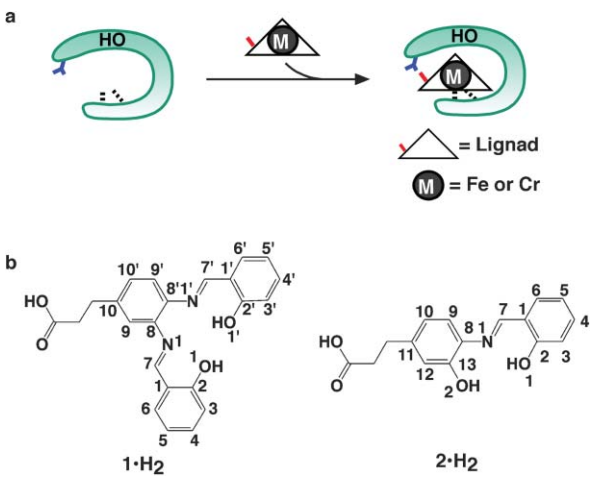
Ligand design for the improvement of stability of metal complex·protein hybrids†

Norihiko Yokoi,[a] Takafumi Ueno,[ab] Masaki Unno,[c] Toshitaka Matsui,[c] Masao Ikeda-Saito[c] and Yoshihito Watanabe*[d]

Received (in Cambridge, UK) 7th September 2007, Accepted 16th October 2007
First published as an Advance Article on the web 24th October 2007
DOI: 10.1039/b713468a

We have succeeded in improving the stability of Fe(Schiff-base)·heme oxygenase (HO) hybrids by ligand design based on the crystal structure of Fe(*N*,*N*′-bis(salicylidene)-3.4-diamino-benzene propionic acid)·HO.

Hybridization of synthetic metal complexes and proteins is an attractive target for the preparation of catalysts,[1–4] sensors,[5] and metal drugs.[6] Non-covalent interactions between metal cofactors and surrounding amino acid residues are very important to accommodate cofactors in the protein cavity as evidenced by the crystal structures of composites.[7–17] However, factors affecting the stability of these composites are still obscure. Recently, we have constructed artificial metalloproteins by insertion of synthetic Schiff-base complexes into the active sites of apo-myoglobin (apo-Mb)[18–22] and heme oxygenase (HO) (Scheme 1).[23] The melting point (T_m) of Fe(Schiff-base)complex·apo-Mb composites was increased by the deletion of steric hindrance between the Schiff-base ligand and amino acid side chains of the active site.[19] We have also reported that the hydrogen bonds between the metal complex (**Fe·1**) and HO induced an increase of the T_m of the Fe(Schiff-base)complex·HO composite (**Fe·1·HO**) (Fig. 1(a)) relative to that of a composite having no hydrogen bonds.[23] In this communication, we report the re-design of Schiff-base metal complex·HO composites to improve the stability on the basis of the crystal structure of **Fe·1·HO**.[23] The result provides fundamental strategy for the construction of stable artificial metalloproteins.



Scheme 1 (a) Reconstitution of HO with a metal complex. (b) Two ligand structures, **1·H$_2$** and **2·H$_2$**, employed in this study.

As reported before, the crystal structure of **Fe·1·HO** indicates that **Fe·1** is fixed in the active site of HO by coordination of the iron atom both to the nitrogen atom (Nε) of His20 and to the carboxylate atom (Oε) of Glu24 as well as the hydrogen bonds of propionic acid at the C-10 position of **Fe·1** with Tyr130 and Arg177 (Fig. 1(a)). The FeN1N1′O1′O1 plane of **Fe·1** is distorted because the O1 atom of **Fe·1** locates at the *trans* position to the Nε(His20) of HO (Fig. 1(a)). On the other hand, upon the reconstitution of apo-Mb with a series of metal Schiff-base complexes, the complexes maintain their planar structures by utilizing His93 of apo-Mb and a water molecule as the axial ligands.[19,20] Apparently, the distortion of **Fe·1** in the active site of HO is caused by the coordination of the Oε(Glu24) to the iron atom to afford the FeN1N1′O1′Oε plane (Fig. 1(a)). The distortion induced destabilization of **Fe·1·HO**. In order to improve the stability of **Fe·1·HO**, we have designed a tridentate ligand **2** that does not have a phenol moiety (O1 and C1–6 of ligand **1**) as shown in Scheme 1.

