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Articles

Directed evolution enhances the peroxidation activity of myoglobin reconstituted with an iron corrole complex

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Abstract

Engineered hemoproteins incorporating synthetic metal porphyrinoids with non-natural tetrapyrrole frameworks are known to exhibit unique catalytic properties which are not observed in native hemoproteins. We previously reported the reconstitution of myoglobin with an iron corrole complex (Mb-FeCor) leading to unique catalytic activity in H_2O_2-dependent peroxidation. To optimize the amino acid sequence of the active site of myoglobin for this noncanonical corrole complex, we performed genetic engineering of Mb-FeCor based on the directed evolution methodology. After 3 rounds of directed evolution, a triple mutant Mb(L89F/H97F/I107F)-FeCor was obtained as a best-performing catalyst, exhibiting a 24-fold higher initial rate for ABTS peroxidation relative to the original Mb-FeCor. Furthermore, the k_{cat}/K_M value of this engineered variant was calculated to be 16 $\mu M^{-1}s^{-1}$, exceeding that of horseradish peroxidase (HRP, 0.12 $\mu M^{-1}s^{-1}$). These results demonstrate that combining synthetic porphyrinoid incorporation with directed evolution of a protein scaffold provides an efficient strategy for improving the catalytic activity of hemoprotein-based artificial metalloenzymes.

Keywords: artificial metalloenzyme, directed evolution, hemoprotein.

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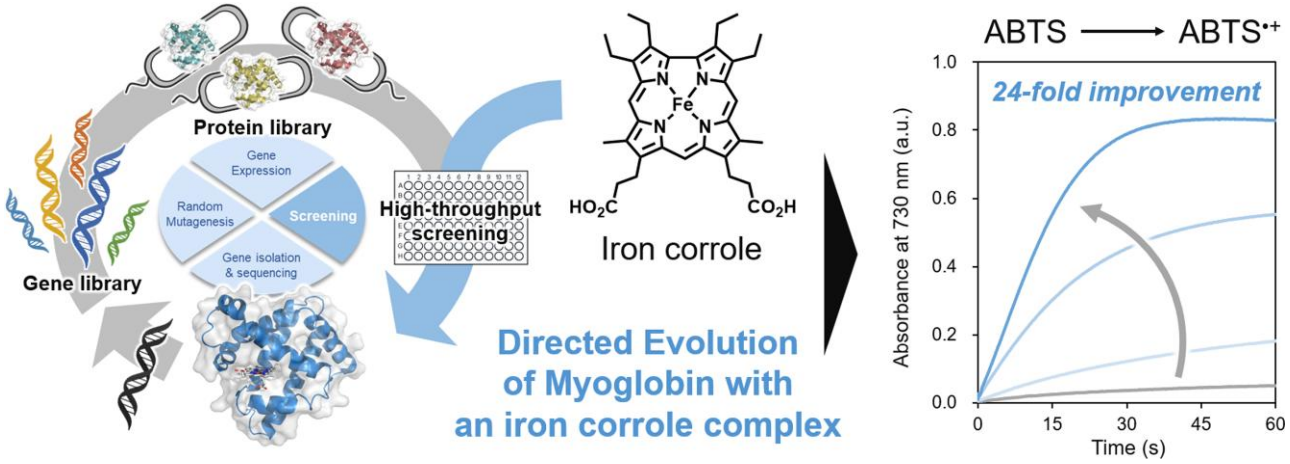
Graphical Abstract



1. Introduction

Hemoproteins reconstituted with synthetic metal porphyrinoids represent one of the major classes of artificial metalloenzymes.[1–6] As exemplified in previous studies of CO_2 reduction,[7,8] sulfoxidation,[9–12] hydroxylation,[13–15] cyclopropanation,[16–21] carbene[22–25] and nitrene[26,27] insertions, reconstituted hemoproteins provide efficient catalytic platforms for both natural and non-natural chemical transformations of various substrates. In particular, hemoproteins incorporating synthetic porphyrinoids with "noncanonical" tetrapyrrole frameworks have been shown to display unique reactivities that are not observed in native hemoproteins.[28–30] Our group previously reported the construction of myoglobin (Mb) reconstituted with an Fe corrole complex (Mb-FeCor) (Fig. 1a,b).[31] Since the trianionic character of the corrole ligand can stabilize high-valent Fe-oxo intermediates, Mb-FeCor was found to exhibit distinctive peroxidase activity which is absent in native myoglobin. Although this result highlights the potential of corrole-based artificial metalloenzymes, the catalytic efficiency of Mb-FeCor remains limited mainly due to the structural mismatch between the native heme pocket and the corrole cofactor. Therefore, we proposed that optimization of the amino acid sequence surrounding the non-natural cofactor would further enhance the catalytic activities of Mb-FeCor.

