass spectrometer. A protein sample, which was dialyzed with water, was diluted with an H_2O–MeCN (1:1) solution containing 0.1% TFA. A mixture of 2,6-dihydroxybenzoic acid with 5-methoxysalicylic acid (9:1) was used as a matrix. The iron and cobalt contents in [cytc^{III}{Co^{III}(dtpa)}] were analyzed with a Hitachi Z-7000 atomic absorption spectrometer at excitation wavelengths of 248.3 nm and 240.7 nm, respectively.

Results and Discussion

Characterization of [cytc^{III}{Co^{III}(dtpa)}]. An HPLC profile for cytc^{III}(dtpa)$_n$ on a CM-5PW column is shown in Fig. 2. Six main fractions (A, B, C, D, E, and F) were obtained and their yields were 46, 21, 5, 2, 2, and 2%, respectively. The m/z values for these fractions were 13105, 12769, 12720, 12734, 12747, and 12385, respectively. Since the m/z value for native cyt$c$ is 12385, it can be concluded that fraction A has two dtpa ions, and that each species from B to E contains one dtpa moiety. Species F was found to be unmodified cyt$c$. In the present work, only the main fraction of the singly modified species B was characterized and used for preparing the Co(III) complex. The ratio A_{409}/A_{280} in the electronic absorption spectrum for the species B was 4.61, and similar to that for native cytc^{III}.[32] The protein concentration was determined from the iron content by atomic-absorption spectroscopy. The molar absorption coefficients for species B were found to be $\varepsilon_{409} = 1.06 \times 10^5$ dm^3 mol^{-1} cm^{-1} and $\varepsilon_{280} = 2.30 \times 10^4$ dm^3 mol^{-1} cm^{-1}, respectively.

Figure 3 shows the HPLC profile for the peptide fragments of species B, cytc^{III}dtpa(B), digested with lysyl endopeptidase

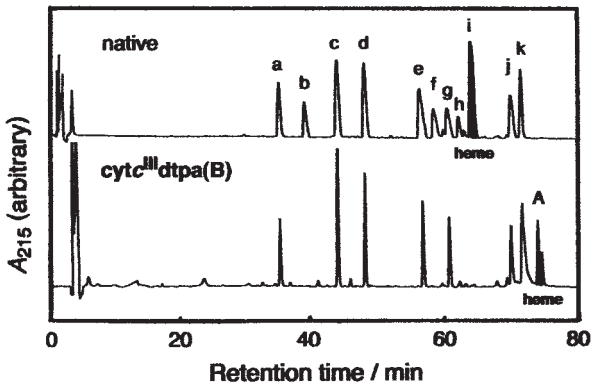

Fig. 3. HPLC profile for lysyl endopeptidase digests of cytc^{III}dtpa(B). Conditions: a linear gradient from a 0.1% aqueous TFA to a mixed solution of aqueous TFA with MeCN (1:1), a flow rate of 1.0 mL/min, and wavelength monitoring at 215 nm.

along with that for native cytc^{III}. A heme-containing fragment i, which was obtained in native cytc^{III}, disappeared in the HPLC profile for species B. A new fragment peak appeared as peak A, which contains the amino-acid sequence from Ile9 to Lys22 with heme. The peptide b (from Ile9 to Lys13) obtained in native cytc^{III} was also lost in the digest for species B. From these results, it is concluded that dtpa in species B binds the Lys13-amino group.

The Lys13-modified cytc^{III}dtpa was treated with a 3-fold excess of $CoCl_2 \cdot 6H_2O$ at 25 °C and pH 8.5 in a 0.010 mol dm^{-3} Tris–HCl buffer for 24 h, followed by oxidation with a 100-fold excess of $K_3[Fe(CN)_6]$. A small amount of cytc^{III}dtpa was found and easily separated from the desired [cytc^{III}{Co^{III}(dtpa)}] by using a CM-cellulose column; the former was found to move faster than the latter on the column. The iron:cobalt content ratio was 1.01 ± 0.06, indicating that the modified cyt$c$ contains one [Co(dtpa)] moiety.