Fe·2·HO was reconstituted by a method reported before with some modifications.[23] When a DMF solution of **Fe·2** (4 μmol) was added to a buffer solution of HO (2 μmol), some of **Fe·2** were expected to be randomly bound to the protein surface. Thus, **Fe·2·HO** was treated with anion exchange column chromatography to remove the **Fe·2** molecules bound to the surface of HO.

The T_m values of **Fe·2·HO**, apo-HO and **Fe·1·HO** were determined by temperature dependent CD measurements to estimate the stability of composites of metal complexes and HO because T_m values of the composites of heme and several myoglobin mutants were related to the rate constants for heme dissociation from myoglobins.[24] Each composite shows two T_ms during the unfolding process (Fig. 2 and Table 1). **Fe·2·HO** gives a low T_m(T_{mL}) and a high T_m(T_{mH}) at 46.1 and 49.6 °C, respectively. Similar CD spectral changes have also been observed for apo-Mb mutants, which exhibit two unfolding processes against pH, and initial spectral change was attributed to the unfolding of some flexible α helixes.[25] The crystal structures of **heme·HO** and apo-HO indicate that the α helix A located at the proximal site of heme is more flexible than the other α helixes, and the α helix A structure

[a]*Department of Chemistry, Graduate School of Science, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, 464-8602, Japan*
[b]*PRESTO, Japan Science and Technology Agency (JST), Japan*
[c]*Institute of Multidisciplinary Research for Advanced Materials, Tohoku University, Katahira, Aoba, Sendai, 980-8577, Japan*
[d]*Research Center for Materials Science, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, 464-8602, Japan. E-mail: yoshi@nucc.cc.nagoya-u.ac.jp; Fax: +81-52-789-2953; Tel: +81-52-789-3049*
† Electronic supplementary information (ESI) available: Experimental details. See DOI: 10.1039/b713468a

