Peroxidase Activity of Myoglobin Is Enhanced by Chemical Mutation of Heme-Propionates

Takashi Hayashi,*,† Yutaka Hitomi,‡ Tsutomu Ando,† Tadashi Mizutani,‡ Yoshio Hisaeda,† Susumu Kitagawa,‡ and Hisanobu Ogoshi§

Contribution from the Department of Chemistry and Biochemistry, Graduate School of Engineering, Kyushu University, Fukuoka 812-8581, Japan, Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Kyoto 606-8501, Japan, and Fukui National College of Technology, Sabae 916-8507, Japan

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Abstract: Peroxidase activity of a myoglobin reconstituted with a chemically modified heme **1** is reported. The heme **1** bearing a total of eight carboxylates bound to the terminal of propionate side chains is incorporated into apomyoglobin from horse heart to obtain a new reconstituted myoglobin, rMb(**1**), with a unique binding domain structure. The UV−vis, CD, and NMR spectra of rMb(**1**) are comparable with those of native myoglobin, nMb. The mixing of rMb(**1**) with hydrogen peroxide yields a peroxidase compound II-like species, rMb(**1**)-II, since the spectrum of rMb(**1**)-II is identical with that observed for nMb. Stoichiometric oxidation of several small molecules by rMb(**1**)-II, demonstrates the significant reactivity. (i) The oxidation of cationic substrate such as $[Ru(NH_3)_6]^{2+}$ by rMb(**1**)-II is faster than that observed for oxoferryl species of nMb, nMb-II. (ii) Anionic substrates such as ferrocyanide are unsuitable for the oxidation by rMb(**1**)-II. (iii) Oxidations of catechol, hydroquinone, and guaiacol are dramatically enhanced by rMb(**1**)-II (14−32-fold) compared to those observed for nMb-II. Thus, the chemical modification of heme-propionates can alter substrate specificity. Steady-state kinetic measurements indicate that both the reactivity and substrate affinity toward guaiacol oxidation by rMb-(**1**) are improved, so that the specificity, k_{cat}/K_m, is 13-fold higher than that in nMb. This result strongly suggests that the artificially modified heme-propionates may increase the accessibility of neutral aromatic substrates to the heme active site. The present work demonstrates that the chemical mutation of prosthetic group is a new strategy to create proteins with engineered function.

Introduction

Myoglobin (Mb) is a familiar protein naturally used for oxygen storage with a single noncovalently bound iron protoporphyrin IX as a prosthetic group in a hydrophobic pocket of protein matrix.[1] Furthermore, it is known that Mb can catalyze the oxidation of ferrocytochrome *c* and several small molecules through oxoferryl heme species,[2] since Mb and peroxidase share partially similar features of the heme active site.[3] The peroxidase activity of Mb is, however, lower than that of native peroxidase.[2] One of the reasons is that native Mb has no specific substrate-binding site.[4,5]

In previous papers, we have reported that a horse heart Mb reconstituted with an artificially created heme bearing a carboxylate cluster forms an electrostatically stabilized complex with cationic methyl viologen[6] or cytochrome *c*[7] on the protein surface. We further monitored photoinduced electron transfer from the reconstituted zinc Mb to methyl viologen or ferricytochrome *c* via noncovalent interaction. Therefore, the reconstitution with chemically modified heme could be useful for introducing an engineered binding domain with substrate specificity into Mb.[8,9] Recently, we have found that the oxoferryl species of reconstituted Mb oxidizes several cationic or small aromatic substrates more effectively compared to that of native Mb (nMb). The present paper demonstrates that the modification of heme-propionates enhances the peroxidase activity of the reconstituted Mb for small molecule substrates.[10,11]

* Address for correspondence: Dr. Takashi Hayashi, Kyushu University. Fax: 81-92-632-4718. E-mail: thayatcm@mbox.nc.kyushu-u.ac.jp.

† Kyushu University.

‡ Kyoto University.

§ Fukui National College of Technology.

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(5) Recently, substrate binding sites of some peroxidases have been clarified by X-ray crystallography: (a) Itakura, H.; Oda, Y.; Fukuyama, K. *FEBS Lett.* **1997**, *412*, 107−110. (b) Henriksen, A.; Schuller, D. J.; Meno, K.; Welinder, K. G.; Smith, A. T.; Gajhede, M. *Biochemistry* **1998**, *37*, 8054−8060.

