Semisynthetic Metalloenzymes

Preparation of Artificial Metalloenzymes by Insertion of Chromium(III) Schiff Base Complexes into Apomyoglobin Mutants**

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Construction of artificial metalloenzymes is one of the most important subjects in bioinorganic chemistry, because metalloenzymes catalyze chemical transformations with high selectivity and reactivity under mild conditions.[1–4] There are several reports on protein design: introduction of metal binding sites,[1,3,5–8] design of substrate binding cavities,[9–12] chemical modification of prosthetic groups,[13–16] and covalent attachment of metal cofactors.[2,17–20] In particular, the covalent modification of proteins is a powerful tool for the generation of new metalloenzymes, while the efficiency of the modification is very much dependent on the position and reactivity of the cysteinyl thiol functional group.[2] Herein, we describe a novel strategy for the preparation of artificial metalloenzymes by noncovalent insertion of metal-complex catalysts into protein cavities. The resulting semisynthetic metalloenzymes, apo-myoglobin (apo-Mb) reconstituted with Cr^{III} Schiff base complexes, are able to catalyze enantioselective sulfoxidation.

Manganese(III) and chromium(III) Schiff base complexes are known to be catalysts for various oxidations in organic solvents.[21,22] Jacobsen,[23] and Katsuki[24] have already reported many examples of asymmetric oxidations catalyzed by chiral Mn Schiff base complexes. In our case, symmetric complexes, $[M^{III}(salophen)]^+$ (M·**1**; M = Mn, Cr; H_2 salophen

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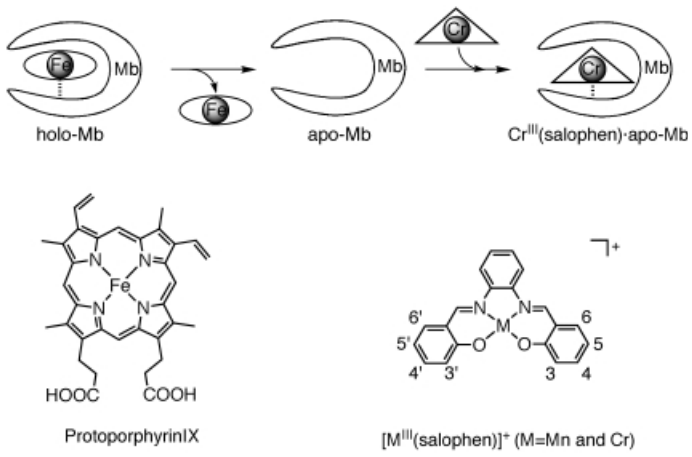

Figure 1. Insertion of a salophen complex into apo-Mb.

(**1**) = *N*,*N*′-bis(salicylidene)-1,2-phenylenediamine) are inserted into a chiral cavity of apo-Mb (Figure 1). In addition, the 5- and 5′- positions of salophen are substituted by a *tert*-butyl group, 5,5′-*t*Bu$_2$-salophen (**2**) to improve the binding affinity to apo-Mb, that is, the binding affinity of heme in apo-Mb is provided by the hydrophobic interaction, and the ligand size.[25] We found that similar factors are important for metal complexes to be inserted into the Mb cavity.

For the creation of artificial metalloenzymes, apo-Mb is an excellent candidate, since reconstitution of apo-Mb with heme has been well studied.[13,26–28] We demonstrated that the catalytic selectivity and reactivity of Mb as a peroxygenase are controlled by appropriate site-directed mutagenesis in the active site.[10,11,29] On the basis of these results, we have designed a suitable apo-Mb mutant for the reconstitution with Schiff base complexes by using the Insight II/Discover 3 program. A designed protein structure, Cr·**2**·apo-H64D/A71GMb, is shown in Figure 2b. If we assume that Cr·**2** binds to His 93 as observed for heme, the replacement of His 64 with Asp (H64D) will provide increased access of substrates and oxidants to a vacant distal site above the Cr^{III} center.[11] At the same time, the alanyl side chain at the position 71 in Mb is expected to locate very close to the 4- and 5- positions of Cr·**2** (Figure 2). Thus, Ala 71 was replaced with Gly (A71G) to improve the binding affinity of Cr·**2** in the active site.

