New Functionalization of Myoglobin by Chemical Modification of Heme-Propionates

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ABSTRACT
The reconstitution of myoglobin with an artificially created prosthetic group is a unique method for introducing a new chemical function into the protein. Particularly, the modification of two heme-propionates gives us an effective binding domain or binding site on the protein surface. This Account traces the design and construction of the highly ordered binding domain around the entrance of the heme pocket. The discussion includes the protein−small molecule or protein−protein recognition, electron transfer reaction within the complex, and enhancement of the chemical reactivity of the myoglobin with a substrate binding site. The synthetic approach to modifying a protein will be a new trend in engineering a novel function in naturally occurring hemoprotein.

1. Introduction

Myoglobin, a member of the hemoprotein family, is a well-known protein which binds a protoporphyrin IX iron complex via noncovalent interactions.[1] The X-ray crystallographic analysis indicates that the myoglobin is a relatively compact and globular protein which consists of eight α-helices and the heme as a prosthetic group.[2] The essential role of myoglobin is storage of oxygen during periods of rest until required for oxidative phosphorylation. Over the past 30 years, myoglobin has been investigated with respect to structure and function by many chemists and biochemists.[3] Particularly, a series of kinetic and spectroscopic studies suggest the role of the distal and proximal residues in modulating O_2, CO, and NO binding to ferrous myoglobin.[4,5]

Moreover, it is of particular interest to focus on the electron-transfer (ET) reaction of myoglobin or the chemical reactivity of ferryl myoglobin,[6] because the small globular myoglobin could be a suitable model to understand the physicochemical properties of hemoproteins. In fact, the heme pocket of myoglobin can stabilize the reduced and oxidized states of the iron complex; therefore, myoglobin has considerable potential for the catalytic oxidation of a substrate or ET with oxidoreductases, although the native myoglobin is less reactive for these reactions. Thus, the next stage in the study will be not only the evaluation of O_2 binding in the hemoprotein but also the engineering of a new function inside and/or on the surface of the myoglobin.

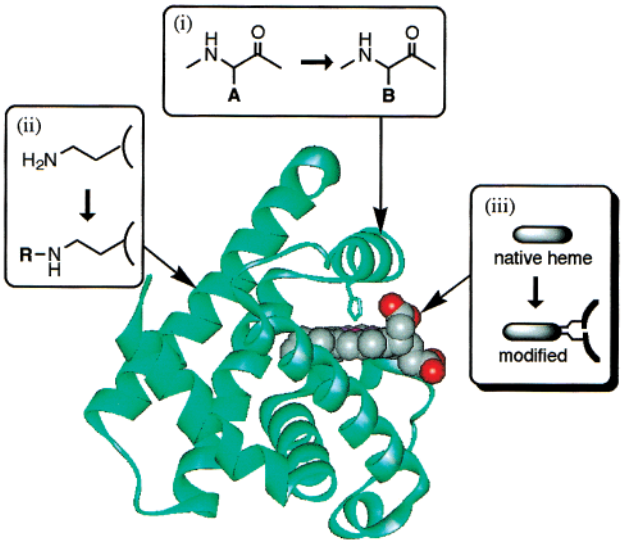

FIGURE 1. Schematic representation of three types of myoglobin modifications. (i) Replacement of an amino acid residue with the other one. (ii) Introduction of a functional group at a chemically active residue. (iii) Insertion of an artificial prosthetic group into apomyoglobin.

The strategy of hemoprotein modification can be divided into at least three approaches as shown in Figure 1: (i) site-directed mutagenesis of a protein, (ii) introduction of a functional group at an active residue on the protein surface, and (iii) replacement of the native heme with a functionalized metalloporphyrin. To modify the environment of the myoglobin heme pocket, a rearrangement of the amino acid residues by the mutagenetic approach is a powerful method.[7] However, it seems not to be easy to construct an interface, such as a substrate binding site, on the protein surface, because single or double amino acid substitutions on the protein surface are not sufficient for the formation of a local interface which recognizes the substrate or partner protein with high affinity. Chemical modification of a lysine residue on the protein surface is also an idea for the construction of a functional site,[8] although it could be sometimes difficult to undergo selective reaction at a special residue. In contrast, we believe that the myoglobin reconstituted

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with the chemically modified heme having functional groups at the terminal of the two heme-propionates exhibits the appropriate binding interface for substrates,[9−11] since we can dramatically change the chemical property of the protein surface by introducing multiple functions into the heme-peripheral side chains.

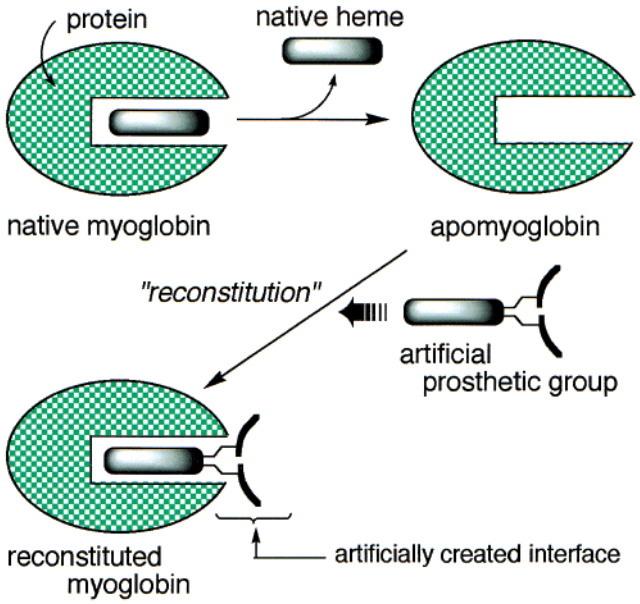
In this Account, we first give a brief introduction to the reconstitutional method of an artificial prosthetic group into the apomyoglobin. We then apply this methodology to the functionalization of myoglobin.

