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Blue Myoglobin Reconstituted with an Iron Porphycene Shows Extremely High Oxygen Affinity

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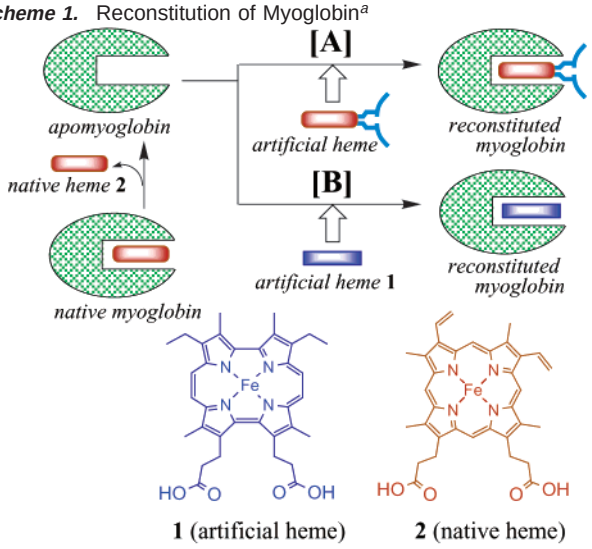
Myoglobin, one of the well-known hemoproteins, will be an attractive biomolecule for engineering a function into the protein, since a prosthetic group, heme, bound in the protein matrix shows a variety of reactivities and unique physicochemical properties. The strategy of the myoglobin modification can be mainly divided into two approaches: (i) amino acid mutation by site-directed mutagenesis and (ii) replacement of the native heme with artificially created metalloporphyrins. The former method enables us to modulate the physiological function of the myoglobin[1,2] or convert the myoglobin into peroxidase or peroxygenase.[3−5] In contrast, the latter method has the advantage of introducing a new function on the myoglobin surface by modification of heme-propionate side chains as shown in Scheme 1[A].[6−12] Furthermore, as can be seen in Scheme 1[B], the modification of the heme framework will be another way to improve the physiological function of myoglobin,[13−16] although there have been few examples that demonstrate drastic changes in the inherent myoglobin function by the chemical modification of the heme. Toward this end, we focused on a study involving the use of an iron porphycene, a structural isomer of iron porphyrin, as an artificial prosthetic group.[17,18] Here, we demonstrate that the reconstituted myoglobin exhibits an extremely high O_2 affinity and low autoxidation rate without any changes in the amino acid sequence.

To establish the affinity of an artificially created prosthetic group into the heme pocket, we designed and prepared an iron porphycene, 2,7-diethyl-3,6,12,17-tetramethyl-13,16-bis-carboxyethylporphycenatoiron (**1**), which has peripheral alkyl groups and two propionate side chains at the porphycene pyrrole rings. The reconstituted metmyoglobin with **1**, met-rMb(**1**), was obtained by a standard reconstitution protocol from horse heart apomyoglobin and purified by gel and ion-exchange chromatographies.[19,20] As can be seen in Figure 1, the solution of met-rMb(**1**) is light blue due to the iron porphycene color, and the electronic absorption spectrum of met-rMb(**1**) shows an axially coordinate iron(III) character with wavelengths of 387, 563, and 624 nm.[21,22] The ESI-TOF mass spectrum of rMb(**1**) gave a mass number of 17 572 which is identical to that expected for the holoprotein. The pH corresponding to 50% unfolding, obtained by monitoring the Soret band at 387 nm of met-rMb(**1**), is 3.1. The value is 1.4 pH units lower than that of the native metmyogobin, met-nMb(**2**), at 25 °C, suggesting that ferric **1** is more stable against dissociation from the protein matrix than is the ferric heme **2**. The UV−vis spectroelectrochemical experiments demonstrate that rMb(**1**) was electrochemically reversible and the redox potential (Fe^{3+}/Fe^{2+}) was found to be −193 mV vs NHE which is significantly lower than the corresponding value of +52 mV determined for nMb(**2**). These results support the fact that the coordination of proximal His93 to **1** is stronger than to **2**.

Scheme 1. Reconstitution of Myoglobin[a]



[a] [A] Modification of heme-propionate and [B] modification of heme framework.

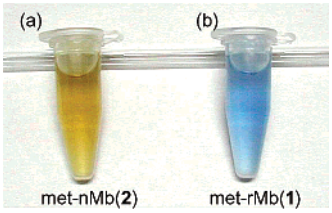


Figure 1. Visual color image of metmyoglobins: (a) nMb(**2**) and (b) rMb(**1**).

The UV−vis spectrum of the reconstituted deoxymyoglobin, deoxy-rMb(**1**), was observed upon the addition of dithionite. After the removal of excess dithionite under aerobic conditions by Sephadex G25, a greenish-blue solution was obtained, indicating the formation of oxymyoglobin, oxy-rMb(**1**). The reduction of met-rMb(**1**) under a CO atmosphere exhibits a spectrum different from oxy-rMb(**1**), showing the formation of CO-myoglobin. The reversibility between the two species was detected by blowing the corresponding gas stream.

