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Structural and biochemical study of *Bacillus subtilis* HmoB in complex with heme

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ABSTRACT

Most bacteria have developed a hemoprotein degradation system to acquire iron from their hosts. *Bacillus subtilis* HmoB, a heme monooxygenase, is involved in the degradation of heme and subsequent release of iron. HmoB contains a C-terminal ABM domain, which is similar in sequence and structure to other heme monooxygenases. Heme degradation assay showed that highly conserved residues (N70, W128, and H138) near the heme-binding site were critical for activity of HmoB. However, HmoB was shown to be different from other bacterial heme oxygenases due to its longer N-terminal region and formation of a biological monomer instead of a dimer. The degradation product of *B. subtilis* HmoB was identified as staphylobilin from mass spectrometric analysis of the product and release of formaldehyde during degradation reaction.

1. Introduction

Almost all bacteria require iron for their survival due to their requirement in biologically essential processes [1]. Therefore, bacteria have developed sophisticated systems to acquire iron from the environment [2]. Some bacteria release siderophores, which capture free Fe^{3+} from the surrounding environment. However, due to the low solubility of free Fe^{3+} at physiological pH in the presence of oxygen, its use by microorganisms is limited [3]. Moreover, humans express high-affinity iron-binding proteins, including hemoglobin, transferrin, lactoferrin, and ferritin, which control the concentration of free Fe^{3+} in the order of 10^{-24} M. This limits the availability of free Fe^{3+} for invading pathogens and plays important roles in the innate immune system of the hosts [4]. To counteract the low availability of free Fe^{3+}, many bacteria have developed systems to acquire it from host hemoproteins and ferritins [5,6]. Especially, iron acquisition systems using heme that holds about 80% of iron in the host have been well characterized [7–10].

The iron-regulated surface determinant (Isd) system of *Staphylococcus aureus* includes well-studied heme monooxygenases [9,10]. The Isd system of *S. aureus* removes heme cofactor from hemoproteins and transports heme to the inside of bacteria. The transferred heme is then degraded by heme monooxygenases such as IsdG and IsdI, resulting in the release of free iron. *Bacillus subtilis* contains two heme monooxygenases, HmoA and HmoB, in its genome [11]. HmoA is similar in size to other heme monooxygenases but contains arginine in its active site instead of asparagine. HmoB has a longer N terminal region (1–63), and the structure and sequence of its C-terminal region is similar to other IsdG family proteins. The C-terminal region (64–166) of HmoB contains a highly conserved essential triad (N70, W128, H138), which is critical for heme degradation in our assay. The structures of wild type and N70A mutant HmoB indicate that these three residues are important for binding to heme. The degradation product of HmoB was identified as staphylobilin using mass spectrometry, which is also the product of IsdG and IsdI. HmoB represents a novel bacterial heme monooxygenase due to its longer N-terminal region and existence as a monomer instead of a homodimer, as observed previously in other IsdG family heme monooxygenases.

2. Materials and methods

2.1. Cloning and protein preparation

The *hmoB* gene was amplified from *B. subtilis* genomic DNA by polymerase chain reaction (PCR) using primers (5′-GCTAGCAT GAAGGTTTATATTACATATGGG-3′ and 5′-GAATTCCTATTCGACAGC

Abbreviations: ABM domain, antibiotic biosynthesis monooxygenase domain; RMSD, root-mean-square deviation.

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GAAATATGTGG-3′). The purified PCR product was cloned into pET28b vector using NheI and EcoRI enzymes with an N-terminal His_6-tag and thrombin-cut site. After thrombin cut, six amino acids (GSHMAS) from the vector sequence were left on the N-terminus of HmoB protein. The construct was then transformed into BL21(DE3) *Escherichia coli* strain (Novagen). Cells were grown in LB medium containing 30 μg/ml of kanamycin at 37 °C until an OD_{600nm} of 0.6, after which 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added for induction. After growth for 16 h, cells were harvested by centrifugation and lysed by sonication in 20 mM Tris-HCl pH 7.5 and 250 mM NaCl (lysis buffer). The lysate was cleared by centrifugation, after which the supernatant was loaded onto a Ni-Sepharose 6 affinity column and eluted with a stepwise gradient of 50–400 mM imidazole in lysis buffer. After the N-terminal His_6-tag from the vector was cut by thrombin at 4 °C for 16 h, HmoB was further purified using a Superdex75 size-exclusion column (GE Healthcare) equilibrated with buffer composed of 20 mM TrisHCl and 200 mM NaCl. Purity of the protein was analyzed by 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

