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Precise Design of Artificial Cofactors for Enhancing Peroxidase Activity of Myoglobin: Myoglobin Mutant H64D Reconstituted with a "Single-Winged Cofactor" Is Equivalent to Native Horseradish Peroxidase in Oxidation Activity

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Abstract: H64D myoglobin mutant was reconstituted with two different types of synthetic hemes that have aromatic rings and a carboxylate-based cluster attached to the terminus of one or both of the heme-propionate moieties, thereby forming a "single-winged cofactor" and "double-winged cofactor," respectively. The reconstituted mutant myoglobins have smaller K_m values with respect to 2-methoxyphenol oxidation activity relative to the parent mutant with native heme. This suggests that the attached moiety functions as a substrate-binding domain. However, the k_{cat} value of the mutant myoglobin with the double-winged cofactor is much lower than that of the mutant with the native heme. In contrast, the mutant reconstituted with the single-winged cofactor has a larger k_{cat} value, thereby resulting in overall catalytic activity that is essentially equivalent to that of the native horseradish peroxidase. Enhanced peroxygenase activity was also observed for the mutant myoglobin with the single-winged cofactor, thus indicating that introduction of an artificial substrate-binding domain at only one of the heme propionates in the H64D mutant is the optimal engineering strategy for improving the peroxidase activity of myoglobin.

Keywords: cofactors · heme proteins · myoglobin · oxidation · reconstitution

Introduction

To create an artificial enzyme with high catalytic activity, we should consider the elementary processes of enzymatic reactions: substrate-binding and the chemical catalytic process. These processes are apparently qualified by K_m and k_{cat} in Michaelis–Menten kinetics, respectively. Having a solid understanding of the general concepts of supramolecular chemistry is necessary to improve a substrate-binding process (decrease in K_m value).[1,2] Enhancement of catalytic steps (increase in k_{cat} value) is related to the regulation of reactive intermediates (their rates of formation, lifetimes, and intrinsic reactivities). Controlling both substrate-binding and chemical processes will be required for effective molecular design of an artificial enzyme that exhibits high catalytic activity equivalent or superior to that of naturally occurring enzymes.

Myoglobin (Mb), an oxygen-storage hemoprotein, is an easily obtainable protein that is suitable for use as a scaffold to engineer the native functions by genetic and/or chemical modifications.[3] The native Mb contains one protoheme IX **1** at the active site (Scheme 1; see the Supporting Information). This prosthetic group is responsible for the reversible binding of dioxygen.[4] Although the protoheme IX of Mb is the same as that of horseradish peroxidase (HRP), the catalytic oxidation activity of Mb is much lower than that of HRP. This is because Mb lacks a specific binding site for substrates and a general acid–base catalytic system to smoothly generate the oxoferryl species, a key intermediate in the catalytic oxidation cycle. Possible strategies for introducing these factors into Mb are 1) genetic mutations to provide an HRP-like environment at the heme distal site,

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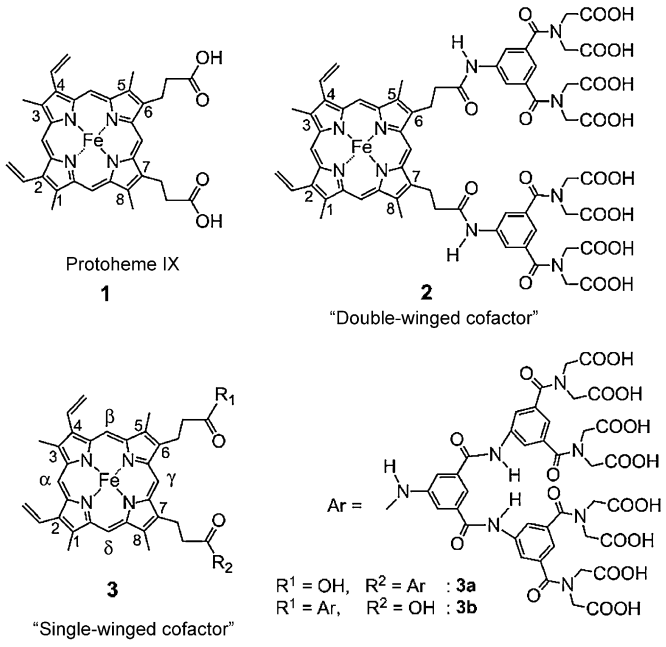


Scheme 1. Native and synthetic heme cofactors.

2) attachment of an artificial substrate binding domain at the termini of the heme propionates, and 3) replacement of the native heme with a more reactive heme analogue. The first approach is attained by the H64D mutant, in which the introduced Asp residue works as a general acid–base unit to activate H_2O_2.[5] In the second strategy, a variety of designed substrate-binding domains have been suggested, including boronic acid,[6] aromatic rings,[7] peptides,[8] and DNA,[9] among others. The third method involves the insertion of an iron complex of porphycene[10] or corrole[11] into the heme pocket of Mb.

