VIII-C Physicochemical Properties of Hemoprotein Reconstituted with Artificially Created Hemins

Hemoprotein is one of the most versatile metalloproteins having a prosthetic group heme, *e.g.* protoheme IX, which shows various physicochemical properties. Most of hemes in hemoproteins are usually bound in the heme pocket via multiple non-covalent interactions with the several amino acid residues. Recently, we have focused on the replacement of the native hemin with an artificially created metal complex. This method is at least two advantages; First, the reconstitution with an artificial hemin gives an insight into the elucidation of hemoprotein function. Second, we have a chance to dramatically modify the function of hemoproteins. In our project, we try to prepare various artificial prosthetic groups and inserted them into apohemoproteins to obtain reconstituted proteins. For example, one of our aims is to elucidate the role of each heme-propionate side chains in a series of hemoproteins by replacing the native hemin with monopropionated hemin. Replacing the native hemin with hemin derivatives such as iron porphycene or iron corrole is also very attractive to modify the function of hemoproteins. From these projects, we wish to understand the physicochemical properties of hemoproteins based on the coordination chemistry in the protein matrix.

VIII-C-1 Preparation and O_2 Binding Study of Myoglobin Having a Cobalt Porphycene

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[*Inorg. Chem.* **44**, 9391–9396 (2005)]

Sperm whale myoglobin, an oxygen-storage hemoprotein, was reconstituted with 2,7-diethyl-3,6,12,17-tetramethyl-13,16-bis(carboxyethyl)porphycenatocobalt (II) in order to investigate the reactivities of a cobalt porphycene in a protein matrix (Figure 1). Similar to the previously reported finding for the myoglobin with the iron porphycene, the reconstituted myoglobin with the cobalt porphycene was also found to have a higher O_2 affinity by two orders of magnitude when compared to the myoglobin possessing cobalt protoporphyrin IX. The EPR spectra of the deoxy and oxy myoglobins having the cobalt porphycene at 77 K also have similar features to the myoglobin with cobalt protoporphyrin IX. These spectra suggest that the porphycene cobalt in the deoxy form is coordinated by one nitrogenous ligand postulated to be the imidazole ring of His93, and that the bond configuration of Co^{II}–O_2 is regarded as the Co^{III}–$O_2^{\bullet-}$ species.

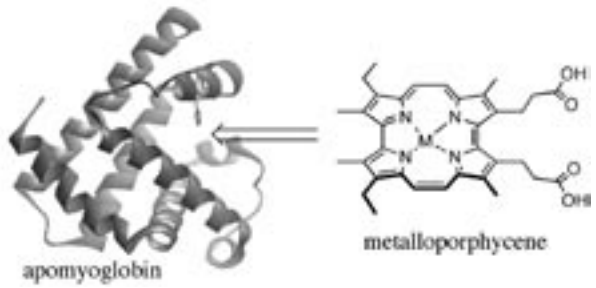

Figure 1. Incorporation of metalloporphycene into apomyoglobin.

VIII-C-2 Iron Porphyrin–Cyclodextrin Supramolecular Complex as a Functional Model of Myoglobin in Aqueous Solution

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[*Inorg. Chem.* **45**, 4448–4460 (2006)]