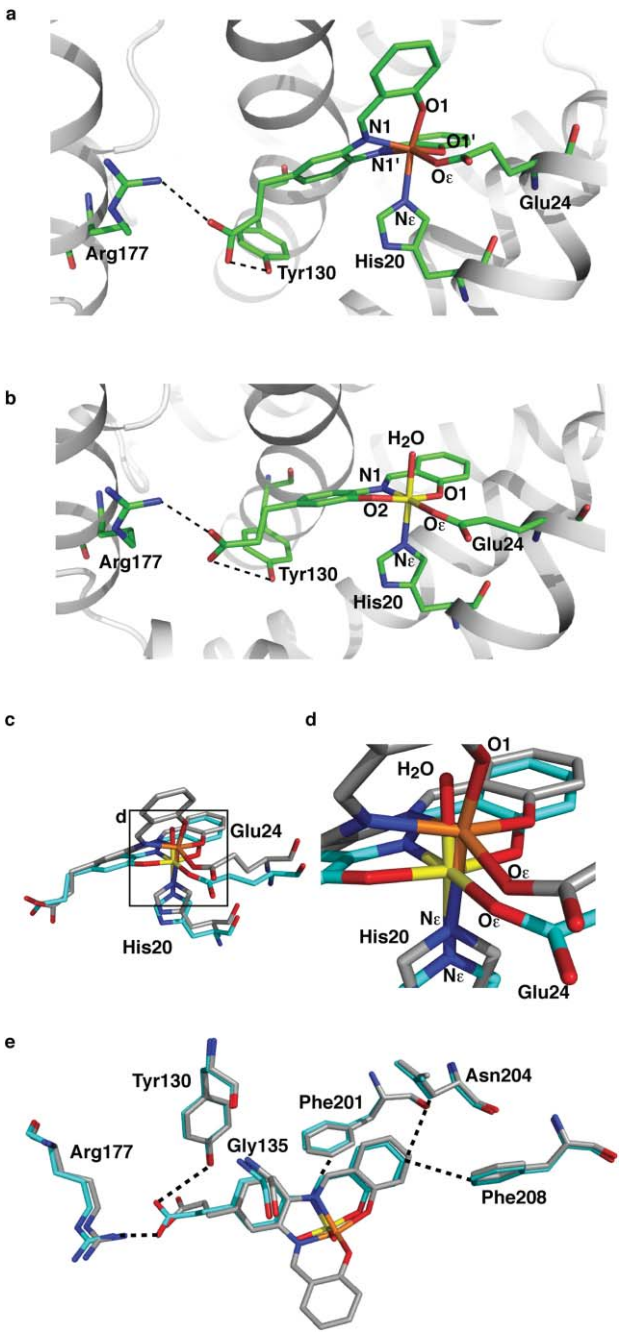


Fig. 1 Crystal structures of metal Schiff-base complex·HO composites; (a) the active site structure of **Fe·1·HO** (ref. 23). (b) The active site crystal structure of **Cr·2·HO**. Green is carbon. Purple, red, orange and yellow are nitrogen, oxygen, iron and chromium, respectively. Protein structures are written in white ribbon model. Superimposed structures of **Cr·2·HO** (carbon color is cyan) with **Fe·1·HO** (carbon color is gray); (c) the coordination structure of each composite. (d) A close-up view of (c). (e) Hydrogen bonds and hydrophobic interactions of each composite with surrounding amino acid residues.

is stabilized by the hybridization of HO with heme.[26] In fact, the T_{mL} values of **Fe·2·HO**, **Fe·1·HO** (T_{mL} = 39.2 °C) and **heme·HO** (T_{mL} = 54.4 °C) are much higher than that of apo-HO (T_{mL} = 32.1 °C). The difference of T_{mL} values of **Fe·2·HO** and **Fe·1·HO** suggests that the ligand **2** contributes to the increase in its stability

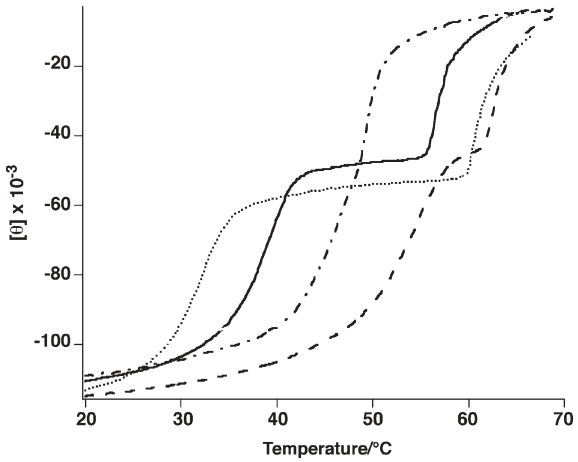


Fig. 2 CD spectral changes at 222 nm against temperature of apo-HO (···) (2.5 μM), **Fe·1·HO** (—) (2.5 μM), **Fe·2·HO** (-·-) (2.0 μM), and **heme·HO** (---) (2.5 μM) in 10 mM Tris/HCl buffer (pH 7.3).

Table 1 T_m Values of metal complex·HO composites

	T_m/°C	
	T_{mL}	T_{mH}
apo-HO	32.1	60.7
Fe·1·HO	39.2	56.5
Fe·2·HO	46.1	49.6
Cr·2·HO	60.2	—[a]
heme·HO	54.4	62.7

[a] T_{mH} of **Cr·2·HO** was not observed.

compared with the ligand **1**, while the stabilization by **Fe·2** does not come close to that of heme.

Although we attempted the crystallization of **Fe·2·HO** to elucidate the reason why **Fe·2·HO** became stable, we failed to obtain crystals. On the other hand, we have succeeded in the crystallization of **Cr·2·HO**, which is assumed to have almost the same coordination geometry and protein folding to **Fe·2·HO** as reported on Cr(Schiff-base)·apo-Mb and Fe(Schiff-base)·apo-Mb.[19,20]

The crystal structure of **Cr·2·HO** was refined with the diffraction data of 1.58 Å resolution. The X-ray data and refinement statistics of **Cr·2·HO** are listed in Table 2. The root-mean-square (rms) deviation of the Cα chain atom of **Cr·2·HO** from **Fe·1·HO** is 0.37 Å. The value indicates that the protein folding of **Cr·2·HO** is very similar to that of **Fe·1·HO**.[23] As shown in Fig. 1(b), **Cr·2** is located at the HO active site with a typical octahedral coordination geometry around the Cr atom. The oxygen atom of a water molecule coordinates to the Cr atom as the sixth ligand while the same position in **Fe·1** was occupied by the O1 atom of the Schiff-base ligand **1** as shown in the superimposed structure of **Fe·1·HO** and **Cr·2·HO** (Fig. 1(c), (d)). The orientation of **2** in the HO cavity is conserved as **1** by several specific interactions as shown in Fig. 1(e). The propionic acid in **2** forms two hydrogen bonds with Tyr130 and Arg177. At the same time, the ligand **2** possesses hydrophobic interactions with Gly135, Phe201, Asn204 and Phe208. These interactions are almost identical with those in **Fe·1·HO** (Fig. 1(e)). Thus, the deletion of the phenol moiety in **Fe·1** contributes for **Cr·2** to preserve the

Table 2 Summary of X-ray data from the crystals of **Cr·2·HO** Atomic coordinates are deposited in the Protein Data Bank under accession number 2Z68 for **Cr·2·HO**.

