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Cupin Variants as Macromolecular Ligand Library for Stereoselective Michael Addition of Nitroalkanes

Nobutaka Fujieda, Haruna Ichihashi, Miho Yuasa, Yosuke Nishikawa, Genji Kurisu, and Shinobu Itoh**

Abstract: *Cupin superfamily proteins (TM1459) work as a macromolecular ligand framework with a double-stranded β-barrel structure ligating to a Cu ion through histidine side chains. Variegating the first coordination sphere of TM1459 revealed that H52A and H54A/H58A mutants effectively catalyzed the diastereo- and enantio-selective Michael addition reaction of nitroalkanes to an α,β-unsaturated ketone. Moreover, in silico substrate docking signified C106N and F104W single-point mutations, which inverted the diastereoselectivity of H52A and further improved the stereoselectivity of H54A/H58A, respectively.*

In modern organic synthesis, asymmetric reactions have been carried out using transition-metal complexes with chiral ligands.[1,2] However, the recognition of compounds bearing multiple chiral centers is still challenging and has garnered research interest, considering the increasing industrial and economic demands.[3] Artificial metalloenzymes are emerging hybrid biocatalysts, in which intrinsic chemical reactivity and selectivity of the introduced metal ion or metal complex can be enhanced by modifying the secondary coordination sphere of the protein or DNA scaffold.[4–14] By using artificial metalloenzymes, several enantioselective carbon–carbon bond forming reactions, such as Diels-Alder,[5,15–17] Michael addition,[18,19] and Friedel–Crafts alkylation[20] reactions, were established in water by employing α,β-unsaturated ketones harboring methylimidazole or pyridine groups.

The power of the DNA-based asymmetric catalysis concept was also demonstrated in Michael addition reactions using a Cu-complex in water. Enantioselectivity of up to 99 and 94 % *ee* was attained by using dimethyl malonate and nitromethane as nucleophiles, respectively, and α,β-unsaturated 2-acylimidazoles as Michael acceptors.[18] The transition-metal complexes[21] and the organocatalysts[22][23] have been developed for stereoselective Michael addition of nitroethane to α,β-unsaturated ketones in organic solvents. Recently, Pedro and Blay's group synthesized pyBOX (pyridine-2,6-bis(oxazolines))-derived ligand-La(III) complex and performed the Michael addition of nitroethane or nitropropane to azachalcone ((*E*)-3-phenyl-1-(pyridin-2-yl)prop-2-en-1-one) in moderate enantio- and diastereoselectivity.[24] Recently, a similar system involving Sc(III) complex with C_2-symmetric Schiff base ligands has been developed for the catalytic asymmetric Michael addition of nitroalkanes to azachalcone *N*-oxides by Wang's group with good diastereo- and enantioselectivity.[25] Thus, owing to the synthetic versatility of nitro-groups, immense progress of the asymmetric Michael additions of nitroalkane has been reported, but the control of diastereoselectivity and enantioselectivity, and catalytic efficiency were still limited.

We have devised artificial metalloenzymes using several proteins as a macromolecular metal ligand.[26,27] We have also harnessed the metal-binding promiscuity of the cupin superfamily protein (TM1459) to develop an Os-cupin complex system for the regioselective *cis*-1,2-dihydroxylation reaction.[28] TM1459 is derived from hyperthermophile, whose scaffold is very thermally stable, and has a molecular mass of only 12,977 Da (residues 1–114) with a metal-binding site consisting of a 4-His metal binding motif (Figure 1A); however, its function is unknown.[29,30] Herein, we have demonstrated that the small cupin variants of TM1459 (Figure 1B) can be effectively used to create efficient and robust biocatalysts for the stereoselective Michael addition reaction of nitroalkanes to α,β-unsaturated ketone, 2-azachalcone **1**, achieving excellent diastereo- and enantioselectivity as well as high TON.

