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PAPER

Semi-synthesis of an artificial scandium(III) enzyme with a β-helical bio-nanotube†

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We have succeeded in preparing semi-synthesized proteins bound to Sc^{3+} ion which can promote an epoxide ring-opening reaction. The Sc^{3+} binding site was created on the surface of $[(gp5\beta f)_3]_2$ (N. Yokoi *et al.*, *Small*, 2010, **6**, 1873) by introducing a cysteine residue for conjugation of a bpy moiety using a thiol–maleimide coupling reaction. Three cysteine mutants $[(gp5\beta f\_X)_3]_2$ (X = G18C, L47C, N51C) were prepared to introduce a bpy in different positions because it had been reported that Sc^{3+} ion can serve as a Lewis-acid catalyst for an epoxide ring-opening reaction upon binding of epoxide to bpy and two –ROH groups. G18C_bpy with Sc^{3+} can accelerate the rate of catalysis of the epoxide ring-opening reaction and has the highest rate of conversion among the three mutants. The value is more than 20 times higher than that of the mixtures of $[(gp5\beta f)_3]_2$/2,2′-bipyridine and L-threonine/2,2′-bipyridine. The elevated activity was obtained by the cooperative effect of stabilizing the Sc^{3+} coordination and accumulation of substrates on the protein surface. Thus, we expect that the semi-synthetic approach can provide insights into new rational design of artificial metalloenzymes.

Introduction

In nature, metalloenzymes promote enzymatic reactions by using various metal ions such as Fe, Mn, Cu, Co, Ni, Zn, among others.[1] Metal ion coordination complexes constructed within the unique molecular environments provided by protein scaffolds help them to overcome their relative instability in aqueous media. The coordination structures are stabilized by ligation of amino acid residues such as His, Cys, Asp and/or natural cofactors such as porphyrin and chlorophyll.[2] In preparation of biomimetic systems, various synthetic metal complexes have been fixed within protein scaffolds in order to construct artificial metalloenzymes that can achieve various catalytic reactions such as hydrogenation, sulfoxidation, and the Diels–Alder reaction.[3–8] Anchoring of the synthetic metal complexes has been achieved by using natural host–guest interactions of heme/apo-myoglobin and biotin/(strept)avidin[9–12] or selective conjugation reactions of cysteinyl thiol/maleimide and lysinyl amine/succinimidyl ester.[13–16] Furthermore, if synthetic metal complexes conjugated to proteins are activated by coordination of amino acid residues originally existed in the proteins, the possibilities of artificial metalloenzymes will be expanded.

Scandium ion (Sc^{3+}) is a potent candidate for anchoring to a protein because of its unique catalytic activity. It can function as a Lewis-acid in catalysis of various reactions such as formation of C–C and C–A bonds (where A = N, O, S, P, and Si).[17] Sc^{3+} retains activity in water, in contrast to other Lewis acids such as $AlCl_3$, BF_3, $TiCl_4$ and $SnCl_4$, which are unstable in water. This suggests that anchoring of Sc^{3+} to a protein would provide a promising strategy for construction of a new class of Lewis-acid metalloenzymes. However, an enzyme containing Sc^{3+} has not yet been reported because it has proven difficult to stabilize the Sc^{3+} coordination structure within a protein.

Herein, we report the construction of an artificial Sc^{3+} enzyme by combining a conjugated synthetic ligand and natural coordinating amino acids on surface of a protein as shown in Fig. 1a. The synthetic ligand is covalently anchored to a position adjacent to the coordinating amino acids by chemical modification of a cysteinyl thiol which is introduced by site-directed mutagenesis. Natural coordinating amino acids on the host protein are used as supporting ligands. It was expected that the coordination structure of Sc^{3+} would be stabilized by the cooperative coordination of the synthetic ligand and the amino acid ligands. The host protein must meet certain criteria as follows; (1) it must have chemical and thermal stability under chemical modification and catalytic reaction conditions, and (2) coordinating amino acids and the synthetic ligand must be located at an appropriate position to promote metal binding. Thus, we employ our previously reported tubular protein $[(gp5\beta f)_3]_2$ which satisfies these requirements. It is a chimeric protein assembly