Parallel to the above-mentioned approaches, the combination of these strategies is also promising for the enhancement of peroxidase activity of Mb. In our previous paper,[12] we described a reconstituted H64D Mb with a "double-winged here cofactor" **2** (Mb(H64D·**2**); wherein the notation "Mb(H64D·**X**)" indicates H64D Mb with cofactor **X**). This is designated "hybrid Mb." The aromatic moiety introduced at both of the heme propionates was found to function as an effective binding site for hydrophobic substrates such as 2-methoxyphenol. This leads to a reduction of the K_m value, thereby indicating that binding is more effective. As described above, the introduction of an effective H_2O_2 activation system by genetic mutation and the attachment of a substrate-binding domain by synthetic chemical strategy are indeed useful to the remarkable overall enhancement of the peroxidase activity of the native Mb. It was disclosed, however, that the k_{cat} values unexpectedly decreased by replacement of the native heme with **2** when these values for the two H64D Mbs, Mb(H64D·**1**) and Mb(H64D·**2**), were compared. This finding suggests that modification of the heme propionates often causes negative effects on the chemical processes in the catalytic cycle. Our independent investigations have also indicated that the interactions between the heme propionates and nearby amino acid residues in some hemoproteins are related to the reactivity of the heme iron as well as the thermostability of the proteins.[13] The next approach to enhance the peroxidase activity of Mb is to improve the overall catalytic activity without negative effects on chemical processes. In this paper, we describe a systematic investigation of the results of heme modification with a "single-winged cofactor" **3**, which was developed for Mb reconstitution to obtain key insights with respect to the creation of artificial enzymes optimized for both substrate-binding and chemical processes.

Abstract in Japanese:

プロトヘムIXの末端にあるプロピオン酸の片方あるいは両方を修飾した「片翼ヘム」および「両翼ヘム」を合成し、H64Dミオグロビン変異体に挿入することにより、再構成ミオグロビン変異体の酸化触媒活性を検討した。片翼ヘムを有するH64Dミオグロビン変異体は、天然西洋ワサビペルキシダーゼに匹敵する酸化触媒活性を示した。基質の結合挙動、高酸化活性種の生成速度および生成効率に関する実験結果から、ミオグロビンの酸化活性を向上させる最も効率のよい方法は、H64D変異体に対して、ヘムプロピオン酸の片方のみを修飾した補因子を導入することであることが明らかとなった。

Results and Discussion

Reconstitution of the H64D Myoglobin Mutant with the Single-Winged Cofactor and NMR Spectroscopic Study of Heme Orientation

Single-winged cofactor **3** was prepared by partial condensation of protoporphyrin IX with aniline derivatives, followed by iron insertion and alkaline hydrolysis (see the Supporting Information for the synthetic scheme and the procedure). Because the condensation at the propionate side chain occurs nonselectively, the 1:1 mixture of regioisomers **3a** and **3b** was obtained, and it is difficult to separate them. Sperm whale H64D Mb expressed by *Escherichia coli* was reconstituted with single-winged cofactor **3** to obtain Mb(H64D·**3**) after removal of the native heme. The UV/Vis spectrum of Mb(H64D·**3**) shows bands at the same wavelengths as those for Mb(H64D·**1**), thus indicating that cofactor **3** occupies the same space in the heme pocket as the native heme **1** (see the Supporting Information). This includes coordination of the His93 imidazole to the heme iron. Although the two regioisomers were inserted into the apoprotein together, the NMR spectroscopic study for the prepared Mbs suggests that each isomer is accommodated so that the unmodified propionate preferentially interacts with Arg45 (vide infra).

Mb(H64D·**3**) could theoretically contain four possible configurations, because a mixture of the two regioisomers, **3a** and **3b**, was employed for reconstitution of the H64D mutant and each regioisomer has two possible orientations

within the heme pocket (normal and reversed orientations) as a result of a 180° rotation of the heme about the heme α–γ *meso* axis ("normal orientation" is a fashion of the major heme orientation, in which 6-propionate forms a hydrogen bond with Arg45, see the Supporting Information). One useful method to elucidate the cofactor orientations in the heme pocket of Mb is the ^{1}H NMR spectroscopic measurement for cyanomet Mb because the paramagnetic shift of the methyl protons on the periphery of the heme framework is affected by orientation of proximal His93 and is useful information of the heme orientation in the myoglobin matrix.[14] This methodology is applicable to mutant Mbs.[14c]

Figure 1 shows the ^{1}H NMR spectra of the cyanomet wild-type and H64D Mbs obtained in deuterium oxide. The assignments of the selected heme methyl peaks were made based on the knowledge obtained in the previous reports.[14] The numbering of the heme framework is indicated in Scheme 1. The assignment indicated in Figure 1c was conducted with reference to the previous data obtained in the NMR spectroscopic measurements before the heme orientation reached equilibrium.[14] The pattern of the singlet peaks for 5-, 1-, and 8-methyl protons for cyanomet Mb(H64D·**1**) (Figure 1b) is similar to that observed for wild-type Mb (Figure 1a). This suggests that hemin **1** in the protein interior of H64D Mb also adopts the normal orientation observed for wild-type Mb.[15] In particular, the chemical shift of 5-methyl proton is affected by the existence of the hydrogen bonding between 6-propionate and Arg45 located in the entrance of the heme pocket: La Mar and co-workers reported that cyanomet Mb reconstituted with 6-methyl-6-despropionate hemin, in which a hydrogen bond between 6-propionate and Arg45 is lacking, has a downfield-shifted peak of the 5-methyl protons at $\delta = 28$ ppm.[14,16] The observation of the 5-methyl proton peak at the same chemical shift ($\delta =$ 26.8 ppm) supports the fact that the hydrogen bonding between 6-propionate and Arg45 in Mb(H64D·**1**) is conserved.