Reduction of [cytc^{III}{Co^{III}(dtpa)}] by MV$^{\bullet+}$. A MV$^{\bullet+}$ ion was produced by the photoreduction of MV^{2+} by $^3([Ru(bpy)_3]^{2+})^*$,[31] given by

$$[Ru(bpy)_3]^{2+} \overset{h\nu}{\rightleftarrows} {}^3([Ru(bpy)_3]^{2+})^*, \quad (2a)$$

$$MV^{2+} + {}^3([Ru(bpy)_3]^{2+})^* \rightarrow MV^{\bullet+} + [Ru(bpy)_3]^{3+}, \quad (2b)$$

$$[Ru(bpy)_3]^{3+} + Hedta^{3-} \rightarrow [Ru(bpy)_3]^{2+} + (edta)_{ox}. \quad (2c)$$

Here, $(edta)_{ox}$ is an oxidation product of $Hedta^{3-}$. With one flash, a 5.0×10^{-7} mol dm^{-3} MV$^{\bullet+}$ ion was produced under the present experimental conditions.

The formation of the reduced cytc^{II} and the decays of the oxidized cytc^{III} and MV$^{\bullet+}$ in the reaction of [cytc^{III}{Co^{III}(dtpa)}] with MV$^{\bullet+}$ (Eq. 3a) were monitored at 415 nm, 360 nm, and 600 nm, respectively. The reaction was completed within 5 ms. The second-order rate constant for Eq. 3a was $(2.4 \pm 0.3) \times 10^8$ dm^3 mol^{-1} s^{-1}, which is similar to that for the reduction of native cytc^{III} by MV$^{\bullet+}$($(2.5 \pm 0.3) \times 10^8$ dm^3 mol^{-1} s^{-1} at $I = 0.50$ mol dm^{-3} (this work) and 2.3×10^8 dm^3 mol^{-1} s^{-1} at $I = 0.2$ mol dm^{-3}[33]). The reduction of the [Co^{III}(dtpa)] moiety could not be detected, because the absorption of the Co(III) complex is much smaller than that

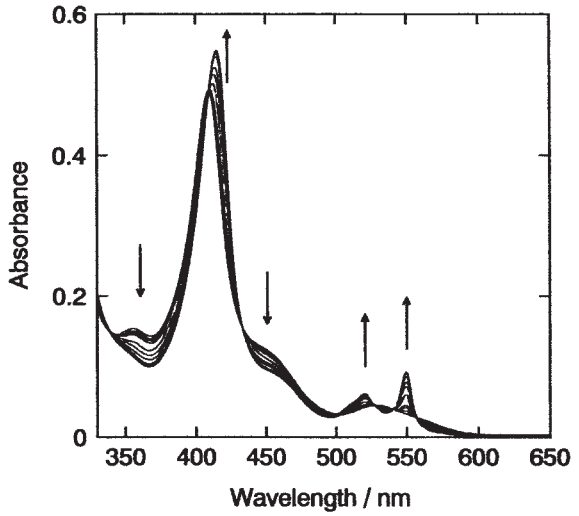

Fig. 4. Absorption spectra after the reduction of $[cytc^{III}\{Co^{III}(dtpa)\}]$ (4.6×10^{-6} mol dm^{-3}) by $MV^{\bullet+}$ which is produced by the photoreduction of MV^{2+} (5.00×10^{-4} mol dm^{-3}) with a Xe flash lamp in the presence of $[Ru(bpy)_3]^{2+}$ (5.0×10^{-6} mol dm^{-3}) and Na_2H_2edta (5.00×10^{-3} mol dm^{-3}) at 25 °C, pH 7.5 (0.010 mol dm^{-3} Tris–HCl buffer), and $I = 0.50$ mol dm^{-3} (NaCl). Each spectrum was recorded at 20 s after flashing.