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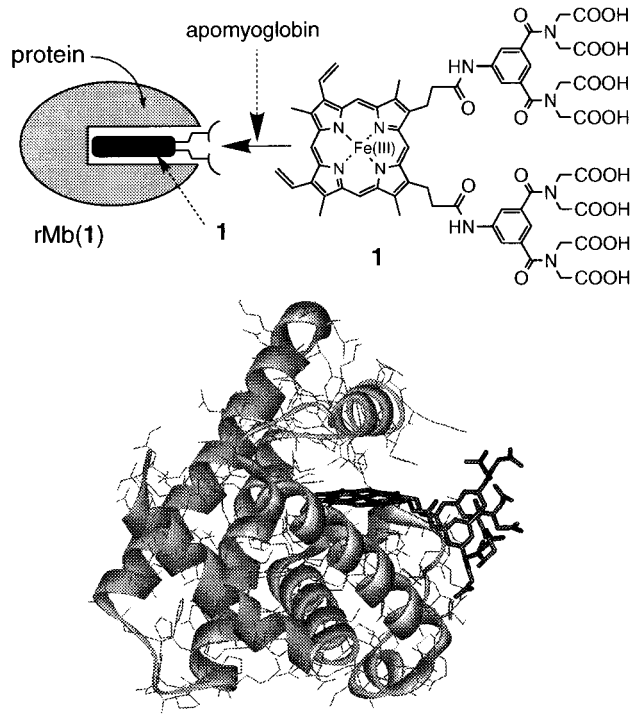

Figure 1. Scheme of chemically modified heme **1** and rMb(**1**) (upper), and hypothetical structure of rMb(**1**) based on Brayer's coordinate (lower).[12]

Results and Discussion

Characterization of Reconstituted Myoglobin. The horse heart myoglobin, rMb(**1**), reconstituted with a modified heme **1** bearing a total of eight carboxylate groups on the edge of two propionate side chains has been prepared by usual reconstitution technique as shown in Figure 1.[7a,12] The ^{1}H NMR spectrum of rMb(**1**) shows the characteristic paramagnetic shift as a high spin state of metaquo Mb.[13] The spectra of metaquo rMb(**1**) and cyanomet rMb(**1**) as a low spin state indicate that the major orientation of **1** in the protein is the same as that of native heme.[14] The electronic absorption spectrum of rMb(**1**) in pH 7.0 also shows the typical metaquo form of horse heart myoglobin, which is essentially identical to that of native protein.[15] As is evident from Figure 2 and NMR study, the heme **1** is located in the normal position of the heme pocket. The pK_a of the coordinated water molecule is slightly higher in rMb-(**1**) (pK_a = 9.0) at 20 °C than that observed for nMb (pK_a = 8.9). However, the deviation from the pK_a in nMb is rather small compared with those values for a series of reconstituted Mbs reported in the literature.[16]

Formation of Oxoferryl Species of Mb. The addition of hydrogen peroxide to a solution of rMb(**1**) caused a rapid change in the shape of UV−vis spectrum as shown in Figure 3. Soret absorption of rMb(**1**) decreases and shifts from 408 to 420 nm with an isosbestic point at 417 nm. The absorption maximum of rMb(**1**) in the presence of hydrogen peroxide is consistent with that of the oxoferryl species of nMb, an equivalent of peroxidase compound II.[2a,17,18] The formation rate of compound II-like intermediate from ferric rMb(**1**) was determined to be $(1.6 \pm 0.1) \times 10^3$ M^{-1} s^{-1} at 20 °C by stopped flow spectroscopy, while the second-order rate constant for nMb was $(6.3 \pm 0.1) \times 10^2$ M^{-1} s^{-1} under the same conditions. The enhancement of the formation rate of oxoferryl species means that hydrogen peroxide could be accessible to the heme pocket due to the change in the structure of the heme pocket.

Specificity of Small Molecule Substrate Oxidation. To determine the reactivities of rMb(**1**)-II and nMb-II,[19] we first monitored the stoichiometric oxidation of several organic and inorganic substrates by use of the oxoferryl species generated with hydrogen peroxide. Figure 4 shows the relative reactivity of rMb(**1**)-II to that of nMb-II in 100 mM phosphate buffer at pH 7.0. Significant features are as follows. (i) The oxidation of cationic substrate such as $[Ru(NH_3)_6]^{2+}$ by rMb(**1**)-II is clearly faster than that observed for nMb-II, since the cationic metal complex can interact with the artificially created binding domain on the protein surface because of the electrostatic contact.[20] (ii) In contrast, the oxidations of anionic substrates, 2,2′-azinobis-(3-ethylbenzothiazoline-sulfonic acid) (ABTS) and ferrocyanide, by rMb(**1**)-II proceed at 31 and 21% of the maximum rates observed by nMb-II, respectively. The anionic substrates are unsuitable for the oxidation by rMb(**1**)-II due to the electrostatic repulsion.[21] (iii) Interestingly, neutral phenol derivatives are good substrates for rMb(**1**)-II. Catechol oxidation by rMb(**1**)-II is 32-fold higher than that observed for nMb. Compared to nMb-II, rMb(**1**)-II also essentially accelerates hydroquinone and guaiacol oxidation. On the other hand, the addition of 1,2-dimethoxybenzene as a control substrate to the solution of rMb-(**1**)-II gave no clear decay of oxoferryl species. Therefore, this series of results indicates that the modification of heme-propionates can alter substrate specificity toward small molecule oxidations.