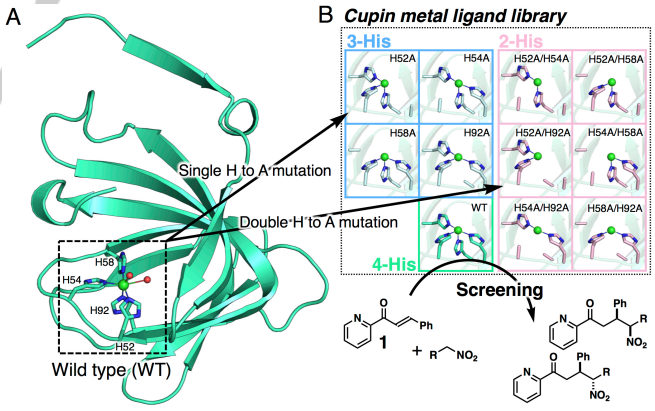

Figure 1. Protein metal ligands for Michael addition reaction of nitroalkane. A) The subunit structure of Cu-bound wild type TM1459 (PDB code: 6L2D). The protein main chain is displayed as ribbon, key amino acid residues as sticks, Cu ion as green sphere and waters as red sphere. B) The model structures of TM1459 mutants produced *in silico* and the Michael addition reaction targeted in this study.

Based on the crystal structure of Cu-bound wild type TM1459 (WT) (Figure 1A, Figures S1 and Table S1-S3), the mutants containing 2-His and 3-His metal-binding motifs were rationally designed (Figure 1B). Such metal binding site is reminiscent of the chiral bidentate or tridentate ligands bearing N atoms as donors such as bis(oxazoline) ligands (*N*,*N*-

[*] Ms. H. Ichihashi, Prof. S. Itoh[+]
Department of Material and Life Science,
Graduate School of Engineering, Osaka University
2-1 Yamada-oka, Suita, Osaka 565-0871 (Japan)
E-mail: shinobu@mls.eng.osaka-u.ac.jp (SI)
Ms. M. Yuasa, Prof. N. Fujieda[+]
Department of Applied Life Sciences, Graduate School of Life and Environmental Sciences, Osaka Prefecture University,
1-1 Gakuen-cho, Naka-ku, Sakai-shi, Osaka 599-8531, Japan
E-mail: fujieda@biochem.osakafu-u.ac.jp (NF)
Dr. Y. Nishikawa, Prof. G. Kurisu
Institute for Protein Research, Osaka University
3-2 Yamada-oka, Suita, Osaka 565-0871 (Japan)

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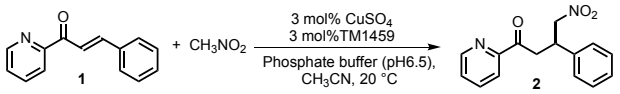
BOX and *N,N,N*-PyBOX derivatives) that are frequently employed in the asymmetric reactions.[1,2] 3-His-motifs of H52A and H58A mutants presented *facial* coordination geometry *in silico*, whereas those of H54A and H92A mutants exhibited a *meridional* coordination geometry (Figure 1B). In 2-His-motif-containing mutants, H54A/H92A presumably formed *trans* dyad, whereas others formed *cis* dyad (Figure 1B). The TM1459 mutants were prepared and purified in the same manner as WT (Figure S2 and S3 and Table S4).

Table 1: Addition of nitromethane to azachalcones **1** catalyzed by Cu-TM1459 and its mutants [a, b]



Entry	TM1459 Variant	Yield (%)[c]	*ee* (%)
1	-	9.5	n. d.
2	Wild type	0.5	n. d.
3	H52A	91	99 (*S*)
4	H54A	2.7	n. d.
5	H58A	15	29 (*S*)
6	H92A	50	85 (*S*)
7	H52A/H54A	18	25 (*R*)
8	H52A/H58A	1.5	n. d.
9	H54A/H58A	83	89 (*R*)
10	H54A/H92A	23	16 (*R*)
11	H58A/H92A	54	55 (*S*)
12	H52A/C106A	31	34 (*S*)
13	H52A/C106S	14	54 (*S*)
14	H52A/C106D	47	90 (*S*)
15	H52A/C106N	14	85 (*S*)
16	I49W/H54A/H58A	45	81 (*R*)
17	H54A/H58A/F104W	62	97 (*R*)

[a] Reaction conditions: TM1459 (0.3 mM), $CuSO_4$ (0.3 mM), **1** (10 mM), CH_3NO_2 (100 equiv.), Potassium phosphate buffer (pH 6.5)/CH_3CN (9:1), 20 °C, 3 h. [b] Yields and enantiomeric excesses (*ee*) were determined by chiral HPLC analysis. [c] Yields were calculated based on the total amount of (*S*) and (*R*).

