Intracomplex Quenching by Copper(II) Ion of Excited Singlet and Triplet States of Zinc Myoglobin Modified with Diethylenetriaminepentaacetic Acid[1]

Keiichi Tsukahara,* Chieko Kimura, and Takeshi Sakurai†
Department of Chemistry, Faculty of Science, Nara Women's University, Nara 630
†*Institute for Molecular Science, Myodaiji, Okazaki 444*

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Both excited singlet and triplet states of zinc myoglobin, whose lysine residue was modified with diethylenetriaminepentaacetic acid, were quenched by copper(II) ions bound at the modified site.

Myoglobin is an oxygen-storage heme protein found in the muscles of vertebrates. Since the structure of myoglobin has been well characterized by an X-ray crystallography,[2] a number of studies of electron-transfer (ET) reactions of myoglobin have been reported for elucidating the ET mechanism, especially on the ET pathway, by chemical modification and mutation techniques.[3,4] Horse heart muscle metmyoglobin (metMb) possesses 19 lysine residues among which 14 lysines are located on the surface of the protein. Succination of lysine residues on metMb has been reported by Antonini et al.[5] and Aono et al.[6] It is, therefore, possible to modify lysine residue(s) with diethylenetriaminepentaacetic acid (DTPA) which is one of the strong metal-chelating reagents. In this work we report the characterization of the complex between DTPA-modified metmyoglobin (metMb-DTPA) and Cu^{2+} ion and the photoinduced ET quenching of its zinc-substituted myoglobin (ZnMbDTPA) by Cu^{2+} ion. Interestingly, both the excited singlet and triplet states of ZnMbDTPA were *intra*molecularly quenched by Cu^{2+} ion which binds DTPA site of myoglobin to form a Cu(dtpa) chelated complex.

Horse heart metMb (Sigma) was treated with excess DTPA dianhydride (Dojindo Lab.) in a 0.1 mol dm^{-3} Tris/HCl buffer at pH 8.6. Four main fractions obtained (F-1—F-4) were found to be singly lysine-modified metmyoglobins in which a terminal carboxylate of DTPA binds a different lysine residue through amide linkage. The detailed procedures for preparation and purification of metMbDTPA will be published elsewhere. ESR spectra were recorded on a JEOL JES-RE1X X-banded spectrometer at 77 K. Zinc-substituted myoglobins modified with DTPA were prepared by a previously reported method[7] and purified by a DEAE Sepharose column chromatography. Fluorescence spectra and life-times were measured with a Hitachi 850 spectrofluorometer and a Horiba NAES-500 nano-second fluorometer, respectively. A single flash photolysis was carried out using a Photal RA-412 pulse flash apparatus with a 30 μs pulse-width Xe lamp (λ >450 nm; a Toshiba Y-47 glass filter) under the same experimental conditions as those in the fluorescence measurements.[7,8]

Figure 1a shows an ESR spectrum for the 1:1 mixture of metMbDTPA(F-2) with $CuSO_4 \cdot 5H_2O$ in a phosphate buffer (pH 8.3) at 77 K. The g values, $g_\perp = 2.064$ and $g_{//} = 2.284$, are very similar to those for free $[Cu(dtpa)]^{2-}$ (see Figure 1b; $g_\perp = 2.076$ and $g_{//} = 2.282$), indicating that the Cu^{2+} ion binds the DTPA moiety on the modified metMb. A signal at $g = 5.660$ for a high spin heme iron in the metMb{Cu^{II}(dtpa)} (spectrum not shown) is similar to that for native metMb, indicating that the heme environment does not change by modification with {Cu^{II}(dtpa)}. The coordination environment around the Cu(II) binding site

