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Self-sufficient Electron Injection from NADH to the Active Center of Flavin-Pendant Myoglobin

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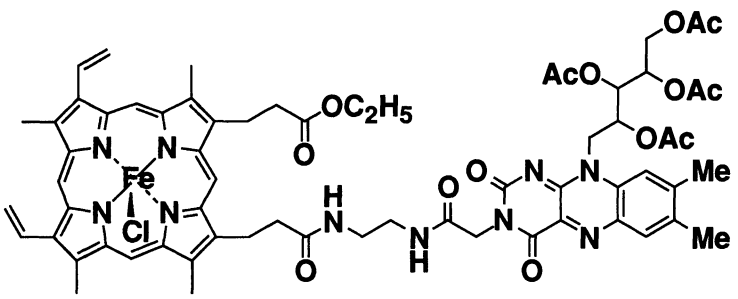
A riboflavin-appended myoglobin was successfully synthesized by the reconstitution of a chemically modified heme with apomyoglobin. Electron transfer from NADH to the heme active site was remarkably accelerated through a pendent riboflavin in the semi-artificial myoglobin.

A redox state of a heme active site directly influences the net reactivity of a hemoprotein. A catalytic cycle of cytochrome P-450 monooxygenase, for example, is initiated by reduction of the oxidized state of heme (Fe(III)).[1] Hemoglobin and myoglobin can absorb dioxygen only in the reduced state of heme (Fe(II)).[2] In natural systems, redox reactions of hemoproteins are mainly regulated by corresponding reductases such as flavoenzymes or cytochromes. As an interesting example of the improved enzymatic activity, a new self-sufficient monooxygenase, cytochrome P-450$_{BM3}$ was recently discovered that combined two domains of the catalytic heme site and the reductase-like flavoprotein site in one enzyme.[3] Kokubo and Kaiser reported that a chemically modified flavohemoglobin could act as a monooxygenase-like enzyme without reductases.[4] We recently found that photo-excited ruthenium tris(bipyridine) complex efficiently reduced the heme active center of a ruthenium-pendent myoglobin, instead of reductase.[5] Here we describe the design and the synthesis of a thermally redox active myoglobin that can accept self-sufficiently electron from dihydronicotinamide adeninedinucleotide (NADH).



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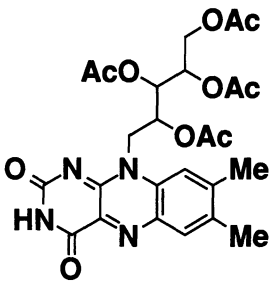

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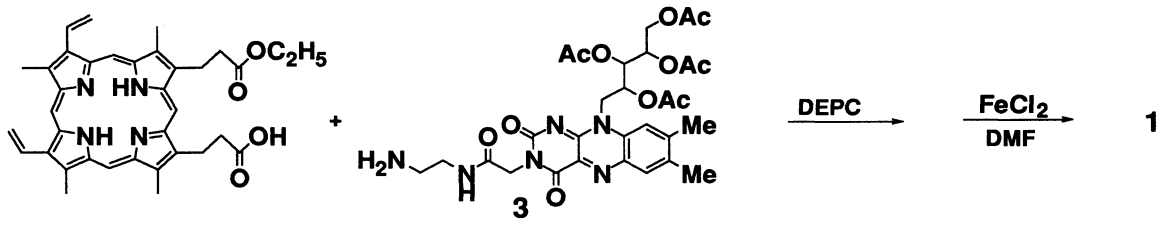
A protoheme derivative bearing tetra-O-acetyl-riboflavin **1** was prepared as shown in Scheme 1. Protoporphyrin IX monoethylester was condensed with a tetra-O-acetylriboflavin derivative **3** in the presence of diethylcyanophosphate, followed by complexation of iron ($FeCl_2$ / DMF under N_2 atmosphere) to afford **1**.[6] The flavin-appended heme **1** was successfully reconstituted with apomyoglobin (apo-Mb ; from horse



Scheme 1.

heart) according to the standard method.[7] The heme **1** (1.2 equiv.) dissolved in ethanolamine and dimethylsulfoxide (1:1 (v:v)) was slowly added dropwise to the aqueous solution of apo-Mb (1.0 equiv., 0.1 mM: 1M = 1 mol dm^{-3}) with gently stirring at 4 °C. The resultant mixture was dialyzed against 10 mM phosphate buffer (pH 6.0) for 12 hours, centrifuged (10000 rpm for 15 min at 4 °C) and passed through gel chromatography (Sephadex G-25, eluent 10 mM phosphate buffer, pH 6.0).