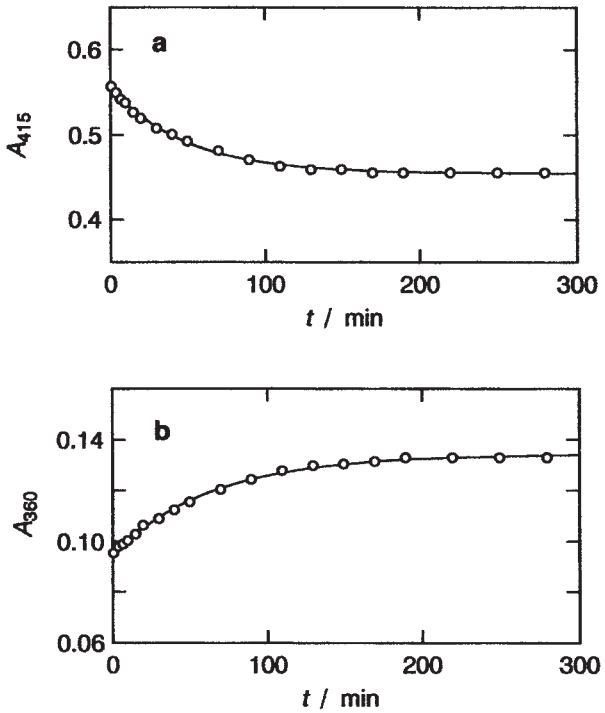

Fig. 5. Changes in absorbance after recording the final spectrum in Fig. 4. (a) Decay of $[cytc^{II}\{Co^{III}(dtpa)\}]$ at 415 nm. (b) Formation of $[cytc^{III}\{Co^{II}(dtpa)\}]$ at 360 nm.

of cytc^{III}. Therefore, we used $[Co^{III}(edta)]^-$ as a model of the Co(III) moiety of $[cytc^{III}\{Co^{III}(dtpa)\}]$. Since the second-order rate constant of the reduction of $[Co^{III}(edta)]^-$ by $MV^{\bullet+}$ was $(2.1 \pm 0.2) \times 10^8$ dm^3 mol^{-1} s^{-1} at $I = 0.50$ mol dm^{-3}, being comparable to that for the reduction of the cytc^{III} moiety in $[cytc^{III}\{Co^{III}(dtpa)\}]$, reaction (3b) also occurs:

$$[cytc^{III}\{Co^{III}(dtpa)\}] + MV^{\bullet+} \rightarrow [cytc^{II}\{Co^{III}(dtpa)\}] + MV^{2+}, \quad (3a)$$

$$[cytc^{III}\{Co^{III}(dtpa)\}] + MV^{\bullet+} \rightarrow [cytc^{III}\{Co^{II}(dtpa)\}] + MV^{2+}. \quad (3b)$$

Figure 4 shows the absorption spectra after the reduction of $[cytc^{III}\{Co^{III}(dtpa)\}]$ by $MV^{\bullet+}$. Each spectrum was obtained at 20 s after flashing with a Xe pulse flash lamp (a 30 μs pulse width). The concentrations of $[cytc^{II}\{Co^{III}(dtpa)\}]$ and $[cytc^{III}\{Co^{III}(dtpa)\}]$ remaining after the reduction by $MV^{\bullet+}$ were estimated. The total concentrations of cytc^{II} ($[cytc^{II}]_T = [[cytc^{II}\{Co^{III}(dtpa)\}]]_0 + [[cytc^{II}\{Co^{II}(dtpa)\}]]_0$) were determined from the change in the absorbance at 415 nm. Because the rates of the reductions of the cytc^{III} and Co(III) sites by $MV^{\bullet+}$ are similar to each other, as mentioned above, it is assumed that the same concentrations of $[cytc^{II}\{Co^{III}(dtpa)\}]$ and $[cytc^{III}\{Co^{II}(dtpa)\}]$ are formed. After the second flash $[cytc^{II}\{Co^{III}(dtpa)\}]$, $[cytc^{III}\{Co^{II}(dtpa)\}]$, and the remaining $[cytc^{III}\{Co^{III}(dtpa)\}]$ react with $MV^{\bullet+}$ to produce $[cytc^{II}\{Co^{II}(dtpa)\}]$, $[cytc^{II}\{Co^{III}(dtpa)\}]$, and $[cytc^{III}\{Co^{II}(dtpa)\}]$. The results are summarized in Table 1.

Reaction of $[cytc^{II}\{Co^{III}(dtpa)\}]$. Considering the free-energy change between $[cytc^{II}\{Co^{III}(dtpa)\}]$ and $[cytc^{III}\{Co^{II}(dtpa)\}]$ ($\Delta G^0 = -0.16$ eV), being estimated from the redox potentials of cytc^{III}/cytc^{II} (0.26 V)[34] and Co^{III}/Co^{II} (0.42 V),[26] the reaction of $[cytc^{II}\{Co^{III}(dtpa)\}]$ to produce $[cytc^{III}$

Table 1. Distribution of the Cytochrome *c* Species and the First-Order Rate Constants for the Reaction of $[cytc^{II}\{Co^{III}(dtpa)\}]$ at 25 °C, pH 7.5 (a 0.010 mol dm^{-3} Tris–HCl buffer), and $I = 0.50$ mol dm^{-3} (NaCl)

