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Intramolecular Electron-Transfer Pathway for Deoxy and Zinc Myoglobins Modified with *N*,*N*,*N*′,*N*″,*N*″-Diethylenetriaminepentaacetatocobaltate(III)

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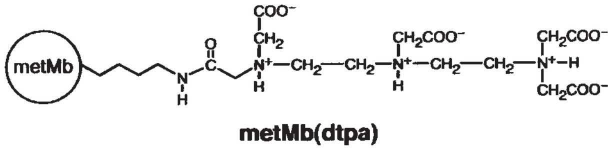
Horse heart metmyoglobin (metMb), whose N-terminal Gly1 is linked by an *N*,*N*,*N*′,*N*″,*N*″-diethylenetriaminepentaacetatocobaltate(III) ion ([Co^{III}(dtpa)]) with an amide bond, was prepared and characterized. A one-electron reduced protein, [deoxyMb{Co^{III}(dtpa)}], was prepared by reduction with 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 (a 0.010 mol dm^{-3} tris(hydroxymethyl)aminomethane–HCl buffer), and an ionic strength of 0.50 mol dm^{-3} (NaCl). The reaction of [deoxyMb{Co^{III}(dtpa)}] to form [metMb{Co^{II}(dtpa)}] obeyed a first-order rate law on the protein concentration. The first-order rate constant was dependent on the concentration of the protein, indicating that both intra- and intermolecular electron-transfer (ET) processes simultaneously occur. The latter is the reaction with excess [metMb{Co^{III}(dtpa)}] remaining. Both intra- and intermolecular ET rates could be explained by the Marcus theory, including the distance dependence of the rate of the reaction, and might arise mainly from the very large reorganization energy for the Co(III)/Co(II) couple. Zinc-substituted myoglobin, [ZnMb{Co^{III}(dtpa)}], was also prepared and the photoinduced ET reaction from the excited triplet state of zinc myoglobin to the Co(III) moiety was examined. The observed ET quenching rate constant is reasonably explained by the Marcus theory through the same ET pathway as that for [deoxyMb{Co^{III}(dtpa)}]. It is suggested that the ET occurs with through-bond and van der Waals interactions from the heme edge to the Co(III) edge via Phe138 and Leu137 over 18.8 Å (1 Å = 1 × 10^{-10} m) both in deoxy and zinc myoglobins.

Recent interest in biological electron-transfer (ET) reactions has been focused on the ET pathway from the donor to the acceptor within a protein matrix.[1–11] In such a system the donor and the acceptor are located at a fixed distance and orientation. The dependence of both the distance and the driving force on the *intra*molecular ET rate has been predicted.[1,12–14] A chemical modification and a mutation of metalloproteins are very useful techniques for elucidating the ET pathway, through space or through bond along with van der Waals and/or hydrogen-bonding interaction(s). 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 in ruthenium-modified systems, where through-bond, van der Waals, and hydrogen bonding interactions are important.[15–19] Dutton et al. predicted an empirical tunneling expression with the packing density between redox cofactors.[11] However, the intramolecular ET reduction of the oxidized form of cytochrome *c* (cyt*c*^{III}) attached to a Co(II) cage complex has been reported to be independent of the separate distance between heme and Co(II); the observed rate constants were much smaller than the expected values based on the Marcus theory.[8,20,21] Generally, a slow self-exchange reaction of Co(III)/Co(II) couples was discussed concerning the large reorganization energy and the spin-multiplicity change.[22–24] However, the above system could not be explained by such factors. It was assumed that ET is controlled by a conformational change of cyt*c*.[8,20,21]

We reported on artificial metmyoglobin, of which Lys residues were modified with one of the strong chelating reagents, *N*,*N*,*N*′,*N*″,*N*″-diethylenetriaminepentaacetic acid (metMb-(dtpa)$_n$, $n = 1$, 2, 4, and 5; Chart 1 for $n = 1$),[25–28] and their cobalt(III) complexes ([metMb{Co^{III}(dtpa)}$_n$], $n = 1$, 2, 4, and 5).[29] We further applied a modification technique by dtpa to cyt*c*, and reported that a slow intramolecular ET of [cyt*c*^{II}{Co^{III}(dtpa)}], where one of the carboxylates in the [Co^{III}(dtpa)] complex is linked with the ε-amino group of Lys13, arises not only from the long distance, but also from the large reorganization energy for the Co(III)/Co(II) couple.[30]

In this work, we examined both thermal and photochemical intramolecular ET reactions of deoxy and zinc myoglobins, of which N-terminal Gly1 was modified with the [Co^{III}(dtpa)] ion, to elucidate whether both ET reactions occur via the same pathway or not. We found that the intramolecular ET in [deoxy-



metMb(dtpa)

Chart 1. Structure.

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Mb{Co$^{\mathrm{III}}$(dtpa)}] is slow in a time scale of hours; therefore, there is no ambiguity as to whether the mechanism is ET controlled or conformationaly gated, because the conformational change of myoglobin is not as slow as in a time scale of hours. Therefore, the present system has a merit to study the ET pathway.

Experimental

Materials. Horse heart metMb (Sigma) was purified as previously described.[31–33] *N*,*N*-Bis[2-(2,6-dioxomorpholino)ethyl]glycine was purchased from Dojindo Laboratories and used without further purification. Cobalt(Ⅱ) chloride hexahydrate was from Wako Pure Chemical Industries, Ltd. Sodium ethylenediaminetetraacetatocobaltate(Ⅲ) tetrahydrate ($Na[Co(edta)] \cdot 4H_2O$) was prepared as reported in the literature.[34] 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 metMb(dtpa). Previously reported method was slightly modified for preparing metMb(dtpa).[27–29] Horse heart metMb was treated with a 3-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. The following procedures were carried out at 4 °C. After the solution was dialyzed with water several times, and then with a 0.010 mol dm^{-3} sodium phosphate buffer at pH 6.0, it was passed through a CM-52 cellulose column (Whatman) at pH 6.0. The singly modified metMb(dtpa) species were then passed through a DM-52 cellulose column (Whatman) at pH 6.0. Multiply modified metMb(dtpa)$_n$ remained on the top of the column. After the effluent was dialyzed with a 0.010 mol dm^{-3} Tris–HCl buffer at pH 8.0, the solution was loaded on a DE-52 cellulose column at pH 8.0. After all of the metMb(dtpa) species were eluted with a 0.010 mol dm^{-3} Tris–HCl buffer containing 1.0 mol dm^{-3} NaCl, the concentrated metMb(dtpa) solution was dialyzed with a 0.010 mol dm^{-3} Tris–HCl buffer, and subjected to an HPLC separation with a Tosoh Super Q-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} Tris–HCl buffer (pH 8.0) to an aqueous 0.10 mol dm^{-3} NaCl solution containing a 0.010 mol dm^{-3} Tris–HCl buffer (pH 8.0), a flow rate of 0.6 cm^3/min, and wavelength monitoring at 280 nm.

