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Crystal Structure and Peroxidase Activity of Myoglobin Reconstituted with Iron Porphycene

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The incorporation of an artificially created metal complex into an apomyoglobin is one of the attractive methods in a series of hemoprotein modifications. Single crystals of sperm whale myoglobin reconstituted with 13,16-dicarboxyethyl-2,7-diethyl-3,6,12,17-tetramethylporphycenatoiron(III) were obtained in the imidazole buffer, and the 3D structure with a 2.25-Å resolution indicates that the iron porphycene, a structural isomer of hemin, is located in the normal position of the heme pocket. Furthermore, it was found that the reconstituted myoglobin catalyzed the H_2O_2-dependent oxidations of substrates such as guaiacol, thioanisole, and styrene. At pH 7.0 and 20 °C, the initial rate of the guaiacol oxidation is 11-fold faster than that observed for the native myoglobin. Moreover, the stopped-flow analysis of the reaction of the reconstituted protein with H_2O_2 suggested the formation of two reaction intermediates, compounds **II**- and **III**-like species, in the absence of a substrate. It is a rare example that compound **III** is formed via compound **II** in myoglobin chemistry. The enhancement of the peroxidase activity and the formation of the stable compound **III** in myoglobin with iron porphycene mainly arise from the strong coordination of the Fe–His93 bond.

Introduction

Modification of the hemoprotein function is the subject of increasing research attention because of its potential applications in bioinorganic chemistry and protein engineering.[1] Particularly, myoglobin, a dioxygen storage hemoprotein, will be especially well suited for studies of functional engineering because its structure and physicochemical properties have been relatively clarified. One of the features of the myoglobin structure is that the prosthetic group, heme, is bound in the globin matrix via a noncovalent interaction. Therefore, one can expect that the replacement of the native heme with an artificially created heme is an attractive strategy to regulate the myoglobin function.[2] Over the past decade, several groups have devoted their efforts to preparing a series of reconstituted myoglobins that demonstrate not only improved ligand-binding properties[3] but also high enzymatic activity.[4] For example, it is known that the introduction of functional groups at the terminal of the heme−propionate side chains enhances the peroxidase activity in the myoglobin,[5] whereas there are few studies that evaluate the enzymatic activity by modifying the porphyrin framework.[6]

In a series of molecular designs of an artificially created prosthetic group for hemoproteins, metalloporphycene, a structural isomer of the metalloporphyrins, has been one of the best candidates because porphycene, which was first

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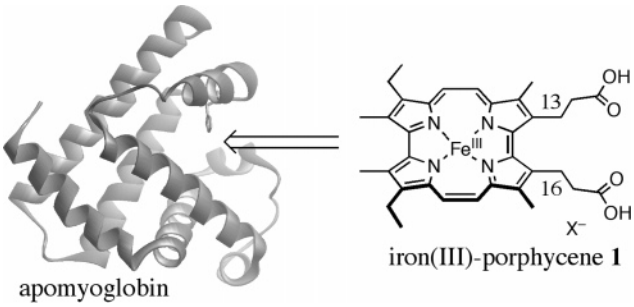
Scheme 1. Reconstitution of Myoglobin with Iron(III) Porphycene **1**



prepared by Vogel and co-workers,[7] revealed that some of the physicochemical properties are sharply different from those of the parent porphyrin because of the decrease in the framework symmetry.[8] These findings in previous reports prompted us to prepare a myoglobin reconstituted with iron porphycene and evaluate its function. Recently, we replaced the native hemin with 13,16-dicarboxyethyl-2,7-diethyl-3,6,-12,17-tetramethylporphycenatoiron(III) (**1**) in sperm whale myoglobin, as shown in Scheme 1.[9] The 1:1 complexation between **1** and the globin was determined by electrospray ionization time-of-flight mass spectroscopy, and the UV−vis spectrum of the reconstituted protein, rMb(**1**), suggested that the imidazole ligand of histidine would bind to the Fe atom of **1**. However, the precise structural data of rMb(**1**) was not available in our previous studies. In contrast, we found that deoxy-rMb(**1**) showed an extremely high dioxygen affinity compared to the native myoglobin.[9,10] Furthermore, the stability of **1** in the myoglobin matrix was significantly improved under acidic conditions, and the Fe(III)/Fe(II) redox potential of **1** in the protein was much lower than that observed for the native heme in myoglobin.[11] On the basis of these results, we proposed that the Fe−His93 bond strength would be enhanced by replacement of the native hemin with **1**.[12,13]