The 1:1 inclusion complex of 5,10,15,20-tetrakis(4-sulfonatophenyl)porphinatoiron(II) (Fe^{II}TPPS) and an *O*-methylated β-cyclodextrin dimer having a pyridine linker (**1**) binds dioxygen reversibly in aqueous solution. The O_2 adduct was very stable ($t_{1/2}$ = 30.1 h) at pH 7.0 and 25 °C. ESI-MS and NMR spectroscopic measurements and molecular mechanics calculations indicated the inclusion of the sulfonatophenyl groups at the 5- and 15-positions of Fe^{III}TPPS or Fe^{II}TPPS into two cyclodextrin moieties of **1** to form a supramolecular 1:1 complex (hemoCD1 for the Fe^{II}TPPS complex), whose iron center is completely covered by two cyclodextrin moieties. Equilibrium measurements and laser flash photolysis provided the affinities ($P^{O2}{}_{1/2}$ and $P^{CO}{}_{1/2}$) and rate constants for O_2 and CO binding of hemoCD1 ($k^{O2}{}_{on}$, $k^{O2}{}_{off}$, $k^{CO}{}_{on}$, and $k^{CO}{}_{off}$). The CO affinity relative to the O_2 affinity of hemoCD1 was abnormally high. Although resonance Raman spectra suggested weak back-bonding of $d\pi(Fe) \rightarrow \pi^*(CO)$ and hence a weak CO–Fe bond, the CO adduct of hemoCD1 was very stable. The hydrophobic CO molecule dissociated from CO-hemoCD1 hardly breaks free from a shallow cleft in hemoCD1 surreounded by an aqueous bulk phase leading to fast rebinding of CO to hemoCD1. Isothermal titration calorimetry furnished the association constant (K^{O2}), $\Delta H°$, and $\Delta S°$ for O_2 association to be (2.71±0.51) × 10^4 M^{-1}, –65.2±4.4 kJ mol^{-1}, and –133.9 ±16.1 J mol^{-1} K^{-1}, respectively. The autoxidation of oxy-hemo CD1 was accelerated by H^+ and OH^-. The inorganic anions also accelerated the autoxidation of oxy-hemo CD1. The O_2–Fe^{II} bond is equivalent to the $O_2^{\bullet-}$–Fe^{III} bond, which is attacked by the inorganic anions or the

water molecule to produce met–hemoCD1 and a superoxide anion.

VIII-C-3 Crystal Structure and Peroxidase Activity of Myoglobin Reconstituted with Iron Porphycene

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[*Inorg. Chem.* to be submitted]

The incorporation of an artificially created metal complex into an apomyoglobin is one of the attractive studies in a series of hemoprotein modifications. Single crystals of 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 artificially created prosthetic group as a heme structural isomer is located in the normal position of the heme pocket (Figure 1). Furthermore, 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 studies of the reconstituted protein with H_2O_2 demonstrated that two reaction intermediates, compounds II and III, were detected 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 could be due to the strong coordination of the Fe–His93 bond.

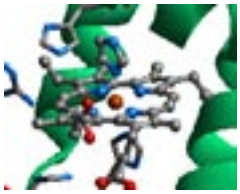

Figure 1. 3D crystal structure of iron(III)-porphycene in myoglobin matrix.

VIII-C-4 Construction of Glycosylated Myoglobin by Reconstitutional Method

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[*Chem. Commun.* **29**, 3131–3133 (2006)]

An artificially created prosthetic group **1** having β-galactosyl moieties (Figure 1) was prepared and inserted into sperm whale apomyoglobin to successfully afford a glycosylated myoglobin. The glycomyoglobin was characterized by ESI-MS and UV-vis spectroscopy and stable in a buffer solution at 4 °C over one week. The immunoprecipitaton experiment was carried out to evaluate the function of the galactose units on the protein surface. The mixture of the glycomyoglobin and commercially available biotin-labelled peanut lectin was treated with streptavidin-modified sepharose to determine the affinity. From this study, the galactose units on the myoglobin surface well work as the interface for forming the myoglobin–lectin complex without any non-specific interaction.

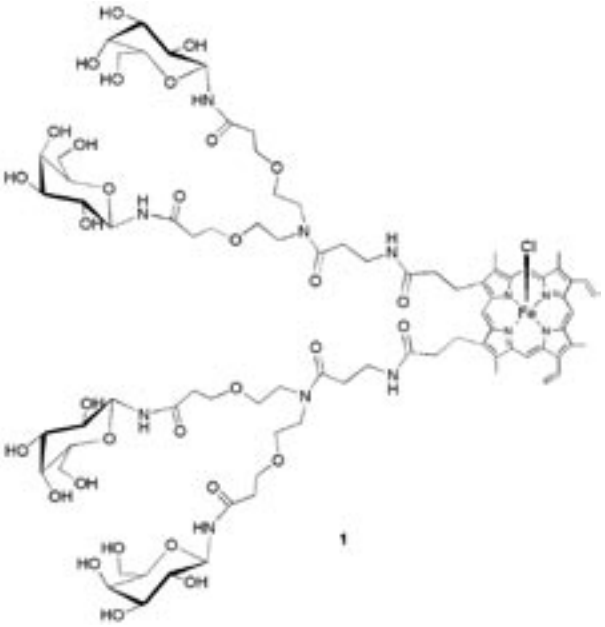

Figure 1. Galactohemin **1** as an artificial prosthetic group for myoglobin.