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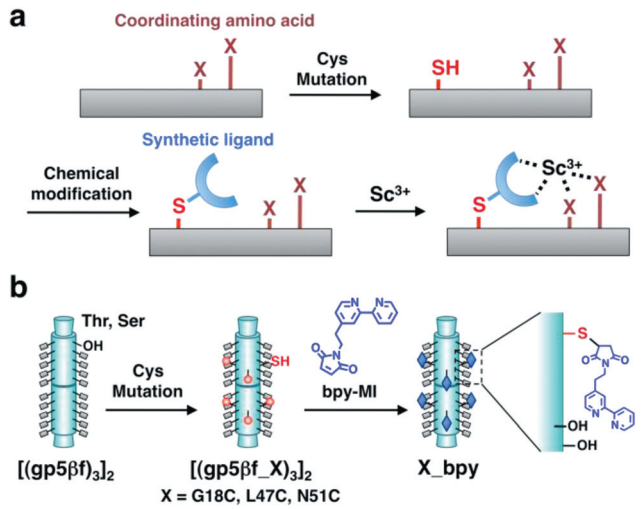


Fig. 1 (a) Scheme of semi-synthesis of a metal-binding site on a protein surface. (b) A schematic drawing indicating conjugation of 2,2′-bipyridine (bpy) to $[(gp5\beta f)_3]_2$. Cys is introduced to a position near Thr and Ser by site-directed mutagenesis (construction of $[(gp5\beta f\_X)_3]_2$, X = G18C, L47C, N51C). Bpy was coupled to the Cys residues by a selective reaction between cysteinyl thiol and a bipyridine-maleimide derivative (bpy-MI) (construction of **X_bpy**).

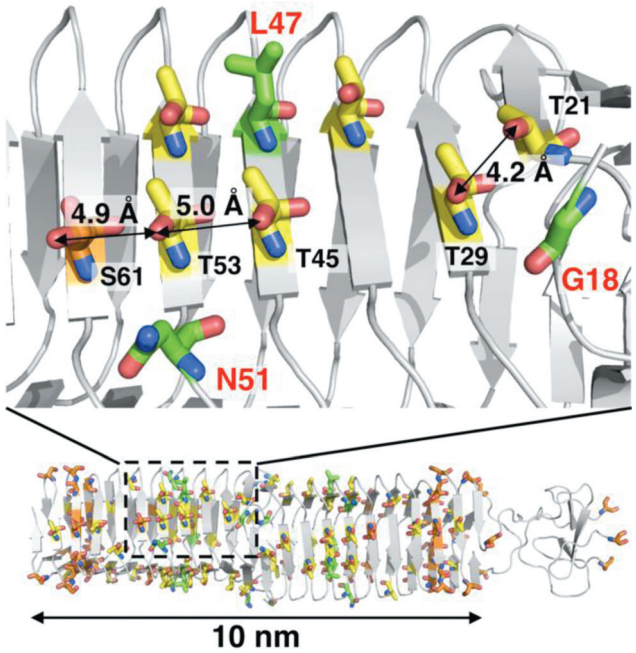


Fig. 2 Side view of the crystal structure of $[(gp5\beta f)_3]_2$.[21] One of the six sites is extended. Green sticks represent G18, L47, and N51. Yellow and orange sticks represent Thr and Ser, respectively. S61 has two alternative conformations.

formed by genetic fusion of the β-helical motif of gene product 5 (gp5β)[18–20] and the foldon domain (f) isolated from bacteriophage T4.[21] $[(gp5\beta f)_3]_2$ adopts a trimer–dimer structure (89 kDa) which is composed of six gp5βf monomers (14.8 kDa). Compared to other host tubular proteins such as tobacco mosaic virus (TMV),[15] M13 bacteriophage,[16] and potato virus X (PVX),[22] $[(gp5\beta f)_3]_2$ has a tubular structure with excellent stability. The crystal structure of $[(gp5\beta f)_3]_2$ indicates regular alignment of amino acids on the surface of the β-helix.[21] We anchored catalytic metal complexes on the surface of $[(gp5\beta f)_3]_2$ by chemical modification of Lys and Cys residues.[21,23] This indicates that $[(gp5\beta f)_3]_2$ can serve as an ideal host protein for catalytic reactions with Sc^{3+} ion.

