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A Site-Selective Dual Anchoring Strategy for Artificial Metalloprotein Design

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Introducing nonnative metal ions or metal-containing prosthetic groups into a protein is a branch of metalloprotein design with considerable impact, as it can dramatically expand the repertoire of protein functionalities and thus their range of applications.[1−9] Since protein scaffolds have not evolved to tightly bind artificial metal complexes in a single conformation, one of the most challenging aspects of this field is control of chemo- and/or enantioselectivity.[8−11] To meet the challenges, two approaches, noncovalent[12−14] and single-point covalent attachments,[8,9] have been successfully demonstrated, either by altering the metal complex and protein to promote binding in the noncovalent case[12−14,15] or by carefully selecting a protein host for covalent attachment of nonnative metal complexes.[16] Here we report a novel site-selective two-point covalent attachment strategy to introduce an achiral manganese salen complex (Mn(salen)), into apo sperm whale myoglobin (Mb) (Figure 1) and to demonstrate the effectiveness of the dual anchoring approach to markedly improve the enantioselectivity of a semi-synthetic enzyme with minimal structural modification to either the metal complex or the protein.

In an attempt to expand the functionality of heme proteins, we[12,13] and Watanabe and co-workers[14,15] have reported the replacement of heme with noncovalently attached achiral Mn(salen) and Cr(salen) complexes in the chiral pocket of cytochrome *c* peroxidase (C*c*P) or Mb, respectively. Metallo−salens are known for their high catalytic activity and versatility,[17] and their planar nature makes them an ideal choice for introduction into heme proteins. Despite their similarity, replacement of heme with metallo−salens has proven challenging. Noncovalent placement of a Cr(salen) inside the heme binding pocket requires the use of modified salens to increase the binding affinity, which results in a low yield of the Cr(salen) protein complex (0.3−15%) and low ee (0.3−13% ee).[14] Further, the method that successfully incorporated Cr(salen) into Mb is reported to be difficult to apply to Mn(salen).[13,14]

Covalent linkage is an alternative approach for site-specific attachment of an artificial metallo complex to a protein with high yield (∼100%) and with minimal structural information. Distefano and co-workers have demonstrated that covalent attachment of a Cu(II)/1,10-phenanthroline complex to a single cysteine in an adipocyte lipid binding protein results in a catalyst that promotes highly enantioselective hydrolysis.[8,16] We employed such a strategy to incorporate Mn(salen) into apo-Mb(Y103C) by methane thiosulfonate groups (Figure 1B) with high selectivity and reactivity toward cysteines.[18] This catalyst shows sulfoxidation activity (Table 1, entry 7); however, the ee remains low (12%) (Table 1).[14] Similarly low ee (<10%) is reported by Reetz and co-workers for a Mn(salen) complex attached to papain via a single maleimide linker.[19] The low ee of the single attachment again suggests that multiple orientations of the metallo complex may be possible. From these results we hypothesized that an improved catalyst could be engineered using a dual anchoring strategy, which allows for precise control of the placement of the artificial metallo complex with specific orientation and limited rotational freedom.

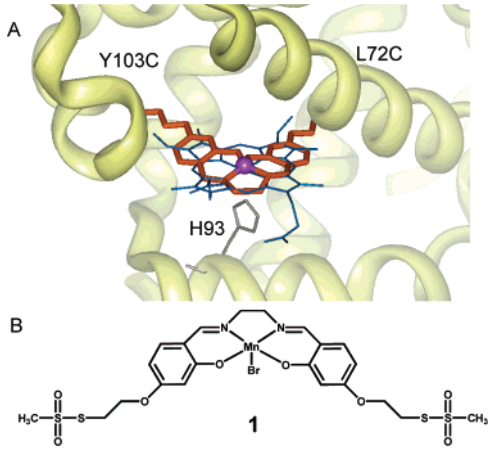

Figure 1. (A) Computer model of Mb(L72C/Y103C) with **1** covalently attached overlayed with heme. (B) Complex **1**.

Table 1. Enantioselective Sulfoxidation of Thioanisole

entry	catalyst[a]	rate[b,c]	ee, %[c]	attach[d]
1	**1**	2.4(0.1)	1 (0.5), S	−
2	apo-WTMb	4.6(0.3)	1 (1.5), S	−
3	apo-Mb(Y103C)	5.7(0.8)	3 (2), R	−
4	apo-Mb(L72C/Y103C)	15(5)	3 (2), S	−
5	apo-WTMb + **1**	10(3)	0 (0.5), S	n
6	Cr·**2**·-apo(H64D/A71G)Mb[e]	78	13, S	n
7	Mn·**1**·apo-Mb(Y103C)	51(14)	12 (1), S	s
8	Mn·**1**·apo-Mb (L72C/Y103C)	390(30)	51 (1), S	d

[a] In 50 mM NH_4OAc pH 5.1 at 4 °C, 130 μM catalyst, thioanisole (5 mM), H_2O_2 (6.5 mM) (for details see Supporting Information). [b] The unit of the rate is 10^{-3} turnover min^{-1}, std dev reported in parentheses. [c] Reaction rates and ee were determined by GC analysis using an ASTEC G-TA cyclodextrin column, and acetophenone was added as an internal standard.[25] [d] Attachment method (n = noncovalent, s = single, d = double). [e] Cr-**2** = Cr-5,5′-tBu_2-salophen (from ref 14).

