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Reductive Activation of Dioxygen by a Myoglobin Reconstituted with a Flavohemin

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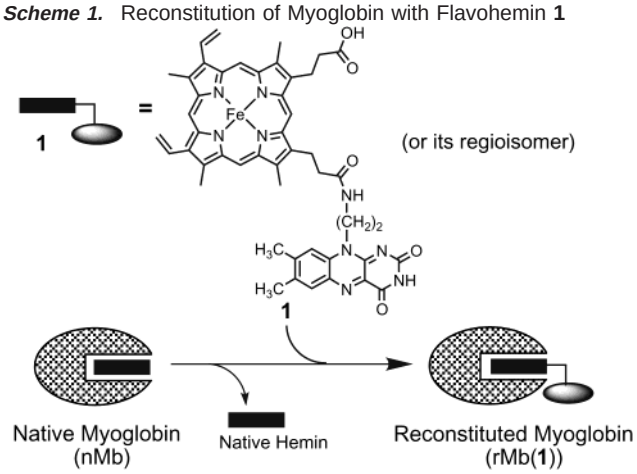
Myoglobin (Mb) and cytochrome P450 (P450) are hemoproteins having the same prosthetic group, protoheme IX, although the bound dioxygen on the heme is utilized in a different way by each protein: Mb simply binds a dioxygen, whereas P450 activates the dioxygen bound to the heme-iron. One reason for the critical differences is that P450 has a unique system where two electrons are sequentially transferred from NAD(P)H to the hemin via a reductase such as a flavoprotein, to generate highly reactive oxidants,[1] whereas Mb does not have such an efficient electron pathway. Therefore, it is expected that the construction of an artificial electron pathway near the heme pocket can convert Mb into a dioxygen-activating protein. To engineer the function of Mb, the replacement of the native hemin with a chemically modified one is a powerful strategy.[2] For the dioxygen activation by Mb, we have recently designed a new myoglobin, rMb(**1**), reconstituted with a flavohemin **1**, where the flavin moiety (isoalloxazine ring) is a mediator of electron transfer as shown in Scheme 1.[3,4] In this report, we demonstrate the preparation of rMb(**1**) and its reactivity toward P450-like dioxygen activation as compared to that of native Mb (nMb).[5]

Scheme 1. Reconstitution of Myoglobin with Flavohemin **1**



The flavohemin **1** was synthesized from riboflavin and protoporphyrin IX. The deconvoluted ESI-TOF-mass spectrum of rMb(**1**) gave the desired mass number of 17 834, which reveals the exact number of the holoprotein. The UV−vis spectrum of rMb(**1**) shows that **1** is located in the normal position of the heme pocket (λ_{max} in the Soret region = 408 nm). The ^{1}H NMR spectrum of cyanomet rMb(**1**) suggests that two hemin conformers (the normal and the reversed form) are present in a ratio of 1:1.[6] The characteristic fluorescence of the flavin (λ_{ex} = 470 nm, λ_{em} = 520 nm) was not observed, indicating that the covalently linked flavin is situated at the proximity of the porphyrin ring.

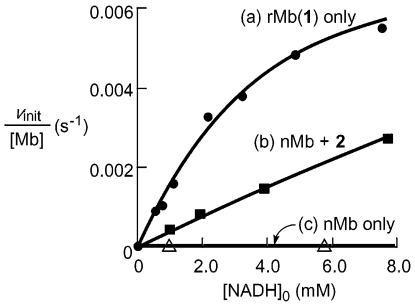


Figure 1. Formation of oxygenated Mbs versus the initial concentration of NADH: (a) rMb(**1**) only, (b) nMb + **2** ([nMb]/[**2**] = 1/1), and (c) nMb only. Conditions: 10 mM phosphate buffer (pH = 7.5), 25 °C, [rMb(**1**)] = [nMb] = 3 μM, SOD 53 units, catalase 252 units. The rate was obtained by following the increase in the absorbance at 580 nm.

At first, the reaction of rMb(**1**) with NADH, an electron donor, was investigated under aerobic conditions in the presence of SOD and catalase. These additives are necessary to eliminate the effects of superoxide anion ($O_2^{\bullet-}$) and/or hydrogen peroxide.[7] Immediately after the addition of NADH to the buffer solution of rMb(**1**), the appearance of two peaks in the Q-band (542 and 581 nm) and a red shift in the Soret band were observed, which are typical spectral changes derived from the formation of the oxy complex.[8] The initial rate of oxymyoglobin formation, v_{init}, depends on the initial concentration of NADH with saturation kinetics (Figure 1a). On the contrary, the formation of the oxygenated nMb in the presence of 10-(*N*-acetylaminoethyl)isoalloxazine (**2**) shows linear kinetics in the range of $[NADH]_0$ employed (Figure 1b), indicating that the apparent K_m is significantly large, because K_m could be reflected by two binding processes, NADH-**2** and **2**-nMb pairings. The overall rate constant, k_{cat}/K_m, calculated from the slope is one-sixth that of rMb(**1**) (Table 1). This is because, in rMb(**1**), the smooth electron transfer from NADH to the hemin is achieved through the covalently linked flavin, as we expected. In the absence of **2**, the formation of the oxy complex in nMb is extremely slow (Figure 1c). Therefore, the direct electron transfer from NADH to the hemin in Mbs is ruled out, although the process is not thermodynamically unfavorable.[9]