VIII-C-5 Structure and Ligand Binding Properties of Sperm Whale Myoglobins Reconstituted with Two Monopropionated Hemin: Role of Each Heme-Propionate Side Chain in Myoglobin

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[*J. Am. Chem. Soc.* to be submitted]

Many hemoproteins have protoheme IX as a prosthetic group. The heme bears two unique propionate side chains at 6- and 7-positions of β-pyrrolic carbons. According to a series of 3D structures of hemoproteins, the propionate side chains interact with polar amino acid residues in the protein matrix, so it has been known that the side chains play a role on the fixation of the prosthetic group in the heme pocket. However, we have recently thought that the role of the two propionate side chains are not only the fixation of heme but also the direct contribution to the regulation of the hemoprotein

function. Based on this viewpoint, we have prepared two monopropionated hemes; 6-methyl-7-carboxyethyl-heme (6M7PHE) and 6-carboxyethyl-7-methyl-heme (6P7MHE) (Figure 1). Furthermore, the hemins were incorporated into apohemoprotein by conventional method to understand each role of the defective propionate side chain.

In the case of sperm whale myoglobin, 6- and 7-propionate side chains interact with Arg45 and Ser92 via hydrogen bonding, respectively. The reconstituted myoglobins with the two monopropionated hemins, rMb (6M7PHE) and rMb(6P7MHE), were characterized by UV-vis, ESI-MS, 1H NMR spectroscopic methods. The dissociation of O_2 from oxymyoglobin with 6M7PHE was accelerated about three times as that of the oxymyoglobin with the native heme. Furthermore, the autoxidation rate of oxymyoglobin with 6M7PHE was approximately six times faster than that of oxymyoglobin with native heme. These results indicate that the 6-propionate side chain plays an important role on the stabilization of oxymyoglobin. In contrast, the acceleration of the CO binding rate was observed for myoglobin with 6P7MHE, suggesting that the 7-propionate side chain regulates the His93–heme iron coordination in the proximal site.

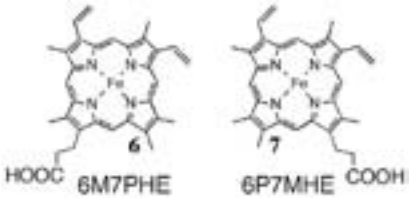

Figure 1. Structures of Two Monopropionated Heme.

VIII-C-6 Ligand Binding Properties of Two Kinds of Reconstituted Myoglobins with Iron Porphycene Having Propionates: Effect of β-Pyrrolic Position of Two Propionate Side Chains in Porphycene Framework

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[*J. Inorg. Biochem.* **100**, 1265–1271 (2006)]

An iron porphycene containing two propionate side chains at the 12th and 17th β-pyrrolic positions of the porphycene ring was synthesized and incorporated into sperm whale apomyoglobin in order to investigate the O_2 and CO binding properties of the reconstituted ferrous myoglobin. The protein showed a slower O_2 dissociation rate by 1/20, compared to the native myoglobin, whereas the CO dissociation rates were found to be almost the same. This tendency is similar to the result of a previous study on the reconstituted myoglobin with a porphycene having the propionates at the 13th and 16th b-pyrrolic positions. However, the present myoglobin showed a faster O_2 dissociation than the previously studied myoglobin. This finding suggests that the position of the two propionates as well as the symmetry of the porphycene framework is an important factor for obtaining a stable oxygenated iron porphycene myoglobin.