<table>
<thead>
<tr>
<th>Title</th>
<th>Intramolecular Electron Transfer of Cytochrome c Modified with N,N,N′,N″,N″-Diethylenetriamintetraacetatocobaltate(III)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author(s)</td>
<td>TSUKAHARA, Keiichi, IIDA, Nao, KAIDA, Yuka, TAKASHIMA, Hiroshi, MIZOBE, Maya, ARAKAWA, Ryuichi</td>
</tr>
<tr>
<td>Citation</td>
<td>Bulletin of the Chemical Society of Japan, 76(7): 1323-1328</td>
</tr>
<tr>
<td>Issue Date</td>
<td>2003</td>
</tr>
<tr>
<td>URL</td>
<td><a href="http://hdl.handle.net/10112/6148">http://hdl.handle.net/10112/6148</a></td>
</tr>
<tr>
<td>Rights</td>
<td>Type</td>
</tr>
<tr>
<td>Textversion</td>
<td>publisher</td>
</tr>
</tbody>
</table>
Intramolecular Electron Transfer of Cytochrome c Modified with N,N,N’,N’’-Diethylenetriaminepentaacetatocobaltate(III)

Keiichi Tsukahara,‡ Nao Iida, Yuka Kaida, Hiroshi Takashima, Maya Mizobe,† and Ryuichi Arakawa‡

Department of Chemistry, Faculty of Science, Nara Women’s University, Nara 630-8506
†Department of Applied Chemistry, Faculty of Engineering, Kansai University, Suita 564-8680

(Received January 23, 2003)

Horse heart cytochrome c (cytc) modified with an N,N,N’,N’’-diethylenetriaminepentaacetatocobaltate(III) ion, [cytc^B(-Co^B(dtpa))], was prepared and characterized. The major component of singly modified [cytc^B(-Co^B(dtpa))] contains a [Co^B(dtpa)] ion at Lys13, of which the ε-amino group is linked with a carboxylate ion of dtpa. A one-electron reduced protein, [cytc^B(-Co^B(dtpa))], was prepared by reduction with a methylviologen-radical cation, which was produced in situ by a photoreduction using a tris(2,2’-bipyridine)rhuthenium(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 [cytc^B(-Co^B(dtpa))] to form [cytc^B^red(-Co^B^red(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 ± 0.5) × 10^-8 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 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

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

Here, \( r \) is the distance between the donor and acceptor centers, \( r_0 \) is the contact distance at which the reaction becomes adiabatic, \( \beta \) is the decay constant which describes the sensitivity of the coupling to changes in distance, \( \Delta G^\circ \) is the diving force, \( \lambda \) 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 \( \beta \) 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 (cytc^B) attached to a Co(II) cage complex is independent of the 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 cytc.

We recently reported on artificial metmyoglobin, of which Lys residues are modified with one of the strong chelating reagents, N,N,N’,N’’-diethylenetriaminepentaacetic acid (metMb(dtpa)) or n = 1, 2, 4, and 5; see Chart 1), and their cobalt(III) complexes ([metMb[Co^B^red(dtpa)]_n]_c, n = 1, 2, 4, and 5).22–26 In this work, we applied a chemical modification by dtpa to cytc, where the ε-amino group of Lys13 is linked with
through-space distance is, however, completely different from in the case of residues (see Fig. 1). It is, therefore, expected to distinguish was purchased from Dojindo Laboratories and was used without was adjusted with NaCl. Preparation of dtpa-modified cyt The modified cyt was digested with lysyl endopeptidase from Achromobacter lyticus (Wako) at 35 °C for 8 h by a standard method (cyt : enzyme = 200:1). 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 0.010 mol dm−3 sodium phosphate buffer at pH 6.0 to a 0.10 mol dm−3 sodium phosphate buffer at pH 7.0, a flow rate of 1.0 mL/min, and wavelength monitoring at 215 nm. The peptide fragments different from those of native cyt were analyzed with a Hitachi L-8500 automated amino-acid analyzer and a Shimadzu PPSQ-23 automated protein/peptide sequencer. Preparation of [cyt]CO3(dtpa)2. The modified cyt 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 CoCl2·6H2O, and the resulting solution was left standing at 25 °C for 24 h. The solution was oxidized by a 100-fold excess of K3[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 ethylenediaminetetra-acetate (Na2H2edta, 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 [cyt]CO3(dtpa)2] 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 cyt dtpa was detected below the [cyt]CO3(dtpa)2] band on the column. The [cyt]CO3(dtpa)2] 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 [cyt]CO3(dtpa)2] was prepared in situ by reduction with a methylviologen-radical cation (MV+), which was produced by a photoreduction of MV2+ in the excited triplet state of tris(2,2′-bipyridine)ruthenium(II) ((Ru(bpy)3)2+). The experimental conditions were: (4.0–5.0) × 10−6 mol dm−3 [cyt]CO3(dtpa)2], 5.0 × 10−6 mol dm−3 [Ru(bpy)3]2+, 5.00 × 10−4 mol dm−3 MV2+, and 5.00 × 10−3 mol dm−3 Na2H2edta 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 Photol RA-412 pulse flash apparatus with a 30 μs pulse-width Xe lamp (λ > 450 nm; a Toshiba