Apo-Mb was chosen as a chiral scaffold because it has been determined to be essentially a folded globular protein[20] and has been used as a template for engineering numerous artificial proteins.[4,7,14,21,22] To covalently attach Mn(salen) to Mb, we again utilized methane thiosulfonate groups (Figure 1).[18] By modeling the *N*,*N*′-bis(4-(2-methanesulfonylthioethoxy)salicylidene)-1,2-ethanediamino-manganese(III) bromide (**1**) molecule (Figure 1B) into Mb using the InsightII (Accelrys) program and searching for its best fit in the heme pocket, we identified two mutations (L72C and Y103C) that would selectively anchor the compound (Figure 1A).

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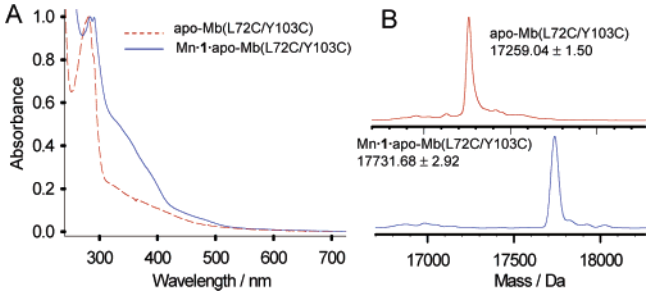

Figure 2. (A) UV−vis spectra of apo-Mb(L72C/Y103C) (red dashed line) and Mn·**1**·apo-Mb(L72C/Y103C) (blue solid line) in 50 mM ammonium acetate buffer pH 5.1. (B) ESI-MS of apo-Mb(L72C/Y103C) and Mn·**1**·apo-Mb(L72C/Y103C).

Construction, expression, and purification of Mb mutant proteins were performed as described previously.[21,23] Addition of **1** to this mutant results in the catalyst called Mn·**1**·apo-Mb(L72C/Y103C). The UV−vis spectrum of this catalyst shows new absorption peaks at 284 and 292 nm, suggesting the formation of a protein−salen adduct (Figure 2A). Further confirmation of the adduct formation comes from electrospray mass spectroscopy (ESI-MS), which shows a single peak corresponding to the apo enzyme plus **1** minus two methyl thiosulfonate groups displaced by covalent attachment (measured: 17731.68 ± 2.92 Da; calcd: 17731.12 Da) (Figure 2 B). The mass spectrum also suggests a complete conversion from apo enzyme to Mn(salen)-modified enzyme. These results are consistent with elemental analysis data and the absence of free cysteines by the Ellman's test.[24]

Comparison of the dual anchor Mn·**1**·apo-Mb(L72C/Y103C) catalyst with previously reported noncovalent and single-point attached protein metallo−salen complexes, as well as various controls with apo protein, **1** only, and noncovalent and single-point attached catalysts reported herein demonstrate the advantages of the dual anchor strategy. Control experiments (Table 1, entry 1−5) show that **1** in water and heme-free apoproteins employed in this study all displayed slow reaction rates and low ee (0−3%). This is not surprising since, in water and without a chiral center (in the case of **1**) or without a cofactor (for the apoproteins), they have previously been shown to be ineffective asymmetric catalysts.[12−14] When **1** was added to apo-WTMb without covalent attachment, the reaction rate and ee are also low (Table 1, entry 5); consistent with observations made by Watanabe and co-workers.[14] In their experiments, increased yield and ee were obtained by modifying both the Cr(salen) complex (*tert*-butyl substitution) and the Mb substrate binding pocket (H64D/A71G mutations). When we incorporated **1** into apo-Mb(Y103C) through a single-point attachment with no further modification of the protein, metal, or salen, we observed an increased rate (0.051 min^{-1}) and higher ee (12.3%). These results are comparable to the rate (0.078 min^{-1}) and ee (13%) reported for the noncovalent strategy used by Watanabe and co-workers (Table 1, entry 6),[14] indicating that covalent attachment is a comparable alternative to the noncovalent attachment strategy for controlling selectivity. More significantly, when **1** was incorporated into Mb through the site-selective dual anchoring strategy using apo-Mb(L72C/Y103C) (Figure 1A), a significant increase in rate (0.390 min^{-1}) and ee (51%) were observed (Table 1, entry 8). These results strongly suggest that the dual covalent anchoring strategy limits the number of conformational states available to the metallo complex inside the protein and by so doing improves both rate of reaction and ee.