Results and discussion

The Sc^{3+} binding site was created on the surface of $[(gp5\beta f)_3]_2$ by the introduction of a cysteine residue to conjugate a 2,2′-bipyridine (bpy) moiety using the thiol–maleimide coupling reaction (Fig. 1b). A previously reported Sc^{3+} ligand containing bpy and two –ROH groups, [bpy–$(ROH)_2$], was employed as a model to coordinate the Sc^{3+} ion in a cooperative manner.[24–27] Tetradentate coordination of the bpy and two –ROH groups to the Sc^{3+} ion is essential to produce the complex, Sc[bpy–$(ROH)_2$], which is capable of catalyzing the epoxide ring-opening reaction.[24,27] Hydroxy protons of the –ROH moieties serve an essential role for the high catalytic activity.[27] The two –ROH groups can be altered by alcohol groups of Thr or Ser residues of $[(gp5\beta f)_3]_2$ because the alcohols (pK_a ~13) retain –ROH form under the catalytic conditions. The crystal structure of Sc[bpy–$(ROH)_2$] shows that the O–O distance between the two –ROH groups is 4.2 Å.[25] We expect that a similar coordination structure can be reconstructed with pairs of T21/T29, T45/T53, and T53/S61 of $[(gp5\beta f)_3]_2$ which have O–O distances of 4.2, 5.0, and 4.9 Å, respectively (Fig. 2). We introduced cysteine residues by site-directed mutagenesis at G18, L47 and N51. These sites are geometrically adjacent to the alcohol pairs. The introduced cysteine residues were selectively conjugated with bpy because $[(gp5\beta f)_3]_2$ itself has no cysteine residues. Three cysteine mutants $[(gp5\beta f\_X)_3]_2$ (X = G18C, L47C, N51C) were prepared for conjugation of the 2,2′-biprydine-maleimide derivative (bpy-MI) (Fig. 1b).

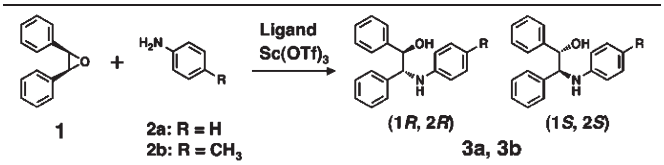
Modification of $[(gp5\beta f\_X)_3]_2$ (X = G18C, L47C, N51C) with bpy-MI was carried out using our previously reported method.[23] A dimethylsulfoxide solution of bpy-MI (6 mM, 2.2 mL) was added to $[(gp5\beta f\_G18C)_3]_2$ (5.3 μM, 41.4 mL) in 20 mM potassium phosphate buffer pH 7.0. After stirring for 15 h at 25 °C, the mixture was purified with Sephadex G-25 (10 mM MOPS buffer pH 7.5). **G18C_bpy** was obtained with an isolated yield of ~100%. The MALDI-TOF mass spectrum of **G18C_bpy** shows a mass increment of 279 Da, which is assigned to bpy-MI (calcd 279 Da), with complete loss of the peak of the original gp5βf_G18C monomer (14 842 Da) (Fig. S1†). The result indicates that all of the Cys residues of **G18C_bpy** were conjugated with bpy-MI molecules. The tubular structure of **G18C_bpy** was confirmed by gel permeation chromatography (GPC). The elution profile of **G18C_bpy** had a peak at 13.5 min. This peak is the same as that of $[(gp5\beta f\_G18C)_3]_2$ (Fig. S2†). This indicates that the tubular assembly structure was maintained even after conjugation of bpy-MI. These results show that six bpy-MI units were completely conjugated to six cysteine residues of $[(gp5\beta f\_G18C)_3]_2$ which retains its original tubular folding. **L47C_bpy** and **N51C_bpy** were also prepared with a ~100% modification yield

and retaining the tubular folding under the same conditions (Fig. S1†). The MALDI-TOF mass spectrum of **G18C_bpy** with $Sc(OTf)_3$ shows new peaks assigned to adducts of a single Sc^{3+} ion to the **G18C_bpy** monomer, although no such new peaks were observed when $Sc(OTf)_3$ was added to unmodified $[(gp5\beta f)_3]_2$ under same conditions (Fig. S3†). These results indicate that a single Sc^{3+} ion can coordinate to the bpy moiety on **G18C_bpy** without any random binding of Sc^{3+} ions on the surface of **G18C_bpy**.

To evaluate the catalytic activity of **X_bpy** (X = G18C, L47C, N51C) with Sc^{3+}, ring-opening reactions of *cis*-stilbene oxide (1) with aniline derivatives (**2a**, **2b**) were examined in 10 mM MOPS buffer pH 7.5, 10% acetonitrile for 48 h at 40 °C in the dark and under an Ar atmosphere. Conversion yields were determined by ^{1}H-NMR. **G18C_bpy** shows the highest rate of conversion of aniline (**2a**) among the reactions listed in Table 1 (42%, entry 1). This value is more than 20 times higher than that of the mixtures of $[(gp5\beta f)_3]_2$/2,2′-bipyridine (entry 5) and L-threonine/2,2′-bipyridine (entry 6). The rates of conversions by **L47C_bpy** (entry 2) and **N51C_bpy** (entry 3) were found to be 3 and 5 times lower, respectively, than that of **G18C_bpy** (entry 1). **G18C_bpy** also catalyzed the reaction with the aniline derivative *p*-toluidine (**2b**) with a conversion rate comparable to that of **2a** (entries 1 and 4). Enantioselectivities were determined by HPLC using a Chiralcel AD column. **G18C_bpy** was found to have higher ee selectively ((1*R*, 2*R*), 17%, entry 1), than the ee selectivity values of **L47C_bpy** ((1*S*, 2*S*), 9%, entry 2) and **N51C_bpy** ((1*S*, 2*S*), 1%, entry 3).