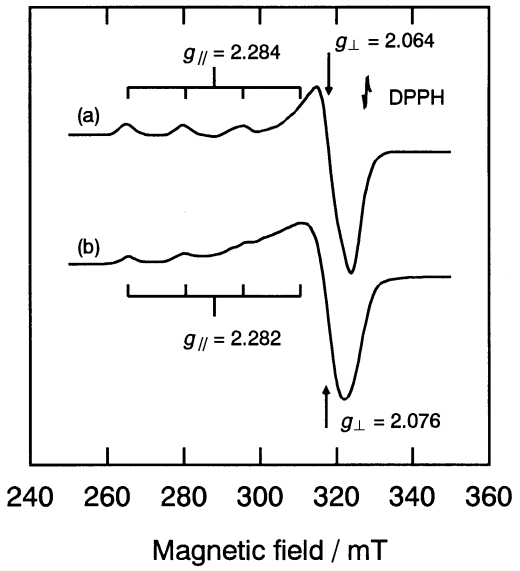


Figure 1. ESR spectra obtained at 77 K for 1:1 mixtures of metMbDTPA(F-2, 4.00 × 10^{-5} mol dm^{-3}) with $CuSO_4 \cdot 5H_2O$ at pH 8.3 (a) and of Na_3H_2dtpa (4.00 × 10^{-5} mol dm^{-3}) with $CuSO_4 \cdot 5H_2O$ at pH 7.3 (b) in 0.016 mol dm^{-3} phosphate buffer solutions. ESR settings were: microwave power, 6.25 mW; field, 300 ± 50 mT (9.215 GHz); modulation, 1.0 mT; time constant, 0.1 s; sweep time, 8 min.

seems to be a 2N2O square planar geometry, being based on the $A_{//}$ values (1.57 × 10^{-2} cm^{-1} and 1.55 × 10^{-2} cm^{-1} for metMb-{Cu^{II}(dtpa)} and free $[Cu^{II}(dtpa)]^{2-}$, respectively).[9]

The fluorescence life-times of ZnMbDTPA (F-1—F-4) are 2.4 ± 0.5 ns at 25 °C, pH 7.0 (a 0.010 mol dm^{-3} phosphate buffer), and ionic strength (I) of 0.03 mol dm^{-3} and similar to that for unmodified ZnMb (2.5 ns). The excited singlet state of Zn(II) protoporphyrin IX (^{1}(ZnPP)*) in ZnMb{Cu^{II}(dtpa)} was quenched by the bound {Cu^{II}(dtpa)} moiety (Figure 2a), although ^{1}(ZnMb)* was not quenched by free $[Cu(dtpa)]^{2-}$. By adding Cu^{2+} ions the life-times became shorter and reached a constant at the $[Cu^{2+}]$ / [ZnMbDTPA] ratio beyond 1.0 (Figure 2b). The *intra*complex quenching rate constants ($k_q^{singlet}$) are (5.0 ± 0.3) × 10^7 s^{-1} (F-1), (8.1 ± 0.5) × 10^7 s^{-1} (F-2), (1.0 ± 0.1) × 10^8 s^{-1} (F-3), and (2.7 ± 0.2) × 10^8 s^{-1} (F-4). Although the back ET reaction was not monitored in the *intra*complex quenching reaction in this work, the quenching reaction might proceed by an ET mechanism.

The excited triplet state of ZnMbDTPA was also quenched by Cu^{2+} ion, which was monitored by T-T absorption spectra at 460 nm.[7] The spontaneous decay rate constant of ^{3}(ZnMbDTPA)* was similar to that for the unmodified ^{3}(ZnMb)* and was independent of the modified species ($k_0^{triplet}$ = (8.0 ± 0.4) × 10 s^{-1}). The observed first-order rate constant depends on the initial