2. Reconstitution of Myoglobin with Unnatural Prosthetic Group

It has been found that the hemin in the myoglobin is readily removed from globin by treatment with acid solution (0.1 N HCl) followed by extraction with 2-butanone,[12] since the hemin is bound in the heme pocket via noncovalent interactions. The reconstituted myoglobin was then obtained by addition of the hemin solution into purified apoprotein solution.[12b] By using this method, several myoglobins reconstituted with metalloporphyrins such as Co, Zn, Mn, and Rh have been prepared and characterized.[13] Furthermore, a number of myoglobins with an unnatural hemin having chemically modified peripheral groups have been reported, in which the kinetics and thermodynamics of the reconstitutional process between the apomyoglobin and unnatural hemin have been discussed on the basis of NMR spectroscopy and X-ray crystallographic analysis.[14,15] According to a series of studies focusing on the thermal stability of the proteins, hydrophobic contacts between peripheral methyl and vinyl groups and heme pocket are more important than the interaction of two heme-propionates and polar residues, although the stability of the myoglobin with ferriprotoporphyrin IX dimethyl ester decreases due to the lack of a hydrogen network between the propionates and protein.[16−18] In contrast, it was found that the substitution of some amines via an amide linkage at the terminal of the propionates has no serious influence on the structure and stability of the protein.[19] This finding encourages us to introduce some functional groups at the terminal of the propionates by chemical synthesis. The preparation of the reconstituted myoglobin with functional groups is shown in Scheme 1.

The advantage of the reconstitutional strategy is summarized by the following three points. First, compared to other methods such as a mutagenetic approach, the modified myoglobin on a large scale is readily available. Second, multiple functional groups can be arranged and localized near the entrance of the heme pocket. The functional groups will play the role of an interface between the protein and an external compound. Third, as discussed above, the introduction of some functional groups into the terminal of two propionates should not give rise to a drastic perturbation of the principal property of a prosthetic group. Here, we wish to describe the construction of unique binding domains on the protein surface by the reconstitutional method.

Scheme 1

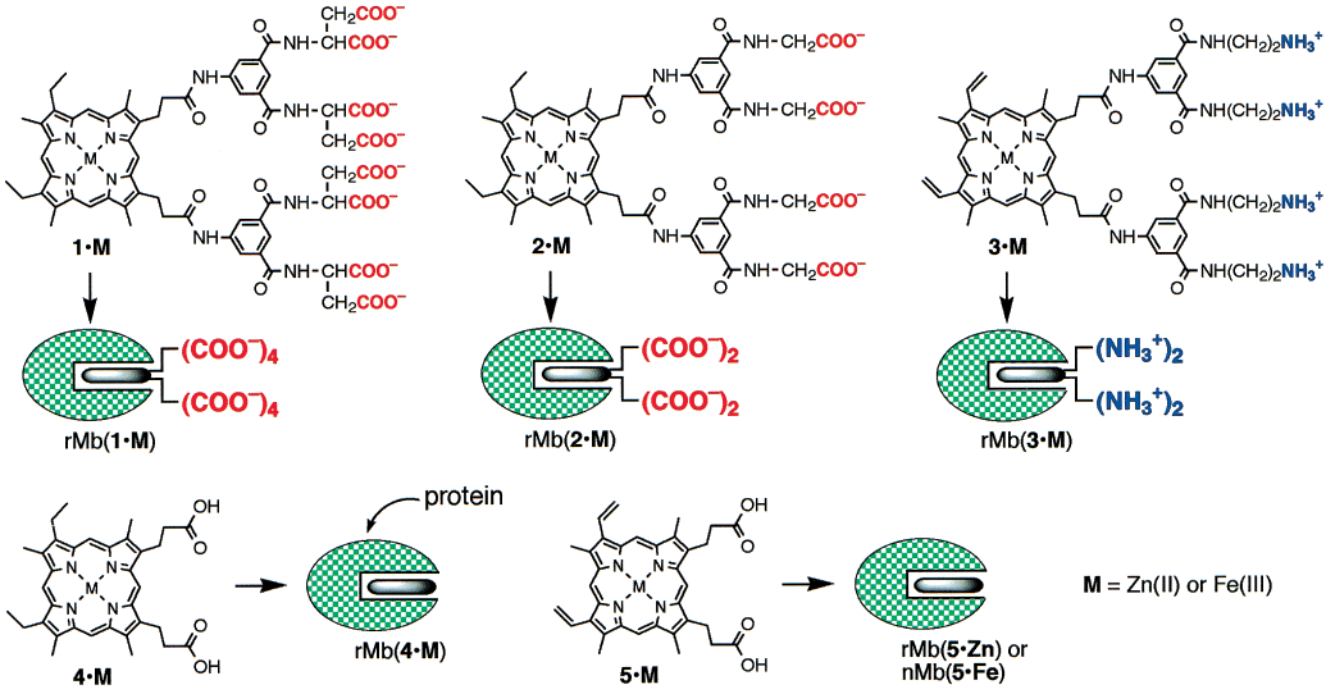


3. Construction of Binding Domain on the Myoglobin Surface

3.1. Modeling of Protein−Protein Recognition. The molecular recognition event on the protein surface plays a critical role in important biological processes, including mitochondrial respiratory system and photosynthesis, since it is well known that long-range ET is often mediated through the specific noncovalent interaction between protein and protein or protein and a small electron carrier. In particular, cytochrome *c*, which is an electron carrier protein with a positively charged patch formed by several lysines, acts as a mobile electron shuttle in the intermembrane space of mitochondria. Redox partners of cytochrome *c* are negatively charged hemoproteins such as cytochrome *b* and cytochrome *c* peroxidase, or huge oxidoreductase complexes such as cytochrome *c* oxidase and CoQ-cytochrome *c* reductase having a negatively charged binding site for the cytochrome *c*.[20] From this viewpoint, in the past decade, there have been a number of molecular recognition and/or self-assembly systems as biological ET models using synthetic small molecules.[21,22] However, it is likely that the information from the small system is sometimes insufficient to discuss the essentials of the protein−protein recognition and biological ET within the complex. Thus, the design and preparation of simple and well-organized models by real biomaterials would be required to clarify the ET mechanism via noncovalent mediating interactions.[23]