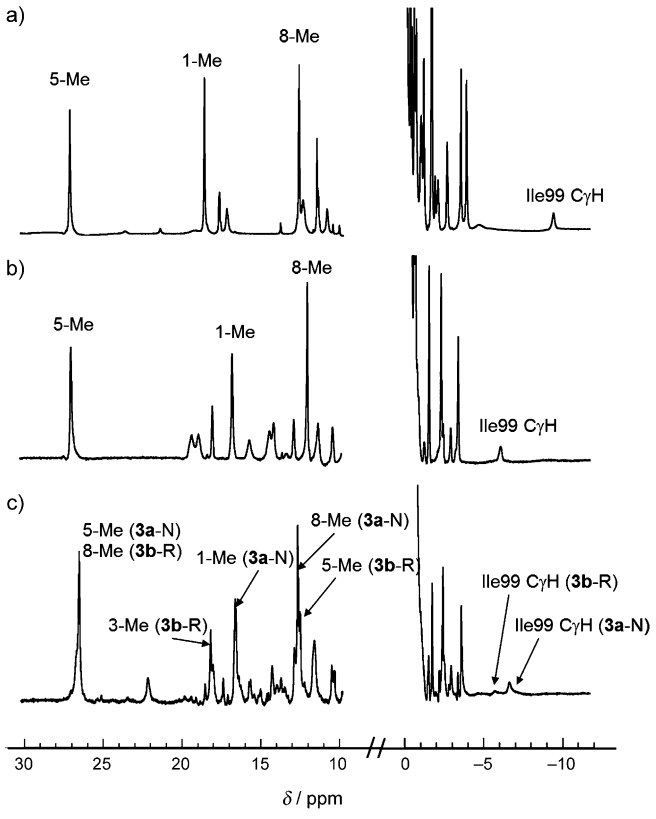
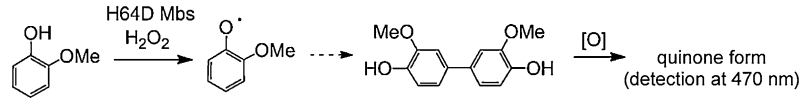


Figure 1. ^{1}H NMR spectra of cyanomet Mbs: a) wild-type Mb; b) Mb(H64D·**1**); c) Mb(H64D·**3**); KPi D_2O buffer (10 mM, pD = 7.4), 25 °C. The numbering of the heme framework is indicated in Scheme 1. The designations **3a-N** and **3b-R** indicate H64D Mb reconstituted with cofactor **3a** in the normal orientation and **3b** in the reversed orientation, respectively.

In contrast, it is expected that cyanomet Mb(H64D·**3**) shows a complicated peak pattern on the ^{1}H NMR spectrum because of the possibility to take several heme configurations. However, as shown in Figure 1c, the characteristic heme methyl peaks are relatively simple. In particular, only one signal appeared at $\delta = 26.5$ ppm. The chemical shift is very similar to that observed for Mb(H64D·**1**). When the H64D mutant was reconstituted with 6-methyl-6-despropionate heme, the 5-methyl proton peak appeared at $\delta =$ 28 ppm in the same manner as the wild-type Mb reconstituted with this propionate-truncated heme (see the Supporting Information), although no peak was observed around $\delta =$ 28 ppm in Figure 1c. This result indicates that the hydrogen bonding between a propionate side chain and Arg45 is conserved also in Mb(H64D·**3**). Therefore, the mechanism to account for this spectrum is that cofactor **3** in the heme pocket should take an orientation that allows interaction between the propionate and Arg45. Isomer **3b** takes the reversed heme orientation to form hydrogen bonding with Arg45 (see the Supporting Information).[17] In the reversed orientation, the 8-methyl group should be located in the position that the 5-methyl group in the normal heme orientation occupies, thereby resulting in the similar chemical shift ($\delta = 26.5$ ppm for the 5-methyl proton in the normal orientation of **3a** and 8-methyl proton in the reversed orientation of **3b**) and vice versa. The rather broad peak at $\delta =$ 26.5 ppm also supports the existence of the two conformations.[18] In contrast, the chemical shifts of the 1-methyl and 3-methyl protons are significantly affected by the accommodated position in the heme pocket because the positions of these methyl groups are not symmetrical about the heme α–γ *meso* axis. These findings suggest that the hydrogen bonding at Arg45 is essential for the stability of a reconstituted Mb with the propionate-modified cofactor[19] and that the hydrogen bonding between a heme propionate and Arg45 might indeed contribute to the regulation of the heme iron reactivity (vide infra).

Steady-State Kinetics for 2-Methoxyphenol Oxidation

For evaluation of the peroxidase activities of H64D Mbs, the oxidation of 2-methoxyphenol (also known as guaiacol) was investigated (Scheme 2).[20]



Scheme 2. 2-Methoxyphenol oxidation mediated by H64D Mbs.

Figure 2 shows the time courses of the absorbance changes at 470 nm during 2-methoxyphenol oxidation catalyzed by the H64D Mbs in the presence of an excess of H_2O_2. The protein that shows the most effective catalytic activity is Mb(H64D·**3**). Table 1 lists the Michaelis–Menten parameters of 2-methoxyphenol oxidation.[21] To compare the effects of cofactor structures on the catalytic activity, the previously reported kinetic parameters for H64D Mb with heme **1** (native heme) and with double-winged heme **2** are also described.[12] For the purpose of discussing the effect of the propionate modification on the catalytic activities (vide infra), the kinetic parameters for Mb(H64D·**4**), in which the four-carboxylate moiety is attached at one heme-propionate, are also included.

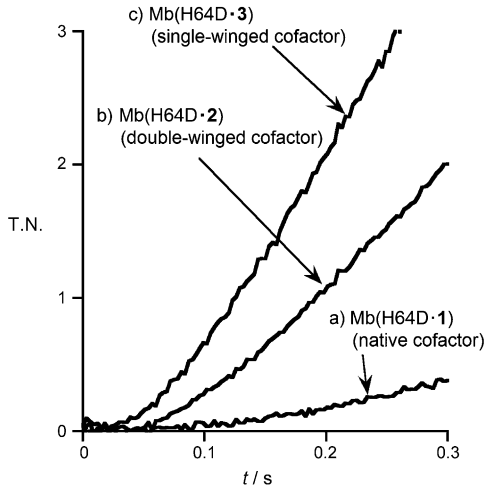
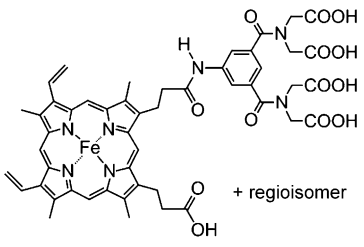


Figure 2. Time-courses of 2-methoxyphenol oxidations catalyzed by H64D Mbs; sodium malonate buffer (20 mM, pH 6.0), 25 °C; [H64D Mb] = 2.0 μM, $[2\text{-methoxyphenol}]_0 = 0.5$ mM, $[H_2O_2]_0 = 15$ mM. a) Mb(H64D·**1**); b) Mb(H64D·**2**); c) Mb(H64D·**3**). T.N. = turnover number.