$[cytc^{III}Co(III)]_0$[a]	$[cytc^{II}]_T$[b]	$[cytc^{II}Co(III)]_0$[c]	$[cytc^{II}Co(II)]_0$[d]	$[cytc^{III}Co(III)]_{remaining}$[e]	k_1
10^{-6} mol dm^{-3}	10^{-6} mol dm^{-3}	10^{-6} mol dm^{-3}	10^{-6} mol dm^{-3}	10^{-6} mol dm^{-3}	10^{-4} s^{-1}
4.0	1.1	0.90	0.20	2.0	2.8
4.0	0.79	0.70	0.09	2.5	2.8
4.0	0.51	0.48	0.03	3.0	3.3
4.0	0.25	0.25	0	3.5	3.4
4.6	2.1	1.4	0.70	1.1	3.0

a) $[cytc^{III}Co(III)]_0 = [[cytc^{III}\{Co^{III}(dtpa)\}]]_0$. b) $[cytc^{II}]_T = [[cytc^{II}\{Co^{III}(dtpa)\}]]_0 + [[cytc^{II}\{Co^{II}(dtpa)\}]]_0$. The $[cytc^{II}]_T$ was determined spectrophotometrically at 415 nm and it was assumed that $MV^{\bullet+}$ reacts with $[cytc^{III}\{Co^{III}(dtpa)\}]$ to produce 1:1 molar ratio of $[cytc^{II}\{Co^{III}(dtpa)\}]$ and $[cytc^{III}\{Co^{II}(dtpa)\}]$. c) $[cytc^{II}Co(III)]_0 = [[cytc^{II}\{Co^{III}(dtpa)\}]]_0$. d) $[cytc^{II}Co(II)]_0 = [[cytc^{II}\{Co^{II}(dtpa)\}]]_0$. e) $[cytc^{III}Co(III)]_{remaining} = [[cytc^{III}\{Co^{III}(dtpa)\}]]_{remaining}$.

{Co^{II}(dtpa)}] is expected to occur exothermically.[35] In fact, the decay of [cytc^{II}{Co^{III}(dtpa)}] and the formation of [cytc^{III}{Co^{II}(dtpa)}] are found at 415 nm and 360 nm, respectively, after reduction by $MV^{\bullet+}$, as is shown in Fig. 5. The reaction obeys the first-order rate law on the protein concentration:

$$-d[[cyt c^{II}\{Co^{III}(dtpa)\}]]/dt = k_1[[cyt c^{II}\{Co^{III}(dtpa)\}]]. \quad (4)$$

The observed first-order rate constants are summarized in Table 1. The observed first-order rate constant (k_1) was independent of the remaining concentrations of [cytc^{III}{Co^{III}(dtpa)}] ([[cytc^{III}{Co^{III}(dtpa)}]]$_{remaining}$). Therefore, the following *intra*molecular ET process (Eq. 5) predominantly occurs; that is, $k_1 = k_{et}$:

$$[cyt c^{II}\{Co^{III}(dtpa)\}] \rightarrow [cyt c^{III}\{Co^{II}(dtpa)\}] \quad k_{et}. \quad (5)$$

The *inter*molecular ET reactions between cytc^{II} and [Co^{III}(dtpa)] moieties are negligible (Eqs. 6–8) under the present experimental conditions:

$$[cyt c^{II}\{Co^{III}(dtpa)\}] + [cyt c^{III}\{Co^{III}(dtpa)\}] \rightarrow [cyt c^{III}\{Co^{III}(dtpa)\}] + [cyt c^{III}\{Co^{II}(dtpa)\}], \quad (6)$$

$$[cyt c^{II}\{Co^{II}(dtpa)\}] + [cyt c^{II}\{Co^{III}(dtpa)\}] \rightarrow [cyt c^{III}\{Co^{II}(dtpa)\}] + [cyt c^{II}\{Co^{II}(dtpa)\}], \quad (7)$$

$$[cyt c^{II}\{Co^{II}(dtpa)\}] + [cyt c^{III}\{Co^{III}(dtpa)\}] \rightarrow 2[cyt c^{III}\{Co^{II}(dtpa)\}]. \quad (8)$$

The reaction of cytc^{II} with [Co^{III}(edta)]$^-$ was also examined under the same experimental conditions to confirm the absence of *inter*molecular ET reactions (6)–(8). After photo-irradiating the solution containing equimolar amounts of cytc^{III} and [Co^{III}(edta)]$^-$(4.00×10^{-6} mol dm^{-3}) in the presence of [$Ru(bpy)_3$]$^{2+}$ and Na_2H_2edta, the formation of cytc^{II} was observed. However, the successive reaction between cytc^{II} and [Co^{III}(edta)]$^-$ was not detected during the same time scale, as shown in Fig. 5.