Preparation of [metMb{Co$^{\mathrm{III}}$(dtpa)}]. One of the modified metMb(dtpa) (fraction no. 5) was dialyzed with a 0.010 mol dm^{-3} Tris–HCl buffer at pH 8.0. To this solution was added a 2-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 potassium hexacyanoferrate(Ⅲ) ($K_3[Fe(CN)_6]$) at 25 °C for 2 h, and then treated with a Chelex-100 resin to remove excess Co(Ⅱ) ions. The effluent was dialyzed with water several times, and then with a 0.010 mol dm^{-3} Tris–HCl buffer (pH 8.0). After the solution was loaded on a DE-52 cellulose column, the desired [metMb{Co$^{\mathrm{III}}$(dtpa)}] was eluted with a 0.010 mol dm^{-3} Tris–HCl buffer at pH 8.0. Small amounts of brown and then yellow species were detected above the [metMb{Co$^{\mathrm{III}}$(dtpa)}] band on the column. The brown species was metMb(dtpa) and the yellow ones were $[Fe(CN)_6]^{4-}$ and $[Fe(CN)_6]^{3-}$ ions.

Peptide Sequence Analysis. The modified [metMb{Co$^{\mathrm{III}}$(dtpa)}] solution was acidified with HCl to pH 1.2, and then hemin was extracted with 2-butanone.[35] The resulting apomyoglobin in the aqueous phase was digested with lysyl endopeptidase from *Achromobacter lyticus* (Wako) at 35 °C for 8 h by a standard method (metMb:enzyme = 200:1).[28,36–38] The peptide fragments were dissolved in an aqueous solution of 0.1% trifluoroacetic acid (TFA), and then 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 cm^3/min, and wavelength monitoring at 215 nm. The cobalt-containing peptide fragments different from those for native metMb were analyzed with a Hitachi L-8500 automated amino-acid analyzer and an Applied Biosystems Procise 492 protein sequencing system.

Preparation of ZnMb and [ZnMb{Co$^{\mathrm{III}}$(dtpa)}]. Unmodified ZnMb was prepared by the same method as reported previously, and was purified with CM-52 cellulose column chromatography at 4 °C in the dark.[39–41] The modified Zn-substituted [ZnMb{Co$^{\mathrm{III}}$(dtpa)}] was prepared by the same method as described for ZnMb, except for by using a DE-52 cellulose column chromatography at pH 8.3 (a 0.010 mol dm^{-3} phosphate solution). The concentrations of ZnMb and [ZnMb{Co$^{\mathrm{III}}$(dtpa)}] were determined spectrophotometrically ($\varepsilon_{428} = 1.53 \times 10^5$ dm^3 mol^{-1} cm^{-1}).[42] The Zn myoglobins, of which the absorption ratio, A_{428}/A_{280}, was greater than 9.5, were used for kinetic measurements.

Kinetic Measurements. The sample solution was gently purged with high-purity Ar gas, and then carefully degassed by freeze-pump-thaw cycles. The reduced [deoxyMb{Co$^{\mathrm{III}}$(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 disodium dihydrogen ethylenediaminetetraacetate (Na_2H_2edta).[43] The experimental conditions were: [metMb{Co$^{\mathrm{III}}$(dtpa)}] (1.00×10^{-5} mol dm^{-3}), $[Ru(bpy)_3]^{2+}$ (5.00×10^{-6} mol dm^{-3}), MV^{2+} (5.00×10^{-4} mol dm^{-3}), and Na_2H_2edta (5.00×10^{-3} mol dm^{-3}) at 25.0 °C, pH 7.5 (a 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 Y-47 glass filter). The reaction of [deoxyMb{Co$^{\mathrm{III}}$(dtpa)}] to form [metMb{Co$^{\mathrm{II}}$(dtpa)}] was very slow and, therefore, followed by conventional spectrophotometry with a Shimadzu UV-240 spectrophotometer. The reaction of deoxyMb with $[Co^{\mathrm{III}}(edta)]^-$ was also followed by the same procedure as described for [deoxyMb{Co$^{\mathrm{III}}$(dtpa)}], where the total concentrations of deoxyMb and $[Co^{\mathrm{III}}(edta)]^-$ were 1.00×10^{-5} mol dm^{-3} each.

Decays of excited triplet states of ZnMb and [ZnMb{Co$^{\mathrm{III}}$(dtpa)}] in degassed aqueous solutions were followed by using a Photal RA-412 pulse flash apparatus under the following conditions: ZnMb or [ZnMb{Co$^{\mathrm{III}}$(dtpa)}] (3.00×10^{-6} mol dm^{-3}) at 25.0 °C, pH 7.0 (a 0.010 mol dm^{-3} phosphate buffer), and $I = 0.020$ mol dm^{-3}.

Other Measurements. The electronic absorption spectra were recorded on Shimadzu UV-240 and MultiSpec-1500 spectrophotometers. The fluorescence spectra and lifetimes were measured with a Shimadzu RF-5300PC spectrofluorometer and a Hitachi-Horiba NAES-500 nano-second fluorometer, respectively. 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 PerSpective Biosystems Voyager DE 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. For peptide samples in MeCN containing 3% TFA, 3,5-dimethoxy-4-hydroxycinnamic acid was used as a matrix. The iron and cobalt contents in [metMb{Co$^{\rm III}$(dtpa)}] and the cobalt content in peptide fragments were analyzed with a Hitachi Z-7000 atomic absorption spectrometer.