In conclusion, we have demonstrated for the first time the effectiveness of a site-selective dual anchoring strategy for attachment of artificial metal complexes to proteins. Dual covalent anchoring of the metal complex to the protein affects catalytic sulfoxidation of thioanisole with much higher ee and rate than either noncovalent or single-point covalent attachment strategies. Since these results were obtained without mutation or selectively modified salen groups, the dual anchoring method provides an excellent platform from which to obtain even higher rate and ee. The method can be generally applied to protein incorporation of other metal complexes with minimal structural information and is a promising approach for generating new artificial enzymes.

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Supporting Information Available: Experimental details for sulfoxidation and the synthesis of **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) DeGrado, W. F.; Summa, C. M.; Pavone, V.; Nastri, F.; Lombardi, A. *Annu. Rev. Biochem.* **1999**, *68*, 779−819.
(2) Hellinga, H. W. *Curr. Opin. Biotechnol.* **1996**, *7*, 437−441.
(3) Kennedy, M. L.; Gibney, B. R. *Curr. Opin. Struct. Biol.* **2001**, *11*, 485−490.
(4) Lu, Y.; Berry, S. M.; Pfister, T. D. *Chem. Rev.* **2001**, *101*, 3047−3080.
(5) Farrer, B. T.; Pecoraro, V. L. *Curr. Opin. Drug Discovery Dev.* **2002**, *5*, 937−943.
(6) Hoffman, B. M. In *The Porphyrins*; Dolphin, D., Ed.; Academic Press: New York, 1979; Vol. VII, pp 403−444.
(7) Hayashi, T.; Hisaeda, Y. *Acc. Chem. Res.* **2002**, *35*, 35−43.
(8) Qi, D.; Tann, C.-M.; Haring, D.; Distefano, M. D. *Chem. Rev.* **2001**, *101*, 3081−3111.
(9) Reetz, M. T. *Tetrahedron* **2002**, *58*, 6595−6602.
(10) Miller, V. P.; DePillis, G. D.; Ferrer, J. C.; Mauk, A. G.; Ortiz de Montellano, P. R. *J. Biol. Chem.* **1992**, *267*, 8936−8942.
(11) Jin, S.; Makris, T. M.; Bryson, T. A.; Sligar, S. G.; Dawson, J. H. *J. Am. Chem. Soc.* **2003**, *125*, 3406−3407.
(12) Ma, S. K.; Lu, Y. *J. Inorg. Biochem.* **1999**, *74*, 217.
(13) Ma, S. K. Ph.D. Thesis, University of Illinois − Urbana-Champaign: Urbana, 2001.
(14) (a) Ohashi, M.; Koshiyama, T.; Ueno, T.; Yanase, M.; Fujii, H.; Watanabe, Y. *Angew. Chem., Int. Ed.* **2003**, *42*, 1005−1008. (b) Wilson, M. E.; Whitesides, G. M. *J. Am. Chem. Soc.* **1978**, *100*, 306−307. (c) Collot, J.; Gradinaru, J.; Humbert, N.; Skander, M.; Zocchi, A.; Ward, T. R. *J. Am. Chem. Soc.* **2003**, *125*, 9030−9031.
(15) Ueno, T.; Ohashi, M.; Kono, M.; Kondo, K.; Suzuki, A.; Yamane, T.; Watanabe, Y. *Inorg. Chem.* **2004**, *43*, 2852−2858.
(16) Davies, R. R.; Distefano, M. D. *J. Am. Chem. Soc.* **1997**, *119*, 11643−11652.
(17) Srinivasan, K.; Michaud, P.; Kochi, J. K. *J. Am. Chem. Soc.* **1986**, *108*, 2309−2320.
(18) Smith, D. J.; Miggio, E. T.; Kenyon, G. L. *Biochemistry* **1975**, *14*, 766−771.
(19) Reetz, M. T.; Rentzsch, M.; Pletsch, A.; Maywald, M. *Chimia* **2002**, *56*, 721−723.
(20) Eliezer, D.; Wright, P. E. *J. Mol. Biol.* **1996**, *263*, 531−538.
(21) Springer, B. A.; Sligar, S. G. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 8961−8965.
(22) Watanabe, Y. *Curr. Opin. Chem. Biol.* **2002**, *6*, 208−216.
(23) Sigman, J. A.; Kwok, B. C.; Lu, Y. *J. Am. Chem. Soc.* **2000**, *122*, 8192−8196.
(24) Ellman, G. L. *Arch. Biochem. Biophys.* **1959**, *82*, 70−77.
(25) Ozaki, S.-I.; Ortiz de Montellano, P. R. *J. Am. Chem. Soc.* **1995**, *117*, 7056−7064.

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