We investigated the catalytic activity of a series of divalent transition metal ions (Mn, Fe, Co, Ni, Cu, and Zn) for the Michael addition reaction of nitromethane to azachalcone **1** and found that $CuSO_4$ exhibited highest yield (Table S5), as in the case of the reported work for the Diels-Alder reaction.[31] Highly selective mutants were examined for the Michael addition reaction with TM1459 variants as mini library consisting of various first coordination spheres (Table 1, entry 2-11). We found a trace amount of the racemic product of **2** with WT, H54A, and H52A/H54A (Table 1, entry 2, 4 and 8). On the other hand, other mutants showed a variety of enantiomeric excess (*ee*). Notably, the *S* enantiomer was obtained with excellent yield and enantioselectivity (*ee* = 99 % (*S*)) by employing H52A, whereas the *R* enantiomer was obtained with good yield and enantioselectivity (*ee* = 89 % (*R*)) with H54A/H58A (Table 1, entries 3 and 9 and Figure S4).

To propose a plausible stereochemical mechanism that can account for the observed enantioselectivity, the crystal structure of the Cu-bound H52A mutant was determined with highly reliable crystallographic statistics at the resolution of 1.20 Å (Figure 2A, Figure S5, and Table S1-S3). The coordination geometry of Cu-H52A mutant showed facial histidine triad and two water molecules located in a *cis*-position, completing the N_3O_2 square pyramidal geometry (Figure 2A and Figure S5C). It is worth noting that cysteine 106 was posttranslationally modified to cysteine sulfinic acid, as previously observed (Figure 2A).[28] The involvement of neighboring amino acid residues was evaluated by performing a docking simulation using azachalcone. In the lowest energy conformation of **1**, the polar edge of the pyridine moiety pointed toward the Cu active site (calculated free energy of binding (ΔG_b): -7.0 (kcal/mol), Figure 2B). In this azachalcone-docked structure, the β-*Si*-face attack (the *Si*-face of the double bond β-carbon of azachalcone) by nitromethane takes place at the position indicated by the dotted circle in Figure 2B. This space was tightly packed by the five amino acid residues, Phe41, Ile49, Phe94, Phe104, and Cys106 (sulfinic acid), while the opposite side is solvent accessible. Thus, we presumed that the preferential attack via β-*Si*-face was enforced by the hydrogen bonds exerted with cysteine sulfinic acid 106 rather than the shielding effect of protein matrix.