Table 1. Kinetic parameters for 2-methoxyphenol oxidations catalyzed by H64D Mb mutants.[a]

Cofactor	K_m [mM]	k_{cat} [s^{-1}]	k_{cat}/K_m [$M^{-1}s^{-1}$]
1	1.8 ± 0.4[b]	9.0 ± 1.2[b]	5100[b]
2	0.052 ± 0.016[b]	1.2 ± 0.1[b]	23000[b]
3	0.29 ± 0.06	24 ± 2	85000
4	0.63 ± 0.08	13 ± 1	21000

[a] Sodium malonate buffer (20 mM, pH 6.0), 25 °C, [Mb] = 4.0 μM, $[H_2O_2]_0 = 100$ mM. [b] Reference [12].



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According to the previous report,[12] the kinetic parameter for the wild-type Mb was found to be $k_{cat}/K_m = 52\ M^{-1}s^{-1}$. When His64 in the wild-type protein was converted into Asp, the obtained Mb(H64D·**1**) showed the approximately 100-fold catalytic activity ($k_{cat}/K_m = 5100\ M^{-1}s^{-1}$) as also reported by Watanabe and coworkers.[5] When the further engineering of H64D mutant was conducted by the replacement of the native heme cofactor with a synthetic cofactor **2** or **3**, the overall catalytic activities of the hybrid proteins, Mb(H64D·**2**) and Mb(H64D·**3**), were remarkably enhanced. The main factor of the enhancement in the catalytic activity is the decrease in K_m values. This is because the introduced aromatic moieties at the termini of the propionate side chains function to facilitate the access of the hydrophobic substrate to the vicinity of the reaction site.

On the other hand, the k_{cat} value for Mb(H64D·**2**) significantly decreased from Mb(H64D·**1**), which is a negative effect of the aspects of the enhancement of the peroxidase activity in Mb. The decrease in k_{cat} observed for Mb(H64D·**2**) implies that the modification of both propionates causes a decrease in the reactivities of the oxoferryl species, a reactive intermediate responsible for a substrate oxidation (see below for the investigation of the reaction intermediates). In contrast, the k_{cat} value of Mb(H64D·**3**) is rather improved from Mb(H64D·**1**). This tendency is also observed in Mb(H64D·**4**), in which one heme propionate is unmodified as in **3**. The result provides useful insights into the design of an ideal synthetic heme: retaining one unmodified propionate is essential to prevent the k_{cat} value from decreasing along with enhancement of the substrate binding process. Consequently, Mb(H64D·**3**) has an extraordinary enhancement of the overall catalytic activity ($k_{cat}/K_m = 85000\ M^{-1}s^{-1}$). The catalytic activity of this engineered Mb is, interestingly, almost equivalent to that of a naturally occurring peroxidase under the same reaction conditions (for HRP, $k_{cat}/K_m = 72000\ M^{-1}s^{-1}$ at pH 6.0, 25 °C, $[H_2O_2] = 1.0$ mM).[21,22] This result indicates that the combination of a point mutation and precise design of an appropriate chemically modified cofactor have enabled us to construct an "ultra-native" biocatalyst.

2-Methoxyphenol Binding

To directly evaluate the effects of the attached moiety on the substrate affinity, the binding of 2-methoxyphenol to the engineered H64D Mbs was observed by UV/Vis spectral titration in the absence of H_2O_2. The calculated dissociation constants (K_{d1} and K_{d2}) are summarized in Table 2. The spectral changes for the H64D Mbs are shown in Figures 3, 4, and 5.

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Table 2. Dissociation constants for 2-methoxyphenol binding to H64D Mb mutants.[a]

Cofactor	K_{d1} [mM]	K_{d2} [mM]
1	0.13 ± 0.01	–[b]
2	0.019 ± 0.002	2.6 ± 0.3
3	0.014 ± 0.002	3.3 ± 0.3

[a] 20 mM sodium malonate buffer (pH 6.0), 25 °C, [Mb] = 8.0 μM, [2-methoxyphenol] = 0–4 mM. [b] Not determined due to single-phase dependency.

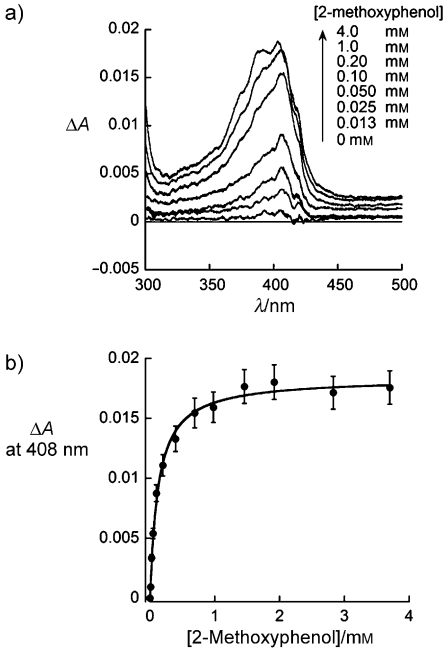


Figure 3. UV/Vis spectral changes in the binding of 2-methoxyphenol to Mb(H64D·**1**); sodium malonate buffer (20 mM, pH 6.0), 25 °C. a) Differential spectra based on the spectrum obtained in the absence of 2-methoxyphenol. b) Dependency of the absorbance at 408 nm on the concentrations of 2-methoxyphenol. Error bars are drawn based on an estimation of 10 % error.