The reconstitution of apo-Mb with Cr·**2** was carried out by applying a method used for modified hemes;[28] [Cr·**2**·2H$_2$O]BF$_4$ dissolved in MeOH (0.9 equiv, 0.7 mM) was added dropwise to an apo-Mb solution in Tris/HCl (10 mM, pH 7.0). The mixture was dialyzed against Bis-Tris/HCl (10 mM pH 6.0) and then loaded on Sephadex G-25. The final purification was performed on a CM52 column with linear gradient Tris/HCl buffer (10–100 mM pH 7.0), which gave a 15% yield of isolated Cr·**2**·apo-Mb. The yield of isolated Cr·**2**·apo-H64D/A71GMb (4.3%) was about 15-fold larger than that of Cr·**1**·apo-H64D/A71GMb (0.3%) and almost twice as large as that of Cr·**2**·apo-H64DMb (2.4%). In contrast, all efforts made to prepare Mn·**1**·apo-H64D/A71GMb failed. These results imply that the type of metal ion and the ligand structure are important factors that influence the binding affinity of the metal complexes with the Mb mutants.

The deconvoluted ESI-TOF mass spectrum of purified Cr·**2**·apo-Mb in ammonium acetate (5 mM; Figure 3a) shows two peaks, which are readily assigned to Cr·**2**·apo-Mb (measured: 17808.3 ± 1.1 Da, calcd: 17808.6 Da) and a small portion of apo-Mb (measured: 17330.0 ± 0.1 Da, calcd: 17330.1 Da), respectively. The appearance of a small peak corresponding to apo-Mb is also observed for purified heme-bound Mb (holo-Mb) under the same conditions, which indicates that the binding affinity of Cr·**2** is comparable to that of heme. To confirm that Cr·**2** is incorporated into the heme position, the following experiments were performed. In the first experiment, a mixture of apo-Mb and two equivalents of hemin was analyzed by ESI-TOF mass spectrometry (MS) and holo-Mb was observed as the main peak of the resulting spectrum (measured: 17945.6 ± 0.1 Da, calcd: 17946.6 Da; Figure 3b). In the second experiment, a solution of hemin (two equiv) in DMF was added to purified Cr·**2**·apo-Mb in ammonium acetate (5 mM). The ESI-TOF mass spectrum of the resulting solution (Figure 3c) shows peaks assigned to holo-Mb and Cr·**2**·apo-Mb + heme (measured: 18421.6 ± 0.3 Da, calcd: 18424.7 Da), which are smaller than the peaks of Cr·**2**·apo-Mb. If the Cr^{III} complex binds to the surface of apo-Mb, we expect to observe Cr·**2**·apo-Mb + heme as the major peak, since the incorporation of heme into apo-Mb is very rapid and the mutation of His 64 and Ala 71 should not be effective for the reconstitution. In addition, the preliminary results of an X-ray crystallographic analysis of

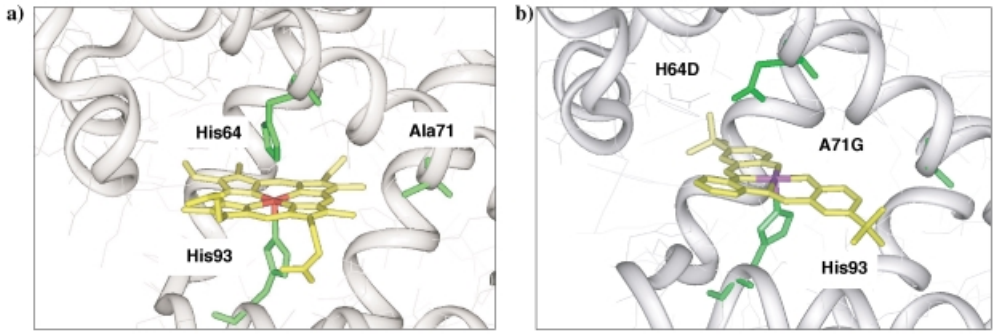

Figure 2. Comparison of a) the crystal structure of wild-type Mb (PDB code 2MBW) with b) a proposed structure of Cr·**2**·apo-H64D/A71GMb calculated by the Insight II/Discover 3 program (ESFF force field). The parts of apo-Mb that interact with Cr·**2** are shown in green, **2** is yellow, Cr is a) red or b) purple.