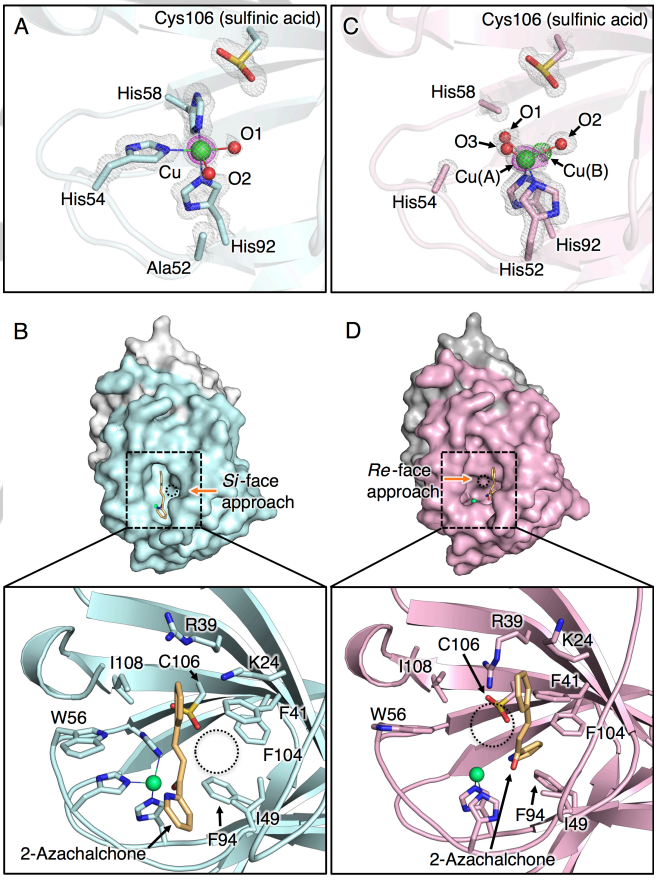


Figure 2. A) Crystal structures of Cu-binding site of H52A (Chain A, PDB code: 6L2E) and C) H54A/H58A (Chain A, PDB code: 6L2F) mutants. B) The surface models and close-up views of Cu site of H52A and D) H54A/H58A mutants of in-silico-obtained azachalcone-TM1459 complex. 2*Fo*-*Fc* and anomalous maps contoured at 1.5 and 6.0 σ are shown in gray and magenta mesh, respectively.

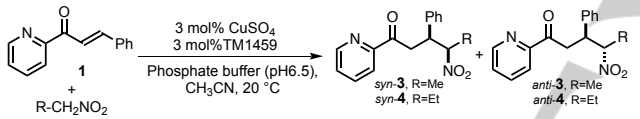
To support this assumption, four H52A/C106X (A, D, N, S) variants were subjected to the same reaction to examine their stereoselectivity. The H52A/C106A mutant exhibited not only lower enantioselectivity

10.1002/anie.202000129

but also lower yield, implying that Cys106 affects chemical reactivity as well as enantioselectivity (Table 1, entry 12). Furthermore, H52A/C106D and H52A/C106N still have excellent enantioselectivity, whereas H52A/C106S showed the lowest enantioselectivity, indicating that the spatial position of the hydrogen-bonded atoms to nitromethane is important for enantioselectivity (Table 1, entries 13-15).

In the same manner, chiral properties of the substrate binding pockets of H54A/H58A mutant were explored. The crystal structure of Cu-bound H54A/H58A mutants was resolved at 1.23 Å resolution (Figure 2C, Figure S5 and Table S1-S3), whose Cu binding site has three water molecules binding to the Cu ion (major species; Cu(A), minor species; Cu(B), see Figure S5) in a facial binding mode, acquiring the N_2O_3 square pyramidal geometry. In the lowest energy structure docked with **1** (ΔG_b: -6.7 (kcal/mol)), the β-*Si*-face was fully covered with the protein matrix, whereas the β-*Re*-face was exposed to the solvent (dotted circle, Figure 2D). This indicates that the *Re*-preferential attack of nitromethane occurs in H54A/H58A, which is consistent with the results of the catalytic reaction (Table 1, entry 9). Considering this result, two amino acids (Ile49 and Phe104) at the *Si*-face of azachalcone were chosen for mutation positions to strengthen the shielding effect with the protein matrix (Table 1, entry 16 and 17). As a result, H54A/H58A/F104W was found to give excellent *ee* of 97 % (*R*), which was much higher than that of H54A/H58A (89 % (*R*)) (Table 1, entry 17 and Figure S4).

Table 2: Addition of nitroalkanes to azachalcones **1** catalyzed by selected Cu-TM1459 mutants. [a, b]



Entry	-R	TM1459 Variant	Yield (%)[b,c]	*d.r.* (*anti:syn*)	*ee* (%)[d] (*anti/syn*)
1	Me	-	13	1:2.0	n.d.
2	Me	H52A	100	1:8.2	*syn*, 99(–)
3	Me	H54A/H58A	100	1:10	*syn*, 92(+)
4	Me	H54A/H58A/F104W	60	1:10	*syn*, 96(+)
5	Me	H52A/C106N	81	2.2:1	*anti*, 96(–)
6	Et	-	34	1:2.5	n.d.
7	Et	H52A	82	1:8.8	*syn*, 99(–)
8	Et	H54A/H58A	92	1:10	*syn*, 95(+)
9	Et	H54A/H58A/F104W	90	1:12	*syn*, 99(+)
10	Et	H52A/C106N	59	8.9:1	*anti*, 99(–)

[a] Reaction conditions: TM1459 (0.3 mM), $CuSO_4$ (0.3 mM), **1** (10 mM), R-CH_3NO_2 (100 equiv.), Potassium phosphate buffer (pH 6.5)/CH_3CN (9:1), 20 °C, 3 h (for nitroethane) or 12 h (for nitropropane). [b] Yields, diastereomeric ratio (*d.r.*) and enantiomeric excesses (*ee*) were determined by chiral HPLC analysis. [c] Yields were calculated based on the total amount of stereoisomers. [d] (+) or (-) was determined by polarimeter on HPLC.