Results and Discussion

Characterization of [metMb{Co$^{\rm III}$(dtpa)}]. An HPLC profile for metMb(dtpa) on a SuperQ-5PW column is shown in Fig. 1. The yield of all of the singly modified metMb(dtpa) was 30%. Eight main fractions (F1–F8) were obtained, and their yields were 0.6, 1.7, 0.9, 4.6, 4.4, 1.1, 4.5, and 1.1%, respectively. The F4 species was Lys87-modified metMb(dtpa), which was previously reported.[28] In this work we collected and characterized the F5 species. The m/z value for this species was 17348. The corresponding value for native metMb is 16950, indicating that the heme dissociates to form apoMb during ionization. Therefore, the value of 17348 is that for apoMb(dtpa) containing one dtpa ion and one Na$^+$ ion. The UV–vis absorption spectrum of metMb(dtpa) (F5) was similar to that of native metMb. From determining the iron content by atomic absorption spectroscopy, the molar absorption coefficients were estimated to be $\varepsilon_{409} = 1.88 \times 10^5$ dm^3 mol^{-1} cm^{-1} and $\varepsilon_{280} = 3.45 \times 10^4$ dm^3 mol^{-1} cm^{-1}, respectively.

Under the present experimental conditions, the reaction of metMb(dtpa) with Co^{2+} ions was not so fast that the reacting solution was incubated at 25 °C for 24 h. The m/z value for [metMb{Co$^{\rm III}$(dtpa)}] for F5 was 17384, and the Fe:Co content ratio was 1.00 ± 0.02 from the atomic absorption spectroscopy. The molar absorption coefficients for [metMb{Co$^{\rm III}$(dtpa)}] were $\varepsilon_{409} = 1.88 \times 10^5$ dm^3 mol^{-1} cm^{-1} and $\varepsilon_{280} = 3.76 \times 10^4$ dm^3 mol^{-1} cm^{-1}, respectively. Although the ratio A_{409}/A_{280} in the electronic absorption spectrum was 5.0, being smaller than that for native metMb or metMb(dtpa), the CD spectrum of [metMb{Co$^{\rm III}$(dtpa)}] was similar to that for metMb(dtpa), and a little smaller than that for native metMb (97% at 224 nm). Therefore, a modification of metMb with the [Co$^{\rm III}$(dtpa)] unit does not alter the conformation of metMb significantly.

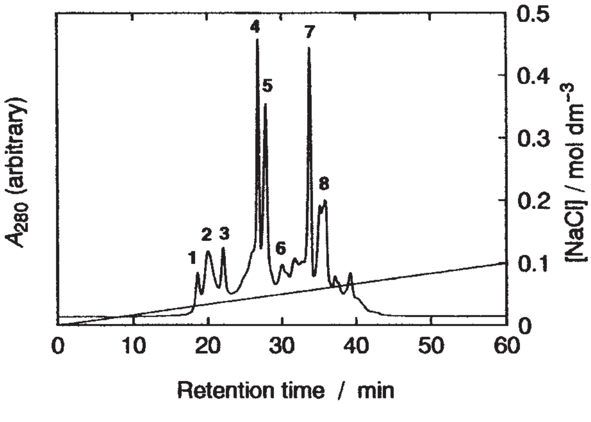

Fig. 1. HPLC profile for the singly modified metMb(dtpa) species with a Tosoh Super Q-5PW column (7.5 mm × 7.5 cm). Conditions: a linear gradient from 0 mol dm^{-3} to 0.10 mol dm^{-3} NaCl in 0.010 mol dm^{-3} Tris–HCl buffers at pH 8.0, a flow rate of 0.6 cm^3/min, and wavelength monitoring at 280 nm.

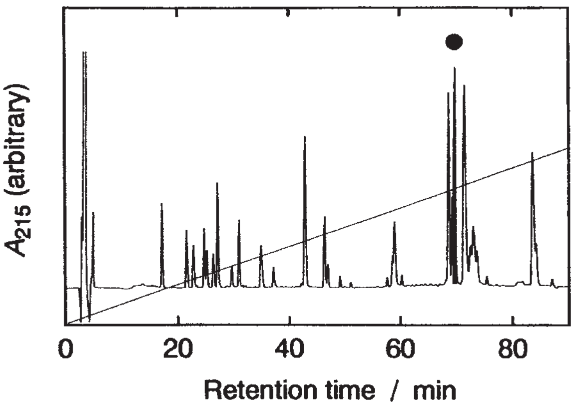

Fig. 2. HPLC profile for the lysyl endopeptidase digests of metMb(dtpa) (F5) with a Tosoh ODS-120T column (4.6 mm × 25 cm). Conditions: a linear gradient from a 0.1% aqueous TFA to a mixed solution of aqueous TFA with MeCN (1:1), a flow rate of 1.0 cm^3/min, and wavelength monitoring at 215 nm.

Figure 2 shows a reversed-phase HPLC profile for the peptide fragment of [metMb{Co$^{\rm III}$(dtpa)}] digested with lysyl endopeptidase. The protein sequencing analysis for the marked peptide in Fig. 2, containing a Co atom, did not show any amino acids. It is suggested that the N-terminal amino acid Gly1 is modified with a [Co$^{\rm III}$(dtpa)] unit. The total amino-acid analysis was then employed to the peptide, suggesting the sequence from 1 to 16 (GLSDGEWQQVLNVWGK). This is confirmed from its m/z values 2247.58 and 2190.72, the latter of which corresponds to the cobalt-dissociated fragment. From these results, we can conclude that the modified amino acid is the N-terminal Gly1.

We attempted to measure the redox potential of [metMb{Co$^{\rm III}$(dtpa)}] by cyclic voltammetry using a gold electrode modified with bis(4-pyridyl)disulfide at 25 °C and pH 6.0 (a 0.010 mol dm^{-3} phosphate buffer). There was no redox wave for both the heme and [Co$^{\rm III}$(dtpa)] units, as well as the previously reported Lys87-modified [metMb{Co$^{\rm III}$(dtpa)}].[29] We assumed that Gly1 is located far from the Au electrode as well as Lys87. We reported that the multiply modified [metMb{Co$^{\rm III}$(dtpa)}$_n$] ($n = 2$, 4, and 5) shows redox waves for the Co(III)/Co(II) couple at 0.42 V vs NHE.[29] Therefore, we employed the value of 0.42 V as the redox potential for the [Co$^{\rm III}$(dtpa)] moiety in [metMb{Co$^{\rm III}$(dtpa)}].

