Hybridization of Modified-Heme Reconstitution and Distal Histidine Mutation to Functionalize Sperm Whale Myoglobin

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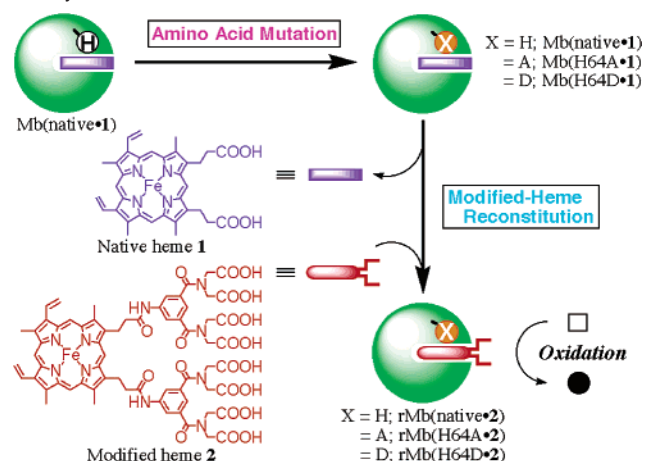
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Hemoproteins, which have an iron porphyrin as a prosthetic group, show a variety of functions in physiological systems due to the unique combination of the heme and protein matrix.[1] Thus, it is of considerable interest to convert the function of a hemoprotein into different ones in order to elucidate hemoprotein mechanisms or create new biomaterials. Over the past decade, a lot of effort has been devoted to the mutagenetic approach, that is, an amino acid residue in an active site is replaced with another one to improve the enzymatic reactivity.[2] For example, a myoglobin in which an amino acid on the heme distal side is selectively mutated to an appropriate one has shown good peroxidase and/or peroxygenase activity.[3,4] In contrast, replacement of a native heme with an artificially created prosthetic group is another way to modify a hemoprotein, although examples that exhibit an enhancement of enzymatic reactivity are quite limited.[5,6] It has recently been found that the introduction of a hydrophobic domain into two heme-propionate termini constructs a substrate binding site in myoglobin.[5] However, an alternate method, the mutation or heme reconstitution, does not always demonstrate sufficient activity compared with the native heme enzyme, because myoglobin has at least two disadvantages over a series of heme-containing oxidases: an unsuitable active site and no substrate binding site. Therefore, to convert sperm whale myoglobin into peroxidase, we now propose a new myoglobin having a suitable amino acid configuration on the heme distal side and a hydrophobic substrate binding site near the heme pocket at the same time (Scheme 1).

An artificially created moiety of **2** linked at each terminal of heme-propionate side chain in protohemin IX **1** has one benzene ring and four carboxylate groups, which should work as a substrate-fixing site and increase the solubility of the hemin, respectively. The reconstitution of myoglobin with **2** was performed by a conventional method.[7] Myoglobin variants with **2**, rMb(H64A·**2**) and rMb(H64D·**2**), were obtained from the combination of the distal histidine mutant apomyoglobins and **2**.[8] Their UV–vis spectra were consistent with those of Mb(H64A·**1**) and Mb(H64D·**1**), respectively. To assess the formation of the heme active species, an oxo-ferryl porphyrin cation radical (Por$^{+•}$Fe^{IV}=O, compound I) and/or an oxo-ferryl heme (PorFe^{IV} = O, compound II), the UV–vis spectral changes were observed using a stopped-flow apparatus. Myoglobin was mixed with excess H_2O_2 at 25 °C, and the formation rates of compounds I (k_1) and II (k_2) were calculated from the decrease in the 408 nm peak and the increase in absorbance at 425 nm, respectively (see Supporting Information). Only the k_2 values could be determined for Mb(native·**1**), rMb(native·**2**), and Mb-

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Scheme 1. Construction of Myoglobins with High Peroxidase Activity

(H64A·**1**), because the decay of compound I of these myoglobins might be too fast to be detected under these conditions. On the contrary, both the k_1 and k_2 values were determined for rMb(H64A·**2**), Mb(H64D·**1**), and rMb(H64D·**2**). The reconstituted myoglobins with **2** formed reactive heme intermediate species as fast as the corresponding myoglobins with the native heme **1**.