3.2. Negatively Charged Myoglobins. To create an artificial binding domain on the myoglobin surface, we first prepared chemically modified prosthetic groups, **1·Zn** and **1·Fe**, which have a total of eight carboxylates at the terminal of the two propionate side chains (Chart 1).[24] The zinc and iron porphyrins were readily inserted into a horse heart apomyoglobin to obtain reconstituted myoglobins, rMb(**1·Zn**) and rMb(**1·Fe**), respectively, in sufficient yields. The UV−vis spectra of rMb(**1·Zn**) and

Chart 1



metaquo-rMb(**1·Fe**) were identical to those of the reference myoglobins, rMb(**4·Zn**) and rMb(**4·Fe**), reconstituted with mesoporphyrin zinc and iron complexes, respectively. The CD spectra of rMb(**1·Zn**) and rMb(**1·Fe**) in the Soret and α-helix regions were also comparable with those of the reference proteins. These findings indicate that the metalloporphyrins will be incorporated into the normal position of the heme pocket and the anionic cluster has no serious effect on the chemical property of the zinc and iron porphyrins.

During the first stage, we monitored the interaction between rMb(**1·Zn**) and the methyl viologen dication $\mathbf{MV}^{2+}$ in a fluorescence study. The characteristic fluorescence emissions of **1·Zn** in the protein at 580 and 635 nm wavelengths were quenched upon the addition of $\mathbf{MV}^{2+}$, whereas no decrease in the fluorescence intensity of rMb(**4·Zn**) was observed, even at a 0.1 M concentration of $\mathbf{MV}^{2+}$. Stern−Volmer plots as shown in Figure 2 reveal that the quenching degree of the emission depends on the ionic strength and pH in the protein solution. These data indicate that the anionic cluster of rMb(**1·Zn**) forms a stable complex with a positively charged molecule via an electrostatic interaction. In addition, there is no change in the porphyrin absorption, suggesting no direct influence on the ground state of **1·Zn** upon the addition of $\mathbf{MV}^{2+}$.

We have also prepared the myoglobin rMb(**2·Zn**) reconstituted with a zinc porphyrin **2·Zn** having a total of four carboxylates at the terminal of the two propionate side chains.[25] Unexpectedly, the fluorescence emission of **2·Zn** in the protein was not quenched by $\mathbf{MV}^{2+}$ in a manner similar for that of rMb(**4·Zn**). The obtained result is discussed in section 3.4.

3.3. Positively Charged Myoglobin. Next, we prepared a cationic prosthetic group **3·Zn** with a total of four amino groups at the terminal of the two propionates (Chart 1).[26] The zinc porphyrin **3·Zn** was then inserted into the apomyoglobin to obtain a positively charged myoglobin rMb(**3·Zn**) over 12 h in approximately 20% yield. Compared to the preparation of the usual reconstituted myoglobin, the difficulty in the reconstitution of rMb(**3·Zn**) might be due to electrostatic repulsion between the ammonium cluster of **3·Zn** and some basic residues near

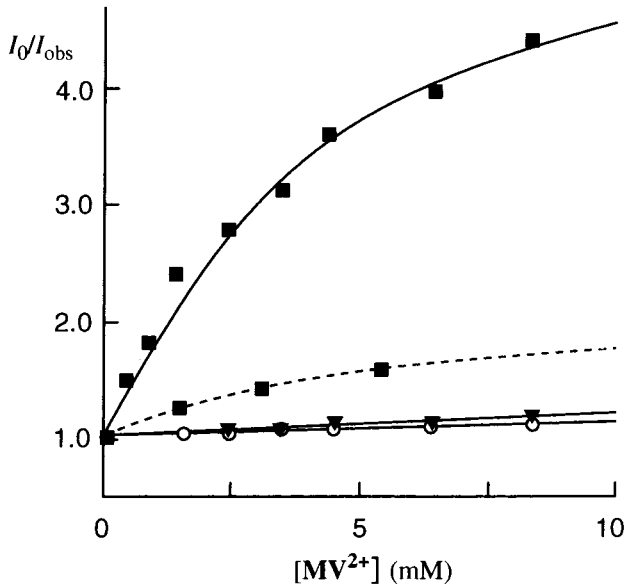


FIGURE 2. Stern−Volmer plots for the fluorescence quenching of the reconstituted zinc myoglobins as a function of $\mathbf{MV}^{2+}$ concentration at 25 °C in phosphate buffer, pH 7.0. The solid and dashed lines correspond to the data obtained in 10 mM and 100 mM phosphate buffers, respectively. (■) rMb(**1·Zn**), (▼) rMb(**2·Zn**), and (○) rMb(**4·Zn**). [myoglobin] = 10^{-6} M. The changes of the fluorescence emission were monitored at 584 nm (λ_{ex} = 543 nm).

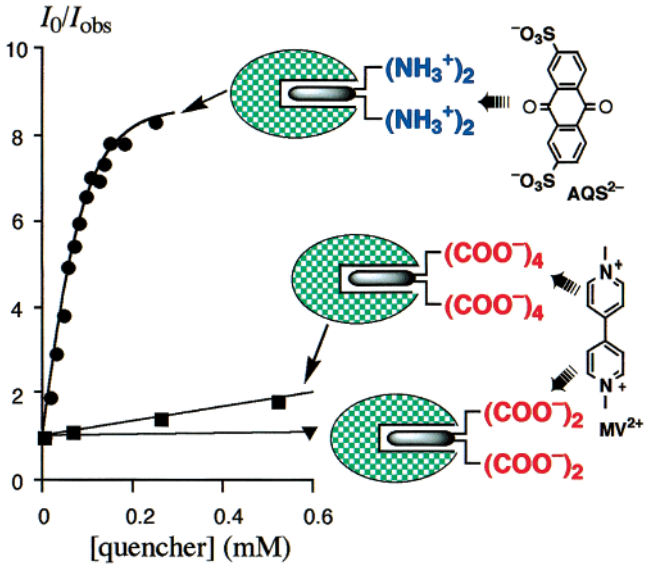


FIGURE 3. Stern−Volmer plots for the fluorescence quenching of the reconstituted zinc myoglobins as a function of MV^{2+} or AQS^{2-} concentration at 20 °C in 10 mM phosphate buffer, pH 7.0. The changes in the fluorescence emission were monitored at 584 nm (λ_{ex} = 543 nm). (■) rMb(**1·Zn**), (▼) rMb(**2·Zn**), and (●) rMb(**3·Zn**). [myoglobin] = 10^{-6} M.