Reduction of metMb{Co$^{\rm III}$(dtpa)}(Gly1) by MV$^{\bullet+}$. The reduction of [metMb{Co$^{\rm III}$(dtpa)}] with MV$^{\bullet+}$ was followed at 409 nm and 434 nm. The observed first-order rate constants at 25.0 °C, pH 7.5 (a 0.010 mol dm^{-3} Tris–HCl buffer), and $I = 0.50$ mol dm^{-3} (NaCl) were $(1.8 \pm 0.2) \times 10^3$ s^{-1} and $(1.9 \pm 0.2) \times 10^3$ s^{-1} for the decay of metMb and the formation of deoxyMb, respectively. We also examined the reactions of MV$^{\bullet+}$ with native metMb and [Co$^{\rm III}$(edta)]$^-$, the latter as a model of the Co(III) moiety. The observed first-order rate constants under the same experimental conditions were $(1.8 \pm 0.2) \times 10^3$ s^{-1} and $(2.1 \pm 0.2) \times 10^3$ s^{-1}, respectively. A similar rate constant for the reaction of metMb with MV$^{\bullet+}$ has been reported by van Leeuwen et al.[44] At lower ionic strengths, the reaction of [Co$^{\rm III}$(edta)]$^-$ with MV$^{\bullet+}$ became faster than that at $I = 0.50$ mol dm^{-3}. Based on the above results, the following reactions occurred:

$$[\text{metMb}\{\text{Co}^{\text{III}}(\text{dtpa})\}] + \text{MV}^{\bullet+} \rightarrow [\text{deoxyMb}\{\text{Co}^{\text{III}}(\text{dtpa})\}] + \text{MV}^{2+}, \quad (1a)$$

$$[\text{metMb}\{\text{Co}^{\text{III}}(\text{dtpa})\}] + \text{MV}^{\bullet+} \rightarrow [\text{metMb}\{\text{Co}^{\text{II}}(\text{dtpa})\}] + \text{MV}^{2+}. \quad (1b)$$

The concentrations of [deoxyMb$\{$Co$^{\text{III}}$(dtpa)$\}$] and [metMb$\{$Co$^{\text{III}}$(dtpa)$\}$] remaining after reduction by MV$^{\bullet+}$ were then estimated. The total concentrations of deoxyMb ([deoxyMb]$_\text{T}$ = [[deoxyMb$\{$Co$^{\text{III}}$(dtpa)$\}$]]$_0$ + [[deoxyMb$\{$Co$^{\text{II}}$(dtpa)$\}$]]$_0$) were determined from the change in the absorbance at 434 nm after flashing. The differences in the molar absorption coefficients at 409 and 434 nm between metMb and deoxyMb were 1.25×10^5 dm^3 mol^{-1} cm^{-1} and 1.00×10^5 dm^3 mol^{-1} cm^{-1}, respectively. With one flash a 9.2×10^{-7} mol dm^{-3} MV$^{\bullet+}$ ion was produced under the present experimental conditions. Because the rates of reactions (1a) and (1b) are similar to each other, it is assumed that the same concentrations of [deoxyMb$\{$Co$^{\text{III}}$(dtpa)$\}$] and [metMb$\{$Co$^{\text{II}}$(dtpa)$\}$] were formed. After the second flash, [metMb$\{$Co$^{\text{III}}$(dtpa)$\}$], [deoxyMb$\{$Co$^{\text{III}}$(dtpa)$\}$], and [metMb$\{$Co$^{\text{II}}$(dtpa)$\}$] reacted with MV$^{\bullet+}$ to produce [deoxyMb$\{$Co$^{\text{III}}$(dtpa)$\}$] and [metMb$\{$Co$^{\text{II}}$(dtpa)$\}$] in a 1:1 ratio and [deoxyMb$\{$Co$^{\text{II}}$(dtpa)$\}$], being dependent on the ratio of the remaining concentration of [metMb$\{$Co$^{\text{III}}$(dtpa)$\}$] ([metMbCo(III)]$_\text{remaining}$) to the concentration of [deoxyMb]$_\text{T}$ or [metMb$\{$Co$^{\text{II}}$(dtpa)$\}$]. The results are summarized in Table 1. Under the conditions that [deoxyMb]$_\text{T}$ are less than 1.0×10^{-6} mol dm^{-3} and 2.8×10^{-6} mol dm^{-3} at [[metMb$\{$Co$^{\text{III}}$(dtpa)$\}$]]$_0$ = 5.0×10^{-6} mol dm^{-3} and 1.0×10^{-5} mol dm^{-3}, respectively, the concentration of the fully reduced [deoxyMb$\{$Co$^{\text{II}}$(dtpa)$\}$] ([[deoxyMb$\{$Co$^{\text{II}}$(dtpa)$\}$]]$_0$) is small.

Reaction of [deoxyMb{Co$^{\text{III}}$(dtpa)}]. Considering the free-energy change between [deoxyMb$\{$Co$^{\text{III}}$(dtpa)$\}$] and [metMb$\{$Co$^{\text{II}}$(dtpa)$\}$] ($\Delta G^\circ = -0.36$ eV), being estimated from the redox potentials of metMb/deoxyMb (0.06 V)[45,46] and Co$^{\text{III}}$/Co$^{\text{II}}$ (0.42 V),[29] ET from the deoxyMb unit to the [Co$^{\text{III}}$(dtpa)] moiety is expected. In fact, the decay of [deoxyMb$\{$Co$^{\text{III}}$(dtpa)$\}$] and the formation of [metMb$\{$Co$^{\text{II}}$(dtpa)$\}$] were observed after reduction by MV$^{\bullet+}$ with several flashes (see Fig. 3). The reaction was very slow and, therefore, the following reactions were possible:

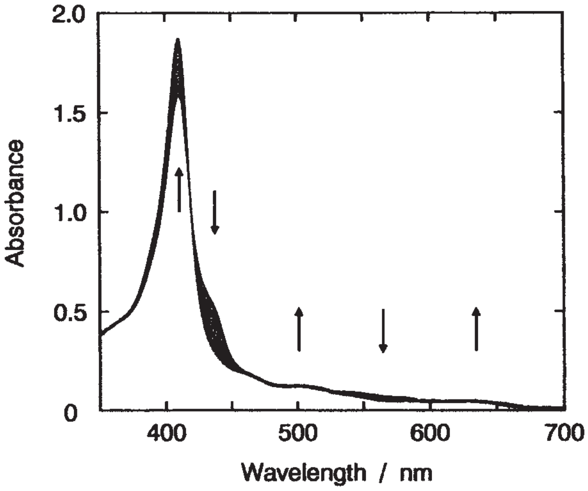

Fig. 3. Spectral changes during the reaction of [deoxyMb$\{$Co$^{\text{III}}$(dtpa)$\}$] after the reduction of [metMb$\{$Co$^{\text{III}}$(dtpa)$\}$] (1.00×10^{-5} mol dm^{-3}) by MV$^{\bullet+}$ with a Xe flash lamp in the presence of MV^{2+} (5.0×10^{-4} mol dm^{-3}), [Ru(bpy)$_3$]$^{2+}$ (5.0×10^{-6} mol dm^{-3}), and Na$_2$H$_2$edta (5.0×10^{-3} mol dm^{-3}) at 25 °C, pH 7.5 (a 0.010 mol dm^{-3} Tris–HCl buffer), and $I = 0.50$ mol dm^{-3} (NaCl).