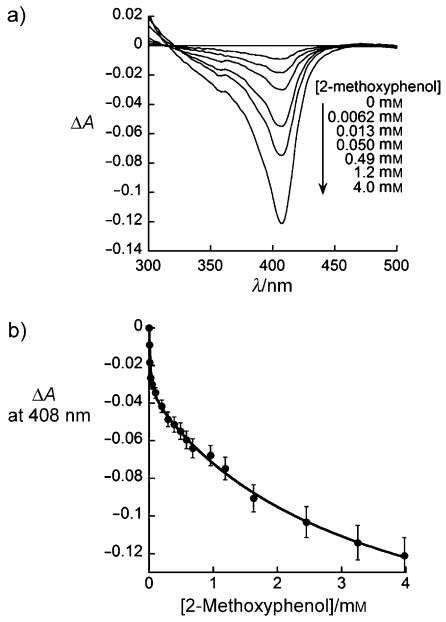


Figure 4. UV/Vis spectral changes occurring during the process of binding of 2-methoxyphenol to Mb(H64D·**2**); sodium malonate buffer (20 mM, pH 6.0), 25 °C. a) Differential spectra based on the spectrum in the absence of 2-methoxyphenol. b) Dependency of the absorbance at 408 nm on the concentrations of 2-methoxyphenol. Error bars are drawn based on an estimation of 10 % error.

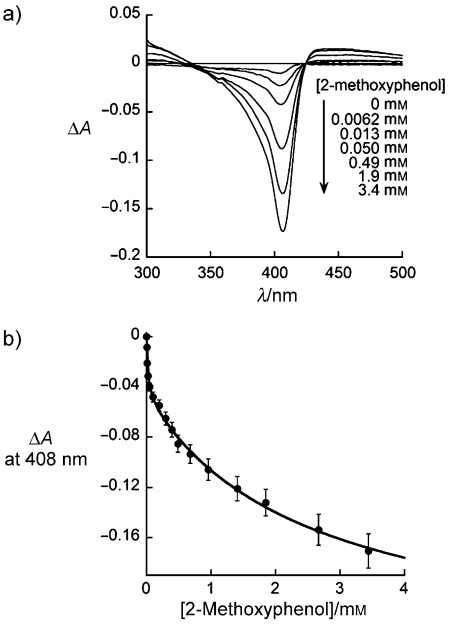
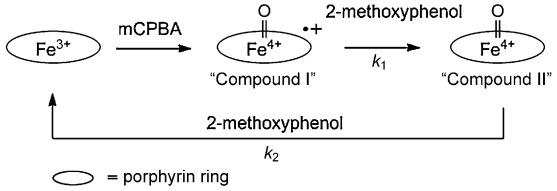


Figure 5. UV/Vis spectral changes occurring during the process of binding of 2-methoxyphenol to Mb(H64D·**3**); sodium malonate buffer (20 mM, pH 6.0), 25 °C. a) Differential spectra based on the spectrum in the absence of 2-methoxyphenol. b) Dependency of the absorbance at 408 nm on the concentrations of 2-methoxyphenol. Error bars are drawn based on an estimation of 10 % error.

For Mb(H64D·**1**), the absorbance of the Soret band slightly increases upon addition of 2-methoxyphenol, which could be caused by small conformational changes in the protein due to the access of the substrate to the vicinity of the heme-propionates. In contrast, H64D Mbs reconstituted with the synthetic cofactors **2** or **3** exhibit quite different spectral changes. The variances of those changes are much larger in the latter two H64D Mbs, thereby indicating that significant perturbations of the π-conjugation system of cofactors **2** and **3** are induced during the access of the organic substrate to the heme pocket of these proteins.[23] Moreover, for Mb(H64D·**1**), the absorbance changes can be analyzed as a single substrate-binding model, whereas the decay of the Soret band for Mb(H64D·**2**) or Mb(H64D·**3**) observed during the titrimetric measurements requires two-phase analysis. With respect to the two dissociation constants obtained for 2-methoxyphenol (K_{d1} and K_{d2}), the lower K_{d1} values for the H64D Mbs with modified cofactor **2** or **3** are attributed to the attached hydrophobic aromatic moieties. These lower K_{d1} values are in agreement with the low K_m values shown in Table 1.

Kinetic Analysis of the Catalytic Cycle

Next, to evaluate the reactivities of the oxoferryl intermediates (Compounds I and II), we determined the two rate constants, k_1 and k_2 (defined in Scheme 3). The generation of



Scheme 3. Reaction scheme for the kinetic analysis of the catalytic cycle of the 2-methoxyphenol oxidation mediated by H64D Mb mutants.

Compound I (oxoferryl species with a porphyrin π-cation radical) was attained by the reaction of the reconstituted ferric H64D Mbs with a slight excess of *meta*-chloroperbenzoic acid (*m*CPBA). The rate constants determined in the kinetic study are summarized in Table 3, and the representative kinetic trace is shown in Figure 6.

Table 3. Rate constants for elemental processes in 2-methoxyphenol oxidation catalyzed by H64D Mb mutants.[a,b]

Cofactor	k_1 [$M^{-1}s^{-1}$][c]	k_2 [$M^{-1}s^{-1}$][d]
1	1.1×10^6	1.3×10^4
2	0.32×10^6	3.5×10^4
3	3.6×10^6	1.2×10^4

[a] Sodium malonate buffer (20 mM, pH 6.0), 15 °C, [Mb] = 2.0 μM, [2-methoxyphenol]$_0$ = 10–100 μM. [b] Experimental errors are within 10%. [c] Reduction rate of Compound I. [d] Reduction rate of Compound II.