Experimental

Materials. Horse heart cyt (Sigma) was purified as previously described. 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 were used 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. Horse heart cyt 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)amino-methane (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) (K3[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 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 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 was digested with lysyl endopeptidase from Achromobacter lyticus (Wako) at 35 °C for 8 h by a standard method (cyt : enzyme = 200:1). 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 were analyzed with a Hitachi L-8500 automated amino-acid analyzer and a Shimadzu PPSQ-23 automated protein/peptide sequencer.

Preparation of [cyt]CO3(dtpa)2. The modified cyt 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 CoCl2·6H2O, and the resulting solution was left standing at 25 °C for 24 h. The solution was oxidized by a 100-fold excess of K3[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 ethylenediaminetetra-acetate (Na2H2edta, 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 [cyt]CO3(dtpa)2] 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 cyt dtpa was detected below the [cyt]CO3(dtpa)2] band on the column. The [cyt]CO3(dtpa)2] 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 [cyt]CO3(dtpa)2] was prepared in situ by reduction with a methylviologen-radical cation (MV+), which was produced by a photoreduction of MV2+ in the excited triplet state of tris(2,2′-bipyridine)ruthenium(II) ((Ru(bpy)3)2+). The experimental conditions were: (4.0–5.0) × 10−6 mol dm−3 [cyt]CO3(dtpa)2], 5.0 × 10−6 mol dm−3 [Ru(bpy)3]2+, 5.00 × 10−4 mol dm−3 MV2+, and 5.00 × 10−3 mol dm−3 Na2H2edta 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 Photol RA-412 pulse flash apparatus with a 30 μs pulse-width Xe lamp (λ > 450 nm; a Toshiba

Fig. 1. Display of heme linked with Cys14, Lys13, and a [Co3(dtpa)] moiety in horse heart cyt.

Values for these fractions were 13105, 12769, 12720, 12734, 12095, and 12075, respectively. The yield of fraction A was 46%, fraction B was 21%, fraction C was 5%, fraction D was 2%, fraction E was 2%, and fraction F was 2%. The iron to cobalt content ratio was 4.61, and similar to that for native cyt\textsuperscript{c}. Species F was found to be unmodified cyt\textsuperscript{c}. 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 cyt\textsuperscript{c}.\textsuperscript{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 \( \varepsilon_{409} = 1.06 \times 10^4 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1} \) and \( \varepsilon_{280} = 2.30 \times 10^3 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1} \), respectively.

Figure 2 shows the HPLC profile for the peptide fragments of species B, cyt\textsuperscript{c}dtpa(B), digested with lysyl endopeptidase along with that for native cyt\textsuperscript{c}. A heme-containing fragment i, which was obtained in native cyt\textsuperscript{c}, 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 cyt\textsuperscript{c} 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 cyt\textsuperscript{c}dtpa was treated with a 3-fold excess of CoCl\textsubscript{2}·6H\textsubscript{2}O at 25 °C and pH 8.5 in a 0.010 mol dm\textsuperscript{-3} Tris–HCl buffer for 24 h, followed by oxidation with a 100-fold excess of K\textsubscript{3}[Fe(CN)]\textsubscript{6}. A small amount of cyt\textsuperscript{c}dtpa was found and easily separated from the desired [cyt\textsuperscript{c}(Co\textsuperscript{II}(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\textsuperscript{c} contains one [Co(dtpa)] moiety.