G18C_bpy has higher catalytic activity than that of a mixture of each of the ligands (entry 1 *vs.* entries 5 and 6, Table 1). This demonstrates the advantage of the approach. It is estimated that $Sc(bpy)(Thr)_2$ complex is not stably formed by simple mixing of bpy and L-threonine in water (entry 6). This observation is consistent with our previous report that a flavin ligand anchored on $[(gp5\beta f)_3]_2$ stabilizes the Cu^+-flavin structure.[21] These results clearly indicate that spatially adjacent bpy and two Thr residues on **G18C_bpy** coordinate the Sc^{3+} ion in a cooperative manner

Table 1 Results of Sc^{3+} catalytic ring-opening reaction



Entry	Ligands	Substrates	Conversion/%	ee/%
1	**G18C_bpy**	**2a**	42	17(1*R*, 2*R*)
2	**L47C_bpy**	**2a**	13	9 (1*S*, 2*S*)
3	**N51C_bpy**	**2a**	8	1 (1*S*, 2*S*)
4	**G18C_bpy**	**2b**	36	0.5 (1*R*, 2*R*)
5	$[(gp5\beta f)_3]_2$/2,2′-bipyridine	**2a**	Trace	—
6	L-Threonine/2,2′-bipyridine	**2a**	2	0.4 (1*R*, 2*R*)

Reaction conditions: [2,2′-bipyridine] = 0.20 mM, $[[(gp5\beta f)_3]_2]$ = 33 μM, $[Sc(OTf)_3]$ = 0.20 mM, [L-threonine] = 1.0 mM, [1] = 4.0 mM, [**2a**, **2b**] = 4.0 mM in 10 mM MOPS buffer pH 7.5, 10% acetonitrile, under dark and Ar, 40 °C, 48 h. Each entry was examined three times.

between bpy and the residues to act the ion as a Lewis acid to activate the epoxide for nucleophilic attack. With respect to the catalytic reactions in aqueous solution, the conversion by **G18C_bpy** (42%) is higher than that by a synthetic ligand reported previously (15%),[26] although the higher conversion of the synthetic ligand was achieved in CH_2Cl_2 (95%).[24] The reaction catalyzed by the previously reported synthetic Sc^{3+} complex was accelerated in water by utilization of $Sc(DS)_3$ (DS = dodecyl sulfate) instead of $Sc(OTf)_3$ due to the formation of a colloidal dispersion of DS that accumulates catalysts and confines substrates to the inside.[26] In the case of **G18C_bpy**, factors responsible for the higher conversion are thought to be the increase in both coordination stability of the Sc^{3+} center and easy accessibility of substrates at the exposed surface of **G18C_bpy** as indicated in our previous report which describes accumulation of substrates at the catalytic center exposed on the surface of $[(gp5\beta f)_3]_2$.[21]

The differences among the catalytic activities of **X_bpy** (X = G18C, L47C, N51C) (entries 1–3, Table 1) are explained by the crystal structure of $[(gp5\beta f)_3]_2$. The high *b*-factor of Cα of G18 (55 Å^2) relative to the *b*-factors of Cα of L47 (23 Å^2) and N51 (22 Å^2) (Fig. S4†) suggests that bpy of **G18C_bpy** has a high level of flexibility. Flexible amino acid residues are known to accommodate demanding conformations that would favor the coordination structure more than rigid residues.[28] It is expected that a bpy conjugated to a cysteine residue at a position 18 can form more suitable Sc^{3+} coordination structure for the catalytic reaction than that at the other positions although all the cysteine residues of the mutants are located at the positions adjacent to two threonine or serine residues which are enable to bind to a Sc^{3+} ion with a bpy ligand as that of a synthetic $Sc[bpy\text{-}(ROH)_2]$ (Fig. 2).[25] The combination of appropriate location of Thr or Ser residues and a bpy moiety anchored to a flexible cysteine residue is essential for the creation of the semi-synthesized Sc^{3+} center on **G18C_bpy**.

