Electronic Supplementary Information

Reconstitution of Full-Length P450BM3 with an Artificial Metal Complex by Utilising the Transpeptidase Sortase A

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Material & Methods

All chemicals and ingredients for expression media, unless otherwise indicated, were obtained from Nacalai tesque, Wako Pure Chemicals Industries, and Kanto Chemical. d-desthiobiotin was purchased from Sigma-Aldrich. Mn(III) Protoporphyrin IX chloride was purchased from Frontier Scientific. Cellufine A-500 was obtained from JNC Corporation. TOYOPEARL DEAE-650S was obtained from Tosoh Corporation. Sephacryl S-200 HR and StrepTrap HP was obtained from GE healthcare. Proteins were purified by using Bioassist eZ (Tosoh). UV-Vis spectra were recorded by Shimadzu UV-2600 PC spectrophotometer. ICP-OES was measured by Varian VISTA-PRO. GC-MS analysis was performed with Shimadzu GCMS-QP2010 SE equipped with a Rtx-1 column fused silica (RESTEK, 60 m, 0.32 μm).

Experimental Section

Preparation of BM3 mutants

- Protein Expression

A single colony of BL21 (DE3) transformed with pET11a_BM3-mutants was inoculated into 100 mL of LB medium containing 100 mg L^{-1} ampicillin and incubated at 37°C, 120 rpm overnight. 30 mL of the culture was added to 2 L of LB medium containing 100 mg L^{-1} ampicillin and incubated at 37°C, 105 rpm. After the OD600 reached approximately 0.7, 0.5 mM 5-aminolaevulinic acid was added. After the OD_{600} reached 0.8–1.1, protein expression was induced with 1 mM IPTG and incubated for a further 6 hr. The cells were harvested by centrifugation at 5500 rpm for 20 min, and the pellets were stored at -80°C.

- Protein Purification

The pellets were resuspended in Buffer A (20 mM Tris-HCl, pH 7.4 at 4°C, containing 100 μM EDTA), and then the cell suspension was homogenized using an ultrasonicator. Cell debris was removed by centrifugation at 17,500 rpm, and the resulting cell lysate was filtered by MF-Millipore membrane using aspirator. The filtered lysate was loaded onto an equilibrated anion-exchange column Cellufine

A-500. After the column was washed with Buffer A, adsorbed BM3 mutants were eluted in Buffer B (Buffer A containing 250 mM KCl) at 4°C. The BM3 mutants were concentrated by Amicon Ultra 30 K NMWL (Merck Millipore Corporation) and then loaded onto DEAE-650S. The BM3 mutants bound to DEAE-650S was eluted by a KCl gradient from 80 to 150 mM. The fraction containing the BM3 mutants was concentrated by Amicon Ultra 30 K NMWL and then further purified by gel filtration on a Sephacryl S-200 column at 4°C equilibrated with Buffer C (20 mM Tris-HCl buffer, pH 7.4 at 25°C, 100 mM KCl). The concentration of the purified BM3 mutants was determined based on the absorbance of pyridine ferrohaemochrome (ε_{557} = 34.7 $mM^{-1}cm^{-1}$)[1].

Preparation of haem domain

- Protein Expression

Glycerol stock of BL21 (DE3) transformed with pET11a_heme-domain-LPATGG was inoculated into 100 mL of LB medium containing 100 mg L^{-1} ampicillin and incubated at 37°C, 120 rpm overnight. 30 mL of the culture was added to 2 L of LB medium containing 100 mg L^{-1} ampicillin and incubated at 37°C, 105 rpm. After the OD_{600} reached about 0.7, 0.5 mM 5-aminolaevulinic acid was added. After the OD_{600} reached 0.8-1.1, 1 mM IPTG was added and the incubation was continued for 6 hr. The cells were harvested by centrifugation at 5,500 rpm for 20 min, and the pellets were stored at -80°C.

- Protein Purification

The pellets were resuspended in Buffer A+DTT (20 mM Tris-HCl, pH 7.4 at 4°C, 100 μM EDTA, 1 mM dithiothreitol), and purified in the same manner as BM3 mutants except the use of Buffer A+DTT in place of Buffer A. The concentration of the purified haem domain was determined based on the absorbance of pyridine ferrohaemochrome.

Preparation of reductase domain

- Protein expression

Glycerol stock of BL21 (DE3) transformed with pET28a_GG-reductase domain was inoculated into 100 mL of LB medium containing 20 mg L^{-1} kanamycin and incubated at 37°C , 120 rpm overnight. 30 mL of the culture was added to 2 L of LB medium containing 20 mg L^{-1} kanamycin and incubated at 37°C, 105 rpm. After the OD_{600} reached 1.15, 1 mM IPTG was added and the incubation was continued for 6 hr. The cells were harvested by centrifugation at 5,500 rpm for 20 min, and the pellets were stored at -80°C

- Protein purification

The pellets were resuspended in Buffer A+DTT (20 mM Tris-HCl, pH 7.4 at 4°C, 100 μM EDTA, 1 mM dithiothreitol), and purified in the same manner as haem domain. The concentration of the purified reductase domain was determined based on the absorbance of flavins (ε_{456} = 21.4 mM^{-1} cm^{-1})[2]

Preparation of SrtA

We used the pentamutant of SrtA P94R/D160N/D165A/K190E/K196T, to obtain the ligated BM3 efficiently, which was reported to exhibit 120-fold higher catalytic activity than wild-type SrtA[3].

- Protein expression

A single colony of *E. coli* strain M15 transformed with pQE30+6His-SrtA (pentamutant) was inoculated into 100 mL of LB medium containing 100 mgL^{-1} ampicillin and 20 mg L^{-1} kanamycin and incubated at 37°C, 120 rpm, overnight. 100 mL of the culture was added to 6 L of LB medium containing 100 mg L^{-1} ampicillin and 20 mg L^{-1} kanamycin and then incubated at 37°C, 105 rpm. After the OD_{600} reached 0.8-1.1, 1 mM IPTG was added and the incubation was continued for 6 hr. The cells were harvested by centrifugation at 5,500 rpm for 20 min, and the pellets were stored at -80°C.