The semi-artificial Mb (oxidized form, met-Fl-Mb) thus obtained, gave a sharp Soret band at 409 nm and Q-bands at 502 and 635 nm due to the heme (Fe(III)) with a broad tail around 440 nm due to the flavin chromophore. Ligand exchange reactions from the axial water to fluoride or azide were monitored by UV-visible spectroscopy (fluoride form: 404 and 605 nm, azide form: 423, 544, and 574 nm), which are quite similar to those of native Mb.[8] The fluorescence spectra of riboflavin derivatives are compared in Fig. 1. The emission intensity of met-Fl-Mb was about 100 times weaker than that of the simple mixture containing tetra-O-acetylriboflavin **2** and native Mb. Such an efficient quenching clearly indicates that the flavin unit is fixed at the proximity of the heme active site in Fl-Mb.

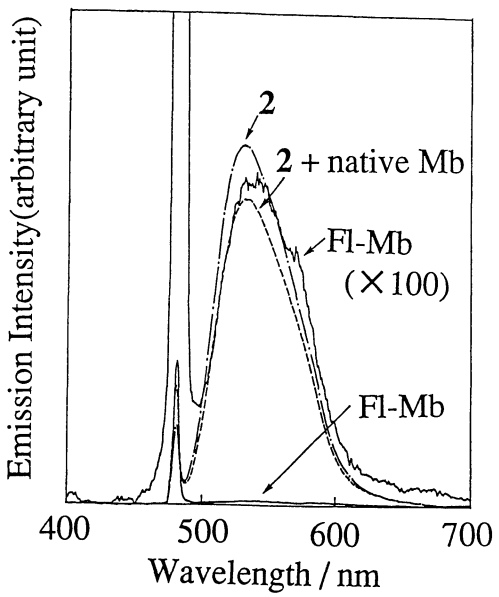


Fig. 1. Fluorescence spectra of riboflavin derivatives. 6.33 x 10^{-6} M: tetra-O-acetylriboflavin **2** only (—·—·—·), equimolar mixture of **2** and native Mb (— — —), met-Fl-Mb(————) in 10 mM phosphate buffer, pH 6. excitation at 470 nm.

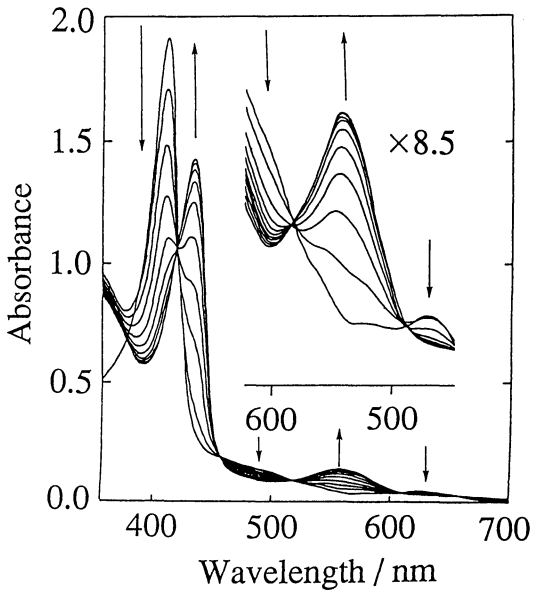


Fig. 2. UV-visible spectral changes of met-Fl-Mb reduction by NADH under N_2 atmosphere. 1.32 x 10^{-5} M met-Fl-Mb, 1.32 x 10^{-4} M NADH in 10 mM phosphate buffer, pH 6.0, 25 °C. Mesurements were made every 1 min.

When NADH as an electron donor was added to the aqueous solution of met-Fl-Mb under unaerobic condition, the reduction smoothly occurred to give deoxy-Fl-Mb (Fe(II)-heme) as monitored by UV-visible spectral change (Fig. 2). The absorbance due to met-Fl-Mb at 409, 502, and 635 nm was gradually lessened with intensifying the absorbance due to deoxy-Fl-Mb at 434 and 559 nm. The obtained deoxy-Fl-Mb rapidly absorbed dioxygen to form a dioxygen complex (oxy-Fl-Mb, λ_{max} = 415, 544, and 580 nm) after it was placed in an aerobic condition.[9]