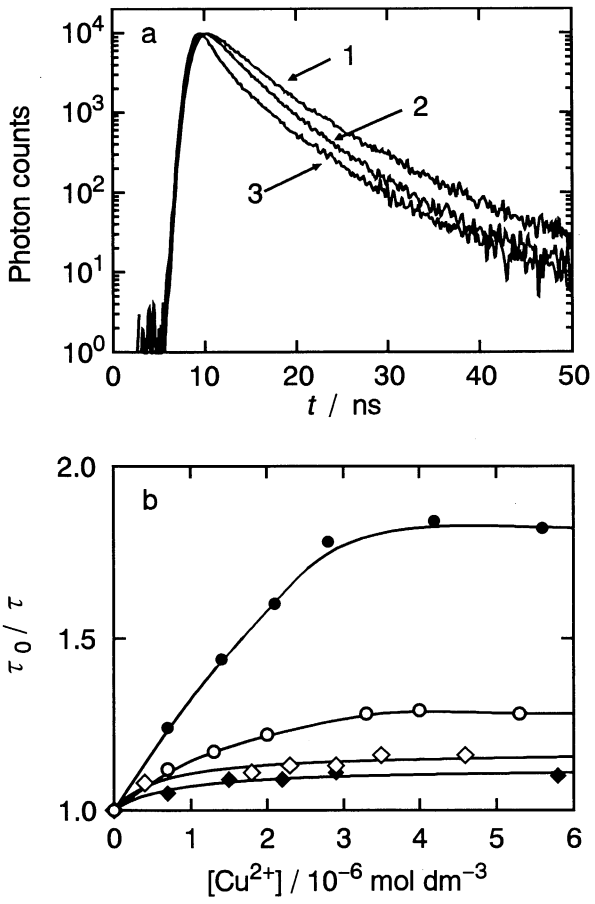
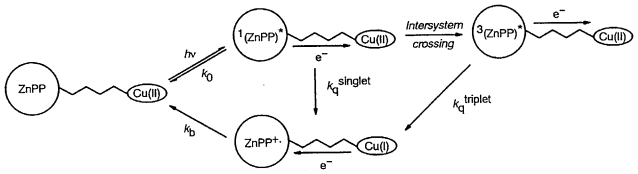


Figure 2. (a) Fluorescence decay of ^{1}(ZnMbDTPA(F-4))* (3.0×10^{-6} mol dm^{-3}) at 25 °C, pH 7.0, and I = 0.03 mol dm^{-3}. (1) In the absence of Cu^{2+} ions, (2) In the presence of equimolar amounts of Cu^{2+} ion, and (3) Lamp. (b) Stern-Volmer plots for the fluorescence life-times of ZnMbDTPA by adding Cu^{2+} ions. (◆) F-1, (◇) F-2, (○) F-3, and (●) F-4.

concentrations of Cu^{2+} ion and a saturation behavior was observed, indicating that ^{3}(ZnPP)* in the modified ZnMb was quenched by the bound {Cu^{II}(dtpa)} moiety. The *intra*complex quenching rate constants ($k_q^{triplet}$) were evaluated from the limited values at higher concentrations of Cu^{2+} ion; $k_q^{triplet}$ = (2.0 ± 0.2) × 10 s^{-1} (F-1), (3.0 ± 0.2) × 10 s^{-1} (F-2), (9.0 ± 0.6) × 10 s^{-1} (F-3), and (1.0 ± 0.1) × 10^2 s^{-1} (F-4). The *inter*molecular quenching of the unmodified ^{3}(ZnMb)* by free $[Cu(dtpa)]^{2-}$ was slow and the quenching rate constant was (8.7 ± 0.6) × 10^4 dm^3 mol^{-1} s^{-1}, which was obtained from the slope of the linear plot of the observed first-order quenching rate constant against the concentrations of free $[Cu(dtpa)]^{2-}$ ion. Although the formation and decay of $ZnMb^{+\cdot}$ were observed at 680 nm in the *inter*molecular system, the modified ZnMb{Cu^{II}(dtpa)} did not show such a signal. This strongly suggests that the back ET reaction in the *intra*complex system is much faster than the quenching step. The reaction mechanism can be represented as follows:



The reactivity in the *intra*complex ET quenching for both excited singlet and triplet states of ZnMb{Cu^{II}(dtpa)} follows the same order that F-1 < F-2 < F-3 < F-4, suggesting that the distance between the binding site of {Cu^{II}(dtpa)} and the Zn(II) porphyrin becomes shorter in this order. We are now determining the binding site of DTPA at lysine residues of the modified myoglobin.

In conclusion we prepared the DTPA-modified ZnMb and found that both excited singlet and triplet states of ZnMb were quenched by the bound {Cu^{II}(dtpa)}.

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References and Notes

1 Dedicated to Professor Ralph G. Wilkins on the occasion of his 70th birthday.
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