- Protein purification

The pellets were resuspended in 50 mM Tris-HCl buffer (pH 8.0 at 4°C) containing 250 mM NaCl and 1 mM dithiothreitol, and then the cell suspension was homogenized by using an ultrasonicator. Cell debris was removed by centrifugation at 17,500 rpm, and the resulting cell lysate was filtered. The filtered lysate was loaded onto Ni-affinity column. After the column was washed with 50 mM Tris-HCl buffer (pH 8.0 at 4°C) containing 250 mM NaCl and 1 mM dithiothreitol, adsorbed SrtA was eluted by 50 mM Tris-HCl buffer containing 250 mM NaCl, 1 mM dithiothreitol and 30–500 mM imidazole. The eluted SrtA was dialyzed to remove imidazole. The concentration of SrtA was determined based on the absorbance at 280 nm (ε_{280} = 14.44 $mM^{-1}cm^{-1}$)[4].

Preparation of haem-substituted haem domain

- Protein expression

Glycerol stock of BL21 (DE3) transformed with pET11a+BM3-mutants was inoculated into 100 mL of M9 minimal medium supplemented with 100 mM glucose, 0.2% casamino acid, and 100 mgL^{-1} ampicillin and incubated at 37°C, 120 rpm, overnight. 100 mL of the culture was added to 6 L of M9 medium containing additives described above and incubated at 37°C, 105 rpm. After the OD_{600} reached 0.8–1.1, 1 mM IPTG was added and cooled down to 27°C, and then the incubation was continued for 15 hr. The cells were harvested by centrifugation at 5,500 rpm for 20 min, and the white pellets were stored at -80°C.

- Reconstitution and purification

The pellets were resuspended in Buffer A containing 1 mg of metal complex per 1 g wet weight of *E. coli* cell pellets. The cell suspension was homogenized by using an ultrasonicator. After a purification with Cellufine A-500, small amount of haem-binding haem domain was removed by DEAE-650S. Haem-substituted haem domain was further purified by a gel filtration column Sephacryl S-200, eluted at 4°C with Buffer C. Purified haem-substituted haem domain was analyzed by ICP-OES and a significant quantity of iron was not observed. The concentration of haem-substituted haem domain

was determined by BCA assay.

SrtA-mediated ligation reaction and the following purification

The reaction was performed in 50 mM Tris-HCl buffer (pH 8.0 at 25°C) containing 150 mM NaCl and 10 mM $CaCl_2$. 10 μM Haem domain and 30 μM reductase domain were added to the reaction solution, and then ligation reaction was initiated by the addition of 10μM SrtA and incubated at 4°C for approximately 15 hr. The reaction solution was loaded onto a Strep-Tactin column and eluted with 2.5 mM desthiobiotin. Elute was concentrated by Amicon Ultra 30 K NMWL and then further purified by gel filtration on a Sephacryl S-200 column, equilibrated with Buffer C at 4°C. The concentration of reconstituted BM3 was determined by BCA assay using wild-type full-length BM3 solution of known-concentration as a standard protein solution.

Crystallization of Mn-substituted haem domain

Purified Mn-substituted haem domain was concentrated to 19 mg mL^{-1}. Crystallization was performed by using the sitting-drop vapor-diffusion method. The reservoir solution contained 12% PEG3350, 100 mM $MgCl_2$ and 100 mM MES (pH 6.0). The crystallization drop was prepared by mixing a 1 μL protein solution and a 1 μL reservoir solution. The crystallization plate was incubated at 20°C for a month.

Data collection and refinement

After crystal was soaked in 4:6 glycerol: reservoir solution for a couple of seconds, the crystal was flash-cooled at 100 K in nitrogen. 1.00 Å and 1.75 Å X-ray diffraction data sets were collected on beam line BL26B2 that was equipped RAYONIX MX225-HS at RIKEN SPring-8 (Hyogo, Japan). The diffraction intensities of 1.00 Å X-ray were integrated by iMosflm[5–7]. Aimless program[8] scaled and merged multiple observations of diffraction peaks into an average intensity. The initial phase of the Mn-substituted haem domain was obtained using MOLREP[9]. The protein model of Mn-substituted haem domain was improved using REFMAC5[10], phenix.refine[11], and COOT[12]. TLS refinement[13] was performed at final stage of the refinement by defining the 15 separate TLS groups (chain A: 4-158, 159-250, 251-455, chain B: 6-108, 109-224, 225-312, 313-454, chain C: 4-108, 109-324, 325-455, chain D: 5-72, 73-198, 199-282, 283-362, 363-455). The resulting model (PDB cord: 5ZIS) had a final R_{work} of 25.9% and an R_{free} of 29.3%. The anomalous diffraction intensities of 1.75 Å X-ray, which is between Mn absorption edge (1.90 Å) and Fe absorption edge (1.74 Å), were integrated and scaled by HKL2000[14]. The protein model for the anomalous map (PDB cord: 5ZLH) was rigid body refinement with occupancies refinement and individual B-factor refinement under the reference model restraints and secondary structure restraints using phenix.refine[11]. The difference of anomalous (DANO) map was calculated by CAD and FFT.