Directed evolution is a powerful protein engineering technique that can systematically optimize the catalytic properties of enzymes.[32–39] This technique was initially developed and widely applied for improving the activity, selectivity, and stability of natural enzymes. More recently, directed evolution has been extended to the engineering of artificial metalloenzymes.[40–45] In these systems, directed evolution has been shown to enable the fine-tuning of protein scaffolds to accommodate synthetic cofactors and to achieve enhanced catalytic functions through iterative cycles of random mutagenesis and high-throughput screening (HTS).[46,47] Such progress demonstrates the broad applicability of evolutionary approaches to both natural and non-natural catalytic systems and is thereby expected to provide a practical route for improving the performance of hemoprotein-based artificial metalloenzymes.

In this study, we applied the directed evolution methodology to engineer Mb-FeCor and dramatically enhanced the peroxidase activity of myoglobin, whose native function is in oxygen storage. Through iterative rounds of mutagenesis and screening, we obtained a highly active Mb variant carrying 3 mutations (L89F, H97F, and I107F) that significantly improved the catalytic performance of Mb-FeCor. The results demonstrate that the combination of incorporation of a synthetic cofactor with genetic engineering of the protein scaffold represents an effective strategy for developing hemoprotein-based artificial metalloenzymes with enhanced catalytic properties.

2. Experimental

2.1 Preparation of the expression plasmid pET-21b-mbpMb

To facilitate the screening process of directed evolution, we first constructed a fusion protein of Mb with maltose binding protein (MBP), designated as mbpMb, based on our previous study.[44,45] The expression plasmid for mbpMb was constructed according to the standard protocol of the NEBuilder HiFi DNA Assembly cloning kit (New England Biolabs Japan). The insert DNA encoding the Mb gene was amplified by PCR from the corresponding plasmid which was used for the expression of myoglobin in our previous study.[48] The PCR products were then treated with DpnI restriction enzyme (New England Biolabs Japan), and purified by agarose gel electrophoresis. The insert DNA was then assembled with a linearized pET-21b vector encoding the MBP gene with an α-helix linker[44] using the NEBuilder HiFi DNA Assembly kit. The assembled product was then transformed into chemically competent *Escherichia coli* DH5α cells. After cultivation in LB media containing 100 μg/mL ampicillin at 37 °C overnight, the plasmids were purified using a NucleoSpin Plasmid kit (Takara Bio Inc.) to afford the expression plasmid for mbpMb.

2.2 Preparation of a gene library of mbpMb variants

A gene library of mbpMb variants was constructed based on the protocol of 22c-trick.[49] Template plasmids encoding the