Table 1. Binding Constants of Reconstituted Myoglobins for MV^{2+} and AQS^{2-} [a]

myoglobin	guest	ionic strength (mM)	binding constant (M^{-1})
rMb(**1·Zn**)	MV^{2+}	10	2.6×10^3
rMb(**1·Zn**)	MV^{2+}	100	2.2×10^2
rMb(**2·Zn**)	MV^{2+}	10	$<1 \times 10^2$
rMb(**3·Zn**)	AQS^{2-}	10	1.5×10^5

[a] At 10 °C, phosphate buffer, pH 7.0.

the entrance of the heme pocket in the horse heart myoglobin.[27] The UV−vis and CD spectra of rMb(**3·Zn**) were similar to those of the reference myoglobin rMb(**5·Zn**) reconstituted with the protoporphyrin IX zinc complex **5·Zn**. For the case of rMb(**3·Zn**), a remarkable fluorescence quenching was observed upon the addition of anthraquinone-2,7-disulfonate AQS^{2-}, and the intensity of the residual fluorescence emission depended on the ionic strength of the protein solution. The curvature of the Stern−Volmer plots with ionic strength dependence indicates that the synthetic ammonium cluster binds an anionic substrate via an electrostatic interaction (Figure 3).

3.4. Comparison of Binding Events between Several Reconstituted Myoglobins. To evaluate the artificially created binding domain, we determined the binding constants between the reconstituted myoglobin and MV^{2+} or AQS^{2-} from a nonlinear curve fit analysis of the Stern−Volmer plots in Figures 2 and 3. From the data in Table 1, it can be inferred that (i) at least a total of eight carboxylates are really required to form the negatively charged binding domain near the entrance of the heme pocket, (ii) the anionic cluster formed by four carboxylates does not completely work as a binding domain on the myoglobin surface, and (iii) interestingly, the cationic cluster formed by four ammonium groups effectively serves as a positively charged binding domain. In particular, the binding constant of rMb(**3·Zn**) for the dianion substrate, AQS^{2-}, is 58 times larger than that of rMb(**1·Zn**) for the dication, MV^{2+}, although **1·Zn** has a total of eight negative charges at the terminal of the side chains.

This contrasting result can be explained by the population of the amino acid residues in the horse heart myoglobin and the evaluation of the electrostatic potentials on the protein surface. According to the X-ray crystallographic analysis of horse heart myoglobin,[27] there are several basic residues such as Lys42, Lys45, Lys47, Lys63, Lys96, and His97 near the entrance of the heme pocket, whereas only two acidic residues, Asp44 and Asp60, are found in the same area. Although myoglobin has no local charged patch as seen on the cytochrome *c* surface, it is known that the myoglobin has a broad reactive surface which encompasses the hemisphere that includes the exposed heme edge for its negatively charged partner, cytochrome b_5.[28] Thus, we evaluated the electrostatic potentials of the reconstituted metaquo-myoglobins using the DelPhi module which is a Poisson−Boltzmann electrostatics simulation engine on the Insight II software (Molecular Simulations Inc.). Figure 4 demonstrates the electron density contour surfaces of rMb(**1·Fe**), rMb(**2·Fe**), nMb(**5·Fe**), and rMb(**3·Fe**). It is clear that rMb(**1·Fe**) has a negatively charged domain (red area) near the entrance of the heme pocket, whereas the local negative patch is not found on the surface of rMb(**2·Fe**), although **2·Fe** has a total of four carboxylates at the terminal of the heme-propionates. For the case of the native myoglobin nMb(**5·Fe**), the surface is almost neutral or slightly negative. It is noted that rMb(**3·Fe**) has a remarkably positive patch (blue area) on the surface, strongly suggesting that four ammonium groups at the terminal of the two heme-propionates might effectively form a favorable binding domain in association with several basic residues near the heme pocket. This finding is consistent with the experimental results obtained from the Stern−Volmer plots as described above (Figure 3).

3.5. Photoinduced Singlet ET from Reconstituted Zinc Myoglobin to MV^{2+}. Time-resolved fluorescence studies made with rMb(**1·Zn**) indicated monoexponential decay kinetics with a lifetime of 2.0 ± 0.1 ns, whereas the addition of MV^{2+} gave the biphasic decay profile with two exponential components of τ_S = 0.4 ± 0.1 ns and τ_L = 2.0 ± 0.1 ns.[9b,24] The shorter- and longer-lived components of the biphasic decay curve are assignable to the fluorescence lifetimes of the rMb(**1·Zn**)−MV^{2+} complex and free rMb(**1·Zn**), respectively. From these data, a rate constant of the forward singlet ET was estimated to be $k_{et} = 2.1 \times 10^9$ s^{-1}. Furthermore, picosecond laser flash photolysis studies showed the characteristic transient absorption spectra of a mixture of the cation radical species and excited singlet and triplet species of rMb(**1·Zn**) in the presence of MV^{2+}. The obtained result provides direct evidence for the photoinduced ET from rMb(**1·Zn**) to MV^{2+}. The decay profile at 447−462 nm led to the kinetic