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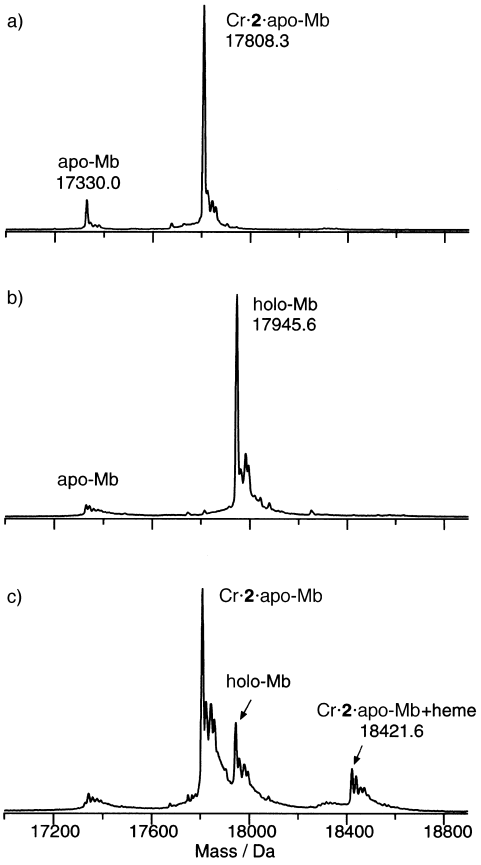

Figure 3. Deconvoluted ESI-TOF mass spectra of a) Cr·**2**·apo-Mb, b) apo-Mb with two equivalents of hemin, and c) Cr·**2**·apo-Mb with two equivalents of hemin in ammonium acetate (5 mM). Sample preparation for b) and c): two equivalents of hemin (1 mM, DMF solution) was added to purified Cr·**2**·apo-Mb (10 μM) and apo-Mb (10 μM), respectively. The mixtures were stirred for 10 min and then dialyzed against 5 mM ammonium acetate for 8 h at 4 °C.

Cr(3,3′-Me_2-salophen)·apo-A71GMb show that the Cr^{III} ion binds to the Nε atom of imidazole in His 93.[30] Thus, we concluded that the His 93 coordination is crucial for Cr·**2** to locate in the active site of Mb.

The H_2O_2-dependent sulfoxidation of thioanisole was examined at 35 °C and pH 5.0 (Table 1). The rate and enantiomeric excess of the sulfoxidation were determined by HPLC analysis (Daicel OD chiral-sensitive column).[9] Catalysts Cr·**1**·apo-H64D/A71GMb, and Cr·**2**·apo-H64D/A71GMb exhibit the highest sulfoxidation activities (entries 4 and 5), whereas Cr·**2**·apo-H64DMb has the lowest activity (entry 3). Catalysts Cr·**1**·apo-H64D/A71GMb and Cr·**2**·apo-H64D/A71GMb showed an approximately sixfold rate increase over Cr·**2** (entries 4,5 versus entry 7). Notably, Cr·**2**·apo-H64D/A71GMb gave (*S*)-methylphenyl sulfoxide with a 13 % *ee*, while the sulfoxidation catalyzed by Cr·**2** proceeded with no enantiomeric discrimination. The other mutants show lower enantioselectivity than that of Cr·**2**·apo-H64D/A71GMb. In a control experiment, the sulfoxidation by Cr·**2**·bovin serum albumin (BSA) was performed (Table 1, entry 6).[31] The enantioselectivity and rate were very low compared with those achieved with Cr·**2**·apo-H64D/A71GMb because of the nonspecific binding ability of Cr·**2** to BSA and the absence of histidine coordination to the Cr^{III} complex. This result indicates that the cavity of an apo-Mb mutant, and His 93 coordination to the Cr^{III} atom are important to improve the enantioselectivity and rate of the sulfoxidation. Furthermore, the EPR spectrum of Cr·**2**·apo-H64D/A71GMb oxidized by *m*-chloroperbenzoic acid at 5 K (pH 7.0) shows a signal at $g = 1.97$ assigned to an oxochromium(v) species.[32] Thus, the intermediate responsible for the catalytic sulfoxidation is expected to be the oxochromium(v) species formed in the Mb cavity. While the enantioselectivity is still low, the results clearly demonstrate that asymmetric reactions can be performed if we use protein cavities, even though symmetric metal complexes are employed.

In summary, we have demonstrated the insertion of symmetric metal complexes into the active site of apomyoglobin by binding to His 93, and that semisynthetic metalloenzymes catalyze enantioselective sulfoxidation by using the chiral protein cavity. Although the semisynthetic metalloenzymes still exhibit low reactivity and enantioselectivity, our experiments suggest the possible use of this method for the design of artificial metalloenzymes. Refinement of the crystal structure and further design to improve the reactivity and selectivity of the metalloenzymes are currently in progress.