Next, to evaluate the reductive activation of the oxyheme, we monitored the deformylation catalyzed by Mbs in the presence of SOD and catalase, because it has been proposed that the deformylation by P450s is initiated by the nucleophilic attack of Fe(III)-peroxoanion (Fe(III)-O_2^{2-}), the one-electron reductant of the oxy complex by NADH, on the formyl carbon.[1b,10,11] When 2-phenyl-

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Table 1. Kinetic Parameters for Formation of an Oxy Complex and Deformylation Activity

myoglobin	k_{cat}/K_m for formation of oxy complex[a] ($M^{-1}s^{-1}$)	initial formation of acetophenone[b] (10^{-6} mol min^{-1})
rMb(**1**)	2.1 ± 0.4[c]	0.23 ± 0.02
nMb + **2**[d]	0.35 ± 0.01	0.051 ± 0.005
nMb	n.d.[e]	n.d.[e]

[a] Conditions are described in the footnote of Figure 1. [b] Conditions: 10 mM phosphate buffer (pH = 7.5), 25 °C, [2-PPA] = 9.6×10^{-4} M, [Mb] = [**2**] = 1.0×10^{-5} M, $[NADH]_0 = 1.1 \times 10^{-3}$ M, SOD 53 units, catalase 252 units. [c] $K_m = (4.3 \pm 0.7) \times 10^{-3}$ M, $k_{cat} = (9.0 \pm 0.8) \times 10^{-3}$ s^{-1}. [d] [nMb]/[**2**] = 1/1. [e] Too slow to determine.

propionaldehyde (2-PPA), a secondary aldehyde, was employed as a substrate, acetophenone was detected as a unique product.[12] The deformylation by rMb(**1**) proceeds more than 4 times faster than that by nMb upon the addition of **2** (Table 1), indicating that the flavin covalently linked to the heme propionic acid functions as an effective mediator of the continuous electron flow from NADH to the hemin.

No formation of 2-phenylpropionic acid, styrene, nor 1-phenylethanol, which are the products observed in the deformylation of 2-PPA by P450 2B4,[10c] was confirmed by GC-MS or HPLC. However, Watanabe and co-workers reported that 2-PPA is converted to acetophenone by Fe(III)(TPP)-oxetane (chemically equivalent to Fe(III)(TPP)-O_2^{2-}; TPP = tetraphenylporphyrin dianion) without any other product,[10d] which is consistent with our results.[13] Therefore, it can be assumed that the reactive species in the deformylation by rMb(**1**) is Fe(III)-O_2^{2-} and the peroxoanion species nucleophilically attacks on the carbonyl group of 2-PPA. It has been proposed that, in the deformylation of 2-PPA by P450 2B4, the nucleophilic attack of Fe(III)-peroxoanion affords Fe(III)-hemiacetal, which rapidly decomposes to the oxoferryl species and an alkyl radical, $PhC^{\bullet}HCH_3$.[10c] The rebound reaction dominantly occurs between these two species, resulting in the formation of 1-phenylethanol. In contrast, if the $PhC^{\bullet}HCH_3$ is generated near the protein surface, the radical could be quenched by dissolved dioxygen, which would lead to acetophenone as a final product.[14] In fact, the formation of acetophenone was observed in our system. Thus, rMb(**1**) certainly catalyzes the deformylation of 2-PPA in the same manner to P450 2B4, whereas the products from the radical intermediate are different between rMb(**1**) and P450 2B4 systems, because the heme in rMb(**1**) is located near the protein surface.

Contrary to 2-PPA, no product was detected when 3-phenylpropionaldehyde (3-PPA), a primary aldehyde, was employed. In the reaction of P450 2B4 with 3-PPA, the more reactive radical, $PhCH_2C^{\bullet}H_2$, produced after the decomposition of Fe(III) hemiacetal, attacks the meso position of the heme, resulting in the formation of the heme adduct and in the inactivation of the protein.[10c,e] Although this reaction probably occurs in rMb(**1**), we are not able to detect the heme adduct from 3-PPA in rMb(**1**) at present.