Guaiacol Oxidation Catalyzed by Myoglobin. Steady-state kinetics were measured for the guaiacol oxidation by rMb(**1**) and nMb in the presence of hydrogen peroxide at 20 °C in 100

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(13) The ^{1}H NMR spectrum of deoxy rMb(**1**) reduced by dithionite also shows the typical deoxy high spin state of horse heart Mb. The spectrum is shown in Supporting Information. Busse, S. C.; Jue. T. *Biochemistry* **1994**, *33*, 10934−10943.

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(15) The CD spectrum of rMb(**1**) is also comparable with that of nMb.

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(19) Abbreviations: rMb(**1**)-II and nMb-II represent compound II-like oxoferryl species of rMb(**1**) and nMb, respectively.

(20) Catalytic oxidation of ferrocytochrome *c* is also accelerated by rMb-(**1**) in the presence of hydrogen peroxide. Hayashi, T.; Hitomi, Y.; Hisaeda, Y.; Ogoshi, H., unpublished results.

(21) Oxidation rate of ferrocyanide by rMb(**1**) depends on the ionic strength of buffer solution. The oxidation activity dramatically decreases to 2% of that observed for nMb at 10 mM ionic strength.

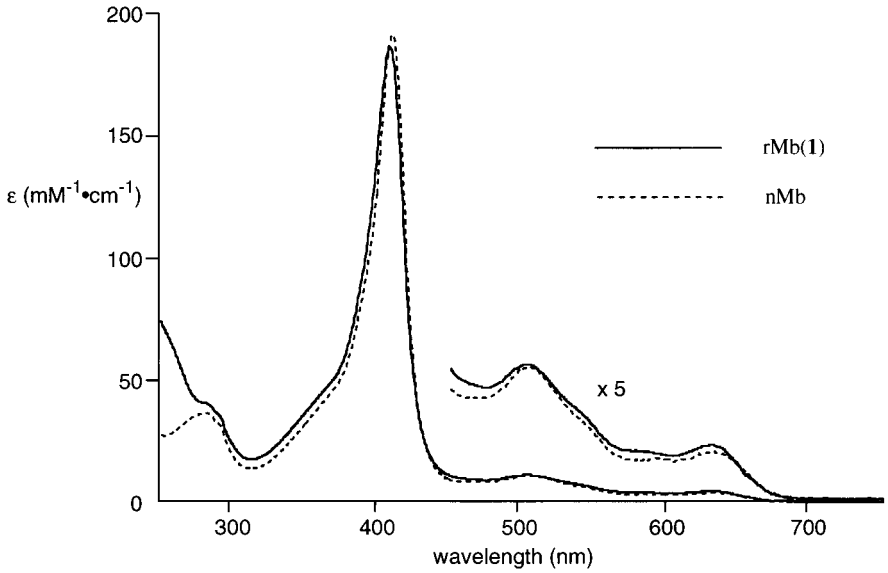

Figure 2. Electronic absorption spectra of rMb(**1**) and nMb in 10 mM potassium phosphate buffer, pH 7.0 at 295 K.