Data collection	
Space group	$P2_1$
a/Å	40.66
b/Å	63.06
c/Å	77.96
β/°	96.88
Molecular per symmetric unit	2
Resolution range (outer cell)[a]/Å	50.0–1.58 (1.64–1.58)
Total observations	194 931
Unique reflections	53 304
Completeness[a] (%)	99.9 (100.0)
R_{merge}[a,b] (%)	6.1 (36.2)
$I/\sigma(I)$[a]	23.2 (2.39)
Refinement statistics	
Resolution/Å	30.0–1.58
R-factor[c] (%)	18.0
R_{free}[d] (%)	22.5
Final model	
No. of non-hydrogen atoms	3803
No. of water molecules	432
No. of SO_4^{2-}	3
No. of Na	2
No. of Cr complexes	2
Rms deviation from ideality	
Bonds/Å	0.013
Angles/°	1.474
Ramachandran plot[e] (%)	
Most favored	94.7
Allowed	5.0

[a] Values in parenthesis are for the highest resolution shell. [b] $R_{merge} = \sum|I - \langle I\rangle|/\sum I$, where I is the integrated intensity of a given reflection. [c] *R*-factor = $\sum||F_o| - |F_c||/\sum|F_o|$, where F_o and F_c are the observed and calculated structure factor amplitudes, respectively. [d] R_{free}: an *R*-factor calculated on a partial set that is not used in the refinement of the structure. [e] Ramachandran plot parameters were calculated using *PROCHECK* (ref. 27).

octahedral coordination structure without relocation of **Cr·2** in the HO active site from the position of **Fe·1** in HO. **Fe·2** is expected to have typical octahedral coordination geometry as **Cr·2** in the HO active site.[19,20] Therefore, a higher T_{mL} value of **Fe·2·HO** than that of **Fe·1·HO** is derived from less distortion of the **Fe·2** geometry and less exposure of **Fe·2** to the solvent than those of **Fe·1** by the elimination of the phenol moiety (C1–6) (Scheme 1). The T_{mL} of **Cr·2·HO** (60.2 °C) is much higher than that of **Fe·2·HO** (Table 1), possibly due to the difference in coordination bond length between Fe(III)–Nε(His) and Cr(III)–Nε(His).[19,20]

We have rationally designed the ligand **2** to improve the stability of Schiff-base complex·HO composites. An important factor for the design of the ligand is construction of a stable coordination structure of metal complexes at the binding site. In our case, the deletion of a phenol moiety in **1** eliminates the distortion structure of **Fe·1·HO**. We have already reported the importance of hydrogen bonds, hydrophobic interactions, and removal of steric hindrance between metal complexes and amino acid residues to improve the stability of artificial metalloproteins.[19,23] Therefore, these results will add further information for the construction of artificial metalloproteins stable enough for practical use.

This work was supported by the 21st Century COE program of Nagoya University for N. Y., a Grant-in-Aid for Scientific Research (Grant 18685019 for T. U.) and on Priority Areas (Grant 16033226, "Chemistry of Coordination Space" for Y. W.) from Ministry of Education, Culture, Sports, Science and Technology, Japan, and PRESTO, Japan Science and Technology Agency (JST).