2.2. Point mutation

N70A, W128A, Y134A, and H138A mutants were constructed using a QuikChange II site-directed mutagenesis kit (Agilent Technologies) and primers (5′-TTGCAGTATTGGC CAATATTGCCGTTACT-3′, 5′-AGTAACGGCAATATTGGCCAATACTGCAAA-3′, 5′-GCCTTTCAA GACGCCCAGCAGTCCGGTTCT-3′, 5′-AGAACCGGACTGCTGGGCGTCT TGAAAGGC-3′, 5′-CAGTCCGATTCTGCCAAAGAAGCCCACAAA-3′, 5′-TTTGTGG GCTTCTTTGGCAGAATCGGACTG-3′ and 5′-TACAAAGAAG CCGCCAAAAAACGCGATACA-3′, 5′-TGTATCGCGTTTTTTGGCGGCTT CTTTGTA-3′).

2.3. Heme degradation assay and product identification

For heme degradation assay, 10 mM hemin (Fe^{3+}-protoporphyrin IX) solution was prepared by dissolving 6.52 mg of hemin (Frontier Scientific) in 100 μl of 1 M NaOH, followed by addition of 100 μl of 1 M TrisHCl pH 7.0, and 800 μl of 20 mM TrisHCl pH 7.5 and 250 mM NaCl. Heme degradation assay was performed in 20 mM TrisHCl pH 7.5 and 250 mM NaCl buffer at room temperature. 10 μM each of wild type HmoB, N70A, W128A, Y134A, and H138A mutant proteins was incubated with 10 μM hemin solution, after which 1 mM ascorbic acid was added as an electron donor to initiate the reaction [12,13]. Each assay was performed in the presence of 1 μM catalase from bovine liver (Sigma) to prevent coupled oxidation reactions. Degradation of heme was monitored at 300–800 nm every 10 min on a spectrophotometer (DU730, Beckman Coulter). All reactions were performed in triplicate.

The identification of hemin and the degradation product was made using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS, Autoflex Speed series; Bruker Daltonics, Leipzig, Germany), in which 2,5-dihydroxybenzoic acid (2,5-DHB) was used as a matrix. For the mass spectroscopic analysis of degradation product, the assay was performed for 4 h to ensure a near completion of the reaction. The release of formaldehyde during heme degradation reaction was monitored using formaldehyde fluorescent detection kit (Arbo assays) and plate reader (Molecular device) following the manufacturer's protocol. The presence of formaldehyde was measured 60 min after the initiation of assay.

Table 1
Data collection and refinement statistics.

Data collection statistics	HmoB wild type	HmoB N70A mutant
Space group	I23	$P2_1$
Unit cell dimensions	a = 99.597, b = 99.597, c = 99.597 Å β = 90.00°	a = 70.821, b = 117.519, c = 70.847 Å β = 91.22°
Resolution (Å)[a]	30.0–2.70 (2.80–2.70)	20.0–1.75 (1.78–1.75)
Observed reflections	1,625,865	4,199,147
Unique reflections	4,635	111,838
Completeness (%)	100.0 (100.0)	95.8 (96.8)
R_{sym} (%)[b]	0.107 (0.635)	0.089 (0.501)
I/σ (I)[c]	45.6 (12.6)	11.0 (5.2)
Refinement statistics		
No. of residues	166	996
R_{cryst} (%)/R_{free} (%)[d]	19.32/27.14	19.88/23.26
rmsd bonds (Å)	0.012	0.018
rmsd angles (°)	1.603	1.670

[a] Resolution range of the highest shell is listed in parentheses.
[b] $R_{sym} = \sum|I - \langle I\rangle|/\sum I$, where I is the intensity of an individual reflection and $\langle I\rangle$ is the average intensity over symmetry equivalents.
[c] I/σ (I) is the mean reflection intensity/estimated error.
[d] $R_{cryst} = \sum||F_o| - |F_c||/\sum|F_o|$, where F_o and F_c are the observed and calculated structure factor amplitudes, R_{free} is equivalent to R_{cryst} but calculated for a randomly chosen set of reflections that were omitted from the refinement process.