We now describe the crystal structure of rMb(**1**) in an imidazole buffer, indicating that the artificially created prosthetic group is exactly located in the normal position of the heme-binding site. The structure is also interesting for metalloporphycene chemistry because there are few examples of the 3D structures of the iron porphycenes.[13] In addition, it was found that the replacement of the native hemin with **1** clearly enhances the peroxidase and peroxygenase activities toward several substrate oxidations. On the basis of the spectroscopic studies of these oxidations, we found that the H_2O_2-dependent oxidation catalyzed by rMb(**1**) competes with the conversion of compound **II** to compound **III** in the presence of excess amounts of H_2O_2.

Results and Discussion

Stability of Ferric Iron Porphycene in a Myoglobin Matrix. Myoglobin reconstituted with ferric iron porphycene **1** was prepared by a conventional method. The characterization of rMb(**1**) has been reported in our previous publications.[9] A UV−vis study suggested that no degradation of rMb(**1**) occurred at 4 °C, pH 7.0, for over 1 week. Furthermore, to compare the stability of the prosthetic groups between **1** and the native hemin in the myoglobin matrix, we evaluated the competitive incorporation of the two prosthetic groups into apomyoglobin. The addition of the mixture of **1** and the native hemin with the molar ratio of 1:1 into a solution of 0.75 equiv of apomyoglobin gave the reconstituted myoglobins with the product ratio of approximately 69:31, respectively, determined by UV−vis spectroscopy, as shown in Figure 1 after reaching equilibrium.[14] This result indicates that **1** is thermodynamically more stable than the native hemin in the protein matrix at pH 7.0. In addition, from the acid titration experiment, we found that ferric **1** is also stable in the myoglobin heme pocket under acidic conditions because we found that the p$K_{1/2}$ value, which represents the pH value corresponding to 50% dissociation of the prosthetic group, was determined to be 3.1 and 4.5 for **1** and the native hemin, respectively.[9] The findings support the fact that the His93 imidazole ring may be bound to the Fe atom of **1** more tightly compared with that observed for the native myoglobin.

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(14) In the short term, it was found that **1** was predominantly inserted into apomyoglobin compared to the native hemin.

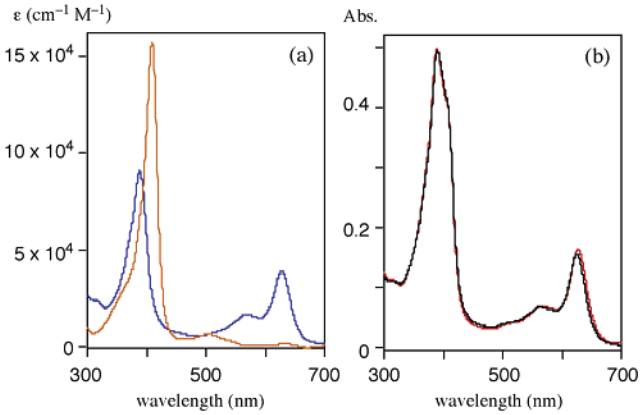

Figure 1. UV−vis spectra of myoglobins. (a) Spectra of native met-myoglobin (brown) and met-rMb(**1**) (blue) at pH 7.0. (b) Result of the competitive incorporation of **1** and the native hemin (1:1) into apomyoglobin. Experimental conditions are as follows: [apomyoglobin] = 9.0 μM, [**1**] = [native hemin] = 12 μM at 4 °C, pH 7.0. The solution of the reconstituted proteins was passed through a Sephadex G-25 column after 2 h to remove the free prosthetic groups. The black and red lines represent the observed and simulated spectra, respectively. The simulated spectrum was obtained from the overlap of both protein spectra with the ratio of 69:31.