The H64D Mb with the double-winged cofactor, Mb(H64D·**2**), has the smallest k_1 value, thereby indicating that the modification of both heme-propionates lowers the reactivity of the Compound I intermediate. This finding may partially account for the drop in the k_{cat} value for the reaction catalyzed by this protein. However, the k_2 value, which indicates the reactivity of Compound II, is approximately threefold greater than that of the other two proteins. Therefore, it is difficult to simply rationalize the catalytic activities of Mb(H64D·**2**) on the basis of the magnitudes of the k_1 and k_2 values. Another possible factor that affects the catalytic activities of the H64D Mbs will be discussed in the next section.

In the determination of the rate constants k_1 and k_2 for Mb(H64D·**3**), the absorbance changes were clearly observed to follow single-phase kinetics. There is a negligible difference in the reactivities of Compound I and Compound II between the proteins with the normal and reversed heme orientations. As indicated by the largest k_1 value in Mb(H64D·**3**), the reactivity of Compound I is improved with respect to the other two proteins, which coincides with the fact that this protein has the highest k_{cat} value among the

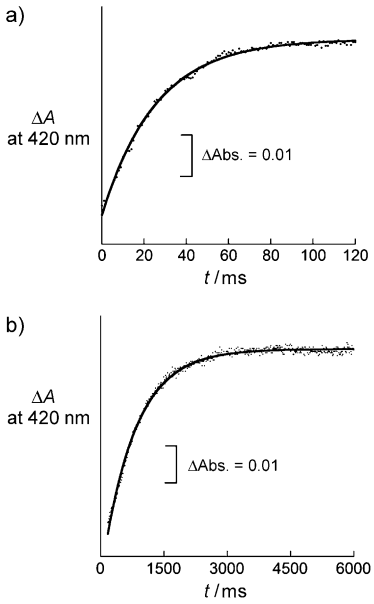


Figure 6. Representative absorbance changes for determination of the rate constants k_1 and k_2 for Mb(H64D·**3**); [Mb]$_0$ = 2 μM, [2-methoxyphenol]$_0$ = 10 μM, [mCPBA] = 1.3 equiv; sodium malonate buffer (20 mM, pH 6.0), 15 °C; a) initial stage (within 120 ms, k_1-step); b) later stage (6 s, k_2-step). The fitting assuming a single-phase kinetics is indicated by solid lines.

Mbs investigated. This suggests that there is a relationship between the heme propionate(s)–protein interactions and the reactivity of Compound I. As indicated in the ^{1}H NMR spectroscopic study (vide supra), cofactor **3** is oriented in the heme pocket of Mb so that the hydrogen-bonding interaction between the heme-propionate and Arg45 is retained at the distal site. These findings suggest that the reactivity of Compound I can be enhanced by two factors: 1) the conservation of the 6-propionate–Arg45 interaction and 2) the removal of the interaction between the 7-propionate and the amino acid residues at the proximal site.[24] The first factor contributes to the maintenance of the distal site structure and the second factor regulates the characteristics of coordination of His93 to the heme iron. According to the resonance Raman measurements of ferric Mb reconstituted with 7-methyl-7-despropionate heme, where the hydrogen bonding interaction between the 7-propionate and Ser92 is disrupted, the Fe–His93 stretching mode is slightly stronger because there is no regulation of proximal ligand coordination by the hydrogen-bonding network at the proximal site.[13a] We expect that a similar mechanism is operating with respect to Mb(H64D·**3**).[25]

Efficiency of Generation of the Compound I Intermediate

The catalytic activity of Mbs may also be controlled by the process of O–O bond cleavage after the formation of the hydroperoxo species (Fe^{III}–OOH) because the Compound I intermediate, the highly reactive oxidizing species, is only produced by heterolytic cleavage of the O–O bond. To eval-

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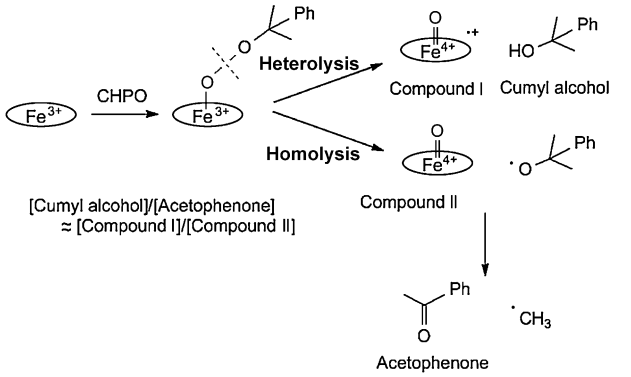
uate the efficiency of Compound I generation, an analysis of the reaction between ferric Mb and cumene hydroperoxide (CHPO) was examined.[5a] Table 4 summarizes the ratio of O–O bond heterolysis and homolysis, based on the product yields of cumyl alcohol and acetophenone, respectively (Scheme 4).

Table 4. Reaction of cumene hydroperoxide (CHPO) with the ferric H64D Mb mutants.[a]

Cofactor	Total amounts of products [μM][b]	Heterolysis/Homolysis[c] (–)
1	25	15
2	146	7
3	100	13

[a] Sodium malonate buffer (20 mM, pH 6.0), 15 °C, [Mb] = 20 μM, [CHPO] = 200 μM. [b] Total amounts of acetophenone and cumyl alcohol as a product produced over 5 min. [c] Calculated from [cumyl alcohol]/[acetophenone].