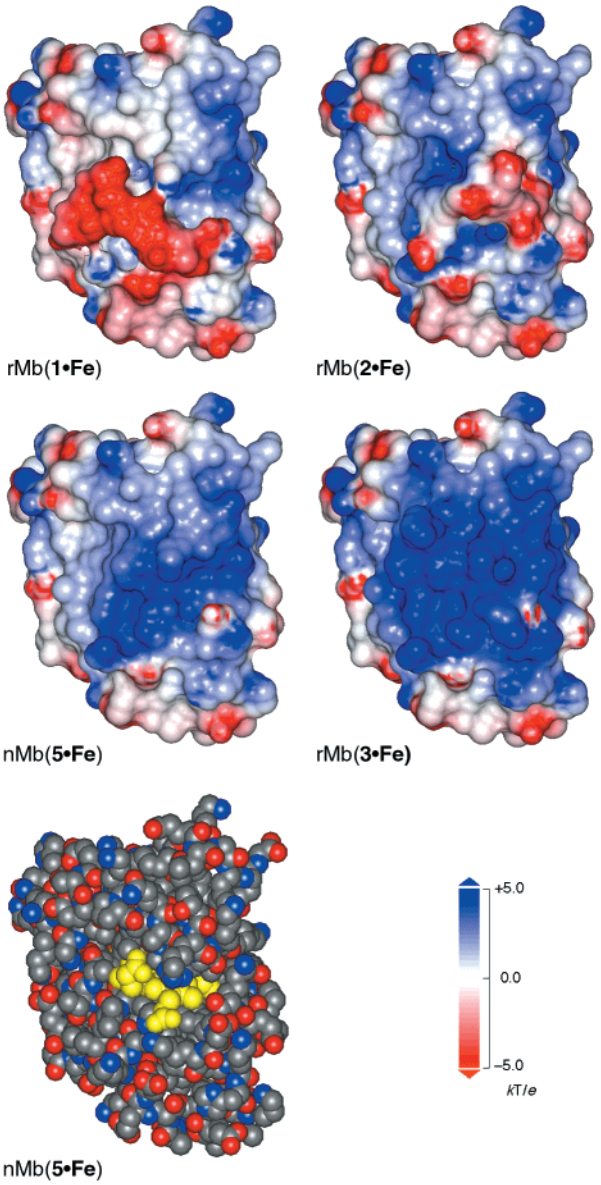


FIGURE 4. Simulation of electrostatic potentials computed from the DelPhi application module in the Insight-II produced by Molecular Simulations Inc., using a finite difference solution to the nonlinear Poisson−Boltzmann equation. The four figures (upper and middle) show that the negative and positive regions are red and blue, respectively, and the neutral region is transparent. Bottom left figure is displayed by normal CPK representation, where the yellow part represents the heme molecule. Structures of reconstituted myoglobins were optimized by MM and MD calculations starting from the crystal structure of horse heart myoglobin reported by Evans and Brayer.

parameters of the cation radical intermediate species: $k_{et} = 2.1 \times 10^9\ s^{-1}$ and $k_{cr} = 3.3 \times 10^8\ s^{-1}$. It has been known that a photoinduced triplet ET occurs from rMb(**5·Zn**) having no special binding domain to $\mathbf{MV^{2+}}$ via diffusional control, [29,30] whereas there was no example regarding singlet ET in the previous literature. Thus, stable rMb(**1·Zn**)−$\mathbf{MV^{2+}}$ complexation via an electrostatic interaction enables us to monitor the fast singlet ET within the protein complex as shown in Figure 5.

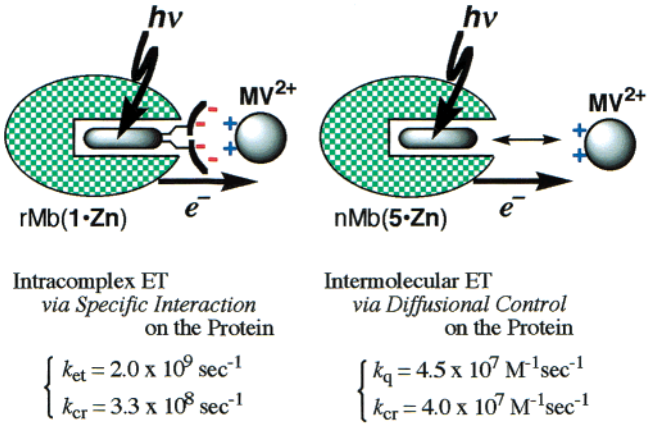


FIGURE 5. Comparison of ET rate constants within the rMb(**1·Zn**)−$\mathbf{MV^{2+}}$ complex with previous data obtained from ET between rMb(**5·Zn**) and $\mathbf{MV^{2+}}$.

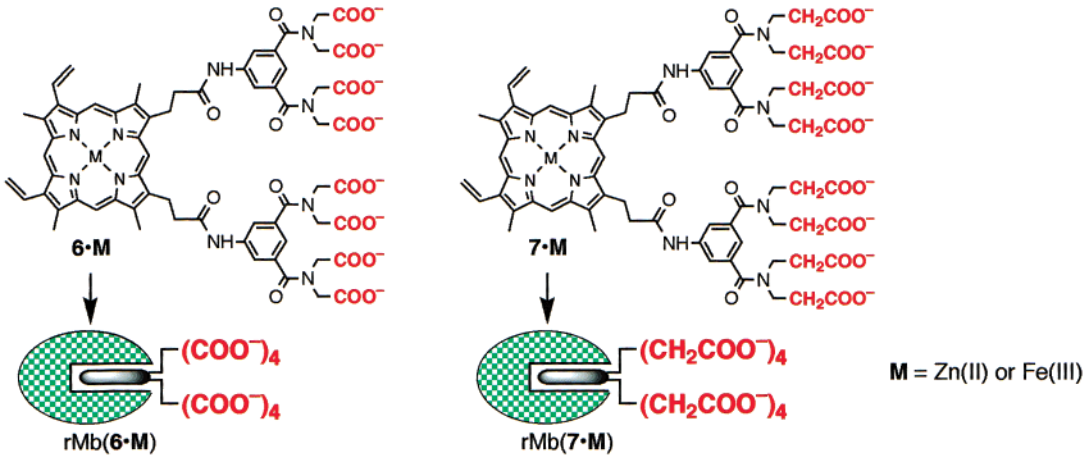
4. Protein−Protein Recognition and Interprotein ET