To evaluate the effects of the hybridization of the distal histidine mutation and the modified-heme reconstitution on the peroxidase activity of the myoglobin, 2-methoxyphenol oxidation was examined in the presence of H_2O_2 in 20 mM sodium malonate buffer (pH 6.0) at 25 °C. The formation of an assay product, tetraguaiacol, was detected as an absorption increase at 470 nm, and initial turnover rates were calculated (Figure 1).[5,9,10] The initial turnover rate of rMb(native·**2**) was increased to 0.18 ± 0.01 s^{-1} by the reconstitution with **2**, and it was 10-fold higher than that of Mb(native·**1**) (0.019 ± 0.001 s^{-1}). Also, the turnover rate of rMb(H64A·**2**) was 0.27 ± 0.02 s^{-1} and it was 14-fold higher than that of Mb(H64A·**1**) (0.020 ± 0.004 s^{-1}). In the case of Mb(H64D·**1**), the turnover rate (1.3 ± 0.1 s^{-1}) was already high due to proper heme pocket environments for the reactive species formation.[3a] Furthermore, rMb(H64D·**2**) showed a significant turnover rate of 5.6 ± 0.6 s^{-1}, which is 300-fold higher than that of Mb(native·**1**). Figure 1 shows that the turnover rate of rMb(H64D·**2**) almost achieved that of horseradish peroxidase (HRP) (32 ± 1 s^{-1}) through combination of the distal histidine mutation and modified-heme reconstitution.[11]

Steady-state kinetics of the 2-methoxyphenol oxidation was examined to depict the effects on the heme modification and the histidine mutation in detail (Table 1). Compared with Mb(native·
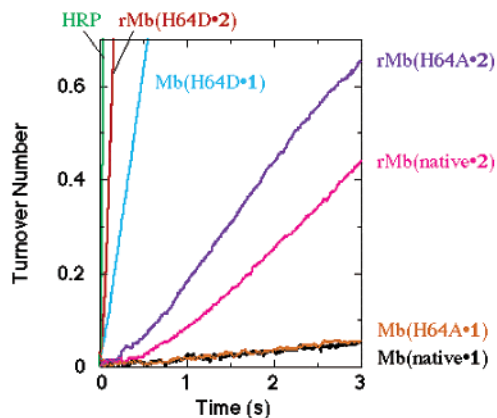
Figure 1. Time course of 2-methoxyphenol oxidation by myoglobin variants and HRP. Conditions: 2.0 μM protein, 0.5 mM 2-methoxyphenol, and 15 mM H_2O_2 in 20 mM sodium malonate buffer (pH 6.0) at 25 °C.

Table 1. Kinetic Parameters of 2-Methoxyphenol Oxidation[a]

myoglobin	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} s^{-1})
Mb(native·**1**)	2.8 ± 0.6	54 ± 15	53
rMb(native·**2**)	6.2 ± 0.6	3.4 ± 0.6	1800
Mb(H64A·**1**)	0.094 ± 0.013	0.98 ± 0.30	95
rMb(H64A·**2**)	2.9 ± 0.3	0.58 ± 0.11	5000
Mb(H64D·**1**)	9.0 ± 1.2	1.8 ± 0.4	5100
rMb(H64D·**2**)	1.2 ± 0.1	0.052 ± 0.016	23 000

[a] Performed with 2.0 μM myoglobin, 100 mM H_2O_2 (15 mM for Mb(H64D·**1**) and rMb(H64D·**2**)), and 20 mM sodium malonate (pH 6.0) at 25 °C. $v/[\text{myoglobin}] = k_{cat}/\{1 + (K_m/[2\text{-methoxyphenol}]) + ([2\text{-methoxyphenol}]/K_i)\}$.