$$[\text{deoxyMb}\{\text{Co}^{\text{III}}(\text{dtpa})\}] \rightarrow [\text{metMb}\{\text{Co}^{\text{II}}(\text{dtpa})\}] \quad k^{\text{intra}}, \quad (2)$$

$$[\text{deoxyMb}\{\text{Co}^{\text{III}}(\text{dtpa})\}] + [\text{metMb}\{\text{Co}^{\text{III}}(\text{dtpa})\}] \rightarrow [\text{metMb}\{\text{Co}^{\text{III}}(\text{dtpa})\}] + [\text{metMb}\{\text{Co}^{\text{II}}(\text{dtpa})\}] \quad k^{\text{inter}}, \quad (3)$$

$$[\text{deoxyMb}\{\text{Co}^{\text{III}}(\text{dtpa})\}] + [\text{deoxyMb}\{\text{Co}^{\text{II}}(\text{dtpa})\}] \rightarrow [\text{deoxyMb}\{\text{Co}^{\text{II}}(\text{dtpa})\}] + [\text{metMb}\{\text{Co}^{\text{II}}(\text{dtpa})\}] \quad k_4, \quad (4)$$

$$2[\text{deoxyMb}\{\text{Co}^{\text{III}}(\text{dtpa})\}] \rightarrow [\text{metMb}\{\text{Co}^{\text{III}}(\text{dtpa})\}] + [\text{deoxyMb}\{\text{Co}^{\text{II}}(\text{dtpa})\}] \quad k_5, \quad (5)$$

$$[\text{deoxyMb}\{\text{Co}^{\text{II}}(\text{dtpa})\}] + [\text{metMb}\{\text{Co}^{\text{III}}(\text{dtpa})\}] \rightarrow 2[\text{metMb}\{\text{Co}^{\text{II}}(\text{dtpa})\}] \quad k_6. \quad (6)$$

In the *inter*molecular ET reactions (3) and (4), [metMb$\{$Co$^{\text{III}}$(dtpa)$\}$] and [deoxyMb$\{$Co$^{\text{II}}$(dtpa)$\}$] are reproduced, respectively. Therefore, reactions (3) and (4) can be treated as a pseudo first-order reaction, where the concentrations of [metMb$\{$Co$^{\text{III}}$(dtpa)$\}$] and [deoxyMb$\{$Co$^{\text{II}}$(dtpa)$\}$] remain constant. As is discussed in the previous section, the concentra-

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

[metMbCo(III)]$_0$$^{\text{a)}}$ / 10^{-6} mol dm^{-3}	[deoxyMb]$_\text{T}$$^{\text{b)}}$ / 10^{-6} mol dm^{-3}	[deoxyMbCo(III)]$_0$$^{\text{c)}}$ / 10^{-6} mol dm^{-3}	[deoxyMbCo(II)]$_0$$^{\text{d)}}$ / 10^{-6} mol dm^{-3}	[Co(III)]$_\text{remaining}$$^{\text{e)}}$ / 10^{-6} mol dm^{-3}	k_1 / 10^{-4} s^{-1}
5.0	1.00	0.84	0.16	3.16	0.69 ± 0.07
10.0	2.76	2.10	0.66	5.14	1.02 ± 0.07
10.0	2.36	1.86	0.44	5.84	1.08 ± 0.08
10.0	1.84	1.58	0.26	6.58	1.22 ± 0.09
10.0	1.38	1.26	0.12	7.36	1.32 ± 0.09
10.0	0.92	0.88	0.04	8.20	1.48 ± 0.10

a) [metMbCo(III)]$_0$ = [[metMb$\{$Co$^{\text{III}}$(dtpa)$\}$]]$_0$. b) [deoxyMb]$_\text{T}$ = [[deoxyMb$\{$Co$^{\text{III}}$(dtpa)$\}$]]$_0$ + [[deoxyMb$\{$Co$^{\text{II}}$(dtpa)$\}$]]$_0$. c) [deoxyMbCo(III)]$_0$ = [[deoxyMb$\{$Co$^{\text{III}}$(dtpa)$\}$]]$_0$. d) [deoxyMbCo(II)]$_0$ = [[deoxyMb$\{$Co$^{\text{II}}$(dtpa)$\}$]]$_0$. e) [Co(III)]$_\text{remaining}$ = [[metMb$\{$Co$^{\text{III}}$(dtpa)$\}$]]$_\text{remaining}$.

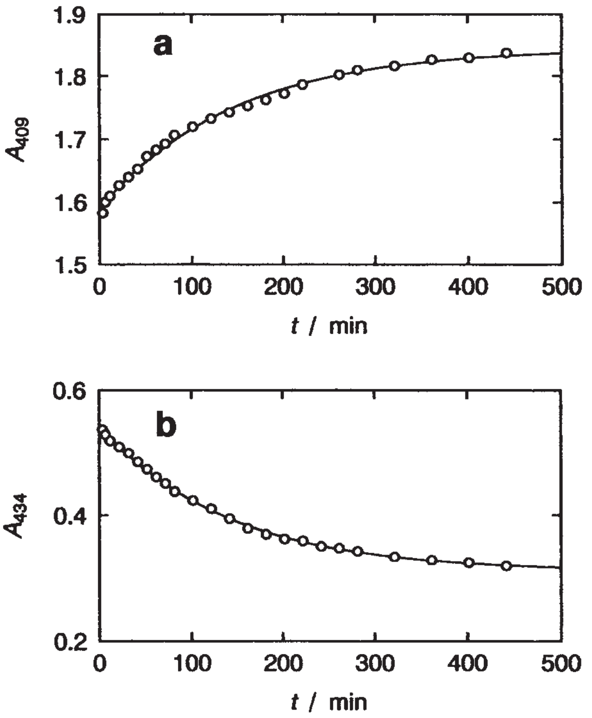

Fig. 4. Spectral changes during the reaction of [deoxyMb{Co^{III}(dtpa)}] under the same conditions as in Fig. 3. (a) Formation of [metMb{Co^{II}(dtpa)}] at 409 nm. (b) Decay of [deoxyMb{Co^{III}(dtpa)}] at 434 nm. The solid lines are fitting to the first-order kinetics.