Table 1 Data reduction and final structural refinement statistics for Mn-substituted heam domain

PDB Cord	5ZIS	5ZLH
Data collection		
Wave length (Å)	1.00	1.75
Resolution (Å)	46.83-3.10 (3.27-3.10) *	50.00-3.40 (3.52-3.40) *
Space group	$C\ 2\ 2\ 2_1$	$C\ 2\ 2\ 2_1$
Unit cell		
a b c (Å)	104.76, 155.32, 208.98	106.36, 155.92, 211.07
$\alpha\ \beta\ \gamma$ (°)	90, 90, 90	90, 90, 90
R_{marge}	0.127	0.134
$I/\sigma(I)$	9.9 (1.8)	14.4 (1.8)
$CC_{1/2}$	(0.580)	(0.922)
Completeness (%)	99.1 (98.9)	100 (100)
Redundancy	7.3 (7.4)	7.4 (7.4)
Wilson B (Å)	85.50	48.00
Refinement		
Resolution (Å)	19.9-3.10	19.9-3.40
No. reflections	30757	22590
R_{work}/R_{free}	0.2587/0.2927	0.3103/0.3462
No. of atoms		
Protein	14550	14550
Ligand	172	172
Water	0	0
RMS deviations		
Bond lengths (Å)	0.004	0.004
Bond angles (°)	0.801	0.826

*Values in parentheses are for the highest-resolution shell., RMS means root mean square

Synthesis of 10-(4-nitrophenoxy)decanoic acid (10-pNCA)

10-pNAC was synthesized based on the previously-reported procedure[15]. 1.1 g of ethyl 10-bromodecanoate, 530 mg of p-nitrophenol, and 530 mg of potassium carbonate were dissolved in 14 mL of dimethyl sulphide and refluxed for 4 hr at 120°C. Products were precipitated by addition of water. The precipitate was collected by suction filtration and dried *in vacuo*. 820 mg of solid was dissolved in 10 mL of acetone and then 10 mL of 1 M NaOH was added. After evaporation to remove acetone, the solution was acidified to pH 3 by 1 M HCl. Precipitate was collected by suction filtration and dried in vacuo. White solid was recrystallized with chloroform and hexane. 1H NMR (CDCl3): δ=11.3 ppm(s, 1H), 8.20 ppm(d, 2H), 6.94 ppm(d, 2H), 4.05 ppm(t, 2H), 2.36 ppm(t, 2H), 1.82 ppm(q, 2H,), 1.64 ppm (m, 2H), 1.47 ppm (m, 2H), 1.33 ppm (m, 8H).

Evaluation of the catalytic activity of BM3s

・pNCA assay

0.5 μM BM3 and 50 μM 10-pNCA were dissolved in 100 mM Tris-HCl buffer (pH 8.2 at 25°C). The reaction was initiated by the addition of NADPH (final concentration; 200 μM), and *p*-nitrophenolate formation was monitored by absorption at 410 nm. The initial rate of this reaction was calculated based on the extinction coefficient ε_{410} = 13.2 $mM^{-1}cm^{-1}$ (pH 8.2)[16].

・propane oxidation

Reaction was conducted based on previously reported procedure[17]. O_2-saturated Buffer C and propane-saturated Buffer C were prepared on ice and adjusted at the ratio of 2:8. 0.5 μM BM3, 100 μM PFC9Trp were dissolved in 1 mL of O_2/propane buffer. The reaction was initiated by the addition of NADPH (final concentration; 5mM) and incubated at r.t. After 10 min, the reaction solution was aliquoted into 500 μL and quenched with 500 μL of 1-butanol. To this mixture, 5 μL of 10 mM 3-pentanol was added as an internal standard. The obtained organic phase was analyzed by using GC-MS and the concentration of propanol was determined from the ratio of propanol and internal standard.

peptide sequence

Wildtype BM3 (full-length)

MTIKEMPQPKTFGELKNLPLLNTDKPVQALMKIADELGEIFKFEAPGRVTRYLSSQRLIKEACDESRFDK
NLSQALKFVRDFAGDGLFTSWTHEKNWKKAHNILLPSFSQQAMKGYHAMMVDIAVQLVQKWERLNADEHI
EVPEDMTRLTLDTIGLCGFNYRFNSFYRDQPHPFITSMVRALDEAMNKLQRANPDDPAYDENKRQFQEDI
KVMNDLVDKIIADRKASGEQSDDLLTHMLNGKDPETGEPLDDENIRYQIITFLIAGHETTSGLLSFALYF
LVKNPHVLQKAAEEAARVLVDPVPSYKQVKQLKYVGMVLNEALRLWPTAPAFSLYAKEDTVLGGEYPLEK
GDELMVLIPQLHRDKTIWGDDVEEFRPERFENPSAIPQHAFKPFGNGQRACIGQQFALHEATLVLGMMLK
HFDFEDHTNYELDIKETLTLKPEGFVVKAKSKKIPLGGIPSPSTEQSAKKVRKKAENAHNTPLLVLYGSN
MGTAEGTARDLADIAMSKGFAPQVATLDSHAGNLPREGAVLIVTASYNGHPPDNAKQFVDWLDQASADEV
KGVRYSVFGCGDKNWATTYQKVPAFIDETLAAKGAENIADRGEADASDDFEGTYEEWREHMWSDVAAYFN
LDIENSEDNKSTLSLQFVDSAADMPLAKMHGAFSTNVVASKELQQPGSARSTRHLEIELPKEASYQEGDH
LGVIPRNYEGIVNRVTARFGLDASQQIRLEAEEEKLAHLPLAKTVSVEELLQYVELQDPVTRTQLRAMAA
KTVCPPHKVELEALLEKQAYKEQVLAKRLTMLELLEKYPACEMKFSEFIALLPSIRPRYYSISSSPRVDE
KQASITVSVVSGEAWSGYGEYKGIASNYLAELQEGDTITCFISTPQSEFTLPKDPETPLIMVGPGTGVAP
FRGFVQARKQLKEQGQSLGEAHLYFGCRSPHEDYLYQEELENAQSEGIITLHTAFSRMPNQPKTYVQHVM
EQDGKKLIELLDQGAHFYICGDGSQMAPAVEATLMKSYADVHQVSEADARLWLQQLEEKGRYAKDVWAG

BM3 Mut-1 (full-length)