Reduction of [cyt\textsuperscript{c}(Co\textsuperscript{II}(dtpa))] by MV\textsuperscript{2+}. A MV\textsuperscript{2+} ion was produced by the photoreduction of MV\textsuperscript{2+} by \( 3[(\text{Ru(bpy)}_3)^{2+}]_s \),\textsuperscript{31} given by

\[
[Ru(bpy)_3]^{2+} \overset{hv}{\longrightarrow} 3[(Ru(bpy)_3)]^{2+}, \quad (2a)
\]

\[
\text{MV}^{2+} + 3[(\text{Ru(bpy)}_3)^{2+}]_s \rightarrow \text{MV}^{++} + [\text{Ru(bpy)}_3]^{3+}, \quad (2b)
\]

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

Here, (edta)\textsubscript{ox} is an oxidation product of Hedta\textsuperscript{3-}. With one flash, a 5.0 × 10\textsuperscript{-7} mol dm\textsuperscript{-3} MV\textsuperscript{2+} ion was produced under the present experimental conditions.

The formation of the reduced cyt\textsuperscript{c} and the decays of the oxidized cyt\textsuperscript{c} and MV\textsuperscript{++} in the reaction of [cyt\textsuperscript{c}(Co\textsuperscript{II}(dtpa))] with MV\textsuperscript{++} (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 ± 0.3) × 10\textsuperscript{8} dm\textsuperscript{3} mol\textsuperscript{-1} s\textsuperscript{-1}, which is similar to that for the reduction of native cyt\textsuperscript{c} by MV\textsuperscript{++} ((2.5 ± 0.3) × 10\textsuperscript{8} dm\textsuperscript{3} mol\textsuperscript{-1} s\textsuperscript{-1} at \( I = 0.50 \text{ mol dm}^{-3} \) (this work) and 2.3 × 10\textsuperscript{8} dm\textsuperscript{3} mol\textsuperscript{-1} s\textsuperscript{-1} at \( I = 0.2 \text{ mol dm}^{-3} \)). The reduction of the [Co\textsuperscript{II}(dtpa)] moiety could not be detected, because the absorption of the Co(II) complex is much smaller than that of the reduced cyt\textsuperscript{c}.
of cyt\textsuperscript{b}. Therefore, we used [Co\textsuperscript{b}](edta)\textsuperscript{−} as a model of the Co(III) moiety of [cyt\textsuperscript{b}][Co\textsuperscript{b}(edta)]\textsuperscript{−}. Since the second-order rate constant of the reduction of [Co\textsuperscript{b}(edta)]\textsuperscript{−} by MV\textsuperscript{2+} was (2.1 ± 0.2) \times 10^{8} \text{ mol} \text{ dm}^{-3} \text{ s}^{-1} at I = 0.50 \text{ mol dm}^{-3}, being comparable to that for the reduction of the cyt\textsuperscript{b} moiety in [cyt\textsuperscript{b}][Co\textsuperscript{b}(dtpa)], reaction (3b) also occurs:

\[
\text{[cyt}^{\text{b}}][\text{Co}^{\text{b}}(\text{dtpa})] + \text{MV}^{2+} \\
\rightarrow \text{[cyt}^{\text{b}}][\text{Co}^{\text{b}}(\text{dtpa})] + \text{MV}^{2+}, \quad (3a)
\]

\[
\text{[cyt}^{\text{b}}][\text{Co}^{\text{b}}(\text{dtpa})] + \text{MV}^{2+} \\
\rightarrow \text{[cyt}^{\text{b}}][\text{Co}^{\text{b}}(\text{dtpa})] + \text{MV}^{2+}. \quad (3b)
\]

Figure 4 shows the absorption spectra after the reduction of [cyt\textsuperscript{b}][Co\textsuperscript{b}(dtpa)] by MV\textsuperscript{2+}. Each spectrum was obtained at 20 s after flashing with a Xe pulse lamp in the presence of [Ru(bpy)]\textsuperscript{2+} (5.00 \times 10^{-4} \text{ mol dm}^{-3}) and Na\textsubscript{2}HEDTA (5.00 \times 10^{-3} \text{ mol dm}^{-3}) at 25°C, pH 7.5, (0.010 \text{ mol dm}^{-3} Tris–HCl buffer), and I = 0.50 mol dm\textsuperscript{−3} (NaCl). Each spectrum was recorded at 20 s after flashing.