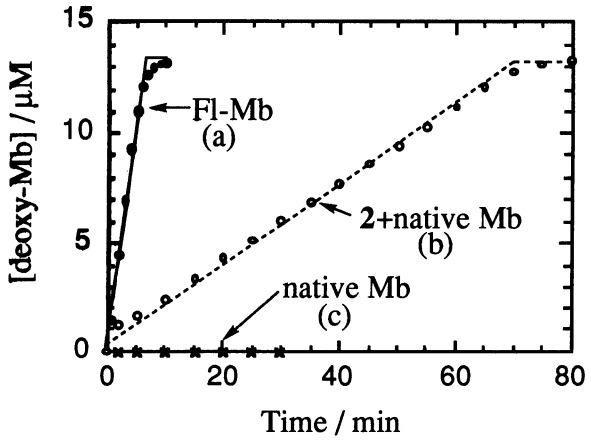
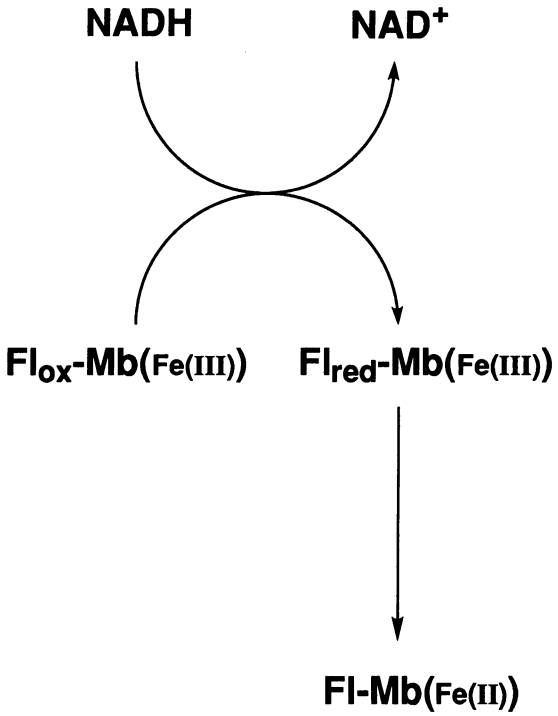


Fig. 3. Time courses of met-Mb reduction by NADH under N_2 atmosphere: 1.32 x 10^{-5} M met-Mb derivatives: native-Mb only (-✱-), equimolar mixture of **2** and native -Mb (-⊖-), met-Fl-Mb (-●-). The reactions were initiated by 1.32 x 10^{-4} M NADH into the Mb-containing aqueous solution in 10 mM phosphate buffer, pH 6.0, 25 °C.



Scheme 2. Electron transfer processes.

The time course of the met-Fl-Mb reduction by NADH in N_2 atmosphere shows the linear increase in deoxy-Fl-Mb (Fig. 3a). Compared to the intermolecular system (i.e., **2** and native Mb; Fig. 3b), the reduction rate of met-Fl-Mb was enhanced by a factor of 13. No reaction occurred in the absence of the flavin **2** (i.e., native Mb only; Fig. 3c). It is clear that the flavin group acts as an efficient electron mediator from NADH to Fe(III)-heme of Mb. The overall reaction proceeds via two steps, that are the reduction of flavin unit by NADH and the following electron transfer to Fe(III)-heme (see Scheme 2). The zeroth-order kinetics on Mb concentration in both the intra- and intermolecular reactions demonstrates that the rate-determining step is not the electron transfer process from flavin to Mb, but the reduction process of flavin by NADH. At higher pH (shifted from pH 6 to pH 8), the rate was lessened to a sixth in Fl-Mb, while the intermolecular reaction rate did not considerably change.[10] These implies that the flavin unit covalently fixed to the cationic Mb surface facilitates the electron uptake from anionic NADH to enhance the net reaction rate.[11]

In summary, we developed a new semi-artificial myoglobin bearing a self-sufficient electron transport system by the cofactor reconstitution method. Since flavin chromophore is known to be an efficient photo sensitizer, a photo-electron injection may also be possible in the present Fl-Mb as well as a thermal one. Detailed properties of Fl-Mb are now investigated in our laboratory.

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6) **1** was purified through column chromatography (silica gel, $CHCl_3$:CH_3OH = 50:1 to 5:1 (gradient, V / V). Anal. Found **1**: C, 59.95; H, 5.62; N, 10.55%. Calcd for $C_{65}H_{70}N_{10}O_{14}FeCl$: C, 59.75; H, 5.40; N, 10.72%.
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8) M. Tamura, T. Asakura, and T. Yonetani, *Biochim. Biophys. Acta*, **295**, 467 (1973).
9) UV-visible spectra of deoxy- and oxy-Fl-Mb are almost identical with those of native Mb.
10) The initial rates of Fl-Mb were 2.3×10^{-6} M/min and 3.8×10^{-7} M/min at pH 6.0 and pH 8.0, respectively. The rates in the intermolecualr reaction were 1.8×10^{-7} M/min and 2.7×10^{-7} M/min at pH 6.0 and pH 8.0, respectively.
11) It was reported that NADH oxidation catalyzed by flavin moieties is accelerated in cationic polyelectrolytes and cationic bilayer surfaces. S. Shinkai, S. Yamada, and T. Kunitake, *Macromolecules*, **11**, 65 (1978); I. Hamachi and Y. Kobuke, *J. Chem. Soc., Chem. Commun.*, **1989**, 130. The redox potential difference between the flavin dissolved in bulk solution and the flavin bound to the protein surface may also influence the rate enhancement .

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