MTIKEMPQPKTFGELKNLPLLNTDKPVQALMKIADELGEIFKFEAPGRVTRYLSSQRLIKEACDESRFDK
NLSQALKFVRDFAGDGLFTSWTHEKNWKKAHNILLPSFSQQAMKGYHAMMVDIAVQLVQKWERLNADEHI
EVPEDMTRLTLDTIGLCGFNYRFNSFYRDQPHPFITSMVRALDEAMNKLQRANPDDPAYDENKRQFQEDI
KVMNDLVDKIIADRKASGEQSDDLLTHMLNGKDPETGEPLDDENIRYQIITFLIAGHETTSGLLSFALYF
LVKNPHVLQKAAEEAARVLVDPVPSYKQVKQLKYVGMVLNEALRLWPTAPAFSLYAKEDTVLGGEYPLEK
GDELMVLIPQLHRDKTIWGDDVEEFRPERFENPSAIPQHAFKPFGNGQRACIGQQFALHEATLVLGMMLK
HFDFEDHTNYELDIKETLTLKPEGFVVKAKSKKIPLGLPATGGTEQSAKKVRKKAENAHNTPLLVLYGSN
MGTAEGTARDLADIAMSKGFAPQVATLDSHAGNLPREGAVLIVTASYNGHPPDNAKQFVDWLDQASADEV
KGVRYSVFGCGDKNWATTYQKVPAFIDETLAAKGAENIADRGEADASDDFEGTYEEWREHMWSDVAAYFN
LDIENSEDNKSTLSLQFVDSAADMPLAKMHGAFSTNVVASKELQQPGSARSTRHLEIELPKEASYQEGDH
LGVIPRNYEGIVNRVTARFGLDASQQIRLEAEEEKLAHLPLAKTVSVEELLQYVELQDPVTRTQLRAMAA
KTVCPPHKVELEALLEKQAYKEQVLAKRLTMLELLEKYPACEMKFSEFIALLPSIRPRYYSISSSPRVDE
KQASITVSVVSGEAWSGYGEYKGIASNYLAELQEGDTITCFISTPQSEFTLPKDPETPLIMVGPGTGVAP
FRGFVQARKQLKEQGQSLGEAHLYFGCRSPHEDYLYQEELENAQSEGIITLHTAFSRMPNQPKTYVQHVM

EQDGKKLIELLDQGAHFYICGDGSQMAPAVEATLMKSYADVHQVSEADARLWLQQLEEKGRYAKDVWAG

BM3 Mut-2 (full-length)

MTIKEMPQPKTFGELKNLPLLNTDKPVQALMKIADELGEIFKFEAPGRVTRYLSSQRLIKEACDESRFDK
NLSQALKFVRDFAGDGLFTSWTHEKNWKKAHNILLPSFSQQAMKGYHAMMVDIAVQLVQKWERLNADEHI
EVPEDMTRLTLDTIGLCGFNYRFNSFYRDQPHPFITSMVRALDEAMNKLQRANPDDPAYDENKRQFQEDI
KVMNDLVDKIIADRKASGEQSDDLLTHMLNGKDPETGEPLDDENIRYQIITFLIAGHETTSGLLSFALYF
LVKNPHVLQKAAEEAARVLVDPVPSYKQVKQLKYVGMVLNEALRLWPTAPAFSLYAKEDTVLGGEYPLEK
GDELMVLIPQLHRDKTIWGDDVEEFRPERFENPSAIPQHAFKPFGNGQRACIGQQFALHEATLVLGMMLK
HFDFEDHTNYELDIKETLTLKPEGFVVKAKSKKIPLGGIPLPSTGGSAKKVRKKAENAHNTPLLVLYGSN
MGTAEGTARDLADIAMSKGFAPQVATLDSHAGNLPREGAVLIVTASYNGHPPDNAKQFVDWLDQASADEV
KGVRYSVFGCGDKNWATTYQKVPAFIDETLAAKGAENIADRGEADASDDFEGTYEEWREHMWSDVAAYFN
LDIENSEDNKSTLSLQFVDSAADMPLAKMHGAFSTNVVASKELQQPGSARSTRHLEIELPKEASYQEGDH
LGVIPRNYEGIVNRVTARFGLDASQQIRLEAEEEKLAHLPLAKTVSVEELLQYVELQDPVTRTQLRAMAA
KTVCPPHKVELEALLEKQAYKEQVLAKRLTMLELLEKYPACEMKFSEFIALLPSIRPRYYSISSSPRVDE
KQASITVSVVSGEAWSGYGEYKGIASNYLAELQEGDTITCFISTPQSEFTLPKDPETPLIMVGPGTGVAP
FRGFVQARKQLKEQGQSLGEAHLYFGCRSPHEDYLYQEELENAQSEGIITLHTAFSRMPNQPKTYVQHVM
EQDGKKLIELLDQGAHFYICGDGSQMAPAVEATLMKSYADVHQVSEADARLWLQQLEEKGRYAKDVWAG

BM3 Mut-3 (full-length)

MTIKEMPQPKTFGELKNLPLLNTDKPVQALMKIADELGEIFKFEAPGRVTRYLSSQRLIKEACDESRFDK
NLSQALKFVRDFAGDGLFTSWTHEKNWKKAHNILLPSFSQQAMKGYHAMMVDIAVQLVQKWERLNADEHI
EVPEDMTRLTLDTIGLCGFNYRFNSFYRDQPHPFITSMVRALDEAMNKLQRANPDDPAYDENKRQFQEDI
KVMNDLVDKIIADRKASGEQSDDLLTHMLNGKDPETGEPLDDENIRYQIITFLIAGHETTSGLLSFALYF
LVKNPHVLQKAAEEAARVLVDPVPSYKQVKQLKYVGMVLNEALRLWPTAPAFSLYAKEDTVLGGEYPLEK
GDELMVLIPQLHRDKTIWGDDVEEFRPERFENPSAIPQHAFKPFGNGQRACIGQQFALHEATLVLGMMLK
HFDFEDHTNYELDIKETLTLKPEGFVVKAKSKKIPLGLPATGGIPSPSTEQSAKKVRKKAENAHNTPLLV
LYGSNMGTAEGTARDLADIAMSKGFAPQVATLDSHAGNLPREGAVLIVTASYNGHPPDNAKQFVDWLDQA
SADEVKGVRYSVFGCGDKNWATTYQKVPAFIDETLAAKGAENIADRGEADASDDFEGTYEEWREHMWSDV
AAYFNLDIENSEDNKSTLSLQFVDSAADMPLAKMHGAFSTNVVASKELQQPGSARSTRHLEIELPKEASY
QEGDHLGVIPRNYEGIVNRVTARFGLDASQQIRLEAEEEKLAHLPLAKTVSVEELLQYVELQDPVTRTQL
RAMAAKTVCPPHKVELEALLEKQAYKEQVLAKRLTMLELLEKYPACEMKFSEFIALLPSIRPRYYSISSS
PRVDEKQASITVSVVSGEAWSGYGEYKGIASNYLAELQEGDTITCFISTPQSEFTLPKDPETPLIMVGPG
TGVAPFRGFVQARKQLKEQGQSLGEAHLYFGCRSPHEDYLYQEELENAQSEGIITLHTAFSRMPNQPKTY