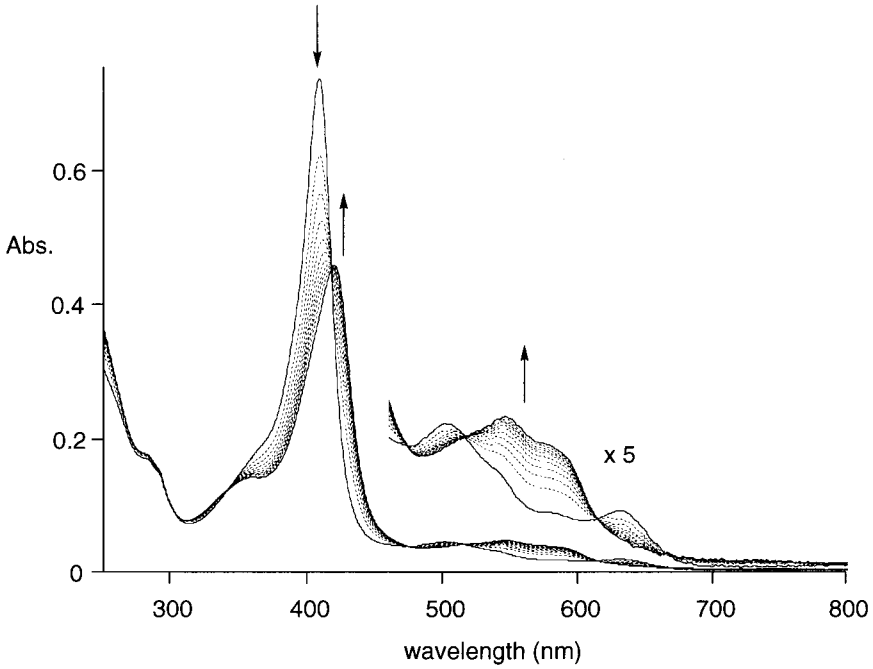

Figure 3. Rapid-scan (0−60 s) of the reaction of rMb(**1**) and nMb with a 25-fold excess of hydrogen peroxide in 100 mM potassium phosphate buffer, pH 7.0 at 295 K. $[rMb(\mathbf{1})]_0 = 4\ \mu M$. $[H_2O_2] = 0.1$ mM.

mM phosphate buffer. The oxidation follows Michaelis−Menten kinetics as shown in Figure 5; $K_m = 7.4 \pm 0.5$ mM and $k_{cat} = 1.1 \pm 0.1\ s^{-1}$ for rMb(**1**), and $K_m = 32 \pm 1$ mM and $k_{cat} = 0.36 \pm 0.01\ s^{-1}$ for nMb.[22] The reactivity and affinity for guaiacol are improved by rMb(**1**), so that the specificity, the value of k_{cat}/K_m, for rMb(**1**) is approximately 13-fold higher than that in nMb. This result suggests that guaiacol is easily accessible to the active site of rMb(**1**). In fact, the Soret band of ferric rMb(**1**) at 408 nm obviously changed with the observation of isosbestic points upon addition of guaiacol, compared to that of ferric nMb. Thus, the artificially created side chain with benzene ring moiety bound to the terminal of two heme-propionates may give the favorable hydrophobic pocket as a binding domain for neutral aromatic substrates as shown in Figure 1.

Conclusion

We have engineered a new binding domain on the Mb surface by modification of heme-propionates to enhance the peroxidase activity with significant substrate specificity. The present *chemical mutation of prosthetic group* will serve as a new strategy for creating novel catalysts with tailored properties.[23] Further studies on kinetic analysis and structural property of rMb(**1**) are currently in progress.

Experimental Section

Horse heart myoglobin (Sigma) was purified by column chromatography through CM-52 (Whatman). Bovine heart catalase (Sigma)

(22) Substrate inhibition appears at high concentration of guaiacol with $K_i = 19 \pm 1$ mM: (a) *Enzyme Assays*; Eisenthal, R., Danson, M. J., Eds.; Oxford University Press: Oxford, 1992. (b) Song. Y.; Yang, C.-M.; Kluger, R. *J. Am. Chem. Soc.* **1993**, *115*, 4365−4366.

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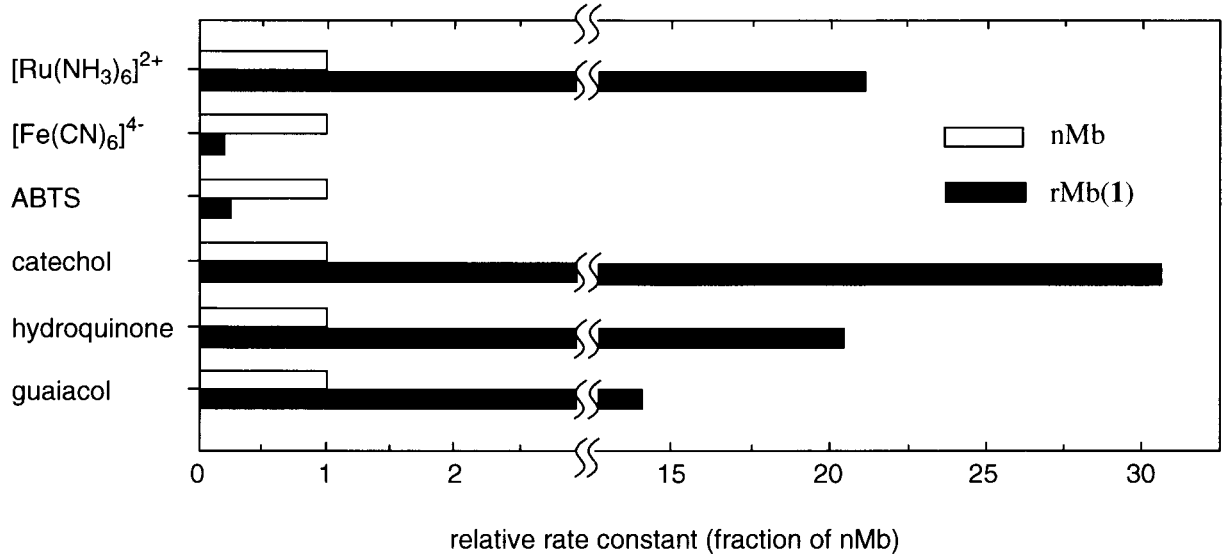