The O_2 binding parameters for ferrous rMb(**1**) are compared in Table 1 with those for nMb(**2**). The O_2 association for rMb(**1**) is 5-fold faster than that observed for nMb(**2**). Furthermore, it is of particular interest that oxy-rMb(**1**) shows a 250-fold reduction in the O_2 dissociation rate constant compared with oxy-nMb(**2**). Thus, the binding kinetics demonstrate the extremely high O_2 affinity of

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Table 1. O_2 Binding Parameters and Autoxidation Rate Constants for Native and Reconstituted Myoglobins

myoglobin	k_{on} ($\mu M^{-1} s^{-1}$)[a,b]	k_{off} (s^{-1})[a,c]	K_{O_2} (M^{-1})[d]	k_{auto} (h^{-1})[e]
nMb(**2**)	22± 1	27 ± 2	8.1×10^5	0.18 ± 0.01
rMb(**1**)	120 ± 10	0.11 ± 0.01	1.1×10^9	0.026 ± 0.001

[a] Reaction conditions: 100 mM phosphate buffer (pH 7.0) at 25 °C. [b] Association process of O_2 ligand was measured by a laser flash photolysis system. [c] Dissociation rates were measured by a stopped-flow spectrophotometry upon the addition of excess amount of ferricyanide. [d] O_2 binding constants were calculated from the measured k_{on} and k_{off} values. [e] The rate of autoxidation was determined by the spectral changes to metmyoglobin at 37 °C in 100 mM phosphate buffer (pH 7.0).

rMb(**1**), 1.1×10^9 M^{-1}, indicating a significant 1400-fold increase in O_2 affinity for rMb(**1**) relative to that for the native horse heart myoglobin. The determined binding constant is as high as that of several oxygen-avid hemoglobins such as *Ascaris* hemoglobin,[23,24] which has special distal Tyr and Gln as strong hydrogen-bonding donors to stabilize the bound O_2, whereas rMb(**1**) has no trick in the protein matrix. However, it is noteworthy that the enhancement of the O_2 affinity is mainly derived from a very slow O_2 dissociation both in *Ascaris* hemoglobin and rMb(**1**). The reason for the strong O_2 binding to rMb(**1**) is suggested as follows. According to several model studies of iron porphyrins without a protein matrix, the O_2 off-rate decreases with increasing electron donation of the axial base ligand, because of the increasing favorable character of the Fe(III)$-O_2^-$ charge separation species.[25,26] In our case, the observed lower redox potential of the iron in rMb(**1**) relative to that in nMb(**2**) supports the fact that the strength of the proximal His93-iron heme coordination increases and then the charge separation species is predominant over Fe(II)$-O_2$ complex due to the increasing π-back-donation to the bound O_2.[27] Indeed, it is known that an iron porphycene binds imidazoles more strongly than an iron porphyrin.[22] In addition, it is likely that the remarkable charge separation gives rise to the tight hydrogen-bonding between the negatively charged oxygen and imidazolyl NH of the distal His64 in rMb(**1**). Therefore, the very small rate constant of O_2 dissociation for rMb(**1**) is clearly explained by the stabilization of the bound O_2 via a strong hydrogen-bonding interaction with His64.[28]

Next, we investigated the stability of oxy-rMb(**1**) by monitoring the autoxidation from oxy-rMb(**1**) to met-rMb(**1**) in 100 mM phosphate buffer, pH 7.0, at 37 °C. The spectral changes in the Q-band region exhibited clear isosbestic points, and the decay at 621 nm provides the exact first-order kinetics. The rate constants of two proteins are summarized in Table 1. Interestingly, the autoxidation of oxy-rMb(**1**) is 7-fold slower than that of nMb(**2**). The significantly slow oxidation will come from the slow dissociation of the O_2 ligand, although the autoxidation mechanism is complicated. This explanation is supported by previous reports where the O_2 affinity is inversely related to the autoxidation rate constant.[29]

In conclusion, the porphycene myoglobin, rMb(**1**), demonstrates a very high oxygen affinity, and the oxy-form is extremely stable compared with the native protein. Over the past decade, several groups have prepared a series of myoglobin mutants by a mutagenesis approach to improve the physiological function of the myoglobin. For example, it is known that a L29F mutant of sperm whale myoglobin shows a 15-fold increase in O_2 affinity compared to that of the native protein.[30] In contrast, our results unequivocally propose that the replacement of the native heme **2** with iron porphycene **1** dramatically improves the function as detailed above without any changes in the amino acid sequence of the horse heart myoglobin. Thus, the modification of the heme framework in the present study will serve as an effective method for the creation of a unique functionalized hemoprotein.

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Supporting Information Available: Experimental details, mass spectrum of rMb(**1**), and kinetic data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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