4.1. Cytochrome *c* Receptor by Reconstituted Myoglobin. A key goal in our work is to construct a well-organized protein model to clarify the behavior of the protein−protein recognition and interprotein ET. Here, cytochrome *c*, one of the well-known electron-transfer hemoproteins, was chosen as the partner of a functionalized myoglobin having a binding domain. We designed reconstituted myoglobins rMb(**6·Zn**) and rMb(**6·Fe**)[31] where the artificially created negative charges might spread over approximately 200 Å^2 to fit the special lysine residues in the binding domain of cytochrome *c* (Chart 2).[32]

Myoglobin−cytochrome *c* complexation was first supported by ^{1}H NMR titration experiments in which changes in the chemical shifts of selective protons in cyanomet-rMb(**6·Fe**) and ferric cytochrome *c* were monitored at different concentrations of cytochrome *c*. In particular, the heme 5-CH_3 and Ile99 CγH protons of rMb(**6·Fe**) clearly shifted upfield, and the heme 3-CH_3 and 8-CH_3 protons of cytochrome *c* also shifted from their original positions, whereas the deviation of the Met80 CϵH protons was quite small. The direction and the size of the shifts are comparable with those seen in the titrimetric measurement by the native pair of cytochrome *c* and cytochrome *c* peroxidase.[33] Thus, the complex fashion of rMb(**6·Fe**) and cytochrome *c* would be similar to that of the native pair, as shown in Figure 6, which is a computer-generated composite based on the crystal structure of horse heart myoglobin and cytochrome *c*.

4.2. Photoinduced ET within the Protein Complex. The photoinduced ET between rMb(**6·Zn**) and ferric cytochrome *c* was followed by monitoring the decay of the triplet excited state of the zinc myoglobins at 460 nm by laser flash photolysis. The addition of ferric cytochrome *c* to the solution of the zinc myoglobins resulted in a dramatic acceleration of the decay of the triplet excited state with a single exponential, as shown in Figure 7. The quenching rate constants depend on the concentration of cytochrome *c* and the ionic strength. In contrast, no acceleration was observed upon the addition of ferrous

Chart 2



cytochrome *c*, suggesting that the quenching is the result of an ET from the photoexcited zinc myoglobin to ferric cytochrome *c*. Furthermore, we observed the transient absorptions at 680 and 550 nm which are identified as the zinc porphyrin cation radical species of rMb(**6·Zn**) and ferrous cytochrome *c*, respectively. These bands increased with the decay of the triplet excited states and decayed slowly on a longer time scale. These findings also support the formation of the stable protein−protein complex, as shown in Figure 6.

4.3. Interprotein ET Regulated by an Artificially Created Binding Domain. The kinetics study on interprotein ET is one of the current topics in biological ET, since it is known that the observed ET rate sometimes reflects not only the true ET process but also several other steps such as protein−protein binding and rearrangement of the protein complex.[34] To evaluate the mechanism of the interprotein ET rate, a systematic model is required. For this subject, we believe that the present myoglobin would be suitable for the investigation of the interprotein ET. Thus, we have prepared the myoglobins rMb(**7·Zn**) having different binding domains on the protein surface

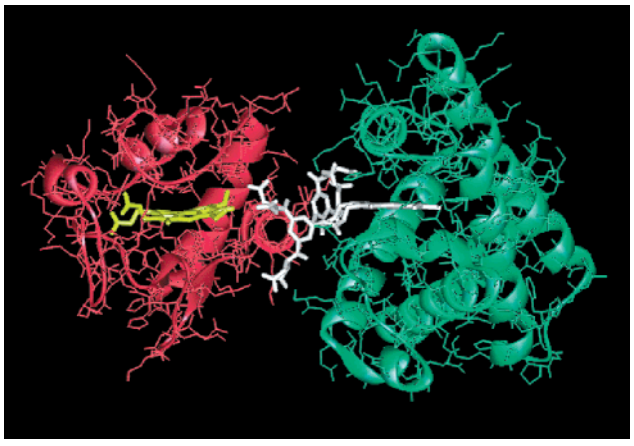


FIGURE 6. One of the plausible structures of the metaquo−rMb(**6·Fe**)−cytochrome *c* complex. The conformation was optimized by MM calculations (Discover/Insight II, MSI) based on the crystal structure of horse heart metmyoglobin and cytochrome *c*. Green and red proteins represent myoglobin and cytochrome *c*, respectively. White and yellow molecules are heme.

Table 2. Affinities and Dynamics of Reconstituted Myoglobin−Cytochrome *c* Complexes[a]

reconstituted myoglobin	ionic strength (mM)	binding constant (M^{-1})	rate constant (s^{-1})[b]
rMb(**6·Zn**)	10	$(6.5 \pm 0.3) \times 10^4$	$(2.2 \pm 0.1) \times 10^3$
rMb(**6·Zn**)	20	$(1.5 \pm 0.6) \times 10^4$	$(2.2 \pm 0.1) \times 10^3$
rMb(**7·Zn**)	10	$(3.1 \pm 0.2) \times 10^4$	$(4.8 \pm 0.1) \times 10^3$
rMb(**7·Zn**)	20	$(0.83 \pm 0.1) \times 10^4$	$(4.8 \pm 0.1) \times 10^3$

[a] At 20 °C in phosphate buffer, pH 7.0. [b] Photoinduced ET from the zinc myoglobin to cytochrome *c* monitored by laser flash photolysis (λ_{ex} = 532 nm).

