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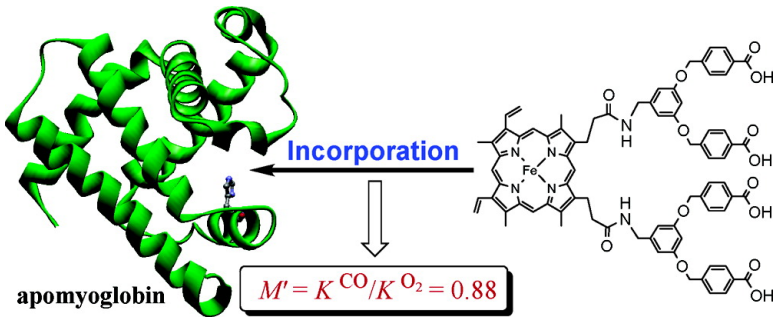

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Unusual Ligand Discrimination by a Myoglobin Reconstituted with a Hydrophobic Domain-Linked Heme

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Myoglobin, one of the most famous dioxygen storage proteins, is a well-investigated hemoprotein.[1] To understand the physiological function of myoglobin, it is important to focus not only on the O_2 affinity for deoxymyoglobin but also on the O_2 discrimination from other ligands such as CO.[2] A series of ligand binding studies of myoglobin mutants indicate that the structure of the distal site strictly regulates the O_2 affinity and O_2/CO discrimination.[3,4] On the basis of this finding, it will be significant to improve the physiological function of myoglobin by molecular engineering. Over the past decade, it has been revealed that several unique mutants such as the L29F and V68N sperm whale myoglobins decreased in the specific affinity of CO over O_2 ($M' = K^{CO}/K^{O_2}$) by 10−20-fold compared to the native protein[5] ($M' = 2.5$ and 1.2 for L29F[6] and V68N,[7] respectively). Furthermore, replacement of the native heme **1** with an artificial prosthetic group is another way to modulate the myoglobin function.[8] For example, the incorporation of a structural isomer of iron porphyrin into the apoprotein dramatically enhances the O_2 affinity and reduces the M' value.[9,10] However, it is likely that the O_2 dissociation is too slow to apply the reconstituted myoglobins to an artificial O_2 carrier. In contrast, there has been no example of improving the physiological function by modification of the heme propionate side chains, although the reconstituted myoglobin could be readily available. Here, we demonstrate the first example to regulate the O_2/CO discrimination by introducing a hydrophobic cluster into the terminal of the two propionates.

The artificial hemes **2**−**4** having hydrophobic moieties linked at each terminal of the two heme propionates were synthesized (Figure 1). Reconstituted horse heart myoglobins were constructed using a standard reconstitution protocol and purified by gel filtration chromatography.[9,11] The aromatic moieties of the modified hemes should form a hydrophobic domain on the protein surface. The UV−vis absorption spectra of the reconstituted myoglobins exhibited the characteristic spectra similar to those observed for the native myoglobin nMb(**1**), indicating that **2**−**4** are located in the normal position of the heme pocket (see Supporting Information). The ESI-mass spectra of the reconstituted myoglobins, rMb(**2**) and rMb(**3**), showed the characteristic peaks corresponding to the multiple ionization states. The deconvoluted mass spectra of rMb(**2**) and rMb(**3**) gave the desired mass numbers of the holoproteins, 18349 ± 1 (calcd 18346) and 19375 ± 1 (calcd 19371), respectively. Furthermore, the autoxidation of oxymyoglobin to metmyoglobin was monitored by the UV−vis spectral changes in 0.1 M potassium phosphate buffer (pH 7.0) at 25 °C. The rate constant (k_{auto}) of oxy-rMb(**4**) (0.18 ± 0.02 h^{-1}) was 6-fold higher than that of nMb(**1**) (Table 1), indicating that the elimination of the salt bridges between the heme propionates and surrounding ionic amino acid residues, such as Lys45, destabilizes oxy-rMb(**4**).[12,13] In contrast, the k_{auto} value of oxy-rMb(**2**) was 4-fold smaller than that of oxy-rMb(**4**) and as small as that of nMb(**1**). Therefore, to prevent oxy-rMb(**2**) from being autoxidized, the hydrophobic domain of **2** may inhibit the penetration of H_2O molecules into the heme pocket, even though oxy-rMb(**2**) also has no salt bridge at the heme propionates.