By using mini ligand library thus obtained including the aforementioned second shell modification based on the molecular docking, the Michael addition reaction of nitroethane and nitropropane to **1** was attempted in the same manner as that of nitromethane (Table 2 and Table S6). In the absence of cupin proteins, diastereoselectivity was not clearly observed (Table 2, entry 1, 6). In stark contrast, in the case of nitroethane, the *syn*-selective product *syn*-**3** was predominantly obtained in the presence of H52A and H54A/H58A mutants (*d.r.* 1:8.2 and 1:10, respectively) with excellent *ee* of 99 % (–) and 92 % (+), respectively (Table 2, entry 2, 3). Moreover, the second shell modification, F104W mutation, further enhanced enantioselectivity. The H54A/H58A/F104W mutant showed improved *ee* from 92 % (+) to 96 % (+) and high diastereoselectivity (Table 2, entry 4). Interestingly, H52A/C106N showed slight *anti*-form preference, retaining excellent enantioselectivity (Table 2, entry 5), whereas H52A/C106D exhibited *syn*-form preference (Table S6, entry 15).

When using nitropropane, higher selectivity was observed and the *syn*-selective reaction proceeded giving *syn*-**4** in the presence of H52A and H54A/H58A (*d.r.* 1:8.8 and 1:10, respectively, Table 2, entry 7, 8) with excellent yield and *ee* 99 % (–) *and* 95 % (+), respectively. As in the case of nitroethane, the second shell modification, F104W mutation, allowed H54A/H58A to increase *ee* from 95 % (+) to 99 % (+) (Table 2, entry 9). Furthermore, C106N mutation was found to cause the complete inversion of diastereo-preference to produce *anti*-form stereoisomers, maintaining the excellent enantioselectivity (*d.r.* 8.9:1, *ee* 99 % (–), Table 2, entry 10).

To the best of our knowledge, this study represents the first example of artificial metalloenzymes that can control *anti*- or *syn*-preference in the Michael addition reaction with 2-azachalcone with distinguished enantioselectivity and excellent TON (Table S7). The generation of the library-like mutant-series bearing various first coordination spheres and second shells in TM1459 allowed for the simultaneous optimization of the substrate binding site as well as the Cu binding site, producing efficient catalysts for the diastereo- and enantio-selective Michael addition reaction of nitroalkanes to an α, β-unsaturated ketone. Thus, the use of such a small cupin library system facilitates a promising approach for creating artificial metalloenzymes that can catalyze diastereo- and enantiodivergent reactions. Hopefully, this system based on the cupin superfamily protein will be applied various reactions that can be used in synthetic organic chemistry.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: artificial metalloenzyme • cupin • macromolecular ligand • stereodivergent • protein library

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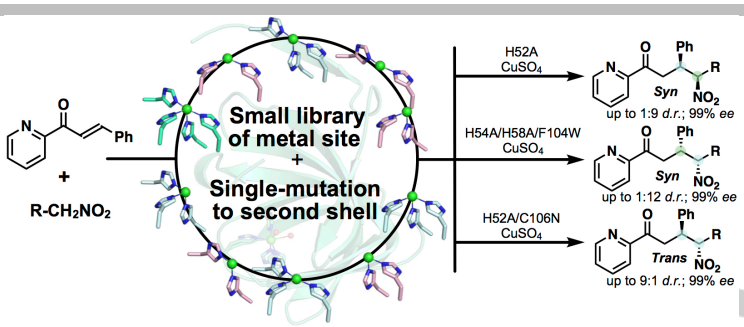

Rational alteration of first and second coordination sphere within cupin protein produced robust artificial metalloenzymes that can approach the enantio- and diastereostereodivergent Michael addition reaction of nitroalkanes.

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