Crystal Structure of Reconstituted Myoglobin. The 3D structure of the heme pocket and the electron density of the prosthetic group **1** in met-rMb(**1**) is shown in Figure 2a. The porphycene is situated between the E and F helices and has the His93 of the F helix as a fifth ligand, which is very similar in appearance to the hemin in the native sperm whale myoglobin. Because the crystals of rMb(**1**) have only been obtained in a solution containing the imidazole buffer, the imidazole molecule coordinated to the ferric porphycene Fe atom was observed in the distal site. Consequently, the distal His64 of the E helix swings out. A similar interaction and displacement of the distal histidine are seen in the structure of the native myoglobin/imidazole complex (PDB code 1MBI). When they are superimposed, most of the C_α atoms can be well fitted with a root-mean-square deviation (rmsd) of 0.5 Å between the C_α atoms of residues 1−151 (Figure 2b). However, the region of the C helix, the CD loop, and the D helix (residues 36−60) of rMb(**1**) shows large conformational differences and relatively high temperature factors in comparison to those of the native myoglobin. In general, the binding of the sixth ligand to the heme-containing protein stabilizes the active site structure, which usually increases the chance of a successful crystallization. The fact that we could obtain a crystal of rMb(**1**) only in the presence of the imidazole may reflect the enhanced motion of the region. The structure of the pocket of rMb(**1**) indicates that the structural difference between porphycene and porphyrin seems to be compensated for by the repositioning of the C/CD/D region. The CD loop of the native myoglobin contains Phe43, whose side chain contacts the porphyrin in the distal site by a hydrophobic interaction. The Phe43 in rMb(**1**) changes the conformation and shifts toward the porphycene to retain the interaction (Figure 2a). The flexible feature of this region may also have an important role in the incorporation of the prosthetic group and creation of a space for the reaction with the substrate. The porphycene plane is almost flat with an rmsd value < 0.1 Å from the plane, and the Fe atom is in the plane. In the superimposed structures, the porphycene is nearly parallel to the heme plane and at the same height along the fifth and sixth coordination axes. Because, unlike the heme, the porphycene has four pyrrole rings, which are not linked by the same number of C atoms in its chemical structure, the positions of the pyrrole rings in rMb(**1**) are different from those in the native myoglobin, resulting in the 0.5-Å shift of the Fe position. Although the deviation of the C_α atom position of His93 is small (0.3 Å), the conformation of its side chain differs between rMb(**1**) and the native myoglobin in order to adjust to the position of the Fe atom. In the proximal site, one of the interesting findings from Figure 2a is that the 16-propionate side chain interacts with the side chains of Lys96 and His97 at the distances of 3.0 and 2.9 Å, respectively, whereas the hydroxyl group of Ser92 is far (5.3 Å) from the propionate. The interaction of the propionate in rMb(**1**) is quite different from that observed in the native myoglobin, where 17-propionate interacts with Ser92 and His97. Despite these differences, the resultant directions of the two propionates of **1** are similar to those of the heme of the native myoglobin.

Peroxidase Activity of Reconstituted Myoglobin. Although both myoglobin and peroxidase, like horseradish peroxidase (HRP), have the same prosthetic group, protoheme IX, myoglobin has a poor peroxidase activity. This is because, compared to HRP, myoglobin has no suitable H_2O_2 activation system at the distal site and coordination from His93 to the heme iron is not strong. To effectively activate the bound H_2O_2 in the heme pocket of myoglobin, several point mutations at the distal site were proposed.[15] In contrast, to the best of our knowledge, there has been no significant trial to enhance the peroxidase activity of myoglobin by modifying the porphyrin framework.[16] First, we carried out the reaction of cumene hydroperoxide with met-myoglobins to evaluate the mode of the O−O bond cleavage.[17,18] The product ratio of cumyl alcohol and acetophenone, which are obtained through the heterolytic and homolytic O−O bond cleavages, respectively, was 42 in rMb(**1**), which is 2.5-fold higher than that observed for the native myoglobin. This finding suggests that, compared to the native myoglobin, rMb(**1**) facilitates the heterolytic O−O bond cleavage, which produces compound **I** ($P^{\bullet+}Fe^{IV}=O$)[19] because of the push effect derived from the strong coordination from His93 to the Fe atom of **1**. This result encouraged us to carry out the

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(19) The porphycene ligand is abbreviated as P in this paper.