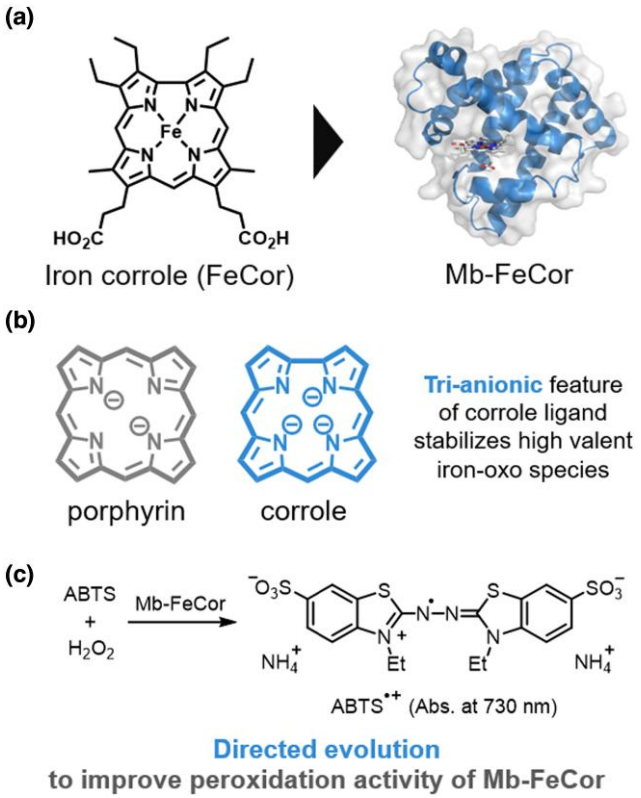


Fig. 1 a) Schematic illustration of Mb-FeCor. b) Ionic properties of tetrapyrrole ligands. c) Peroxidation of ABTS catalyzed by Mb-FePc variants.

mbpMb gene and its variants were amplified by PCR using primers that contain NDT, VHG, and TGG codons at the target positions for site-saturation mutagenesis (SSM) (Supplementary Tables S1, S2). The resulting PCR products were treated with DpnI restriction enzymes (New England Biolabs Japan) and transformed into chemically competent *E. coli* DH5α cells. After cultivation in LB media containing 100 μg/mL ampicillin at 37 °C overnight, the plasmids were purified using a NucleoSpin Plasmid kit (Takara Bio Inc.) to afford a gene library of mbpMb variants.

2.3 HTS of mbpMb variants reconstituted with FeCor

The gene libraries of the mbpMb variants were transformed into the chemically competent *E. coli* BL21-Gold(DE3) strain, and the cells were incubated on LB agar plates supplemented with 100 μg/mL ampicillin. The obtained single colonies were then transferred into a 96-well microplate filled with 100 μL of M9 medium containing 100 μg/mL ampicillin, and the cells were cultivated at 37 °C and 1500 rpm for 8 h. A portion of the precultures (70 μL) was transferred into another 96-well plate and stored at −80 °C as a master plate after adding 70 μL of the sterile glycerol solution (40 v/v%). Another portion of the precultures (7.5 μL) was inoculated into 750 μL of M9 medium containing 100 μg/mL ampicillin in another four 96-deep-well plates, and incubated at 37 °C and 1500 rpm for 3 h. Expression was induced by adding 50 μL of M9 medium containing 8.0 mM IPTG and 100 μg/mL ampicillin, and the cultivation was further continued at 15 °C and 1500 rpm for 16 h. The *E. coli* cells were then harvested by centrifugation at 4 °C and 3500 g for 5 min. The collected cells were resuspended in 100 μL of KPi buffer (20 mM KPi, 200 mM NaCl, 1 mM EDTA, pH 7.5) and stored at −20 °C for the screening step. Next, the frozen cell suspensions were slowly thawed at 4 °C for 2 h. The cells were then lysed by adding 50 μL of KPi buffer (20 mM KPi, 200 mM NaCl, 1 mM EDTA, pH 7.5) containing lysozyme (5.0 μg) and Benzonase nuclease (1.25 U). After incubation for 1 h at 37 °C, the mixtures were centrifuged at 4 °C and 2200 *g* for 5 min. Supernatants from the four 96-deep-well plates were combined and loaded onto a 96-well filter plate packed with 1.5 mL of starch-agarose resin equilibrated with KPi buffer (20 mM KPi, 200 mM NaCl, pH 7.0). After incubation at room temperature for 10 min to strongly bind apo-mbpMb variants, the resins were washed with 4 × 670 μL of KPi buffer (20 mM KPi, 200 mM NaCl, pH 7.0), and the apo-mbpMb variants were eluted with 200 μL of KPi buffer (20 mM KPi, 200 mM NaCl, pH 7.0) containing 25 mM maltose. A part of the elution (150 mL) was then transferred into another 96-well microplate, and 3.0 μL of Fe corrole solution (100 μM) dissolved in a 1:1 mixed solution of MeCN and KPi buffer (20 mM KPi, 200 mM NaCl, pH 7.0) was added to the solution. The mixtures were incubated at room temperature for 8 h to complete the incorporation of FeCor into the apo-mbpMb variants. Finally, the solution of 250 μM ABTS and 2 mM H_2O_2 (150 μL) was added into the mixture, and the reaction progress was monitored by UV-vis absorbance at 730 nm using a microplate reader.