In conclusion, we successfully constructed an effective dioxygen activation system in Mb having a flavohemin **1**. To the best of our knowledge, this is the first example that shows the generation and reactivity of the Fe(III)-peroxoanion intermediate in Mb. The above result demonstrates that the replacement of the native hemin with a chemically modified one having a functional group is useful for creating a new function in hemoproteins.[2]

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Supporting Information Available: Experimental procedures, ESI-TOF-MS, UV−vis, and fluorescence spectra of met rMb(**1**), ^1H NMR spectrum of cyanomet rMb(**1**), transient spectra during the reactions, and the kinetic data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Sono, M.; Roach, M. P.; Coulter, E. D.; Dawson, J. H. *Chem. Rev.* **1996**, *96*, 2841−2887. (b) Watanabe, Y. *J. Biol. Inorg. Chem.* **2001**, *6*, 846−856. (c) Ogliaro, F.; de Visser, S. P.; Cohen, S.; Sharma, P. K.; Shaik S. *J. Am. Chem. Soc.* **2002**, *124*, 2806−2817.

(2) (a) Hayashi, T.; Hitomi, Y.; Ando, T.; Mizutani, T.; Hisaeda, Y.; Kitagawa, S.; Ogoshi, H. *J. Am. Chem. Soc.* **1999**, *121*, 7747−7750. (b) Hayashi, T.; Hisaeda, Y. *Acc. Chem. Res.* **2002**, *35*, 35−43. (c) Hayashi, T.; Dejima, H.; Matsuo, T.; Sato, H.; Murata, D.; Hisaeda, Y. *J. Am. Chem. Soc.*, 11226−11227, in this issue.

(3) It has been reported that the hemoglobin in which the flavin is linked with Cys^{95} has hydroxylase activity. Kokubo, T.; Sassa, S.; Kaiser, E. T. *J. Am. Chem. Soc.* **1987**, *109*, 606−607.

(4) Hamachi and co-workers have reported that myoglobin with a flavohemin smoothyl reduced by NADH under anaerobic conditions, although an experiment under aerobic conditions was not shown. Hamachi, I.; Nomoto, K.; Tanaka, S.; Tajiri, Y.; Shinkai, S. *Chem. Lett.* **1994**, 4439−4442.

(5) In most of the previous reports on the chemical reactivity of Mbs, wild-type or mutant Mbs catalyze oxidations upon the addition of *activated* oxygen (e.g., H_2O_2). Raven, E. L.; Mauk, A. G. *Adv. Inorg. Chem.* **2001**, *51*, 1−49 and references therein.

(6) The signal peaks originating from 5-Me and 1-Me in the hemin are respectively split into two peaks with the same intensity. (a) La Mar, G. N.; Toi, H.; Krishnamoorthi, R. *J. Am. Chem. Soc.* **1984**, *106*, 6395−6401. (b) Hauksson, J. B.; La Mar, G. N.; Pandey, R. K.; Rezzano, I. N.; Smith, K. M. *J. Am. Chem. Soc.* **1990**, *112*, 6198−6205.

(7) Mieyal, J. J.; Ackerman, R. S.; Blumer, J. L.; Freeman, L. S. *J. Biol. Chem.* **1976**, *251*, 3436−3441.

(8) Antonini, E.; Brunori, M. *Hemoglobin and Myoglobin in Their Reaction with Ligands*; North-Holland: Amsterdam, 1971; pp 16−21.

(9) It has been reported that methemoglobin is not reduced by NADH in the absence of phenazine-methosulfate. Kajita, A.; Noguchu, K.; Shukuya, R. *Biochem. Biophys. Res. Commun.* **1970**, *39*, 1199−1204.

(10) (a) Watanabe, Y.; Ishimura, Y. *J. Am. Chem. Soc.* **1989**, *111*, 410−411. (b) Wertz, D. L.; Sisemore, M. F.; Selke, M.; Driscoll, J.; Valentine, J. S. *J. Am. Chem. Soc.* **1998**, *120*, 5331−5332. (c) Raner, G. M.; Chiang, E. W.; Vaz, A. D. N.; Coon, M. J. *Biochemistry* **1997**, *36*, 4895−4902. (d) Goto, Y.; Wada, S.; Morishima, I.; Watanabe, Y. *J. Inorg. Biochem.* **1998**, *69*, 241−247. (e) Kuo, C.-L.; Raner, G. M.; Vaz, A. D. N.; Coon, M. J. *Biochemistry* **1999**, *38*, 10511−10518.

(11) The inability of organic peracids to undergo deformylation suggests that the deformyaltion activity is a good indication of net dioxygen activation on the heme. Vaz, A. D. N.; Roberts, E. S.; Coon, M. J. *J. Am. Chem. Soc.* **1991**, *113*, 5886−5887.

(12) It was confirmed by HPLC that the reduction of acetophenone by NADH is negligible under the conditions employed.

(13) In an enzymatic system, the one-electron reductant of the oxyheme is proposed to involve Fe(III)-O_2^{2-}, not Fe(III)-oxetane, due to the existence of the stabilization by the imidazole on the distal His. Davydov, R.; Macdonald, I. D. G.; Makris, T. M.; Sligar, S. G.; Hoffman, B. M. *J. Am. Chem. Soc.* **1999**, *121*, 10654−10655 and references therein.

(14) Kim, C.; Dong, Y.; Que, L., Jr. *J. Am. Chem. Soc.* **1997**, *119*, 3635−3636.

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