2.4. Crystallization, data collection, and structure determination

Purified wild type and N70A mutant HmoB proteins were concentrated to 15.5 and 14.4 mg/ml, respectively, by centrifugal ultrafiltration (Amicon). Crystals of wild type HmoB and N70A were obtained by the hanging-drop vapor-diffusion method at 20 °C. Well solution for wild type HmoB crystals was composed of 0.5% polyethylene glycol (PEG) 2000, 1.3 M sodium citrate, 0.1 M HEPES pH 7.0, and 1 mM hemin solution. Well solution for N70A crystals was composed of 0.5% polyethylene glycol (PEG) 2000, 1.2 M sodium citrate, 0.1 M HEPES pH 7.0, and 1 mM hemin solution. For cryoprotection, wild type HmoB crystals were transferred into 0.5% PEG 2000, 1.3 M sodium citrate, 0.1 M HEPES pH 7.5, 1 mM hemin solution, and 10% glycerol while N70A mutant crystals were placed in 0.5% PEG 2000, 1.2 M sodium citrate, 0.1 M HEPES pH 7.5, 1 mM hemin solution, and 10% glycerol, followed by flash-freezing in liquid nitrogen.

X-ray diffraction data of wild type HmoB crystals were collected at a 2.7 Å resolution at PAL beamline 7A (Korea), whereas data of N70A crystals were collected at a 1.75 Å resolution at PAL beamline 5C (Korea). Data were processed with HKL2000 [14], and initial models of wild type HmoB and N70A were obtained using the molecular replacement program of CCP4 package [15] with apo HmoB structure (PDB ID: 3TVZ) as a search model. The space group of wild type HmoB was I23, and the asymmetric unit contained one subunit with a Matthews coefficient (Vm) of 2.13 $Å^3$/Da, and the estimated solvent content was 42.2%. The space group of N70A crystal was $P2_1$ with six subunits in the asymmetric unit. The Matthews coefficient (Vm) of N70A was 2.54 $Å^3$/Da with an estimated solvent content of 51.6%. The models were refined with REFMAC [16], and manual model building was performed using the COOT program [17]. Eleven residues out of 166 from wild type HmoB were not observed in the electron density and not included in the final model. Ninety-five residues out of 996 from N70A were not included in the final model (Table 1). The Ramachandran plot produced by MolProbity showed that there is no outlier in the structure of wild type HmoB or N70A [18]. The coordinate and structure factors for *B. subtilis* wild type and N70A HmoB have been deposited in the RCSB with PDB IDs of 4OZ5 and 4FVC, respectively.

3. Results and discussion

3.1. Overall structure

The structures of wild type and N70A mutant HmoB in complex with heme were determined to 2.7 and 1.75 Å. The asymmetric

unit of wild type and N70A mutant contained one and six molecules of HmoB, respectively, and they showed similar structures with root mean square deviations of 0.329, 0.305, 0.342, 0.454, 0.309, and 0.449 Å. The overall structure of HmoB consisted of a β barrel made of eight β strands surrounded by four α helices (Fig. 1). The C-terminal region was shown to be a feredoxin-like fold with a typical βαββαβ motif, and contained the heme group. The interactions between heme and HmoB are mostly via hydrophobic residues, including I72 from β5, F82, K85, and F86 from α2, R89 and V93 from the loop region, I102 from β6, I115 from β7, F125, W128, Q129, and Y134 from α3, F153 from the loop region, and V159 from β8 (Fig. 1C). The propionic acid group of heme formed hydrogen bonds with the backbone amide group of K92 and carbonyl group of A137. N70 is a part of the triad (N70, W128, and H138) that is important for catalytic activity. However, it did not directly interact with the heme group but was involved in forming hydrogen bonds with water molecules (Fig. 1C, red spheres). These water molecules are close to the heme but do not coordinate the iron atom (water molecule closest to iron is 3.9 Å away). In the wild type HmoB: heme complex structure, the conserved H138 residue was coordinating the iron atom of the heme group (2.1 Å) like other heme monooxygenases (Fig 1C). Altogether, the iron atom of the heme group in the wild type HmoB: heme complex was coordinated by 5 atoms including the 4 nitrogens of the porphyrin ring and a nitrogen atom of H138.