Conclusions

A Sc^{3+} binding site was semi-synthesized by chemical modification to place 2,2′-bipyridine in a position adjacent to two alcohol groups of Thr aligned on surface of the β-helical tubular protein. The Sc^{3+} binding site can accelerate the rate of catalysis of the epoxide ring-opening reaction. The high activity was accomplished by taking advantage of the cooperative effect of stabilizing the Sc^{3+} coordination and accumulation of substrates on the protein surface. This is the first example of construction of an artificial Sc^{3+} enzyme by cooperative coordination of bpy and threonine residues. Although there is still scope for improvement of catalytic activity and selectivity, this work indicates that the semi-synthetic approach is one of the useful strategies for construction of new artificial metalloenzymes. We are undergoing further optimization of the artificial enzyme.

Experimental

Physical measurement

UV-vis spectra were recorded on a SHIMADZU UV-2400PC UV-vis spectrometer. ^{1}H-NMR spectra were recorded on a

Bruker UltraShield™ 500 Plus spectrometer. MALDI-TOF mass spectra were recorded on an Autoflex III (Bruker Daltonics).

Materials

Unless otherwise stated, all chemicals were purchased from commercial suppliers and used without further purification. 4-ethylmaleimide-2,2′-bipyridine (bpy-MI) were prepared according to the literature methods.[23] Cys mutants of $[(gp5\beta f)_3]_2$ were prepared by using a Quick Change site-directed mutagenesis kit (Qiagen). DNA sequences of the mutants were determined by an ABI3100 (Applied Biosystems). Expression and purification of the mutants were performed as described previously.[21,23] In the measurement of MALDI-TOF, samples were dialyzed against 5 mM ammonium acetate aqueous solution and then mixed with an equal volume of 70% v/v acetonitrile–water solution containing 0.03% w/v sinapic acid and 0.1% v/v trifluoroacetic acid. GPC was carried out with a HPLC system and columns (Asahipack GF-510HQ, Shodex, Tokyo, Japan), at room temperature with 20 mM Tris buffer pH 8.0 as the eluent. HPLC analyses to determine enantioselectivities were performed with a chiral stationary phase column (Chiralcel AD purchased from Dicel Co., Ltd).

Modification of cysteines with bpy-MI

G18C_bpy; A DMSO solution of **bpy-MI** (6 mM, 2.2 mL) was slowly added to an aqueous solution of $[(gp5\beta f\_G18C)_3]_2$ (5.3 μM, 41.4 mL in 20 mM potassium phosphate buffer pH 7.0) and the mixture was gently stirred at 25 °C for 15 h. **G18C_bpy** was purified by Sephadex G-25 equilibrated with 10 mM MOPS buffer pH 7.5. **L47C_bpy** and **N51C_bpy** were prepared by the same procedure. The protein recovery after the modification was ~100%. UV/Vis: λ_{max} 283 nm ($\varepsilon \cdot M^{-1}$ cm^{-1} 255 000). MS (MALDI-TOF) (Fig. S1†): **G18C_bpy** monomer $[gp5\beta f\_G18C + bpy\text{-}MI]^+$, calcd,15 118; found, 15121, **L47C_bpy** monomer $[gp5\beta f\_L47C + bpy\text{-}MI]^+$, calcd,15 062; found, 15076, **N51C_bpy** monomer $[gp5\beta f\_N51C + bpy\text{-}MI]^+$, calcd,15 061; found,15 065.

Ring-opening reaction of *meso*-epoxide

Under Ar atmosphere, to a solution of $Sc(OTf)_3$ (90 μL, 1.2×10^{-7} mol) **X_bpy** (450 μL, 1.2×10^{-7} mol of 2,2′-bipyridine) or L-threonine (450 μL, 6.0×10^{-7} mol) in 10 mM MOPS buffer pH 7.5 were added. Subsequently acetonitrile (30 μL) or 2,2′-bipyridine (30 μL, 1.2×10^{-7} mol) in acetonitrile were added. After addition of *cis*-stilbene oxide (1) (15 μL, 2.4×10^{-6} mol) and aniline derivative (**2a**, **2b**) (15 μL, 2.4×10^{-6} mol) in acetonitrile, the mixture was incubated for 48 h at 40 °C with stirring under dark and Ar atmosphere. The substrates and products were extracted with ethyl acetate (1 mL) three times after addition of saturated $NaHCO_3$ aqueous solution (1 mL). Conversions of **2a** and **2b** to **3a** and **3b**, respectively, and enantioselectivities of **3a**, **3b** were determined by ^{1}H-NMR and HPLC analysis, respectively, as reported previously.[24,26,29]

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