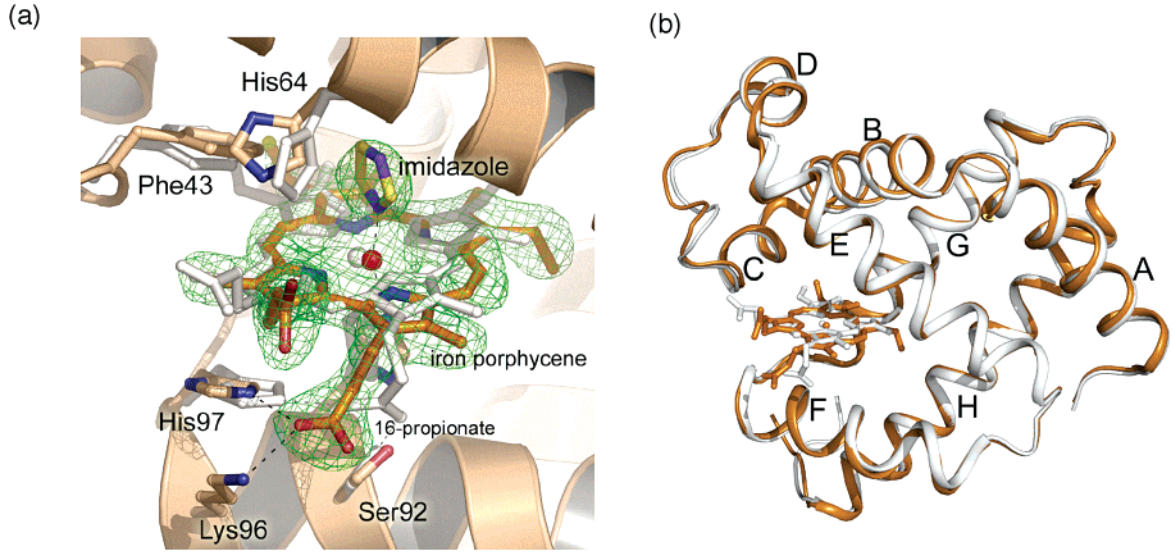


Figure 2. Structure of iron porphycene bound to sperm whale apomyoglobin. (a) Comparison of the iron porphycene **1** in rMb(**1**) (gold) and the heme in the native myoglobin complex (white, PDB ID 1MBI). The $2F_{obs} - F_{calc}$ electron density (1.0 σ contours) around the iron porphycene is also shown. The imidazole molecule in the native myoglobin−imidazole structure is omitted. (b) Superposition of all C_α atoms of rMb(**1**) (gold) and native myoglobin (white).

Table 1. pH-Dependent Peroxidase Activity[a−c]

	pH			
	5.5	6.0	6.5	7.0
native myoglobin	0.102	0.063	0.042	0.021
rMb(**1**)	0.069	0.119	0.183	0.230
relative[d]	0.68	1.9	4.4	11

[a] H_2O_2-dependent guaiacol oxidation catalyzed by myoglobins. [b] Initial turnover number (s^{-1}) of guaiacol oxidation. [c] Conditions: [myoglobin] = 1.0 μM, [H_2O_2] = 20 mM, [guaiacol] = 1 mM, at 20 °C. [d] Relative activities between the reconstituted and native myoglobins.

rMb(**1**)-catalyzed oxidations of small substrates in the presence of H_2O_2.

After incubation of rMb(**1**) with guaiacol, 2-methoxyphenol, as a substrate, the addition of the H_2O_2 solution produced the guaiacol tetramer as the oxidation product as detected by UV−vis spectroscopy at pH 7.0 and 20 °C. The initial rate of the guaiacol oxidation catalyzed by rMb(**1**) is 11-fold faster than that observed for the native myoglobin, suggesting that the replacement of the native hemin with **1** enhances the peroxidase activity toward the guaiacol oxidation. Furthermore, styrene epoxidation and thioanisole sulfoxidation are also remarkably 5-fold accelerated by rMb(**1**) compared to the native myoglobin. This is the first example that demonstrates the enhancement of the peroxidase and peroxygenase activity of myoglobin by modifying the tetrapyrrolic framework of the prosthetic group, although the present activity of rMb(**1**) is still lower than that of the native peroxidases.[20] Furthermore, the initial rates of the guaiacol oxidation catalyzed by the myoglobins at various pH values are shown in Table 1. It is known that the oxidation is normally accelerated at the lower pH value, whereas the activity of the pH-dependent oxidation catalyzed by rMb(**1**) is significantly different from that observed for the native myoglobin.