In the N70A mutant structure, part of the loop region containing H138 was disordered (residues 135–150 in molecules A, B, C, and D, 135–149 in E and 135–145 in F) and the iron of heme group was coordinated by Y134 (average distance of 2.2 Å) instead of H138 in all six molecules in the asymmetric unit (Supplemental Fig. 1B). This is different from IsdG, where His ligation of iron is maintained in N7A mutant [19]. This could be explained by the movement of heme group away from the histidine residue (about 0.8 Å) in N70A mutant of HmoB (Supplemental Fig. 1C) and replacement of H138 by Y134 that can reach and coordinate iron atom.

3.2. Heme degradation assay and product identification

HmoB (formerly known as YhgC) was identified as a heme monooxygenase [11], and our apo structure for HmoB [20] showed that its C terminal region was very similar to IsdI, an enzyme involved in heme degradation [9]. To identify residues that are critical for enzymatic activity, heme degradation assay was performed using wild type as well as N70A, W128A, Y134A, and H138A mutant proteins. Degradation of hemin was monitored at 300–800 nm every 10 min using a spectrophotometer. Solution, including all components except HmoB, was used as a control (Fig. 2A). The spectra of wild type HmoB decreased with time due to degradation of heme (Fig. 2B). Y134A mutant retained its enzymatic activity, whereas N70A, W128A, and H138A mutant proteins almost completely lost their activities (Fig. 2C–F). These results suggest that N70, W128, H138 are critical residues for enzymatic function of HmoB, as shown in *S. aureus* IsdG [21]. On the other hand, Y134 did not seem to play a critical role in heme degradation, although this residue coordinated the iron atom in the N70A mutant structure.

To identify the degradation product of *B. subtilis* HmoB, we have performed mass spectrometric analysis. The *m/z* values of the most abundant molecular ions in the MALDI-TOF spectrum acquired before and after the heme degradation reaction of wild type HmoB were 616.2 and 599.7, which matched the *m/z* values of heme (exact mass: 616.177) and staphylobilin (599.665 Da), respectively (Supplemental Fig. 2). Other possible products of heme oxygenases, such as biliverdin (583.3 *m/z*), bilirubin (585.3 *m/z*), and mycobilin (611.3 *m/z*) were not detected in the MALDI-TOF spectrum. Staphylobilin is the degradation product of *S. aureus* IsdI and IsdG [22], close homologues of HmoB. It was reported that *S. aureus* IsdI and IsdG released formaldehyde instead of CO or O_2 during heme degradation [23]. To investigate whether *B. subtilis* HmoB also releases formaldehyde, its production was monitored using a detection kit. The release of formaldehyde was detected in the presence of HmoB, while it was not detected in a control reaction without HmoB. The production of staphylobilin and release of formaldehyde suggests that *B. subtilis* HmoB shares similar catalytic mechanism with its structural homologues such as *S. aureus* IsdI and IsdG.

3.3. Conformational changes upon heme binding

To investigate conformational changes in HmoB after heme binding, we compared the apo HmoB structure to the HmoB: heme complex structure. The overall structures were very similar, with a RMSD of 0.312 Å. The largest conformational change was observed in the α2–α3 helix region of the apo structure (Fig. 3). After heme binding, the α2 helix moved outwards up to 2.5 Å (measuring the distance of K87) and the α3 helix of apo HmoB became a loop (Fig. 3). HmoB: heme complex structure contained less number of disordered residues (141–151) than that of apo HmoB structure (residues 131–150 are disordered) and α4 helix (residues 132–137) became visible in HmoB: heme complex structure.

3.4. Comparison with other bacterial heme monooxygenases

Structural comparison of the monomer of *B. subtilis* HmoB and the homodimer of *S. aureus* IsdI showed that the C-terminal region of HmoB (64–166) and each subunit of IsdI had very similar structures with root mean square deviations of 0.882 and 0.729 Å, respectively (Fig. 4A). The positions of the essential triads of HmoB and IsdI relative to the heme group were very close to each other (Fig. 4B). However, distortion of the heme group from planarity [24] was less severe in HmoB with out-of-plane distortion of 0.9 Å compared to 2.3 Å observed in IsdI [19]. This difference is most likely due to the close distance between the W66 of IsdI and the heme. This distortion or "ruffling" of the heme group was shown to play an important role in catalysis [25] and might explain why the tryptophan residue, which did not extensively interact with the heme group, was so critical for enzyme activity.