VQHVMEQDGKKLIELLDQGAHFYICGDGSQMAPAVEATLMKSYADVHQVSEADARLWLQQLEEKGRYAKD
VWAG

BM3 Mut-4 (full-length)

MTIKEMPQPKTFGELKNLPLLNTDKPVQALMKIADELGEIFKFEAPGRVTRYLSSQRLIKEACDESRFDK
NLSQALKFVRDFAGDGLFTSWTHEKNWKKAHNILLPSFSQQAMKGYHAMMVDIAVQLVQKWERLNADEHI
EVPEDMTRLTLDTIGLCGFNYRFNSFYRDQPHPFITSMVRALDEAMNKLQRANPDDPAYDENKRQFQEDI
KVMNDLVDKIIADRKASGEQSDDLLTHMLNGKDPETGEPLDDENIRYQIITFLIAGHETTSGLLSFALYF
LVKNPHVLQKAAEEAARVLVDPVPSYKQVKQLKYVGMVLNEALRLWPTAPAFSLYAKEDTVLGGEYPLEK
GDELMVLIPQLHRDKTIWGDDVEEFRPERFENPSAIPQHAFKPFGNGQRACIGQQFALHEATLVLGMMLK
HFDFEDHTNYELDIKETLTLKPEGFVVKAKSKKIPLPATGGIPSPSTEQSAKKVRKKAENAHNTPLLVLY
GSNMGTAEGTARDLADIAMSKGFAPQVATLDSHAGNLPREGAVLIVTASYNGHPPDNAKQFVDWLDQASA
DEVKGVRYSVFGCGDKNWATTYQKVPAFIDETLAAKGAENIADRGEADASDDFEGTYEEWREHMWSDVAA
YFNLDIENSEDNKSTLSLQFVDSAADMPLAKMHGAFSTNVVASKELQQPGSARSTRHLEIELPKEASYQE
GDHLGVIPRNYEGIVNRVTARFGLDASQQIRLEAEEEKLAHLPLAKTVSVEELLQYVELQDPVTRTQLRA
MAAKTVCPPHKVELEALLEKQAYKEQVLAKRLTMLELLEKYPACEMKFSEFIALLPSIRPRYYSISSSPR
VDEKQASITVSVVSGEAWSGYGEYKGIASNYLAELQEGDTITCFISTPQSEFTLPKDPETPLIMVGPGTG
VAPFRGFVQARKQLKEQGQSLGEAHLYFGCRSPHEDYLYQEELENAQSEGIITLHTAFSRMPNQPKTYVQ
HVMEQDGKKLIELLDQGAHFYICGDGSQMAPAVEATLMKSYADVHQVSEADARLWLQQLEEKGRYAKDVW
AG

StrepTag-haem domain-LPATGG

MWSHPQFEKSSGENLYFQGTIKEMPQPKTFGELKNLPLLNTDKPVQALMKIADELGEIFKFEAPGRVTRY
LSSQRLIKEACDESRFDKNLSQALKFVRDFAGDGLFTSWTHEKNWKKAHNILLPSFSQQAMKGYHAMMVD
IAVQLVQKWERLNADEHIEVPEDMTRLTLDTIGLCGFNYRFNSFYRDQPHPFITSMVRALDEAMNKLQRA
NPDDPAYDENKRQFQEDIKVMNDLVDKIIADRKASGEQSDDLLTHMLNGKDPETGEPLDDENIRYQIITF
LIAGHETTSGLLSFALYFLVKNPHVLQKAAEEAARVLVDPVPSYKQVKQLKYVGMVLNEALRLWPTAPAF
SLYAKEDTVLGGEYPLEKGDELMVLIPQLHRDKTIWGDDVEEFRPERFENPSAIPQHAFKPFGNGQRACI
GQQFALHEATLVLGMMLKHFDFEDHTNYELDIKETLTLKPEGFVVKAKSKKIPLGLPATGG

GG-reductase domain

MGGIPSPSTEQSAKKVRKKAENAHNTPLLVLYGSNMGTAEGTARDLADIAMSKGFAPQVATLDSHAGNLP
REGAVLIVTASYNGHPPDNAKQFVDWLDQASADEVKGVRYSVFGCGDKNWATTYQKVPAFIDETLAAKGA
ENIADRGEADASDDFEGTYEEWREHMWSDVAAYFNLDIENSEDNKSTLSLQFVDSAADMPLAKMHGAFST
NVVASKELQQPGSARSTRHLEIELPKEASYQEGDHLGVIPRNYEGIVNRVTARFGLDASQQIRLEAEEEK
LAHLPLAKTVSVEELLQYVELQDPVTRTQLRAMAAKTVCPPHKVELEALLEKQAYKEQVLAKRLTMLELL
EKYPACEMKFSEFIALLPSIRPRYYSISSSPRVDEKQASITVSVVSGEAWSGYGEYKGIASNYLAELQEG
DTITCFISTPQSEFTLPKDPETPLIMVGPGTGVAPFRGFVQARKQLKEQGQSLGEAHLYFGCRSPHEDYL
YQEELENAQSEGIITLHTAFSRMPNQPKTYVQHVMEQDGKKLIELLDQGAHFYICGDGSQMAPAVEATLM
KSYADVHQVSEADARLWLQQLEEKGRYAKDVWAG