Reaction Intermediates. The transient spectral changes of rMb(**1**) upon the addition of H_2O_2 were monitored in the absence of guaiacol within a few minutes. The rapid-mixing experiments showed that at least two characteristic intermediates were generated, as shown in Figure 3. After the addition of H_2O_2 to the rMb(**1**) solution, the Q band at 624 nm underwent a blue shift of 3 nm within 2 s. Figure 4a represents the differential spectra via clear isosbestic points within 1.8 s upon the addition of H_2O_2. The first intermediate (green spectra in Figures 3a and 4a) was assigned as a compound **II**-like species, PFe^{IV}═O,[21] because the green spectrum was consistent with that observed by reaction with *m*-chloroperbenzoic acid (mCPBA). Furthermore, we found that the addition of guaiacol to the first intermediate led to the oxidation product using a double-mixing apparatus. From the stopped-flow technique, the formation rate of the compound **II** intermediate from met-rMb(**1**) was determined to be 32 M^{-1} s^{-1} at 4 °C, pH 7.0, indicating that the compound **II** formation for rMb(**1**) is 4-fold slower than that observed for the native myoglobin. Therefore, one of the possibilities that accounts for the acceleration of the substrate oxidation catalyzed by rMb(**1**) will be due to the predominant formation of compound **I**-like species from the hydroperoxo species of myoglobin with H_2O_2 as described above, although we cannot directly detect the compound **I** formation and monitor the reaction of the intermediate with guaiacol.

Corresponding to the decay of the first intermediate, the second species with a peak top at 620 nm appeared after 20 s, as shown in Figure 3. Spectral changes from the apparent first to second intermediates also occur with some clear isosbestic points, as shown in Figure 4b. The spectrum of the second intermediate (red spectrum of Figure 3a) is completely consistent with the spectrum of the oxygenated

(20) The initial rate of the guaiacol oxidation catalyzed by the native HRP is approximately 85 s^{-1} under the same conditions as shown in Table 1 (pH 7.0).

(21) For the native myoglobin, the compound **I** intermediate is not easily detected by the conventional method. Egawa, T.; Shimada, H.; Ishimura, Y. *J. Biol. Chem.* **2000**, *275*, 34858−34866.

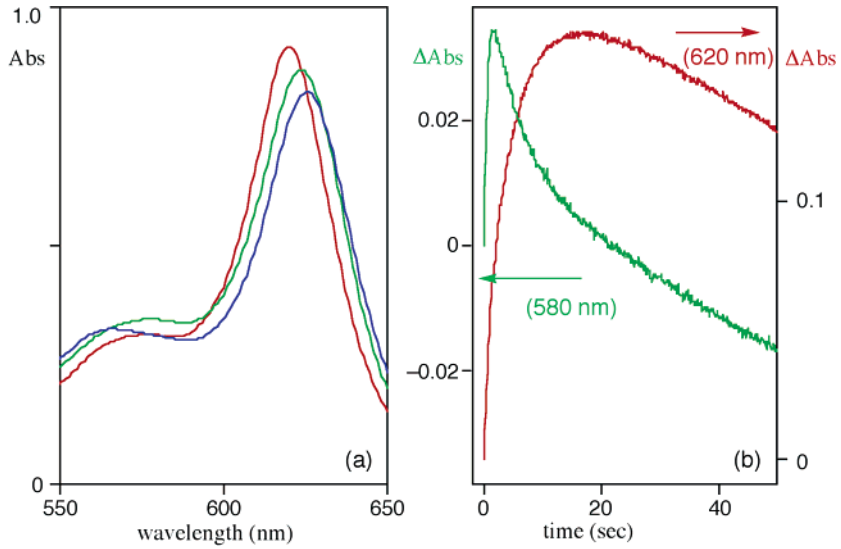


Figure 3. (a) Spectral changes after the mixing of met-rMb(**1**) with H_2O_2. The blue, green, and red spectra were recorded at 0, 1.8, and 19.8 s, respectively, after the addition of H_2O_2 by the stopped-flow mixing technique at 4 °C, pH 7.0. (b) The time course of absorbance changes of rMb(**1**) recorded at 580 nm (green) and 620 nm (red). [rMb(**1**)] = 22 μM; [H_2O_2] = 50 mM.