In this study, we have shown that *B. subtilis* HmoB contains a C-terminal ABM domain that is similar in structure and sequence to other bacterial heme monooxygenases. The C-terminal domain contains the heme binding site, where the heme group is mainly bound by hydrophobic residues. Heme degradation assays using wild type and mutant proteins showed that the well-conserved triad (N70, W128, and H138) is essential for enzymatic activity. The degradation product of *B. subtilis* HmoB was identified as staphylobilin based on the mass spectrometry data and the release of formaldehyde during the degradation assay. The production of staphylobilin and formaldehyde was also observed in other monooxygenases such as IsdI and IsdG, which are the close homologues of HmoB. *B. subtilis* HmoB represents a novel heme monooxygenase due to its longer N-terminal region and formation of a monomer instead of a homodimer.

Interestingly, a monomer of HmoB showed a similar structure to the homodimer of IsdI with a root mean square deviation of 2.778 Å (Supplemental Fig. 3C). Especially, the eight-stranded β barrel of the HmoB monomer was similar in structure to the central β barrel of IsdI dimer. The sequences of the first four (β1–β4) and last four β strands (β5–β8) of the eight-stranded β barrel of the HmoB are quite different (16.5% identity) and do not support a gene fusion event where the homodimer is fused together to form a monomer. It is more likely that HmoB has diverged from