Notes and references

1 D. Qi, C.-M. Tann, D. Haring and M. D. Distefano, *Chem. Rev.*, 2001, **101**, 3081.
2 C. M. Thomas and T. R. Ward, *Chem. Soc. Rev.*, 2005, **34**, 337.
3 Y. Lu, *Angew. Chem., Int. Ed.*, 2006, **45**, 5588.
4 T. Ueno, S. Abe, N. Yokoi and Y. Watanabe, *Coord. Chem. Rev.*, 2007, **251**, 2717.
5 I. Willner, *Science*, 2002, **298**, 2407.
6 Y. K. Yan, M. Melchart, A. Habtemariam and P. J. Sadler, *Chem. Commun.*, 2005, 4764.
7 T. Hayashi, D. Murata, M. Makino, H. Sugimoto, T. Matsuo, H. Sato, Y. Shiro and Y. Hisaeda, *Inorg. Chem.*, 2006, **45**, 10530.
8 B. R. Crane, A. J. Di Bilio, J. R. Winkler and H. B. Gray, *J. Am. Chem. Soc.*, 2001, **123**, 11623.
9 I. J. Dmochowski, B. R. Crane, J. J. Wilker, J. R. Winkler and H. B. Gray, *Proc. Natl. Acad. Sci. U. S. A.*, 1999, **96**, 12987.
10 D. P. Barondeau, C. J. Kassmann, J. A. Tainer and E. D. Getzoff, *J. Am. Chem. Soc.*, 2002, **124**, 3522.
11 D. Alexeev, H. Z. Zhu, M. L. Guo, W. Q. Zhong, D. J. B. Hunter, W. P. Yang, D. J. Campopiano and P. J. Sadler, *Nat. Struct. Biol.*, 2003, **10**, 297.
12 W. Q. Zhong, D. Alexeev, I. Harvey, M. L. Guo, D. J. B. Hunter, H. Z. Zhu, D. J. Campopiano and P. J. Sadler, *Angew. Chem., Int. Ed.*, 2004, **43**, 5914.
13 I. W. McNae, K. Fishburne, A. Habtemariam, T. M. Hunter, M. Melchart, F. Y. Wang, M. D. Walkinshaw and P. J. Sadler, *Chem. Commun.*, 2004, 1786.
14 V. Calderone, A. Casini, S. Mangani, L. Messori and P. L. Orioli, *Angew. Chem., Int. Ed.*, 2006, **45**, 1267.
15 J. E. Debreczeni, A. N. Bullock, G. E. Atilla, D. S. Williams, H. Bregman, S. Knapp and E. Meggers, *Angew. Chem., Int. Ed.*, 2006, **45**, 1580.
16 A. Muller, A. J. Wilkinson, K. S. Wilson and A. K. Duhme-Klair, *Angew. Chem., Int. Ed.*, 2006, **45**, 5132.
17 M. V. Cherrier, L. Martin, C. Cavazza, L. Jacquamet, D. Lemaire, J. Gaillard and J. C. Fontecilla-Camps, *J. Am. Chem. Soc.*, 2005, **127**, 10075.
18 M. Ohashi, T. Koshiyama, T. Ueno, M. Yanase, H. Fujii and Y. Watanabe, *Angew. Chem., Int. Ed.*, 2003, **42**, 1005.
19 T. Ueno, M. Ohashi, M. Kono, K. Kondo, A. Suzuki, T. Yamane and Y. Watanabe, *Inorg. Chem.*, 2004, **43**, 2852.
20 T. Ueno, T. Koshiyama, M. Ohashi, K. Kondo, M. Kono, A. Suzuki, T. Yamane and Y. Watanabe, *J. Am. Chem. Soc.*, 2005, **127**, 6556.
21 T. Ueno, T. Koshiyama, S. Abe, N. Yokoi, M. Ohashi, Y. Satake, H. Nakajima and Y. Watanabe, *J. Organomet. Chem.*, 2007, **692**, 142.
22 S. Abe, T. Ueno, P. A. N. Reddy, S. Okazaki, T. Hikage, A. Suzuki, T. Yamane, H. Nakajima and Y. Watanabe, *Inorg. Chem.*, 2007, **46**, 5137.
23 T. Ueno, N. Yokoi, M. Unno, T. Matsui, Y. Tokita, M. Yamada, M. Ikeda-Saito, H. Nakajima and Y. Watanabe, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 9416.
24 C. L. Hunter, E. Lloyd, L. D. Eltis, S. P. Rafferty, H. Lee, M. Smith and A. G. Mauk, *Biochemistry*, 1997, **36**, 1010.
25 E. E. Scott, E. V. Paster and J. S. Olson, *J. Biol. Chem.*, 2000, **275**, 27129.
26 M. Sugishima, H. Sakamoto, Y. Kakuta, Y. Omata, S. Hayashi, M. Noguchi and K. Fukuyama, *Biochemistry*, 2002, **41**, 7293.
27 R. A. Laskowski, M. W. Macarthur, D. S. Moss and J. M. Thornton, *J. Appl. Crystallogr.*, 1993, **26**, 283.