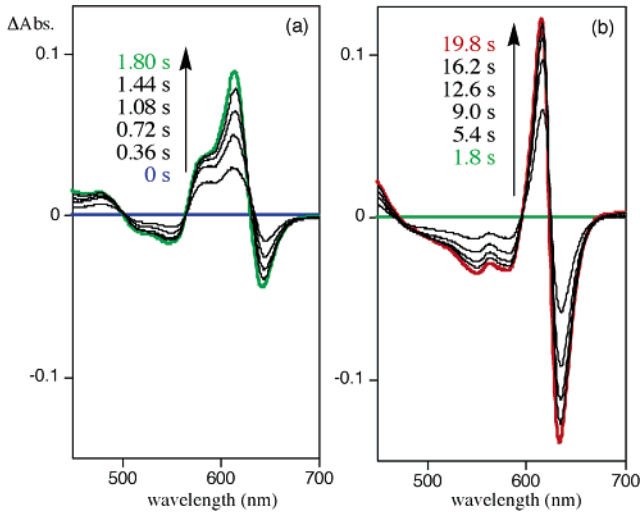
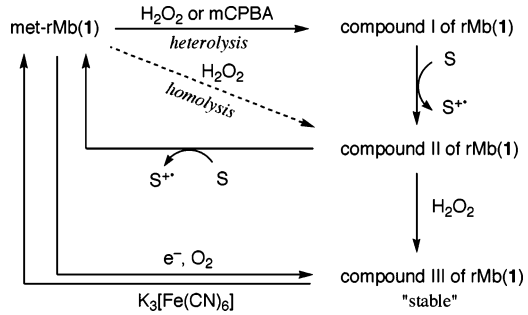


Figure 4. Spectral changes in rMb(**1**) upon the addition of H_2O_2 in the absence of the substrate. Difference spectra were obtained based on the spectrum at 0 s (a) and 1.8 s (b), at 4 °C, pH 7.0 (100 mM potassium phosphate buffer), [rMb(1)] = 22 μM, and [H_2O_2] = 50 mM.

rMb(**1**), which was prepared from deoxy-rMb(**1**) and O_2.[9] In addition, the intermediate smoothly returned to met-rMb(**1**) after the sequential addition of catalase and ferricyanide. In contrast, the addition of mCPBA instead of H_2O_2 only produced the spectrum of a compound **II**-like species, and the second intermediate species was undetectable. These results support the fact that ferric **1** in the protein converts to a stable oxygenated species, compound **III** ($PFe^{III}-O_2^{\bullet-}$), via compound **II** upon the addition of excess amounts of H_2O_2.[22−24] It is known that the compound **III** species in several peroxidase proteins, such as lignin peroxidase or

Scheme 2. Summary of Reaction Scheme of rMb(**1**)



HRP, is observable in the presence of excess amounts of H_2O_2,[25] whereas clear evidence of the compound **III** formation from compound **II** with H_2O_2 has never been reported for the native myoglobin.[26] From a series of reactions of rMb(**1**) summarized in Scheme 2, it is suggested that the formation of compound **III** inhibits the catalytic reaction of the substrate oxidation. In fact, the UV−vis study indicated that the oxidation products of guaiacol were not detected upon the addition of guaiacol to an incubated mixture of rMb(**1**) and H_2O_2, although the native myoglobin produces an oxidation product monitored at 470 nm under the same conditions. Furthermore, Table 1 demonstrates that the turnover number of the guaiacol oxidation deceases at the lower pH value, suggesting that the initial step in the conversion to compound **III** seems to be the protonation of

(22) Oxygenated rMb(**1**) is remarkably stable against autoxidation; $k_{auto(rMb(1))}$ = 0.024 h^{-1}, at 37 °C, pH 7.0, under aerobic conditions. The rate constant of the native myoglobin is 4-fold higher than that of rMb(**1**).[9]

(23) The formation rate of compound **III** from compound **II** in rMb(**1**) under a N_2 atmosphere was consistent with that observed under aerobic conditions. This result rules out the formation of the compound **III** species by the oxygenation of deoxy-rMb(**1**).