1), rMb(native·**2**) showed a 16-fold lower K_m value and 34-fold higher k_{cat}/K_m value, the catalytic efficiency. The large decrease in the K_m value of rMb(native·**2**) suggests that the hydrophobic site of **2** may act as a substrate binding site.[5] On the basis of the spectral titration experiments, the replacement of the native heme **1** in Mb(native·**1**) with **2** decreased the dissociation constant (K_d) by 83-fold (0.083 ± 0.028 mM for rMb(native·**2**)). These data support the fact that the introduced hydrophobic domain linked covalently at the heme-propionate side chains can become a binding site for hydrophobic substrates. The mutant Mb(H64A·**1**) showed a 55-fold lower K_m value compared with the Mb(native·**1**) (Table 1), corresponding to the fact that Mb(H64A·**1**) showed a much lower K_d value (0.036 ± 0.015 mM) than that of Mb(native·**1**). Through reconstitution with **2**, rMb(H64A·**2**) achieved a k_{cat} value that was 30-fold greater than that of the original Mb(H64A·**1**) to give a 100-fold higher catalytic efficiency compared with Mb(native·**1**). The modification of the heme side chains might induce slight conformational changes to favor the peroxidase activity.

Compared with Mb(native·**1**), the other mutant Mb(H64D·**1**) decreased the K_m value by 30-fold to readily accept the substrate (Table 1). The k_{cat}/K_m value of the mutant was higher than that of Mb(native·**1**) by 2 orders of magnitude. Through both the histidine mutation and the heme modification, rMb(H64D·**2**) decreased the K_m value by 1000-fold compared with Mb(native·**1**). The catalytic efficiency of rMb(H64D·**2**) was increased up to 23 000 M^{-1} s^{-1},[12] which was 430-fold higher than that of Mb(native·**1**), significantly higher than that of cytochrome c peroxidase (200 M^{-1} s^{-1}),[10] and only 3-fold less than that of HRP (72 000 M^{-1} s^{-1}).[13] Additionally, rMb(H64D·**2**) showed a significant decrease in the K_d value (0.027 ± 0.004 mM). It is suggested that rMb(H64D·**2**) obtained not only the proper active site but also a good substrate binding site to increase the catalytic efficiency.

In addition, myoglobin-catalyzed degradation of bisphenol A, another hydrophobic substrate, was examined by HPLC analysis. Compared with Mb(native·**1**), rMb(H64D·**2**) showed drastic acceleration (>35-fold) of bisphenol A degradation (See Supporting Information), indicating that rMb(H64D·**2**) could be a good biomolecule catalyst.

In summary, it was found that the K_m values significantly decrease and the k_{cat}/K_m values remarkably increase for 2-methoxyphenol oxidation catalyzed by myoglobins that are prepared by the appropriate hybridization between a mutant and a modified heme. In this system, a highly oxidized heme reactive species is smoothly generated and a substrate is effectively bound within the artificially created substrate binding domain, while the native myoglobin only reversibly binds O_2. The present results indicate that the combination of a modified-heme reconstitution and an amino acid mutation should offer interesting perspectives toward developing a useful biomolecule catalyst from a hemoprotein.

Supporting Information Available: Experimental procedures, UV−vis spectra, heme active species formation rates, 2-methoxyphenol titration, and bisphenol A degradation. This material is available free of charge via the Internet at http://pubs.acs.org.

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(11) With 50 μM of 2-methoxyphenol, the difference in the initial rates between rMb(H64D·**2**) (1.4 ± 0.1 s^{-1}) and HRP (4.8 ± 0.1 s^{-1}) was further decreased.
(12) Compared with Mb(H64D·**1**), rMb(H64D·**2**) showed an unexpected lower k_{cat} value (1.2 s^{-1}). For only rMb(H64D·**2**), a "burst" kinetics was observed due to the slow product release, relating to the high affinity of the product to the active site (data not shown).
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