tion of [deoxyMb{Co^{II}(dtpa)}] was small and, therefore, reactions (4) and (6) are negligible. Since the observed absorbance changes at 409 nm and 434 nm (see Fig. 4) obey the first-order kinetics given by

$$-\mathrm{d}[[\mathrm{deoxyMb\{Co^{III}(dtpa)\}}]]/\mathrm{d}t = k_1[[\mathrm{deoxyMb\{Co^{III}(dtpa)\}}]], \tag{7}$$

the second-order reaction (5) does not contribute appreciably. From these results, reactions (2) and (3) predominantly occurred under the present experimental conditions. Therefore, the rate constant, k_1, can be represented as

$$k_1 = k^{\mathrm{intra}} + k^{\mathrm{inter}}[\mathrm{Co(III)}]_{\mathrm{remaining}}, \tag{8}$$

where $[\mathrm{Co(III)}]_{\mathrm{remaining}}$ represents the concentration of [metMb{Co^{III}(dtpa)}] remaining after reduction by $MV^{\bullet +}$. The values of the rate constant (k_1) are summarized in Table 1. The first-order rate constant (k_1) is linearly dependent on $[\mathrm{Co(III)}]_{\mathrm{remaining}}$, as is shown in Fig. 5. The rate constants for the *intra*- and *inter*molecular ET reactions (k^{intra} and k^{inter}) were obtained from the intercept and the slope of the straight line, respectively: $k^{\mathrm{intra}} = (2.0 \pm 0.5) \times 10^{-5}\ \mathrm{s^{-1}}$ and $k^{\mathrm{inter}} = (1.5 \pm 0.2) \times 10\ \mathrm{dm^3\ mol^{-1}\ s^{-1}}$.

The reaction of deoxyMb with $[\mathrm{Co^{III}(edta)}]^-$, reaction (9), was also examined as a model of the *inter*molecular ET reaction under the same experimental conditions:

$$\mathrm{deoxyMb} + [\mathrm{Co^{III}(edta)}]^- \rightarrow \mathrm{metMb} + [\mathrm{Co^{II}(edta)}]^- \quad k. \tag{9}$$

The second-order rate constant (k) was evaluated by fitting to the following equation (see Fig. 6),

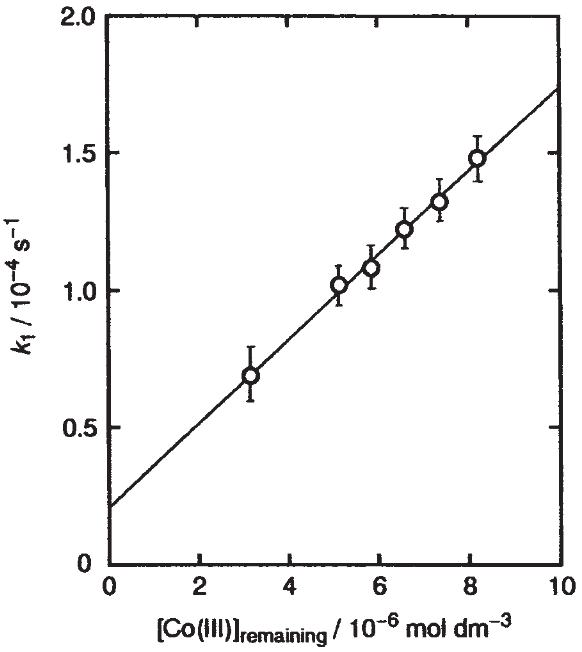

Fig. 5. Plots of k_1 vs the concentrations of [metMb{Co^{III}-(dtpa)}] remaining ($[\mathrm{Co(III)}]_{\mathrm{remaining}}$) for the reaction of [deoxyMb{Co^{III}(dtpa)}].

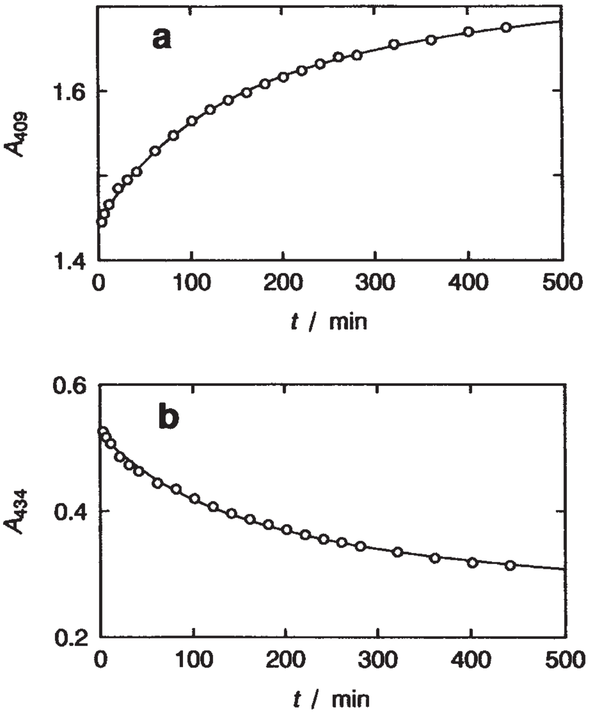

Fig. 6. Spectral changes during the reaction of deoxyMb with $[\mathrm{Co^{III}(edta)}]^-$ after the reductions of metMb and $[\mathrm{Co^{III}(edta)}]^-$ (1.00×10^{-5} mol dm^{-3} each) by $MV^{\bullet +}$ with a Xe flash lamp in the presence of MV^{2+} (5.0×10^{-4} mol dm^{-3}), $[\mathrm{Ru(bpy)_3}]^{2+}$ (5.0×10^{-6} mol dm^{-3}), and Na_2H_2edta (5.0×10^{-3} mol dm^{-3}) at 25 °C, pH 7.5 (a 0.010 mol dm^{-3} Tris–HCl buffer), and $I = 0.50$ mol dm^{-3} (NaCl). (a) Formation of metMb at 409 nm. (b) Decay of deoxyMb at 434 nm. The solid lines are fitting to Eq. 11 based on the second-order kinetics.