![Absorption spectra](image)

**Figure 4.** Absorption spectra after the reduction of [cyt\textsuperscript{b}][Co\textsuperscript{b}(dtpa)] (4.6 \times 10^{-6} \text{ mol dm}^{-3}) by MV\textsuperscript{2+} which is produced by the photoreduction of MV\textsuperscript{2+} (5.00 \times 10^{-4} \text{ mol dm}^{-3}) with a Xe flash lamp in the presence of [Ru(bpy)]\textsuperscript{2+} (5.00 \times 10^{-6} \text{ mol dm}^{-3}) and Na\textsubscript{2}HEDTA (5.00 \times 10^{-3} \text{ mol dm}^{-3}) at 25°C, pH 7.5, (0.010 \text{ mol dm}^{-3} Tris–HCl buffer), and I = 0.50 mol dm\textsuperscript{−3} (NaCl). Each spectrum was recorded at 20 s after flashing.

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

<table>
<thead>
<tr>
<th>[cyt\textsuperscript{b}][Co\textsuperscript{b}(dtpa)]\textsuperscript{0\textsuperscript{a}}</th>
<th>[cyt\textsuperscript{b}][Co\textsuperscript{b}(dtpa)]\textsuperscript{T\textsuperscript{b}}</th>
<th>[cyt\textsuperscript{b}][Co\textsuperscript{b}(dtpa)]\textsuperscript{0\textsuperscript{c}}</th>
<th>[cyt\textsuperscript{b}][Co\textsuperscript{b}(dtpa)]\textsuperscript{0\textsuperscript{d}}</th>
<th>[cyt\textsuperscript{b}][Co\textsuperscript{b}(dtpa)]\textsuperscript{remaining\textsuperscript{e}}</th>
<th>k\textsuperscript{1}</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^{-6} \text{ mol dm}^{-3}</td>
<td>10^{-6} \text{ mol dm}^{-3}</td>
<td>10^{-6} \text{ mol dm}^{-3}</td>
<td>10^{-6} \text{ mol dm}^{-3}</td>
<td>10^{-6} \text{ mol dm}^{-3}</td>
<td>10^{-4} \text{ s}^{-1}</td>
</tr>
<tr>
<td>4.0</td>
<td>1.1</td>
<td>0.90</td>
<td>0.20</td>
<td>2.0</td>
<td>2.8</td>
</tr>
<tr>
<td>4.0</td>
<td>0.79</td>
<td>0.70</td>
<td>0.09</td>
<td>2.5</td>
<td>2.8</td>
</tr>
<tr>
<td>4.0</td>
<td>0.51</td>
<td>0.48</td>
<td>0.03</td>
<td>3.0</td>
<td>3.3</td>
</tr>
<tr>
<td>4.0</td>
<td>0.25</td>
<td>0.25</td>
<td>0.09</td>
<td>3.5</td>
<td>3.4</td>
</tr>
<tr>
<td>4.6</td>
<td>2.1</td>
<td>1.4</td>
<td>0.70</td>
<td>1.1</td>
<td>3.0</td>
</tr>
</tbody>
</table>

\( a) \text{[cyt}^{\text{b}}][\text{Co}^{\text{b}}(\text{dtpa})]_{0} = \text{[[cyt}^{\text{b}}][\text{Co}^{\text{b}}(\text{dtpa})]]_{0} \), \( b) \text{[cyt}^{\text{b}}][\text{Co}^{\text{b}}(\text{dtpa})]_{T} = \text{[[cyt}^{\text{b}}][\text{Co}^{\text{b}}(\text{dtpa})]]_{0} + \text{[[cyt}^{\text{b}}][\text{Co}^{\text{b}}(\text{dtpa})]]_{0} \), \( c) \text{[cyt}^{\text{b}}][\text{Co}^{\text{b}}(\text{dtpa})]_{0} = \text{[[cyt}^{\text{b}}][\text{Co}^{\text{b}}(\text{dtpa})]]_{0} \), \( d) \text{[cyt}^{\text{b}}][\text{Co}^{\text{b}}(\text{dtpa})]_{0} = \text{[[cyt}^{\text{b}}][\text{Co}^{\text{b}}(\text{dtpa})]]_{0} \), \( e) \text{[cyt}^{\text{b}}][\text{Co}^{\text{b}}(\text{dtpa})]_{remaining} = \text{[[cyt}^{\text{b}}][\text{Co}^{\text{b}}(\text{dtpa})]]_{remaining} \).
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 [cyt$^b$(dtpa)]$^-$ ([cyt$^b$(dtpa)]$^-$remaining). Therefore, the following intramolecular ET process (Eq. 5) predominantly occurs; that is, $k_1 = k_{et}$:

$$\text{[cyt}^b\text{][Co}^3\text{(dtpa)}] \rightarrow \text{[cyt}^b\text{][Co}^3\text{(dtpa)}] \rightarrow \text{[cyt}^b\text{][Co}^3\text{(dtpa)}] \rightarrow \text{[cyt}^b\text{][Co}^3\text{(dtpa)}] \rightarrow 2\text{[cyt}^b\text{][Co}^3\text{(dtpa)}].$$

The reaction of cyt$^b$ with [Co$^3$(edta)$^-$] was also examined under the same experimental conditions to confirm the absence of intermolecular ET reactions (6)–(8). After photo-irradiating the solution containing equimolar amounts of cyt$^b$ and [Co$^3$(edta)$^-$]$^-$ (4.00 × 10$^{-6}$ mol dm$^{-3}$) in the presence of [Ru(bpy)$_3$]$^{2+}$ and Na$_2$H$_2$edta, the formation of cyt$^b$ was observed. However, the successive reaction between cyt$^b$ and [Co$^3$(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 [cyt$^b$(Co$^3$(dtpa))].** The rate constant 3.1 × 10$^{-7}$ s$^{-1}$ at 25 °C is extraordinarily small compared with the previously reported values of the intramolecular ET reaction of cyt$^b$. The Lys residue is flexible and lies over 6–10 Å in the edge-to-edge distance from the donor and the acceptor becomes 9.4 Å from the crystal structure of horse heart cyt$^b$. The Lys residue is flexible and lies over 6–10 Å in the edge-to-edge distance from the heme and the e-amino group of Lys13. 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: $k_0 = 3.1 \times 10^{-14}$ s$^{-1}$, $λ = 2.56$ eV (the values of $λ$ are 1.0 eV and 4.12 eV for cyt$^c$ and Co(III), respectively), and $ΔG^0 = -0.16$ eV. These $β$ values are much larger than the previously reported ones (0.8 Å$^{-1} \leq β \leq 1.2$ 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 intramolecular 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 ($H_{RP}$):

$$k_{et} = (4π/\hbar^2λRT)^{1/2} \cdot (H_{RP})^2 \cdot \exp[-(ΔG^0 + λ)^2/4λRT].$$

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$^b$.

In the case of the ET reaction of Co(III)/Co(II) couples, nonadiabaticity has been discussed. Larsson et al. have demonstrated that ET occurs via the $\pi^E$ state between [Co(N-H$_2$)$_2$]$^{3+}$ and [Co(NH$_3$)$_5$]$^{3+}$ by a spin-adiabatic 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$^3$(dtpa)] complex is weaker than the ammine complex, the spin multiplicity change might not contribute to the activation energy for the ET of [cyt$^b$][Co$^3$(dtpa)]. Therefore, the slow intramolecular ET rate for [cyt$^b$][Co$^3$(dtpa)] might arise mainly from the large reorganization energy for the Co(III)/Co(II) couple ($λ = 4.12$ eV) and the long distance of 18.6 Å.

In conclusion, we found a slow intramolecular ET reaction of [cyt$^b$][Co$^3$(dtpa)] with a time scale of hours. The reaction proceeds through a through-bond mechanism via Cyts14 and Lys13 residues over 18.6 Å. The slow intramolecular 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. Therefore, the present system is a very interesting biomimetic model of the photosynthetic charge-separation state.

This research was partly supported by a Grant-in-Aid for Scientific Research on Priority Areas (417) from the Ministry of Education, Culture, Sports, Science and Technology.

**References**

35 We assume that both redox potentials for cyt c\textsuperscript{b}/cyt c\textsuperscript{a} and Co(II)/Co(III) are not much different from native cyt c and the Co(II)(dtpa) complex attached to myoglobin.