HisTag-SrtA

MRGSHHHHHHSSGLVPRGSMQAKPQIPKDKSKVAGYIEIPDADIKEPVYPGPATREQLNRGVSFAEENES
LDDQNISIAGHTFIDRPNYQFTNLKAAKKGSMVYFKVGNETRKYKMTSIRNVKPTAVGVLDEQKGKDKQL
TLITCDDYNEETGVWETRKIFVATEVK

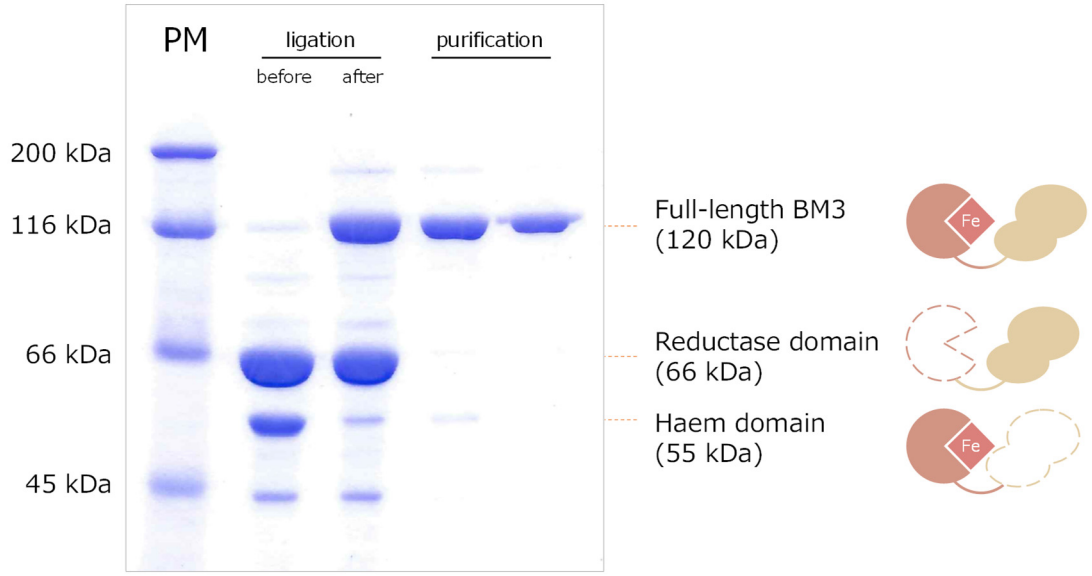


Figure S1. SDS-PAGE before and after protein ligation followed by protein purification. All bands were stained by Coomassie brilliant blue (CBB). Bands are assigned as follows; 55 kDa: haem domain, 66 kDa: reductase domain, 120 kDa: full-length BM3 which appeared after SrtA-mediated ligation.

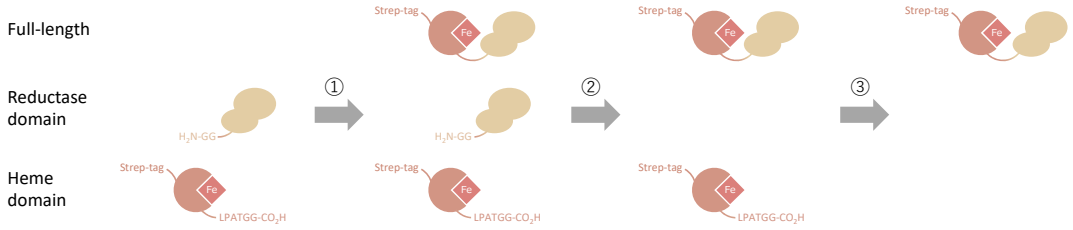
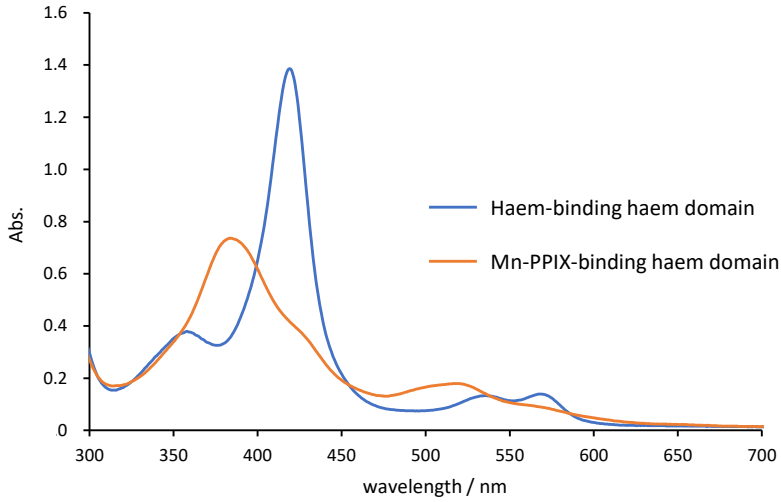


Figure S2. Scheme of SrtA-mediated ligation and protein purification.

① Ligation by SrtA; small amount of haem domain and reductase domain remain to be unreacted, which is removed by following purification.

② Strep-tag purification; reductase domain without Strep-tag is removed in this step and Strep-tagged full-length BM3 and haem domain are recovered.

③ Gel-filtration; haem domain is removed in this step.



	Soret band (nm)	Q band (nm)	Reference
Fe-haem domain	418	537, 567	
Mn-haem domain	385	519	This work
	386	516	[18]
	384	516	[19]

Figure S3. UV-vis spectra of haem-bindingand Mn-PPIX-binding haem domain.

