Interfacial Recognition between Reconstituted Myoglobin Having Charged Binding Domain and Electron Acceptor *via* Electrostatic Interaction

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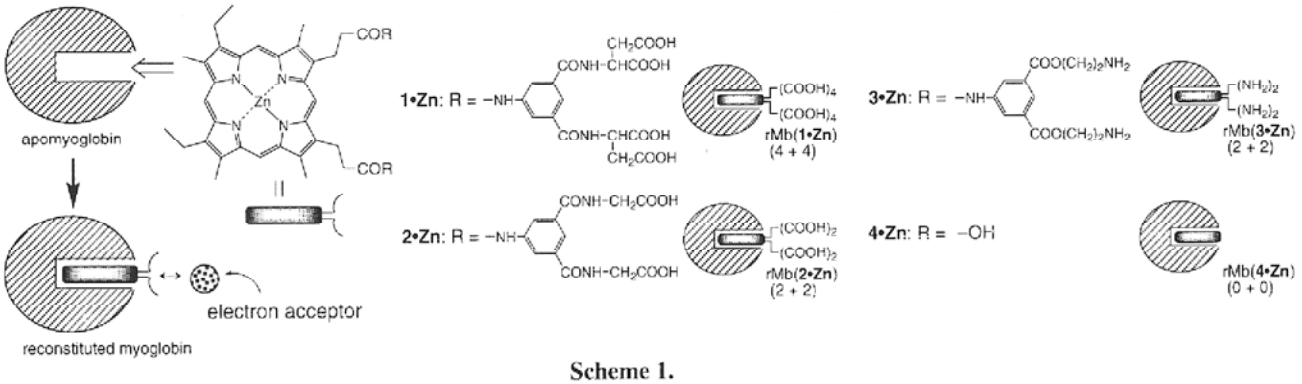
Myoglobins reconstituted with synthetic zinc porphyrins having multiple carboxylic acid or alkylamine groups bound to the terminal of peripheral side chains can bind cationic methyl viologen or anionic hexacyanoferrate, respectively, *via* electrostatic interaction, where the distribution of the charged amino acid residues on the myoglobin surface has an influence on complex formation between the protein and its partner.

Molecular recognition on protein surface is essential to biological oxidoreduction processes, since a variety of specific protein–protein or protein–cofactor pairs are found in photosynthetic and mitochondrial respiratory systems.[1,2] In these electron transfer (ET) systems, each protein has specific binding domain for redox partner to induce effective ET within the complex. This has stimulated a variety of studies, especially on the construction of an artificial binding domain on the protein surface, in an attempt to elucidate the mechanism of the recognition event of proteins and these long-range ET reactions through the highly ordered binding interface. However, the noncovalently linked donor–acceptor model systems focusing on the specific interaction on the protein surface remain limited.[3] We have previously reported a new myoglobin rMb(**1•Zn**) reconstituted with synthetic zinc porphyrin **1•Zn** having a total of eight (4 + 4) carboxylates at the terminal of 6- and 7-propionate side chains.[4] The reconstituted myoglobin is capable of interacting with a cationic compound such as methyl viologen dichloride (MV^{2+}) *via* artificial carboxylate assembly located on the protein surface around the heme pocket. Here, we wish to report the evaluation of the structural basis of interfacial recognition on the protein surface by use of several reconstituted myoglobins to understand the importance of protein–protein electrostatic interactions in biological systems.

Recently, we prepared two zinc porphyrins **2•Zn** and **3•Zn** having four (2 + 2) carboxylic acid or primary alkylamine groups substituted at the terminal of peripheral propionate side chains of mesoporphyrin, respectively, as shown in Scheme 1. These zinc porphyrins were then incorporated into the apoprotein from horse heart myoglobin by usual reconstitution method.[5] The obtained proteins were purified by a CM-Cellulose and Sephadex G-25 column.[6] The characteristic visible absorption spectra and fluorescence spectra of the resultant proteins, rMb(**2•Zn**) and rMb(**3•Zn**), were similar to those of reference protein rMb(**4•Zn**) reconstituted with zinc mesoporphyrin **4•Zn** (0 + 0). Isoelectric focusing was carried out for the reconstituted myoglobins with marker proteins. The isoelectric point, p*I*, of rMb(**1•Zn**) is 5.6 ± 0.2, while those of rMb(**2•Zn**) and rMb(**3•Zn**) are 6.1 ± 0.2 and 7.0 ± 0.2, which are about 0.6 and 0.3 pH units lower and higher than that of rMb(**4•Zn**) having no additional functional groups.[3d] These results indicate that the reconstitution with **2•Zn** or **3•Zn** converts neutral myoglobin to the acidic or basic protein due to the additional four functional groups bound to the terminal of heme propionates, respectively.