(24) The rate of the compound **III** formation in rMb(**1**) was $^1/_{10}$ or less of that observed for the native peroxidases. (a) Jakopitsch, C.; Wanasinghe, A.; Jantschko, W.; Furtmüller, P. G.; Obinger, C. *J. Biol. Chem.* **2005**, *280*, 9037−9042. (b) Hiner, A. N. P.; Ruiz, J. H.; López, J. N. R.; Cánovas, F. G.; Brisset, N. C.; Smith, A. T.; Arnao, M. B.; Acosta, M. *J. Biol. Chem.* **2002**, *277*, 26879−26885 and references cited therein.

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the iron−oxo species of compound **II**.[27] Thus, the strong electron donation from the proximal His93 to the Fe atom of **1** increases the basicity of the O atom of the oxoferryl species and leads to the stable compound **III** through the ligand exchanges between $PFe^{IV}{=}OH^{+}$ and $PFe^{IV}{-}OOH$.[28]

Conclusion

In conclusion, we directly revealed that the incorporation of the iron porphycene **1** into the apoprotein did not produce any remarkable changes in the 3D structure of the parent protein and **1** is relatively stable in the protein matrix compared to the native hemin. Furthermore, we have demonstrated that the peroxidase activity toward several substrate oxidations catalyzed by myoglobin reconstituted with **1** is higher than that observed for the native myoglobin, although the obtained structure with a 2.25-Å resolution cannot account for the enhancement of the peroxidase activity. The results of this study suggest that the replacement of the native heme with an artificially created metalloporphyrin isomer can be an effective way of modifying the hemoprotein function. The appropriate molecular design of a prosthetic group will give us a unique and active biocatalyst.

Experimental Section

Instruments. The UV−vis experiments were conduced using a Shimadzu UV-3150 double-beam spectrophotometer equipped with a thermostated cell holder with a 0.1 °C deviation. Purification of the proteins was performed by an Amersham Biosciences ÄKTA$_{FPLC}$ system with a fraction collector Frac-920 at 4 °C. High-performance liquid chromatography (HPLC) analysis was performed on a Shimadzu SCL 10Avp HPLC system. The fast reaction kinetics was monitored at 4 °C with a stopped-flow rapid-mixing system constructed by Unisoku Co., Ltd. (Osaka, Japan). The pH values were monitored by a Beckman Φ71 pH meter.

Materials. The native sperm whale myoglobin was purchased from Biozyme Laboratories, Ltd. All reagents of the highest guaranteed grade available were obtained from commercial sources and were used as received unless otherwise indicated. Distilled water was demineralized by a Millipore Milli-Q Academic A10. H_2O_2 was purchased from Wako Pure Chemical Industries. The proteins were purified by column chromatography through CM-52 (Whatman), Sephadex G-25 (Amersham Biosciences), Hi-Trap Desalting (Amersham Biosciences), and/or Superdex 75 (Amersham Biosciences). Iron porphycene **1** was synthesized by the method described in a previous paper.[9] The crystal of the reconstituted myoglobin for the X-ray structural analysis was prepared as described below.

Protein Reconstitution with Iron Porphycene. The sperm whale apomyoglobin was prepared from the native met-myoglobin by Teale's 2-butanone method.[29] A 10 mM KOH solution of **1** was added to a solution of apomyoglobin with slow shaking at 4 °C. The solution was dialyzed against a 100-fold volume of a 100 mM phosphate buffer over 9 h (3 h × 3) at pH 7.0 and 4 °C. After centrifugation (4000 rpm, 10 min, at 2 °C) and concentration, the solution was passed through a Sephadex G-25 column. Further purification was performed by an Amersham Biosciences ÄKTA$_{FPLC}$ system equipped with a Superdex 75 column. The purified protein solution was concentrated, kept at 4 °C, and used within 2 days. The concentration of rMb(**1**) was determined by the molar absorption coefficient (ϵ_{388} = 91 000 M^{-1} cm^{-1}).[30]

Table 2. Data Collection and Refinement Statistics[a]