Table 2. Second-Order Rate Constants for the Reaction between deoxyMb and $[\mathrm{Co^{III}(edta)}]^-$ at 25 °C, pH 7.5 (a 0.010 $\mathrm{mol\,dm^{-3}}$ Tris–HCl buffer), and $I = 0.50\ \mathrm{mol\,dm^{-3}}$ (NaCl)

$[\mathrm{metMb}]_\mathrm{T}$	$[\mathrm{metMb}]_0$	$[\mathrm{deoxyMb}]_0$	$[\mathrm{Co(II)}]_0$	$[\mathrm{Co(III)}]_\mathrm{remaining}$	k
$10^{-6}\ \mathrm{mol\,dm^{-3}}$	$10^{-6}\ \mathrm{mol\,dm^{-3}}$	$10^{-6}\ \mathrm{mol\,dm^{-3}}$	$10^{-6}\ \mathrm{mol\,dm^{-3}}$	$10^{-6}\ \mathrm{mol\,dm^{-3}}$	$10\ \mathrm{dm^3\,mol^{-1}\,s^{-1}}$
10.0	8.16	1.84	1.84	8.16	1.9
10.0	7.24	2.76	2.76	7.24	1.8
10.0	6.32	3.68	3.68	6.32	1.6

$$A_\mathrm{t} = (A_0 - A_\infty)[\mathrm{Co}]_0[1 - \exp\{([\mathrm{Co}]_0 - [\mathrm{Mb}]_0)kt\}]/[[\mathrm{Mb}]_0 - [\mathrm{Co}]_0 \cdot \exp\{([\mathrm{Co}]_0 - [\mathrm{Mb}]_0)kt\}]. \quad (10)$$

Here, $[\mathrm{Co}]_0$ and $[\mathrm{Mb}]_0$ are the concentrations of $[\mathrm{Co^{III}(edta)}]^-$ and deoxyMb just after reduction by $\mathrm{MV}^{\bullet+}$, respectively. The obtained second-order rate constants are summarized in Table 2 along with the concentrations of deoxyMb, $[\mathrm{Co^{III}(edta)}]^-$, and $[\mathrm{Co^{III}(edta)}]^-$ remaining after reduction by $\mathrm{MV}^{\bullet+}$. The rate constant ($k = (1.8 \pm 0.2) \times 10\ \mathrm{dm^3\,mol^{-1}\,s^{-1}}$) is similar to the value of k^inter, being reasonable for the *inter*molecular ET reaction.

Evaluation of the Slow Rate Constant for the Intramolecular ET Reaction of [deoxyMb{Co$^{\mathrm{III}}$(dtpa)}]. The rate constant of $(2.0 \pm 0.5) \times 10^{-5}\ \mathrm{s^{-1}}$ at 25 °C is very small for the previously reported *intra*molecular ET reaction of myoglobin.[3,4,6,7,10] We discuss the validity of the slow rate constant based on Eq. 11:[1]

$$k_\mathrm{et} = 10^{13} \cdot \exp[-\beta(r - r_0)] \cdot \exp[-(\Delta G^\circ + \lambda)^2/4\lambda RT]. \quad (11)$$

Here, r is the distance between the donor and acceptor centers, r_0 is the contact distance, β is the decay constant which describes the sensitivity of the coupling to changes in distance, ΔG° is the diving force, λ is the reorganization energy which equals the mean value of the contribution from donor and acceptor, $(\lambda_\mathrm{D} + \lambda_\mathrm{A})/2$, R is the gas constant, and T is the absolute temperature. If ET from the heme to the Co(Ⅲ) moiety occurs via a heme → Phe138 → Leu137 → $[\mathrm{Co^{III}(dtpa)}]$(Gly1) pathway, which contains van der Waals interactions of the heme edge with Phe138 and of Leu137 with the $[\mathrm{Co^{III}(dtpa)}]$ moiety and the through-bond interactions from Phe138 to Leu137, the distance $r - r_0$ between the donor and the acceptor becomes 18.8 Å (see Figs. 7 and 8). By using the parameters $\lambda = 3.21$ eV (the values of λ are 2.30 eV and 4.12 eV for deoxyMb[47,48] and Co(Ⅲ),[49] respectively), $\Delta G^\circ = -0.36$ eV, $r - r_0 = 18.8$ Å, and $k^\mathrm{intra} = 2.0 \times 10^{-5}\ \mathrm{s^{-1}}$, we obtained a β value of 0.86 $\mathrm{Å^{-1}}$. This value lies within $0.8\ \mathrm{Å^{-1}} \leq \beta \leq 1.3\ \mathrm{Å^{-1}}$ for the previously reported myoglobin systems.[4,6,10] In the case of the ET reaction of Co(Ⅲ)/Co(Ⅱ) couples, nonadiabaticity has been discussed.[1,22–24] We concluded that the spin-multiplicity change does not mainly contribute to the activation energy for ET of $[\mathrm{cyt}c^\mathrm{II}\{\mathrm{Co^{III}(dtpa)}\}]$.[30] Therefore, the spin multiplicity change of the Co(Ⅲ)/Co(Ⅱ) couple might not contribute appreciably in the present system. The *intra*molecular ET reaction of $[\mathrm{deoxyMb}\{\mathrm{Co^{III}(dtpa)}\}]$ can be explained by the van der Waals and covalent-bond interactions over 18.8 Å, and the slow rate constant arises mainly from the large reorganization energy for the Co(Ⅲ)/Co(Ⅱ) couple ($\lambda = 4.12$ eV).

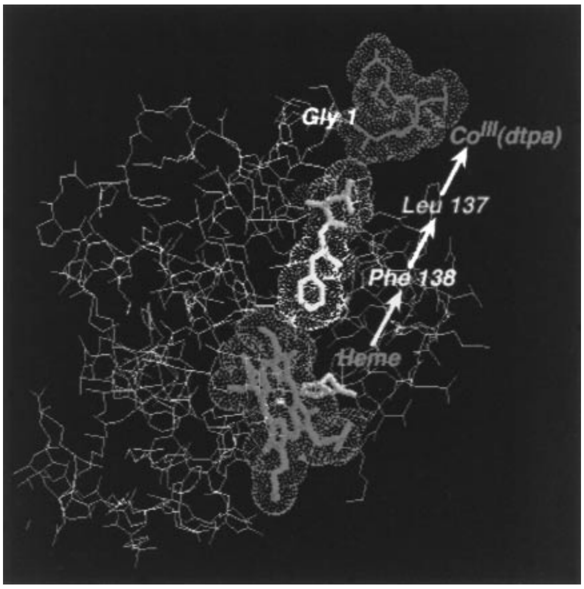

Fig. 7. Electron-transfer pathway from heme to the $[\mathrm{Co^{III}(dtpa)}]$ moiety via Phe138 and Leu137 residues. Data from the PDB of Brookhaven National Laboratory are displayed by a RasMol v2.7 molecular visualization program.