Table 1: The different rates and enantiomeric excesses obtained in the sulfoxidation of thioanisole by using a range of catalysts based on chromium Schiff base complexes in a range of proteins.[a]

Entry	Catalyst	Rate[b]	*ee* [%]
1	Cr·**2**·apo-Mb	46	4.3 (*R*)
2	Cr·**2**·apo-A71GMb	54	6 (*S*)
3	Cr·**2**·apo-H64DMb	27	0.3 (*S*)
4	Cr·**1**·apo-H64D/A71GMb	83	8.3 (*S*)
5	Cr·**2**·apo-H64D/A71GMb	78	13 (*S*)
6	Cr·**2**·BSA	12	0.5 (S)
7	Cr·**2** in buffer	13	0

[a] Sulfoxidations were carried out in sodium acetate buffer (50 mM, pH 5.0) at 35 °C in the presence of a Mb complex (10 μM), thioanisole (1 mM), and H_2O_2 (1 mM). [b] The unit of the rate is 10^{-3} turnover min^{-1}.

Experimental Section

The mutants were constructed by cassette mutagenesis. Expression and purification of the mutants were performed as reported previously.[33] Ligands **1** and **2**, and their Cr^{III} complexes were synthesized by methods described in the references [34, 35].

[Cr·**2**·2H_2O]BF_4: ESI-TOF MS: Cr·**2**·2CH_3CN 560 (calcd 560); UV/Vis: λmax (ε $M^{-1}cm^{-1}$) 480 (0.93×10^4), 337 nm (2.35×10^4).

[Cr·**1**·2H_2O]BF_4: ESI-TOF MS: Cr·**1**·$(CH_3CN)_2$ 448 (calcd 449); UV/Vis: λmax (ε $M^{-1}cm^{-1}$) 469 (1.04×10^4), 334 nm (2.54×10^4).

Cr·**2**·apo-Mb: ESI-TOF MS: 17808.3 ± 1.1 (calcd 17808.3);UV/Vis: λmax (ε $M^{-1}cm^{-1}$) 485 (1.02×10^4), 341 (2.45×10^4), 270 nm (3.75×10^4).

Cr·**2**·apo-A71GMb: ESI-TOF MS:17793.9 ± 0.9 (calcd 17794.3); UV/Vis: λmax (ε $M^{-1}cm^{-1}$) 483 (1.02×10^4), 341 (2.88×10^4), 281 nm (4.38×10^4).

Cr·**2**·apo-H64DMb: ESI-TOF MS: 17786.0 ± 0.6 (calcd 17786.2); UV/Vis: λmax (ε $M^{-1}cm^{-1}$) 474 (0.95×10^4), 338 (2.39×10^4), 321 (2.41×10^4), 282 nm (3.52×10^4).

Cr·**2**·apo-H64D/A71GMb: ESI-TOF MS: 17772.1 ± 0.3 (calcd 17772.2); UV-Vis: λmax (ε $M^{-1}cm^{-1}$) 471 (1.02×10^4), 339 (2.61×10^4), 280 nm (4.18×10^4).

Cr·**1**·apo-H64D/A71GMb: ESI-TOF MS: 17658.2 ± 0.7 (calcd 17660.1); UV/Vis: λmax (ε $M^{-1}cm^{-1}$) 460 (1.18×10^4), 320 (1.2×10^3), 282 nm (2.3×10^3).

The concentration of Cr ion in the purified enzymes was determined by inductively coupled plasma atom emission spectrometry (ICPAES). The spectra were recorded on a SEIKO SPS 7000 that was calibrated with $K_2Cr_2O_7$ in 0.1 mol L^{-1} HNO_3 (100.3 mg L^{-1}, Wako).

ESI-TOF MS were measured on a LCT (Micromass, UK). Typical parameters: capillary, 3 kV; cone, 60 V; source temperature, 60 °C; flow-rate, 5 μL min^{-1}; mass scale calibration, CsI (1 mg mL^{-1}) in 50 % H_2O/CH_3CN. All samples were dialyzed against 5 mM ammonium acetate for 8–10 h at 4 °C.

EPR spectra were recorded at 5 K on a Bruker E500 (X band) with an Oxford helium cryostat. Samples were prepared by addition of 100 equivalents of *m*-chloroperbenzoic acid (1M in MeOH) to purified Cr·**2**·apo-H64D/A71GMb (1 mM in 10 mM Tris/HCl at pH 7.0). The reaction mixtures were then frozen in liquid nitrogen.

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