(Chart 2).[35] Table 2 displays the affinities and ET rate constants upon complexation between the reconstituted zinc myoglobin and ferric cytochrome *c*. From the data in this table, the following can be inferred: (i) Compared to **6·Zn**, the longer side chains in **7·Zn** decrease the affinity between the reconstituted myoglobin and cytochrome *c* due to the increase in flexibility at the binding site. (ii) In

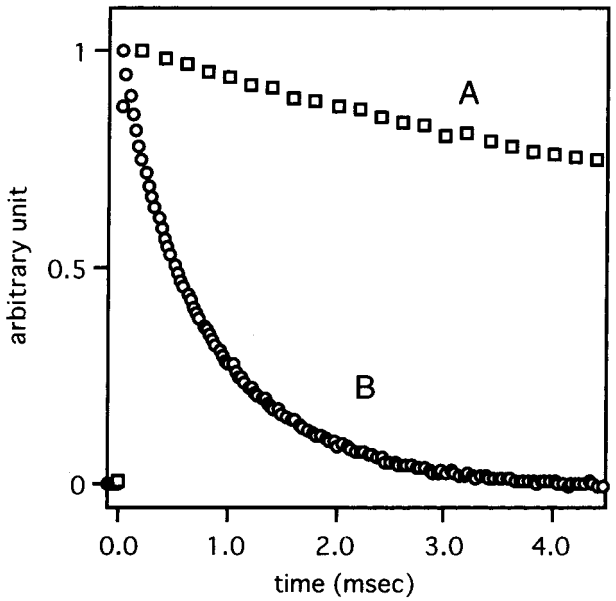


FIGURE 7. Triplet decay profiles of rMb(**7·Zn**) at 460 nm by laser flash photolysis in the absence (trace A) and presence (trace B) of cytochrome *c* in phosphate buffer (μ = 10 mM, pH 7.0) at 20 °C. [rMb(**7·Zn**)] = 3.8×10^{-6} M; [cytochrome *c*] = 1.0×10^{-6} M.

contrast, the forward ET rate constant from the photoexcited rMb(**7·Zn**) to cytochrome *c* increased 2.2-fold more than that of the rMb(**6·Zn**)−cytochrome *c* pairing. (iii) Although the rate constants are not influenced by the ionic strength, the binding affinity depends on the ionic strength, since the complex is formed by an electrostatic interaction. From these data, we can suggest that the ET is regulated by a configurational rearrangement in the complex, since the apparent ET from rMb(**7·Zn**) with a more flexible recognition interface to cytochrome *c* is faster than that of the rMb(**6·Zn**)−cytochrome *c* complex. Therefore, it is likely that the apparatus rate is controlled by a non-ET process such as conformational rearrangement on the protein surface. To our knowledge, the artificially created protein−protein complex presented here is the first synthetic ET model that directly demonstrates the control of an interprotein ET by the flexibility of the recognition interface. Further work will be done to reveal the detailed mechanism of the ET reaction.

5. Chemical Reactivity of Reconstituted Myoglobin

5.1. Comparison between Myoglobin and Peroxidase. Heme peroxidases such as horseradish peroxidase and cytochrome *c* peroxidase have two histidines, the proximal and distal histidines, in the heme pocket. The former residue coordinates to a heme iron, and the latter assists the cleavage of the O−O bond of the hydroperoxoiron species. Myoglobin also has proximal and distal histidines, where the geometry of the proximal histidyl residue is slightly different with the peroxidases. In addition, it is known that myoglobin reacts with hydrogen peroxide to form ferryl species (Fe(IV)═O), which corresponds to compound II in the peroxidases, although the myoglobin compound I is usually undetectable. Thus, myoglobin has a potential for catalytic oxidation of various substrates in the presence of H_2O_2. In fact, the H_2O_2-dependent catalytic oxidation of several substrates has been reported.[36] The peroxidase activity of myoglobin is, however, much lower than that of the native peroxidases. To improve the activity of the myoglobin, one can expect two strategies: (i) rearrangement of several residues at the active site by mutagenetic methods,[7] and (ii) construction of binding sites around the heme pocket via a mutagenetic or synthetic approach.[37−39] In this section, we introduce the improvement in the peroxidase activity of myoglobin by a nongenetic method where the functionalized heme is inserted into the apomyoglobin.

5.2. Reactivity of Ferryl Myoglobins. Figure 8 summarizes the reactivity of ferrylmyoglobins, rMb(**6·Fe^{IV}═O**) and nMb(**5·Fe^{IV}═O**), which are generated by H_2O_2.[38] These results will be separated into three categories. First, the oxidation of positively charged substrates such as cytochrome *c* by rMb(**6·Fe^{IV}═O**) is 2.2-fold faster than that observed by nMb(**5·Fe^{IV}═O**), because of the electrostatic interaction between the substrate and rMb(**6·Fe**) with a negatively charged interface on the protein surface. Second, the oxidation of negatively charged