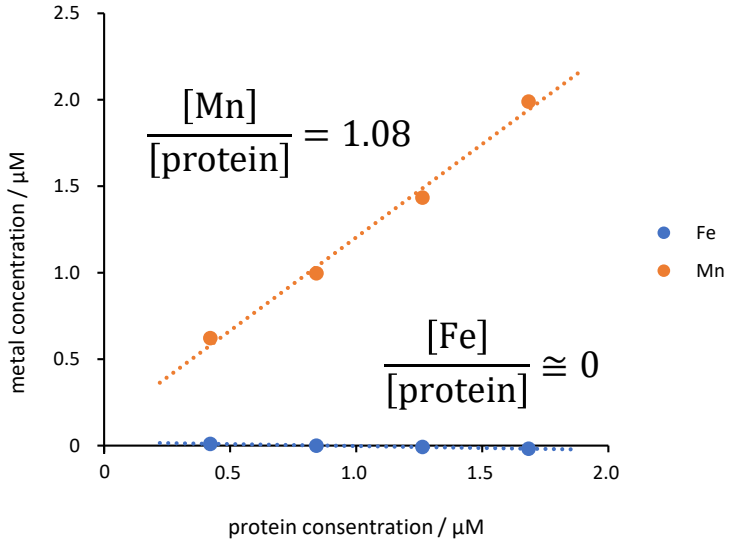
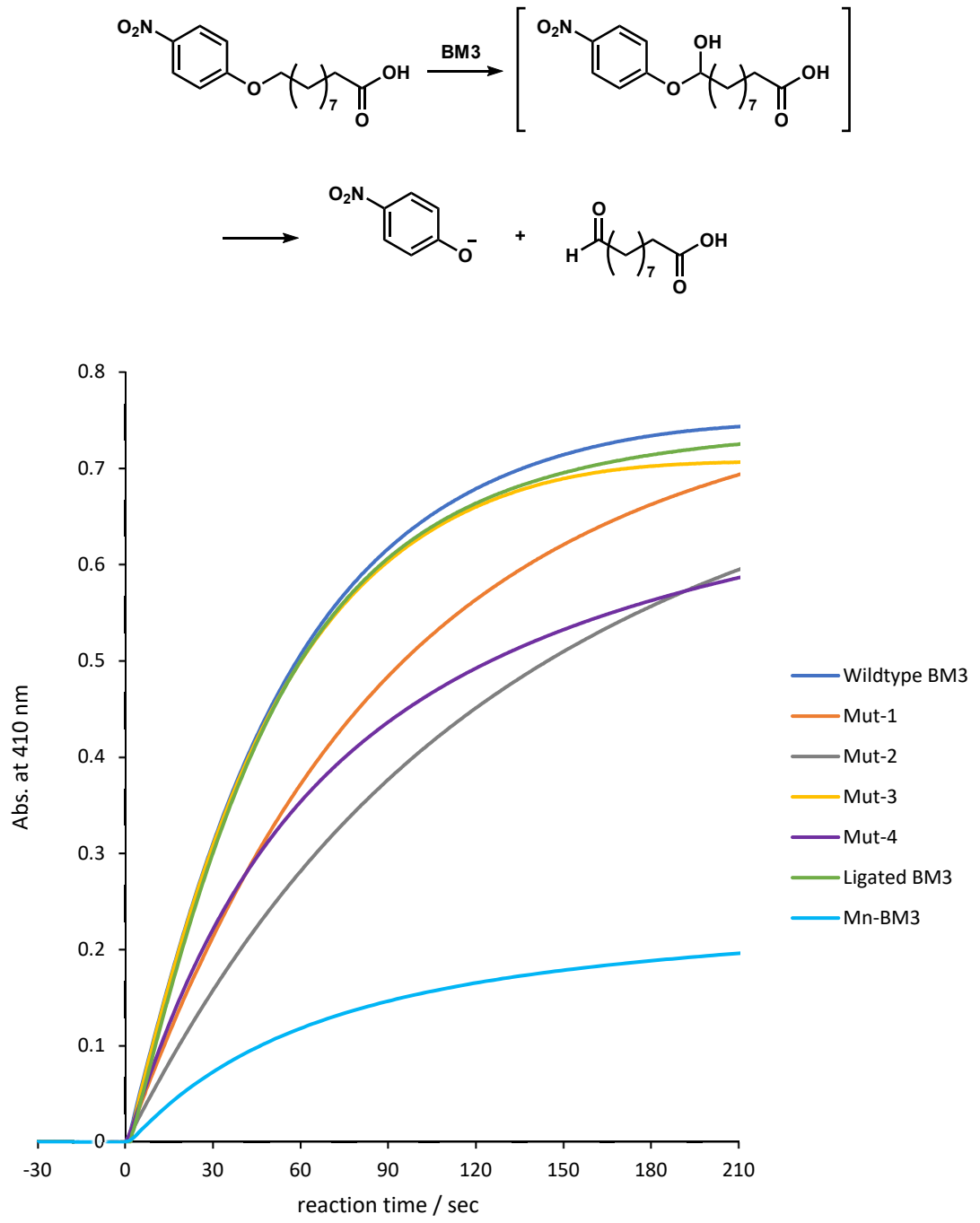


Figure S4. ICP-OES analysis of Mn-PPIX-binding haem domain. Mn concentration was corresponded to protein concentration, whereas significant quantity of Fe was not detected.



FigureS5. Time course analysis of p-nitrophenolate formation. Reactions were initiated by addition of NADPH (0 sec). Initial rates were calculated from the increment of absorbance at 410 nm immediately after initiation.