Data Collection	
X-ray source (beamline)	SPring-8 (BL44B2)
wavelength (Å)	1.000
resolution (Å)	50−2.25 (2.33−2.25)
space group	$P2_1$
unit cell param	a = 42.5 Å, b = 79.9 Å, c = 54.6 Å, β = 110.9°
obsd reflns	47 295
unique reflns	15 759
redundancy	3.0 (2.5)
completeness (%)	97.8 (95.6)
R_{sym} (%)	7.1 (27.5)
$I/\sigma(I)$	12.5 (2.9)
Refinement and Model Quality	
resolution (Å)	20−2.25
total atoms	2756
R_{cryst}/R_{free} (%)	25.1/29.4
rmsd, bond (Å)	0.005
rmsd, angle (deg)	1.13
Ramachandran plot	
most-favored regions (%)	88.2
additional allowed regions (%)	11.4
average B factor (Å^2)	
protein	34.06
main chain	31.84
side chain	36.31
porphycene	23.41
water	37.72

[a] Numbers in parentheses are for the highest resolution shell. $R_{sym} = \Sigma_{hkl}\Sigma_i|I_i(hkl) - \langle I(hkl)\rangle|/\Sigma_{hkl}I_i(hkl)$, where $\langle I(hkl)\rangle$ is the average intensity of i observations. $R_{cryst} = \Sigma_{hkl}||F_{obs}(hkl)| - |F_{calc}(hkl)||/\Sigma_{hkl}|F_{obs}(hkl)|$, where F_{obs} and F_{calc} are observed and calculated structure factor amplitudes, respectively. R_{free} is calculated for 5% of the reflections randomly selected and excluded from structure refinement. R_{cryst} is calculated for the remaining 95% of the reflections.

Reaction with Cumene Hydroperoxide. A reaction mixture containing 100 μM myoglobin and 1 mM cumene hydroperoxide was incubated at 20 °C in a 100 mM potassium phosphate buffer at pH 7.0 for 5 min. The reaction mixture was analyzed by an HPLC system equipped with a YMC Pro C18 column to determine the ratio of the products, acetophenone and cumyl alcohol; the eluent was water/methanol (1:1) at a flow rate of 0.8 mL min^{-1}.

Guaiacol Oxidation Catalyzed by Myoglobins. Measurements of the oxidation activities were performed at 20 °C in a 100 mM potassium phosphate buffer (pH 7.0). A mixture of the protein (1 μM, 0.97 mL) and guaiacol (100 mM, 10 μL) was incubated for 10 min, and then 20 μL of 1 M H_2O_2 was added. The initial rate of the guaiacol oxidation was determined from the increase in absorbance at 470 nm (ϵ_{470} = 26 600 M^{-1} cm^{-1}).[31]

Crystallization and Structure Determination. The protein was crystallized using the sitting vapor diffusion technique. The imidazole-bound crystals were obtained using a reservoir solution containing 3.0 M ammonium sulfate and 0.1 M imidazole, pH 7.0. It belongs to the space group $P2_1$ with the cell dimensions of a = 42.5 Å, b = 79.9 Å, c = 54.6 Å, and β = 110.9° and includes two monomers per asymmetric unit. The crystal lattice does not show

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any similarity to the reported crystal structure of myoglobin in the Protein Data Bank (PDB). The X-ray diffraction data were collected using an ADSC Q210 detector at BL44B2 SPring-8, Japan. The data were integrated and scaled using the HKL2000 program.[32] The structure was determined by molecular replacement with the program MOLREP[33] using the native myoglobin (PDB ID 1A6K) as a search model. Iterative model fitting and refinement cycles were carried out using O[34] and CNS.[35] The stereochemical quality of the model was analyzed by the PROCHECK[33] and WHAT IF[36] programs. The data collection and refinement statistics are summarized in Table 2. The final refined model contains the residues 1−153 for the A chain, the residues 1−151 for the B chain, 225 water molecules in an asymmetric unit, and one imidazole-bound iron porphycene for each molecule. The superposition calculation of the C_α atoms between the A and B chains of rMb(**1**) results in a rmsd of 0.6 Å. The deviation in the C/CD/D region is relatively large. Several residues in C/CD/D fall in the additional allowed region of the Ramachandran plot. The figures for the structural model are created by PyMol (www.pymol.org). The calculation of the superposition was performed by LSQKAB.[33] The atomic coordinate has been deposited in the Protein Data Bank, www.pdb.org (PDB ID 2D6C).

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