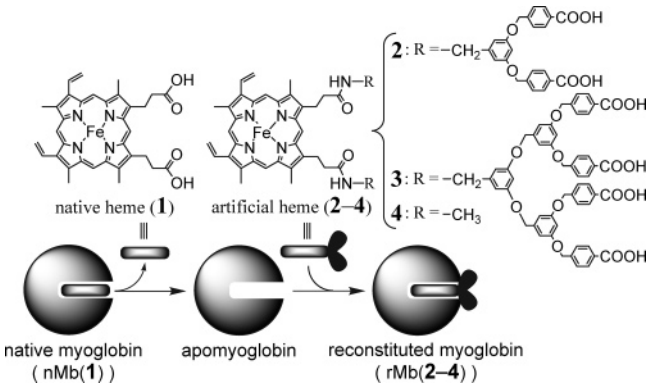

Figure 1. Construction of reconstituted myoglobins with a hydrophobic domain-linked heme.

To evaluate the effect of the introduction of the artificial hydrophobic domain onto the heme pocket, the O_2 and CO bindings were examined in detail by laser flash photolysis and stopped-flow rapid mixing techniques.[9] The kinetic parameters for the O_2 binding to the deoxymyoglobins are summarized in Table 1. The O_2 dissociation rate constant ($k_{off}^{O_2}$) for oxy-rMb(**2**) is as small as that obtained for oxy-nMb(**1**). Oxy-rMb(**3**) also showed a slightly smaller $k_{off}^{O_2}$ (14 ± 1 μM^{-1} s^{-1}) than that of oxy-nMb(**1**). It is suggested that, even in oxy-rMb(**2**) and oxy-rMb(**3**), the heme-bound O_2 should be stabilized by the preserved hydrogen bonding between the heme-bound O_2 and distal His64 imidazole. The observed time courses for the O_2 association to deoxy-nMb(**1**), deoxy-rMb(**2**), and deoxy-rMb(**4**) by laser flash photolysis were monophasic and fit well to single-exponential expressions which led to pseudo-first-order rate constants under aerobic conditions (see Supporting Information). The O_2 associations to the heme iron of deoxy-rMb(**2**) and deoxy-rMb(**4**) are 44-fold and 2.4-fold slower than that observed for deoxy-nMb(**1**), respectively. These results indicate that the constructed hydrophobic domain at the entrance of the heme pocket hinders the approach of an O_2 molecule into the heme pocket. Thus, the binding constant of O_2 (K^{O_2}) for rMb(**2**) decreased by 38-fold as compared to nMb(**1**).

The kinetic parameters for the CO binding to the deoxymyoglobins are also shown in Table 1. The reconstitution with the modified heme **2** decreased the CO association rate constant (k_{on}^{CO}) to the heme iron of deoxymyoglobin by 41-fold and increased the CO dissociation rate constant (k_{off}^{CO}) from the heme iron by 20-fold. Thus, the binding constant of CO (K^{CO}) for rMb(**2**) was significantly decreased (810-fold), compared to that of nMb(**1**). It is suggested that the introduced hydrophobic domain at the entrance

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Table 1. Autoxidation Rate Constants and O_2 and CO Binding Parameters for the Native and Reconstituted Myoglobins at 25 °C[a]

myoglobin	k_{auto} (h^{-1})	$k_{on}^{O_2}$ ($\mu M^{-1}\,s^{-1}$)	$k_{off}^{O_2}$ (s^{-1})	K^{O_2} (μM^{-1})	k_{on}^{CO} ($\mu M^{-1}\,s^{-1}$)	k_{off}^{CO} (s^{-1})	K^{CO} (μM^{-1})	M'
nMb(**1**)	0.029 ± 0.002	21 ± 1	24 ± 1	0.87	0.61 ± 0.02	0.035 ± 0.002	17	20
rMb(**2**)	0.043 ± 0.005	0.48 ± 0.01	20 ± 1	0.024	0.015 ± 0.001	0.70 ± 0.09	0.021	0.88