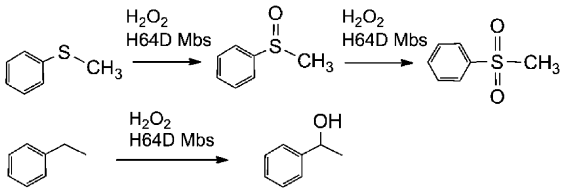
Scheme 4. Reaction of ferric Mb with cumene hydroperoxide (CHPO).

The total amounts of organic products obtained by Mb(H64D·**2**) and Mb(H64D·**3**) were increased relative to the amounts of products obtained by Mb(H64D·**1**). This is because CHPO can easily bind to the artificial binding domain. However, the ratio in Mb(H64D·**2**) is relatively small, thereby indicating that the efficiency of Compound I generation is lower than that of the other engineered proteins. Based on the data described in Tables 3 and 4, the modification of both propionates gives rise to negative effects with respect to generation of Compound I.

Two-Electron Oxidations Catalyzed by H64D Mb Reconstituted with Single-Winged Cofactor 3

Next we observed the H_2O_2-dependent oxidation of thioanisole (oxygen-atom transfer) and ethylbenzene (hydroxylation) catalyzed by Mbs (Scheme 5).

As expected, Mb(H64D·**3**) also exhibits the highest catalytic activity toward the two-electron oxidations among the engineered Mbs (Table 5). In the case of oxygen transfer to thioanisole, a four-electron oxidation product, sulfone, was also observed for all of the reconstituted Mbs proteins ex-



Scheme 5. Two-electron oxidations catalyzed by H64D Mbs.

Table 5. Turnover numbers for thioanisole and ethylbenzene oxidation catalyzed by H64D Mb mutants.[a]

Cofactor	Thioanisole oxidation[b]		Ethylbenzene oxidation[c]
	Sulfoxide[d] [min^{-1}]	Sulfone[e] [min^{-1}]	2-Phenylethanol[f] [min^{-1}]
1	25[g]	2.6[h]	n.d.[i]
2	33[g]	2.0[h]	n.d.[i]
3	49[g]	3.8[h]	0.3[j]

[a] KPi buffer (100 mM, pH 7.0), 25 °C. [b] [Mb] = 2.0 μM, $[H_2O_2]_0$ = 1.0 mM, $[thioanisole]_0$ = 0.5 mM. [c] [Mb] = 20 μM, $[H_2O_2]_0$ = 1.0 mM, $[ethylbenzene]_0$ = 0.5 mM. [d] Product of oxygen transfer to thioanisole. [e] Product of oxygen transfer to methylphenyl sulfoxide. [f] Product of hydroxylation at the benzyl position (as racemic forms). [g] Calculated from the amount of the sulfoxide divided by [Mb]. The ratio of *R* and *S* isomers was not determined. See Ref. [27]. [h] Calculated from the amount of the sulfone divided by [Mb]. [i] Not determined because there was no observation. [j] Calculated from the amount of 2-phenylethanol produced divided by [Mb].

amined. The protein with the highest peroxygenase activity that yielded both sulfoxide and sulfone is Mb(H64D·**3**). Interestingly, catalytic hydroxylation was also observed in Mb(H64D·**3**), although such activity is very rare for most Mb mutants.[26] Catalysis of hydroxylation of a C–H bond by heme enzymes in nature has only been observed for *S*-coordinated hemoproteins such as cytochrome P450 s and chloroperoxidase. Therefore, it is a remarkable finding that Mb(H64D·**3**), the heme iron of which is coordinated by a neutral imidazole, demonstrates hydroxylation activity. These results reflect the synergetic effects derived from the enhanced reactivity of Compound I (increased k_1) and efficient substrate binding (decreased K_m).[25–27]

Conclusion

In this research, it was demonstrated that the incorporation of a single-winged cofactor into apo-H64D Mb enhances the reactivities of the oxidizing intermediates as well as facilitates the binding of substrates. The replacement of His64 with Asp by genetic mutation contributes to the smooth H_2O_2 activation mediated by a general acid–base catalysis of the Asp residue (improvement of chemical processes). The introduction of the aromatic moiety at the termini of the heme propionate side chains is useful to create a substrate-binding domain (improvement of substrate-binding process). The kinetic analysis for each step of the catalytic cycle, however, suggests that retaining one unmodified propionate is important to control the reactivity of Compounds I and II,

thus leading to more effective improvement of the peroxidase activity in Mb. The significance of one unmodified propionate is the formation of hydrogen bonding between the heme propionate and Arg45 and the stabilization of the heme orientation in the heme pocket of Mb. Furthermore, precise design of a synthetic cofactor enables us to endow an appropriate myoglobin mutant with enhanced catalytic activity toward two-electron oxidation through oxo transfer and C–H bond activation. The findings demonstrated in this paper will provide important insights into the methodologies required for engineering of chemically and biologically attractive biocatalysts.

Experimental Section

Materials and Protein Purification

All reagents and chemicals were obtained from commercial sources and were used as received unless otherwise noted. The H64D myoglobin mutant was expressed from *E. coli*. and purified by column chromatography through CM-52 (Whatman) and Sephadex G-25 (GE Healthcare) columns.[5a] The preparation of cofactor **2** was described in the previous report.[7] Cofactors **3** and **4** were synthesized as described in the Supporting Information. The reconstituted H64D Mbs were prepared as described in the previous report,[12] after the removal of the native heme by Teale's 2-butanone method.[28] The reconstituted Mbs were purified with a Sephadex G-25 column (2×50 cm, 100 mM KPi, pH 7.0, 4 °C) and were found to be stable at −80 °C for at least one month as a frozen solution (≈1 mM).

