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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