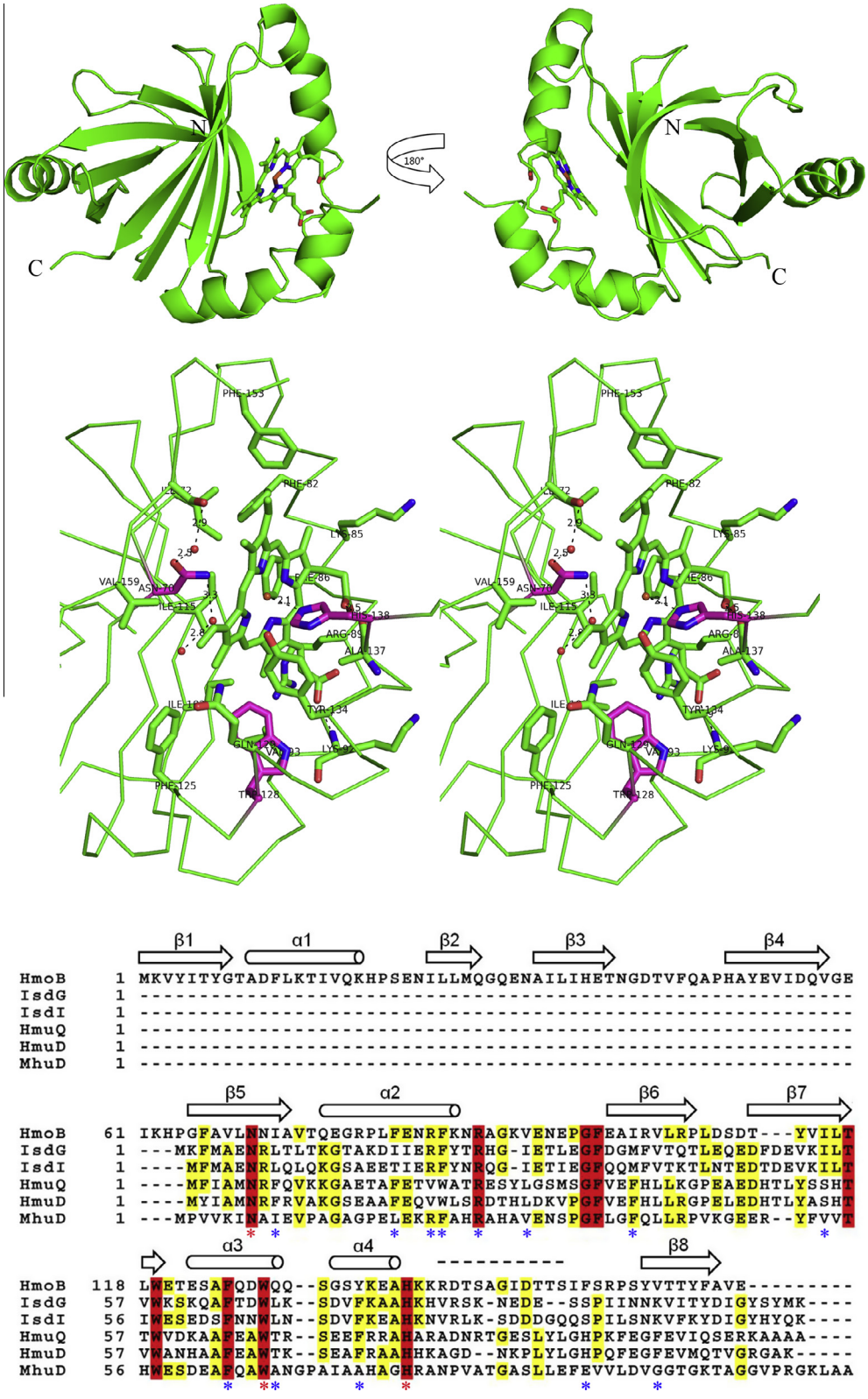

Fig. 1. Overall structure of HmoB. (A) HmoB is colored in green with the bound heme molecule shown in ball-and-stick model. The secondary structures are labeled. (B) The 180° rotated view of (A). (C) Stereoview of heme binding site. Residues interacting with heme are shown in ball-and-stick model. Essential triad (N70, W128 and H138) is colored in magenta. Hydrogen bonds are drawn in dashed line and the distances are indicated in Å. Water molecules that form hydrogen bonds with N70 are drawn as red spheres. (D) Multiple sequence alignment of bacterial heme monooxygenases including *B. subtilis* HmoB, *S. aureus* IsdG, IsdI, *Bradyrhizobacterium japonicum* HmuQ, HmuD and *Mycobacterium tuberculosis* MhuD. The essential triad and hydrophobic residues interacting with the heme group are indicated with red and blue asterisks, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