The binding fashion of the reconstituted myoglobins with MV^{2+} was investigated by titrimetric measurement of fluorescence spectra as shown in Figure 1. Fluorescence quenching in rMb(**1•Zn**) was clearly observed upon addition of MV^{2+} ($< 10^{-2}$ M) due to the singlet ET from photoexcited zinc myoglobin to MV^{2+} within the ground-state rMb(**1•Zn**)–MV^{2+} complex.[7] The curvature of Stern-Volmer plots depends on the pH and ionic strength.[4] In contrast, Stern Volmer plots in Figure 1 show that the fluorescence **2•Zn** in the protein was not quenched in the presence of MV^{2+} even at low ionic strength and pH 7.0, suggesting that ET reaction dose not occur under these condition. This finding indicates that rMb(**2•Zn**) shows no affinity for cationic MV^{2+} *via* electrostatic contact, although rMb(**2•Zn**) has additional four carboxylates as an anionic binding domain which would interact with MV^{2+} on the surface.[8] According to the structure analysis of horse heart myoglobin, the several basic residues are really located on the heme pocket



Scheme 1.

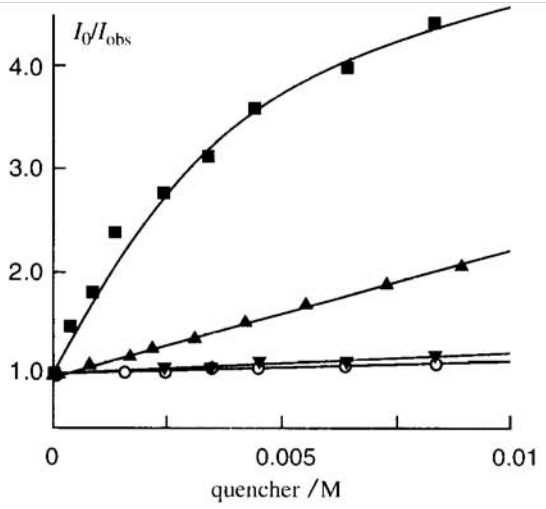


Figure 1. Stern-Volmer plots for the fluorescence quenching of rMb(**1**•**Zn**)–rMb(**4**•**Zn**) at 25 °C in pH 7.0, 10 mM phosphate buffer. The changes of fluorescence emission were monitored at 584 nm (λ_{ex} = 543 nm). The concentration of the protein is ca. 10^{-6} M. Quencher is methyl viologen for rMb(**1**•**Zn**), rMb(**2**•**Zn**) and rMb(**4**•**Zn**) and potassium hexacyanoferrate(III) for rMb(**3**•**Zn**). rMb(**1**•**Zn**): (■), rMb(**2**•**Zn**): (▼), rMb(**3**•**Zn**): (▲), rMb(**4**•**Zn**): (○).

edge;[9] particularly Lys45, Lys63 and Lys96 should be capable of association with some of the carboxylates as the binding interface. Thus, the basic residues which are close to the heme pocket edge can inhibit the interaction between MV^{2+} and the artificial carboxylate interface on the protein surface.

Compared to rMb(**2**•**Zn**), fluorescence of rMb(**3**•**Zn**) was clearly quenched at pH 7.0 upon addition of potassium hexacyanoferrate(III), $K_3[Fe(CN)_6]$, as an anionic electron acceptor.[10] Stern-Volmer plots in Figure 1 suggest that the quenching occurs due to photoinduced singlet ET from positively charged rMb(**3**•**Zn**) to anionic electron acceptor, $[Fe(CN)_6]^{3-}$. In contrast, no fluorescence quenching was observed in $[Fe(CN)_6]^{3-}$ and rMb(**2**•**Zn**) or rMb(**4**•**Zn**) systems in the low concentration range of $[Fe(CN)_6]^{3-}$ (< 0.01 M). These results indicate that the artificial binding domain formed by four alkylammonium groups can bind $[Fe(CN)_6]^{3-}$ on the protein surface *via* electrostatic interaction.

In summary, when we design a negatively or positively charged binding domain on the surface of myoglobin by functionalized metalloporphyrin, the distribution of basic or acidic amino acid residues near the heme edge is quite important; al least eight carboxylate groups as a component of binding domain are required for stable complexation between the myoglobin and MV^{2+}, whereas a total of four alkylammonium groups bound to the terminal of peripheral side chains could be enough for complexation between the myoglobin and $[Fe(CN)_6]^{3-}$ under our experimental condition. To our knowledge, the present work is the first attempt to evaluate the molecular recognition on the protein surface with a special charged domain by use of the intelligible and simplified protein model. Further work on quantitative analysis of binding fashion and ET reaction within the present systems are in progress.

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8 We have also prepared a myoglobin reconstituted with zinc porphyrin **5**•**Zn** having four carboxylates (4 + 0) substituted at one of two propionates. Remarkable fluorescence quenching of **5**•**Zn** in the protein was not observed upon addition of MV^{2+}.

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