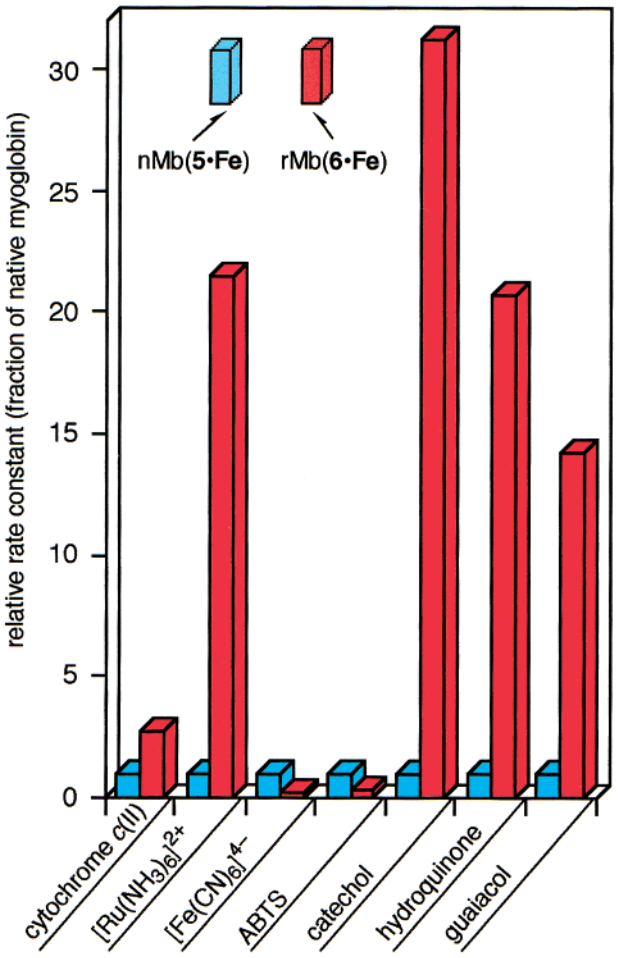


FIGURE 8. Relative reactivities of two ferryl species (Fe(IV)═O) generated with H_2O_2. After the solution of ferryl species was treated with catalase, the substrate (>10 μM) was added to the solution to ensure the pseudo-first-order kinetics at 20 °C. Dark and light bars represent the relative reactivities of rMb(**6·Fe^{IV}═O**) and nMb(**5·Fe^{IV}═O**) toward cytochrome *c* and small-molecule oxidations, respectively.

substrates such as ABTS and ferrocyanate by rMb(**6·Fe^{IV}═O**) is significantly reduced to approximately 20% relative to nMb(**5·Fe^{IV}═O**) due to the electrostatic repulsion between the substrate and rMb(**6·Fe**). Third, surprisingly, the reactivity of the rMb(**6·Fe^{IV}═O**) for neutral phenols such as catechol, hydroquinone, and guaiacol is remarkably higher than that of nMb(**5·Fe^{IV}═O**). Particularly, the rate constant for catechol oxidation by rMb(**6·Fe^{IV}═O**) is more than 30-fold larger than that for nMb(**5·Fe^{IV}═O**). This result comes from the specific binding between the phenol derivatives and hydrophobic pocket formed by the artificial heme side chains involving two benzene moieties as shown in Figure 9. In fact, spectral changes in the absorption of the Soret band of metaquo-rMb(**6·Fe**) were monitored upon the addition of guaiacol, whereas no clear changes were monitored in metaquo-nMb(**5·Fe**), suggesting that guaiacol is bound in the expanded heme pocket near the heme ring. These findings indicate that modification of the heme-propionates will be effective for creating a binding site with a specificity for a substrate.

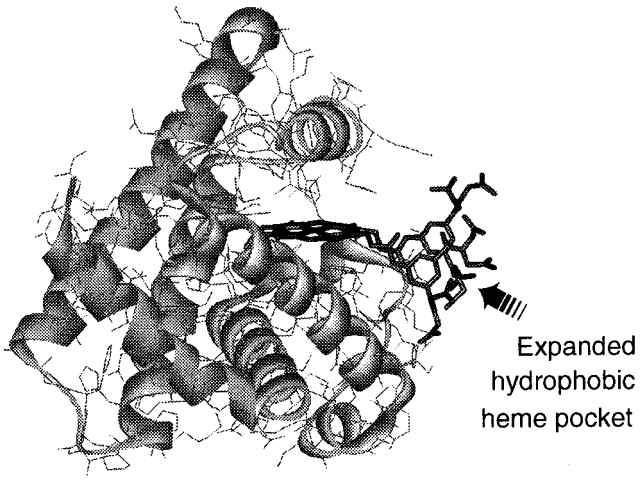


FIGURE 9. Plausible structure of rMb(**6·Fe**) having hydrophobic domain.

Table 3. Steady-State Kinetic Parameters for Guaiacol Oxidation[a]

myoglobin	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
rMb(**6·Fe**)	7.4 ± 0.5	1.1 ± 0.1	1.5×10^{-1}
nMb(**5·Fe**)	32 ± 1	0.36 ± 0.01	0.11×10^{-1}

[a] At 20 °C in 100 mM phosphate buffer, pH 7.0.

5.3. Peroxidase Activity of Reconstituted Myoglobin. Guaiacol oxidation catalyzed by rMb(**6·Fe**) and nMb(**5·Fe**) in the presence of H_2O_2 was monitored by detecting the formation of tetraguaiacol. Steady-state kinetics led to the Michaelis−Menten parameters shown in Table 3.[38] Both K_m and k_{cat} for guaiacol are clearly improved by rMb(**6·Fe**). Thus, the value of k_{cat}/K_m, which represents the specificity of the enzyme, is approximately 14-fold higher than that of nMb(**5·Fe**). This finding indicates that guaiacol is readily accessible into the ferryl species (Fe(IV)=O) through an artificially created binding site. It is noted that the appropriate modification of the heme-propionate enhances the peroxidase activity of myoglobin. Thus, this result demonstrates that myoglobin is converted into a peroxidase.

6. Concluding Remarks and Future Prospects

The reconstitution of myoglobin with an artificially created prosthetic group will give us a wide variety of functions in the protein. In particular, modification of the heme-propionates is a useful method for the design and construction of an efficient binding domain on the protein surface, since this makes it possible to easily introduce a number of functional groups or a hydrophobic cavity near the heme pocket by the synthetic method as compared with the genetic technique. In fact, this Account has described several examples of our reconstituted myoglobins having unique binding domains or sites which lead to the new construction of a protein−protein complex, ET via the binding domain, and enhancement in chemical reactivity. The initial concept of a semisynthetic enzyme by chemical mutation, modification of the cofactor, was reported in the 1980s.[40] However, there have been few examples which demonstrate the systematic construction of a binding domain on the protein surface. Molecular recognition or supramolecular assembly on the protein surface is one of the most fundamental processes in biological systems, so it is likely that the introduction of an artificially created binding domain on the protein surface is a promising way to convert the myoglobin into a new functionalized enzyme.

Finally, the principal advantage of the reconstitutional method is to engineer the tailor-made function on the myoglobin surface, and the methodology can be extended to other proteins having a prosthetic group. The present results will serve as a new way for creating novel functions, such as catalysts or biosensors, as well as improvement in the inherent protein function.

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