2.4 Preparation of Mb-FeCor variants

Mb variants were expressed and purified following the protocol of our previous study.[48] The removal of heme and the incorporation of FeCor into the apoproteins were performed according to the literature procedures with slight modifications.[31,50,51] Briefly, an ice-cold solution of Mb variants was acidified to a pH below 2.0 with 1.0 M HCl_{aq}. After the extraction of heme with 2-butanone, the protein solution was sequentially dialyzed at 4 °C against (i) 10 mM KPi buffer (pH 6.0), (ii) 10 mM KPi buffer (pH 6.5), (iii) 10 mM KPi buffer (pH 7.0), and (iv) 100 mM KPi buffer (pH 7.0) to obtain a solution of apo-Mb. Next, to the solution of apo-Mb on ice, 1.2 equiv of FeCor in MeCN was added dropwise. After incubation for 8 h, the resulting protein solution was passed through HiTrap desalting columns (Cytiva) to remove free FeCor which is not bound to the Mb scaffold. The obtained Mb-FeCor variants were characterized by UV-vis spectroscopy and SDS-PAGE.

2.5 Measurement of kinetic parameters for ABTS oxidation

Into a 500 μL solution of apo-Mb variants (ca. 4 μM), 5 μL of FeCor solution (200 μM) dissolved in MeCN was added. After incubation at room temperature for 8 h, various concentrations of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) dissolved in 500 μL of KPi buffer (100 mM KPi, pH 7.0) were added. The resulting mixture was then combined with 1 mL of H_2O_2 (100 mM) solution with a stopped-flow single mixing apparatus to initiate the reaction. The time-course of the reaction was immediately recorded, and the kinetic parameters were calculated based on the Michaelis-Menten equation. Reaction conditions: apo-Mb

variants (1.0 μM), FeCor (0.5 μM), ABTS (20, 40, 80, 100, 200, 300, 400, 500 μM), H_2O_2 (50 mM) at 25 °C.

2.6 Measurement of kinetic parameters for guaiacol oxidation

Into 300 μL of apo-Mb variant solution (ca. 2 μM), 3 μL of FeCor solution (100 μM) dissolved in a 1:1 mixed solution of MeCN and KPi buffer (100 mM KPi, pH 7.0) was added. After incubation at room temperature for 8 h, various concentrations of guaiacol in 200 μL of KPi buffer (100 mM KPi, pH 7.0) and 100 μL of H_2O_2 solution (300 mM) were added into the mixture. The time-course of the reaction was recorded by monitoring UV-vis absorbance at 730 nm, and the kinetic parameters were calculated based on the Michaelis-Menten equation. Reaction conditions: apo-Mb variants (1 μM), FeCor (0.5 μM), guaiacol (0.1, 0.5, 1.0, 2.0, 3.0, 5.0, 8.0, 10 mM), H_2O_2 (50 mM), at 25 °C.

3. Results and discussion

A key requirement for efficient directed evolution is to establish a reliable platform for HTS. In our previous work, we developed an HTS platform which included an affinity purification step utilizing MBP-tag and starch-agarose resin. In this study, we applied this previous HTS platform with several modifications for the directed evolution of Mb-FeCor (Supplementary Fig. S1). Briefly, a fusion protein mbpMb, in which the *N*-terminus of Mb is genetically fused with an MBP-tag was constructed, and the gene libraries of mbpMb variants were constructed through SSM based on the 22c-trick method.[49] The prepared libraries of mbpMb variants were then expressed as an apo-form using M9 minimal media in a 96-well format.[52] After purification using the starch-agarose resin (Supplementary Figs. S2 and S3), apo-mbpMb variants in the elution were incorporated with an Fe corrole complex to obtain a purified library of artificial metalloenzyme (Supplementary Fig. S4). To validate this HTS platform, we performed a series of control experiments (Supplementary Fig. S5). While apo-mbpMb supplemented with the Fe corrole complex clearly exhibits catalytic activity for the ABTS oxidation, negative controls using apo-mbpMb or the FeCor alone do not exhibit any peroxidation activity. The HTS platform completely excludes the background reaction catalyzed by mbpMb with the heme cofactor and the free Fe corrole complex.