[a] Conditions: 0.1 M potassium phosphate buffer (pH 7.0). k_{auto} is the autoxidation rate constant of oxymyoglobin to metmyoglobin. $k_{on}^{O_2}$ and k_{on}^{CO} are the association rate constants of O_2 and CO to heme iron, respectively. $k_{off}^{O_2}$ and k_{off}^{CO} are the dissociation rate constants of O_2 and CO from heme iron, respectively. K^{O_2} and K^{CO} are the binding constants of O_2 and CO, respectively, and were calculated from the measured k_{on} and k_{off} values. $M' = K^{CO}/K^{O_2}$.

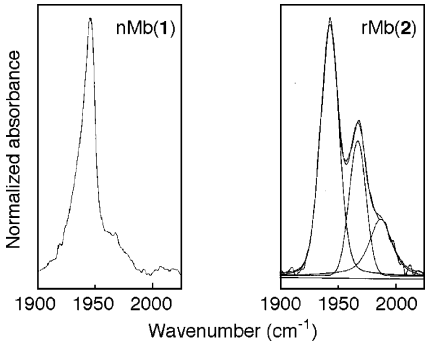

Figure 2. IR spectra of CO-bound nMb(**1**) and rMb(**2**). Conditions: 1.3 mM nMb(**1**) or 0.5 mM rMb(**2**), 0.1 M potassium phosphate (pH 7.0), 20 mM $Na_2S_2O_4$. Resolution = 2 cm^{-1}, light path = 0.1 mm, accumulation = 1024, at room temperature. The smooth lines for the spectrum of rMb(**2**) represent three bands separated by a curve-fitting analysis.

of the heme pocket should modify the environment at the heme distal site to hamper the CO association to the heme iron of deoxy-rMb(**2**) and accelerate the CO dissociation from the CO-bound rMb(**2**). To obtain further information about the distal pocket structure of the reconstituted myoglobin, the infrared (IR) spectra of CO-bound nMb(**1**) and rMb(**2**) were obtained (Figure 2). The IR spectrum of the CO-bound rMb(**2**) exhibited three major bands at 1942 (56%), 1967 (25%), and 1987 cm^{-1} (19%) for the C−O stretching frequency (ν_{CO}) at ambient temperature, while the CO-bound nMb(**1**) showed a major peak at 1944 cm^{-1}.[14] The higher frequency bands for the CO-bound rMb(**2**) appeared to be due to the loss of the hydrogen-bonding interactions between the ligated CO and His64 and/or some steric repulsion at the distal site.[14] These data suggest that the construction of the hydrophobic domain by the modified heme reconstitution perturbs the distal site structure of rMb(**2**) and strengthens the C−O bond.

Moreover, the ligand discrimination in myoglobin can be evaluated using the M' value. Compared to nMb(**1**), the M' value of rMb(**2**) decreased to 0.88 due to the relatively large k_{off}^{CO} value for rMb(**2**) (Table 1), indicating the preferential binding of O_2 over CO. The smallest M' value reported thus far for the myoglobin mutants was 1.2 by the V68N mutation at the distal site of sperm whale myoglobin due to the relatively small $k_{off}^{O_2}$ value.[5,7] The present result indicates that the perturbation of the distal site by modification of the heme propionates clearly regulates the ligand dissociation.

In summary, we constructed new reconstituted myoglobins possessing a hydrophobic domain at the terminal of the two heme propionate side chains. The artificially created domain worked as a barrier against exogenous ligand penetration into the heme pocket, whereas the bound O_2 was stabilized in the reconstituted myoglobin as well as in the native one. The present study concludes that the O_2 selectivity of myoglobin over CO is markedly improved by chemically modifying the heme propionates without any mutation of the amino acid residues at the distal site. This approach will be useful to construct a new supramolecular composite from a hemoprotein and to develop unique biomaterials.

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Supporting Information Available: Syntheses of hemes **2**−**4**, preparation of reconstituted myoglobins, experimental procedures, UV−vis spectra, mass spectra of rMb(**2**) and rMb(**3**), flash photolysis and stopped-flow data of rMb(**2**), ligand binding parameters (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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