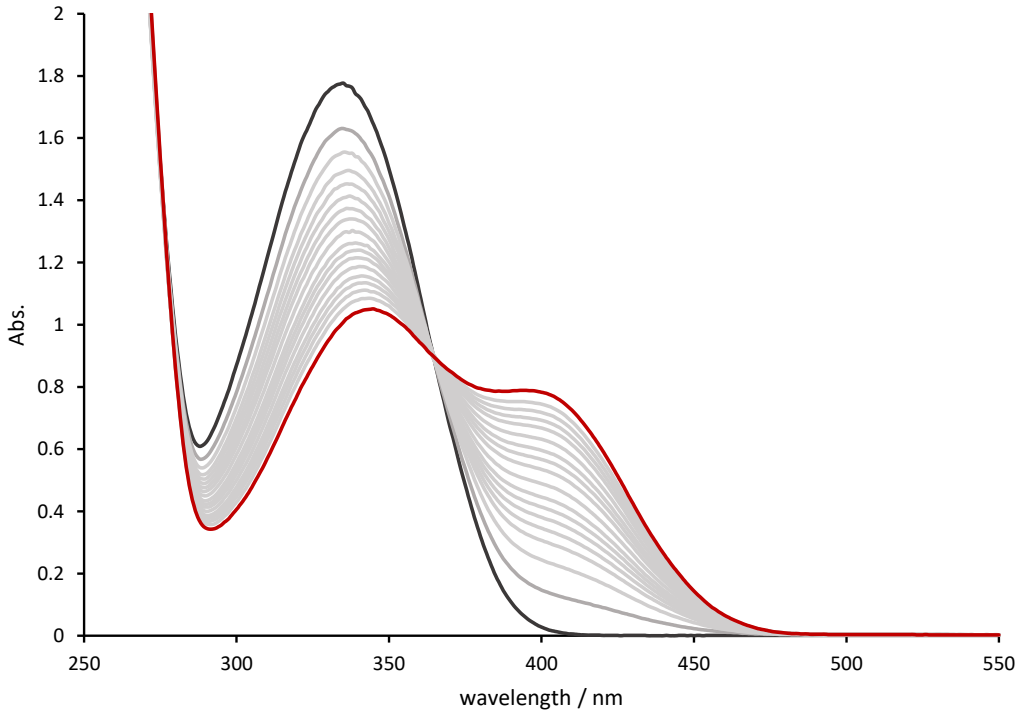


Figure S6. Time course analysis of NADPH consumption and p-nitrophenolate generation in the presence of Mn-BM3. Reaction conditions: BM3 (0.1 μM), 10-pNCA (50 μM), NADPH (200 μM). Reaction was initiated by the addition of NADPH (0 min). Measurement was performed from 0 min (black) to 120 min (red).

References

1 E. A. Berry and B. L. Trumpower, *Anal. Biochem.*, 1987, **161**, 1–15.

2 J. S. French and M. J. Coon, *Arch. Biochem. Biophys.*, 1979, **195**, 565–577.

3 I. Chen, B. M. Dorr and D. R. Liu, *Proc. Natl. Acad. Sci. U. S. A.*, 2011, **108**, 11399–404.

4 H. Hirakawa, S. Ishikawa and T. Nagamune, *Biotechnol. Bioeng.*, 2012, **109**, 2955–2961.

5 T. G. G. Battye, L. Kontogiannis, O. Johnson, H. R. Powell and A. G. W. Leslie, *Acta Crystallogr. Sect. D Biol. Crystallogr.*, 2011, **67**, 271–281.

6 A. G. W. Leslie and H. R. Powell, in *Evolving Methods for Macromolecular Crystallography*, 2007, pp. 41–51.

7 I. Steller, R. Bolotovsky and M. Rossmann, *J. Appl. Crystallogr.*, 1997, **30**, 1036–1040.

8 M. D. Winn, C. C. Ballard, K. D. Cowtan, E. J. Dodson, P. Emsley, P. R. Evans, R. M. Keegan, E. B. Krissinel, A. G. W. Leslie, A. McCoy, S. J. McNicholas, G. N. Murshudov, N. S. Pannu, E. A. Potterton, H. R. Powell, R. J. Read, A. Vagin and K. S. Wilson, *Acta Crystallogr. Sect. D Biol. Crystallogr.*, 2011, 67, 235–242.

9 A. Vagin and A. Teplyakov, *J. Appl. Crystallogr.*, 1997, **30**, 1022–1025.

10 Garib N. Murshudov; Alexei A. Vagin; Eleanor J. Dodson, *Acta Crystallogr. Sect. D Biol. Crystallogr.*, 1997, **D53**, 240–255.

11 P. V Afonine, R. W. Grosse-Kunstleve, N. Echols, J. J. Headd, N. W. Moriarty, M. Mustyakimov, T. C. Terwilliger, A. Urzhumtsev, P. H. Zwart, P. D. Adams and IUCr, *Acta Crystallogr. Sect. D Biol. Crystallogr.*, 2012, **68**, 352–367.

12 P. Emsley and K. Cowtan, *Acta Crystallogr. Sect. D Biol. Crystallogr.*, 2004, **60**, 2126–2132.

13 M. D. Winn, M. N. Isupov and G. N. Murshudov, *Acta Crystallogr. Sect. D Biol. Crystallogr.*, 2001, **57**, 122–133.

14 Z. Otwinowski, D. Borek, W. Majewski and W. Minor, *Acta Crystallogr. Sect. A Found. Crystallogr.*, 2003, **59**, 228–234.

15 Q. Lam, A. Cortez, T. T. Nguyen, M. Kato and L. Cheruzel, , DOI:10.1016/j.jinorgbio.2015.12.005.

16 U. Schwaneberg, C. Schmidt-Dannert, J. Schmitt and R. D. Schmid, *Anal. Biochem.*, 1999, **269**, 359–66.

17 Z. Cong, O. Shoji, C. Kasai, N. Kawakami, H. Sugimoto, Y. Shiro and Y. Watanabe, *ACS Catal.*, 2015, **5**, 150–156.

18 V. S. Lelyveld, E. Brustad, F. H. Arnold and A. Jasanoff, *J. Am. Chem. Soc.*, 2011, **133**, 649–51.

19 N. Kawakami, O. Shoji and Y. Watanabe, *Chembiochem*, 2012, **13**, 2045–7.