Taking advantage of this HTS platform, we next performed a directed evolution campaign on mbpMb-FeCor. Based on the crystal structure of myoglobin (PDB: 5YCE),[53] twelve amino acid residues (L29, L32, F43, H64, T67, V68, L89, H93, H97, I99, L104, and I107) located around the cofactor were selected for SSM (Fig. 2). When the proximal H93 residue was mutated into other metal-coordinating nucleophilic residues (serine, cysteine, tyrosine, and aspartic acid), no significant variants with improved activity were obtained (Supplementary Fig. S6). This indicates the crucial role of the proximal histidine residue for the ABTS peroxidation catalyzed by the FeCor cofactor. In the SSM focusing on other amino acid residues within the active site, the L89F variant of mbpMb-FeCor was identified as an improved variant after screening more than 600 variants. The L89F variant was found to show a 3.3-fold higher initial rate for ABTS oxidation (Fig. 3, Table 1). Furthermore, we performed additional rounds of directed evolution using this improved L89F variant as a template. Consequently, mbpMb(L89F/H97F/I107F)-FeCor was identified as the best-performing catalyst. This variant has 3 phenylalanine residues incorporated within the heme binding site of Mb. Notably, this evolved variant exhibits a 24-fold higher initial rate compared to wild-type mbpMb-FeCor (Fig. 3, Table 1). Since these phenylalanine residues are located within the binding site of the FeCor complex rather than the general acid-base site for H_2O_2, improved affinity for the non-natural corrole cofactor or stabilization of the π-radical cation intermediates by aromatic side chains is expected to be one of the main features contributing to the enhanced peroxidation activity of L89F/H97F/I107F variant.

To obtain deeper insights into the kinetics of this class of evolved artificial metalloenzymes, we next prepared Mb-FeCor variants Mb(L89F)-FeCor, Mb(L89F/107F)-FeCor, and Mb(L89F/H97F/I107F)-FeCor without the MBP-tag. These variants were expressed in *E. coli* cells, and reconstituted with the Fe corrole complex after the removal of heme according to the protocol of our previous studies.[31,48] These evolved Mb-FeCor variants have UV-vis absorption spectra similar to that of wild-type Mb-FeCor

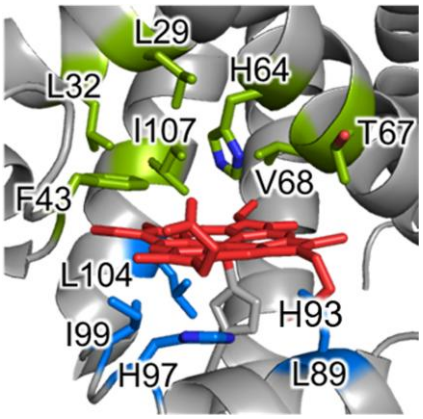


Fig. 2 Crystal structure of native myoglobin (PDB: 5YCE). Heme and the 12 amino acid residues subjected to SSM are highlighted as sticks.

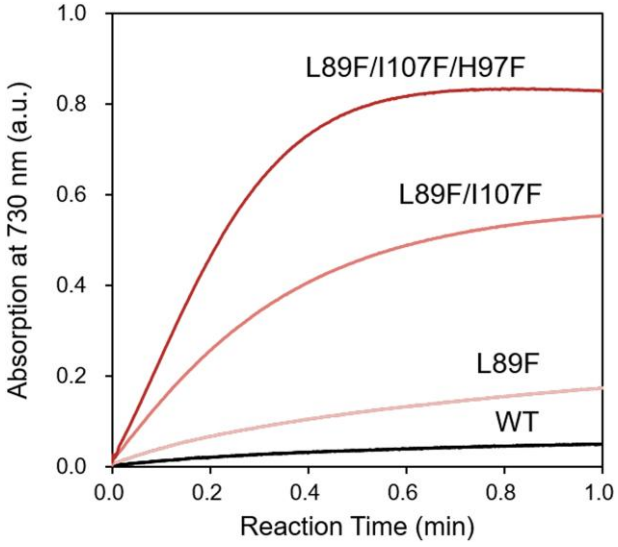


Fig. 3 Time-course of ABTS peroxidation catalyzed by mbpMb-FeCor variants. Reaction conditions: Mb variants (0.75 μM) Fe corrole (0.50 μM), ABTS (125 μM), H_2O_2 (1.0 mM) in KPi buffer (pH 7.0), 25 °C.