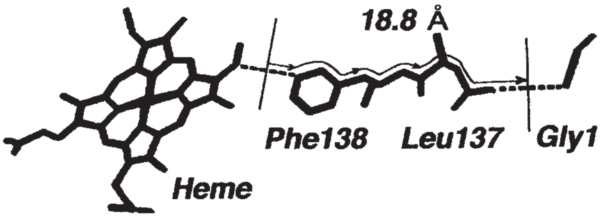

Fig. 8. Estimated distance for $r - r_0$ from the heme edge to the N-terminal Gly1.

Photoinduced ET Reaction of [ZnMb{Co$^{\mathrm{III}}$(dtpa)}]. The UV–vis spectrum of $[\mathrm{ZnMb}\{\mathrm{Co^{III}(dtpa)}\}]$ was similar to that of ZnMb.[27,39–42,50] The florescence properties were not different from those of ZnMb: fluorescence maxima $\lambda_\mathrm{em} = 598$ and 645 nm and a fluorescence lifetime $\tau_\mathrm{f} = 2.0$ ns at 25.0 °C, pH 7.0 (a 0.010 $\mathrm{mol\,dm^{-3}}$ phosphate buffer), and $I = 0.020\ \mathrm{mol\,dm^{-3}}$, indicating that the excited singlet state is not quenched by the bound $[\mathrm{Co^{III}(dtpa)}]$ moiety. On the other hand, the lifetime of the excited triplet state of $[\mathrm{ZnMb}\{\mathrm{Co^{III}(dtpa)}\}]$ became shorter than that of ZnMb, as is shown in Fig. 9. The decay of the T–T absorption of $[^3(\mathrm{ZnMb})^*\{\mathrm{Co^{III}(dtpa)}\}]$ was of first order. The spontaneous decay of the $^3(\mathrm{ZnMb})^*$ unit in $[^3(\mathrm{ZnMb})^*\{\mathrm{Co^{III}(dtpa)}\}]$ is considered to be the same as that for ZnMb ($k_0 = 92\ \mathrm{s^{-1}}$). Therefore, the rate constant of the *intra*molecular ET quenching reaction (12),

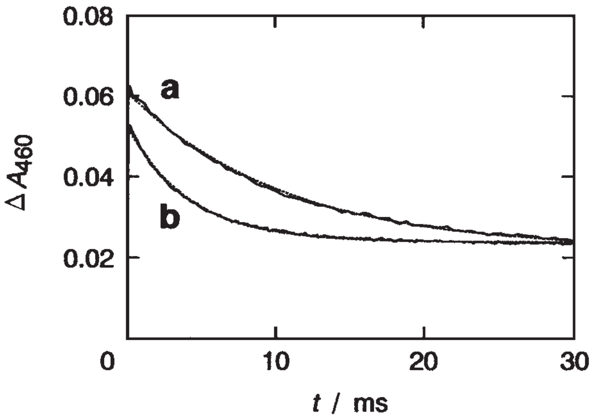

Fig. 9. Decay curves of the excited triplet states of ZnMb and [ZnMb{Co$^{\rm III}$(dtpa)}] (each of 3.00×10^{-6} mol dm^{-3}) at 25 °C, pH 7.0 (a 0.010 mol dm^{-3} sodium phosphate buffer), $I = 0.020$ mol dm^{-3}, and $\lambda = 460$ nm. (a) ZnMb. (b) [ZnMb{Co$^{\rm III}$(dtpa)}].

$$[^3(\mathrm{ZnMb})^*\{\mathrm{Co^{III}(dtpa)}\}] \rightarrow [\mathrm{ZnMb}^{\bullet+}\{\mathrm{Co^{II}(dtpa)}\}] \quad k_{\rm et}, \quad (12)$$

can be estimated: $k_{\rm et} = k_{\rm obsd} - k_0 = (1.5 \pm 0.2) \times 10^2$ s^{-1}. Since a subsequent spectral change at 680 nm, being the absorption maximum of the radical cation of the ZnMb$^{\bullet+}$ unit, was not appreciably observed during the time window (1 s), we assumed that the back ET reaction,

$$[\mathrm{ZnMb}^{\bullet+}\{\mathrm{Co^{II}(dtpa)}\}] \rightarrow [\mathrm{ZnMb}\{\mathrm{Co^{III}(dtpa)}\}], \quad (13)$$

is much slower than the quenching reaction. This might be reasonable from the difference in the driving force: $\Delta G^\circ = -0.56$ eV ($E^\circ = 0.98$ V for ZnMb$^{\bullet+}$/ZnMb) for the back ET and -1.22 eV for the ET quenching.[51]

We assumed that the ET pathway is the same as that for the [deoxyMb{Co$^{\rm III}$(dtpa)}] system, and thus the β value can be calculated from Eq. 11 by using the following parameters: $\lambda = 2.72$ eV (the values of λ are 1.32 eV and 4.12 eV for ^{3}(ZnMb)* and Co(III), respectively),[4,49] $\Delta G^\circ = -1.22$ eV, $r - r_0 = 18.8$ Å, and $k_{\rm et} = 1.5 \times 10^2$ s^{-1}. The calculated β value of 0.90 Å^{-1} is similar to that estimated for [deoxyMb{Co$^{\rm III}$(dtpa)}], and is reasonable for myoglobin. It is, therefore, concluded that ET from the metalloporphyrin (MP) to the Co(III) moiety in both [deoxyMb{Co$^{\rm III}$(dtpa)}] and [^{3}(ZnMb)*{Co$^{\rm III}$(dtpa)}] occurs via a MP → Phe138 → Leu137 → [Co$^{\rm III}$(dtpa)](Gly1) pathway, which contains van der Waals interactions of the MP edge with Phe138 and of Leu137 with the [Co$^{\rm III}$(dtpa)] moiety and the through-bond interactions from Phe138 to Leu137.

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