Figure 4. Relative reactivities of rMb(**1**)-II toward small molecule oxidations compared to those of nMb-II.

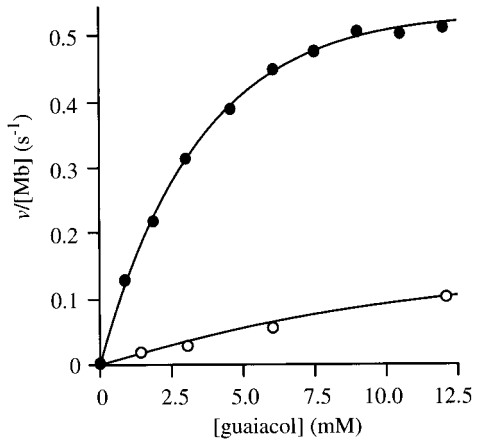

Figure 5. Initial rate, v, of myoglobin-catalyzed oxidation as a function of guaiacol concentration for a fixed amount of myoglobin (1 μM): rMb(**1**) (●), nMb (○).

was used without any purification. Synthesis of **1** and the following reconstitution procedure were described in our previous paper.[7a] Hydrogen peroxide (30% in water) was purchased from Wako Chemicals. All other chemicals were from Wako Chemicals or Nacalai Tesque.

Electronic absorption spectra were recorded on a Hewlett-Packard 8452A diode array spectrophotometer. ^{1}H NMR spectra were obtained using a JEOL A-500 spectrometer and a Bruker AMX-500 spectrometer and chemical shifts are reported relative to DSS at 0 ppm. CD spectra were measured with a Jasco model J-720 spectropolarimeter.

Determination of the Formation Rate of Oxoferryl Species by Stopped Flow Spectroscopy. The rate of oxoferryl species formation was determined at 20 °C by following the decay of the absorption at 408 or 410 nm using a stopped flow spectrophotometer (Otsuka Electronics RA-401 photodiode array accessory) with a 1-cm path length cell. Oxoferryl species was monitored after rapid mixing of H_2O_2 (5 μL, 40 mM) into a solution of myoglobin (2.0 mL, 4.0 μM).

Second-Order Rate Constants for the Reaction of Oxoferryl Myoglobin with Various Substrates. Hydrogen peroxide (200 μM) was added to a solution of either rMb(**1**) or nMb (1 μM) in 100 mM phosphate buffer at pH 7.0. After 5 min incubation at 20 °C, catalase was added, and the solution was further incubated for 2 min to completely quench the excess of H_2O_2. Substrate (>10 μM) was then added to the solution to ensure the pseudo-first-order kinetics. The oxidation reaction was monitored by the increase of ferric Mb at 408−410 nm.

Steady-State Kinetics. The steady-state kinetics of guaiacol oxidation were measured by incubating myoglobin (1 μM) with guaiacol (1−20 mM) and H_2O_2 (9.7 mM) in 0.1 M potassium phosphate buffer (pH 7.0) at 20 °C. The initial rate of the reaction was determined by the formation of tetraguaiacol at 470 nm using a molar absorbance coefficient of $\epsilon = 26.6\ \mathrm{mM^{-1}\ cm^{-1}}$.[24] The curves in Figure 5 obey the following equation: $v/[\mathrm{Mb}] = k_{cat}/\{1 + (K_m/[\mathrm{guaiacol}]) + ([\mathrm{guaiacol}]/K_i)\}$.[22]

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Supporting Information Available: ^{1}H NMR spectra of rMb(**1**), CD spectra of rMb(**1**) and nMb, and optical difference spectra obtained by titration of rMb(**1**) with guaiacol (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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