Table 1 Initial rate of ABTS peroxidation.

Catalysts	Initial rate (μM/s)	Improvement from wild-type
mbpMb-FeCor	19	1.0
mbpMb(L89F)-FeCor	63	3.3
mbpMb(L89F/I107F)-FeCor	2.6×10^2	14
mbpMb(L89F/H97F/I107F)-FeCor	4.5×10^2	24

Reaction conditions: mbpMb variants (0.75 μM) Fe corrole (0.50 μM), ABTS (125 μM), H_2O_2 (1.0 mM) in KPi buffer (20 mM KPi, 200 mM NaCl, 25 mM maltose, pH 7.0), 25 °C.

Table 2 Michaelis-Menten parameters for ABTS oxidation.

Catalysts	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M ($\mu M^{-1}s^{-1}$)
Mb-FeCor	150	60	2.5
Mb(L89F)-FeCor	131	51	2.6
Mb(L89F/I107F)-FeCor	166	38	4.4
Mb(L89F/H97F/I107F)-FeCor	540	33	16
native Mb	6.5	1.3×10^4	1.3×10^{-4}
HRP	224	1.8×10^3	0.12

Reaction conditions: Mb variants (1.0 μM), FeCor (0.50 μM), ABTS (20, 40, 80, 100, 200, 300, 400, 500 μM), H_2O_2 (50 mM) in KPi buffer (100 mM KPi, pH 7.0), 25 °C.

(Supplementary Fig. S7). In addition, the increasing trend in the peroxidation activity is essentially identical to that of the mbpMb-FeCor variants which were fused with MBP-tag. Although Mb(L89F)-FeCor has slightly decreased activity which is similar to the wild-type due to the instability of apo-Mb scaffold without MBP-tag, Mb(L89F/H97F/I107F)-FeCor clearly exhibits higher catalytic activity than the others (Supplementary Fig. S8). Taking these results into consideration, we next determined Michaelis-Menten parameters for the steady-state reactions with varying concentrations of ABTS (Supplementary Fig. S9, Table 2). Mb(L89F/H97F/I107F)-FeCor was revealed to show a 3.6-fold higher k_{cat} value for ABTS oxidation compared to the wild-type (Table 2). The K_M value was also found to be slightly improved though the course of directed evolution, resulting in a 6.4-fold improvement in the k_{cat}/K_M value. The myoglobin scaffold might also contribute to the binding of ABTS in combination with the introduced phenylalanine residues. Surprisingly, compared to native Mb with the natural heme cofactor, Mb(L89F/H97F/I107F)-FeCor provides a 10^5-fold higher k_{cat}/K_M value, and this k_{cat}/K_M value of Mb(L89F/H97F/I107F)-FeCor is even higher than that of horseradish peroxidase (HRP), indicating the significance of the FeCor cofactor (Supplementary Fig. S10). Furthermore, the evolved variants of Mb-FeCor were also found to exhibit improved catalytic activity for the peroxidation of guaiacol (Supplementary Table S3). These results highlight the significance of the directed evolution methodology in optimizing the environment around the non-natural cofactor, thereby achieving improvements in catalytic efficiency.

4. Conclusion

In summary, we have performed directed evolution of Mb-FeCor, an engineered Mb reconstituted with FeCor. Through 3 rounds of a directed evolution campaign, the catalytic activity of Mb-FeCor was significantly improved with respect to H_2O_2-dependent oxidation of ABTS and guaiacol. Compared with wild-type Mb-FeCor, the evolved variant Mb(L89F/H97F/I107F)-FeCor has a 24-fold higher initial rate for ABTS peroxidation. Furthermore, the k_{cat}/K_M value of this engineered variant is 16 $\mu M^{-1}s^{-1}$, exceeding that of native HRP, a representative peroxidase enzyme. These findings highlight the potential of directed evolution methodology in efforts to improve the compatibility of myoglobin with synthetic non-natural cofactors, thereby providing a practical design principle for developing high-performance artificial metalloenzymes.

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Supplementary material

Supplementary material is available at *Bulletin of the Chemical Society of Japan* online.

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Conflicts of interest

None declared.

Data availability

The data supporting this article has been included as part of the Supplementary material.

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