Evaluation of Slow Rate Constant for the Intramolecular ET Reaction of [cytc^{II}{Co^{III}(dtpa)}]. The rate constant 3.1×10^{-4} s^{-1} at 25 °C is extraordinarily small compared with the previously reported values of the *intra*molecular ET reaction of cyt*c*.[1–4,6–8,10,20,21] We confirmed the validity of the rate constant based on Eq. 1. If the ET from heme to the Co(Ⅲ) moiety occurs by a through-space interaction, the edge-to-edge distance between the donor and the acceptor becomes 9.4 Å from the crystal structure of horse heart cytc^{III}.[36] The Lys residue is flexible and lies over 6–10 Å in the edge-to-edge distance from the heme and the ε-amino group of Lys13.[37,38] If the values of 6–10 Å are assumed for $r - r_0$, the β values are estimated to be 1.6–2.7 Å^{-1} by using the following parameters: $k_{et} = 3.1 \times 10^{-4}$ s^{-1}, $\lambda = 2.56$ eV (the values of λ are 1.0 eV and 4.12 eV for cyt*c*[1] and Co(Ⅲ),[39] respectively), and $\Delta G^0 = -0.16$ eV. These β values are much larger than the previously reported ones (0.8 Å$^{-1} \leq \beta \leq$ 1.2 Å^{-1}). If the mean value of β (=1.0 Å^{-1}) is adopted, the estimated k_{et} values are 0.13 s$^{-1} \leq k_{et} \leq$ 7.4 s^{-1}. These are much larger than the observed rate constant.

On the other hand, the through-bond mechanism from the heme to the Co(Ⅲ) complex via the Cys 14 and Lys 13 residues over 18.6 Å (see Fig. 1) results in the calculated β value of 0.87 Å^{-1}, being reasonable for the above region of β. Therefore, the *intra*molecular ET reaction for the present system might be explained by the through-bond mechanism over 18.6 Å.

Equation 1 can be replaced by Eq. 9 by using the electronic matrix element between reactants and products at the transition state (H_{RP}),[1]

$$k_{et} = (4\pi^3/h^2\lambda RT)^{1/2} \cdot (H_{RP})^2 \cdot \exp[-(\Delta G^0 + \lambda)^2/4\lambda RT]. \quad (9)$$

Here, h is Planck's constant. By using the parameters mentioned above and the observed k_{et} value, we can estimate the H_{RP} value to be 0.079 cm^{-1}, which is quite reasonable for the ET reactions of cyt*c*.[4,6,10]

In the case of the ET reaction of Co(Ⅲ)/Co(Ⅱ) couples, nonadiabaticity has been discussed.[1,40–42] Larsson et al.[42] have demonstrated that ET occurs via the ^{2}E state between [$Co(NH_3)_6$]$^{3+}$ and [$Co(NH_3)_6$]$^{2+}$ by a spin-preequilibrium mechanism, and that the system is close to the limits of nonadiabatic ET; that is, the spin multiplicity change is not a main factor. Since the ligand field of the [Co^{III}(dtpa)] complex is weaker than the ammine complex, the spin multiplicity change might not mainly contribute to the activation energy for the ET of [cytc^{II}{Co^{III}(dtpa)}]. Therefore, the slow *intra*molecular ET rate for [cytc^{II}{Co^{III}(dtpa)}] might arise mainly from the large reorganization energy for the Co(Ⅲ)/Co(Ⅱ) couple ($\lambda = 4.12$ eV) and the long distance of 18.6 Å.

In conclusion, we found a slow *intra*molecular ET reaction of [cytc^{II}{Co^{III}(dtpa)}] with a time scale of hours. The reaction proceeds by a through-bond mechanism via Cys14 and Lys13 residues over 18.6 Å. The slow *intra*molecular ET rate arises not only from the very large reorganization energy for the Co(Ⅲ)/Co(Ⅱ) couple, but also from the long distance of 18.6 Å. This slow ET reaction in the Marcus normal region is also found for the back ET in the final step of photosynthetic bacterial reaction centers.[43] Therefore, the present system is a very interesting biomimic model of the photosynthetic charge-separation state.

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