Instrumentation

^{1}H NMR spectra were measured with a Bruker DPX 400 spectrometer. The measurements of UV/Vis spectra and the titrations of 2-methoxyphenol to monitor the binding of substrate to protein were carried out with a Shimadzu UV-3210 double-beam spectrophotometer. Kinetic measurements were conducted with an RSP-1000 stopped-flow system constructed by Unisoku, Co., Ltd. (Osaka, Japan). The HPLC analyses were conducted with a Shimadzu HPLC LC-VP system. The GC/FID measurements were made with a Shimadzu GC-2014 gas chromatography system. The mass analyses (FAB-MS and ESI-MS) were conducted with a JEOL JMS-700 mass spectrometer.

Determination of Dissociation Constants (K_{d1} and K_{d2})

H64D Mb (8 μM) was dissolved in KPi (20 mM; pH 6.0) and titrated with 2-methoxyphenol. The UV/Vis spectra were measured in the range 350 to 450 nm with dropwise additions of 2-methoxyphenol. The dependency of the absorbance change at 408 nm (Δabs_{408}) on concentrations of 2-methoxyphenol was analyzed by the following equation [Eq. (1]:

$$\Delta abs_{408} = (A \times K_{d2} \times [S] + B \times [S]^2)/(K_{d1} \times K_{d2} + K_{d2} \times [S] + [S]^2) \quad (1)$$

in which $[S]$ is the concentration of 2-methoxyphenol, A and B are constants, and K_{d1} and K_{d2} are the first and second dissociation constants of 2-methoxyphenol, respectively.

Steady-State Kinetics for 2-Methoxyphenol Oxidation

Steady-state kinetic measurements for 2-methoxyphenol oxidation catalyzed by H64D Mb mutants were carried out using the stopped-flow method at 25 °C. A mixture of Mb and various concentrations of 2-methoxyphenol in sodium malonate buffer (20 mM; pH 6.0) was rapidly mixed with H_2O_2 in the same buffer after incubation at 25 °C in the sample reservoirs. The final concentrations are as follows: [Mb] = 4 μM, [2-methoxyphenol] = 0.025–3 mM, and [H_2O_2] = 100 mM. The oxidation reactions were monitored by observing the increase in absorbance at 470 nm. The initial rates were calculated using the molar absorption coefficient of the oxidation product; $\varepsilon = 26\,600\ M^{-1}cm^{-1}$.[29]

Determination of the Rate Constants for the Elemental Processes in 2-Methoxyphenol Oxidations

The reactions were carried out in sodium malonate buffer (20 mM, pH 6.0) at 15 °C with a double-mixing stopped-flow apparatus equipped with a PDA detector. A Mb solution (8.0 μM) was mixed with *m*CPBA (10.4 μM) in a first mixing shot to generate the Compound I species. After an aging period of 700–1200 ms, the generated intermediate was mixed with 2-methoxyphenol (20–200 μM) in a second mixing shot. The spectral changes after the second mixing shot were monitored by collecting the transient spectra every 10–100 ms. The absorbance changes at 408 nm were analyzed according to pseudo-first-order kinetics.

Catalytic Activities toward Thioanisole and Ethylbenzene Oxidations

The reactions were carried out in KPi buffer (100 mM, pH 7.0) at 25 °C. A buffer solution of Mb, thioanisole, and benzyl alcohol (internal standard) was incubated prior to the addition of H_2O_2 to initiate the reaction. The final concentrations were: [Mb] = 2.0 μM, [thioanisole] = 0.5 mM, [benzyl alcohol] = 5.0 μM, and [H_2O_2] = 1.0 mM. After a reaction period of 5 min, ether was added, and the reaction mixture was vigorously shaken using a vortex mixer to extract the organic materials. The separated organic phase was concentrated by evaporation with streaming N_2 gas, and the residues were analyzed with a GC/FID system equipped with a DB-1 column. The oxidation of ethylbenzene was carried out according to the same procedure.

Reaction of Myoglobins with Cumene Hydroperoxide (CHPO)

A Mb solution was treated with cumene hydroperoxide ([Mb] = 20 μM, [CHPO] = 200 μM) in sodium malonate buffer (1 mL, 20 mM, pH 6.0) at 25 °C for 5 min. After the addition of benzyl alcohol (10 μM), the reaction mixture was filtered with a Centricon concentrator, and the filtrate was analyzed with an HPLC system equipped with a YMC Pro-C18 column, 150×4.6 mm at a flow rate of 0.8 mL min^{-1} with elution by addition of a 1:1 mixture of H_2O/MeOH to determine the amounts of acetophenone and cumyl alcohol formed by cleavage of CHPO based on the intensity ratios of these materials against that of a benzyl alcohol standard.

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[25] The strong coordination of the proximal ligand to the heme iron is recognized as a "push-effect" that controls the reactivities of the high-valent species in native heme-containing oxidases. See Ref. [3d].
[26] The intramolecular hydroxylation of the amino acid residue at the distal site has been reported for the F43W/H64D/V68I myoglobin mutant, see Ref. [5b].
[27] For thioanisole oxidation using $H_2{}^{18}O_2$, the complete incorporation of ^{18}O into the products was confirmed by GC-MS. The enantiometric excess value (*ee*) for methyl phenyl sulfoxide was not determined in this research, because the value would be time-dependent. Both stereoisomers of the sulfoxide product can be converted into the sulfone through the second oxygen transfer. The sequential oxidation will bring about the change in the ratio of the stereoisomers in the transiently formed sulfoxide product unless the second oxidation becomes quite negligible. The previous paper reported the slightly preferential production of *S* isomer (6% *ee*) in the oxidation of thioanisole mediated by Mb(H64D·**1**) (Reference [5a] and correction: *Biochemistry* **2008**, *47*, 2700).
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