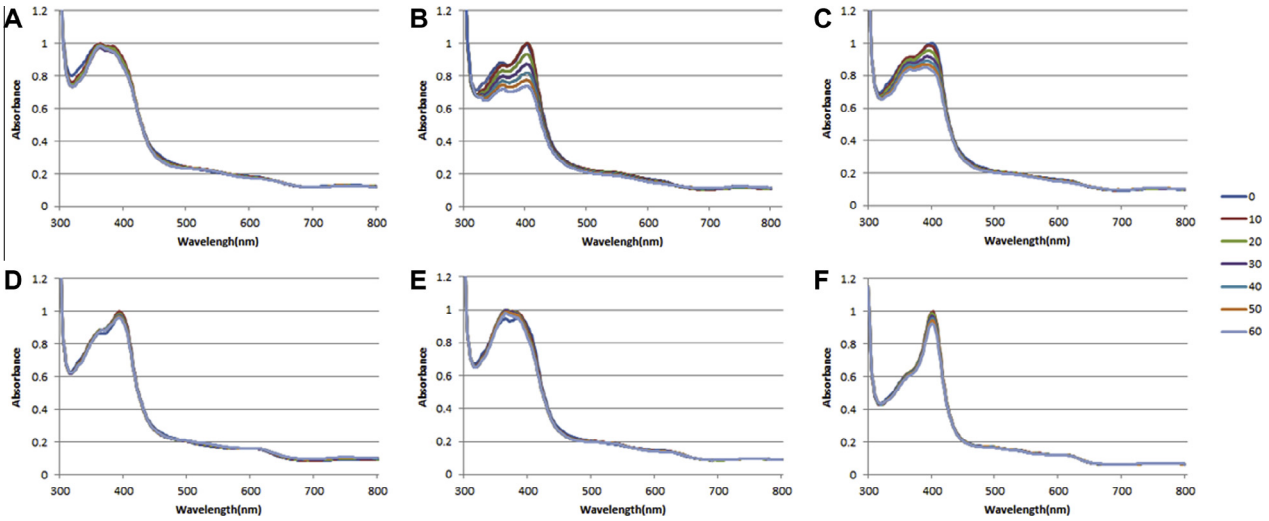

Fig. 2. Heme degradation assay of HmoB. Heme degradation assays of (A) control, (B) wild type, (C) Y134A, (D) H138A, (E) W128A and (F) N70A mutants were performed in the presence of catalase. Degradation of heme was monitored at 300–800 nm wavelength every 10 min using spectrophotometer. Every component except HmoB was included in the control experiment. All reactions were performed in triplicate.

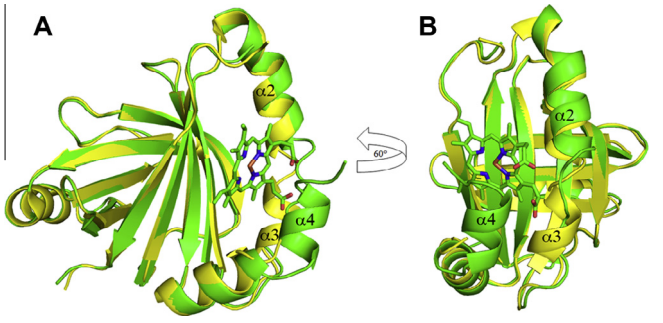

Fig. 3. Superposition of apo and heme bound HmoB structures. (A) HmoB apo structure and heme bound structures are colored in yellow and green, respectively. Bound heme is shown in ball-and-stick model. The α2 and α3 helices of apo structure and α4 helix of the HmoB: heme complex structure are labeled. (B) The 60° rotated view of (A). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the original heme oxygenase structure. The structurally closest protein to HmoB identified by DALI search [26] was not *S. aureus* IsdI but another *S. aureus* protein called target of RNAIII-activating protein (TRAP), which showed RMSD of 1.015 Å with a DALI score of 22.3 (Supplemental Fig. 4) [27]. Although their structures are similar, TRAP contains only conserved histidine (H138) among the essential triad shared by other monooxygenases and the orientation of this histidine is different from those of other heme monooxygenases in the heme binding site. TRAP is known as a signal transduction protein with a function of protecting DNA from oxidative stress.

In conclusion, we have shown that *B. subtilis* HmoB is a heme monooxygenase with a heme binding site in the C-terminal region including a highly conserved triad (N70, W128, and H138). Heme degradation assays using mutant proteins demonstrated that these triad residues are critical for enzymatic activity. Mass spectrometric analysis of the product and detection of released formaldehyde indicated that the degradation product is staphylobilin. *B. subtilis* HmoB is unique in that it has longer N-terminal region and exists as a monomer instead of a homodimer.

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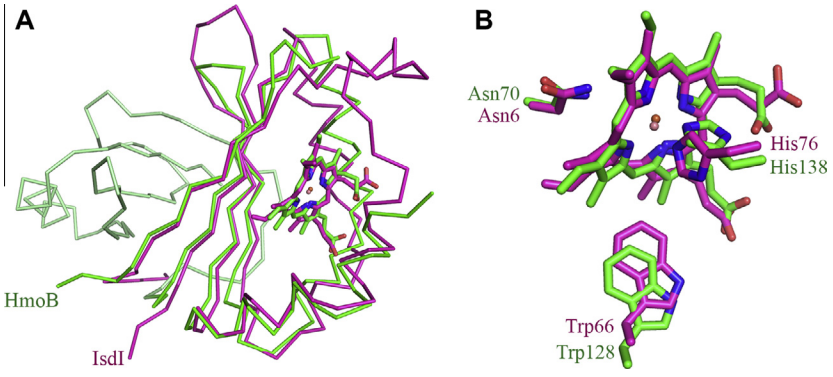

Fig. 4. Comparison of heme binding site of HmoB and IsdI. (A) Superposition of HmoB (green) and IsdI (magenta) with bound heme molecules. The N terminal region (1–63) of HmoB is colored in pale green to highlight the C terminal region (B) Superposition of essential triad and bound heme of HmoB and IsdI. HmoB and IsdI are colored in green and magenta, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.02.092.

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