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Bacillus subtilis HmoB is a heme oxygenase with a novel structure

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Iron availability is limited in the environment and most bacteria have developed a system to acquire iron from host hemoproteins. Heme oxygenase plays an important role by degrading heme group and releasing the essential nutrient iron. The structure of *Bacillus subtilis* HmoB was determined to 2.0 Å resolution. *B. subtilis* HmoB contains a typical antibiotic biosynthesis monooxygenase (ABM) domain that spans from 71 to 146 residues and belongs to the IsdG family heme oxygenases. Comparison of HmoB and IsdG family proteins showed that the C-terminal region of HmoB has similar sequence and structure to IsdG family proteins and contains conserved critical residues for heme degradation. However, HmoB is distinct from other IsdG family proteins in that HmoB is about 60 amino acids longer in the N-terminus and does not form a dimer whereas previously studied IsdG family heme oxygenases form functional homodimers. Interestingly, the structure of monomeric HmoB resembles the dimeric structure of IsdG family proteins. Hence, *B. subtilis* HmoB is a heme oxygenase with a novel structural feature. [BMB reports 2012; 45(4): 239-241]

INTRODUCTION

Most pathogenic bacteria require iron for survival, growth and pathogenecity (1). However, free iron is scarce within the human host because the majority of iron is bound by hemoproteins like hemoglobin and myoglobin, and other high-affinity iron binding proteins such as transferrin and lactoferrin (2). Bacterial pathogens have developed sophisticated system to utilize host iron source to meet their iron requirement. For example, *Staphylococcus aureus* has an iron-regulated surface determinant (Isd) system to bind hemoglobin and remove the heme cofactor and traffic heme into the bacterium (3, 4). Once inside the bacterium, heme is degraded by two heme oxygenases, IsdG and IsdI, to release the nutrient iron. Two heme oxygenases (HmoA and HmoB) are identified in *B. subtilis* (5). These two proteins have been shown to bind and degrade heme in the presence of electron donor. HmoB (formerly known as YhgC) belongs to the IsdG family heme oxygenases and sequence alignment showed that HmoB contains conserved active site residues for heme binding and degradation. However, HmoB is about 60 amino acids longer in the N-terminus than other IsdG family members and comparison of structures showed that monomer of HmoB is similar to the dimer of IsdG family heme oxygenases.

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RESULTS AND DISCUSSION

Overall structure

The crystal structure of *B. subtilis* HmoB was determined at 2.0 Å resolution. The overall structure showed that HmoB has eight anti-parallel β strands in the center surrounded by four α helices on the outside (Fig. 1). This protein belongs to the ferredoxin-like fold with the typical βαββαβ motif in the C-terminal region (6). There were three HmoB molecules in the asymmetric unit with approximate 3-fold symmetry (Fig. 1). The three subunits were quite similar to each other with root mean square deviations of 0.289 Å between subunit A and B, 0.239

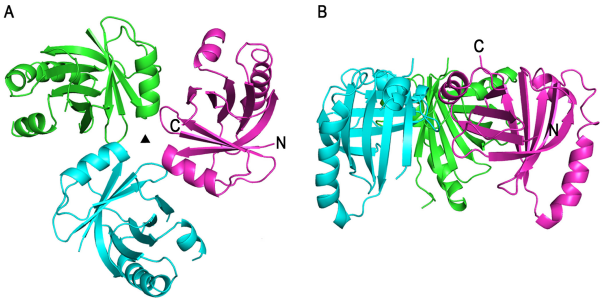

Fig. 1. Overall structure of *B. subtilis* HmoB (A) Three molecules of HmoB's in the asymmetric unit are shown. The three molecules are related by 3-fold axis perpendicular to the page indicated by a triangle. Chain A is colored in green, chain B in cyan and chain C in magenta, respectively. (B) The 90° rotated view of (A).

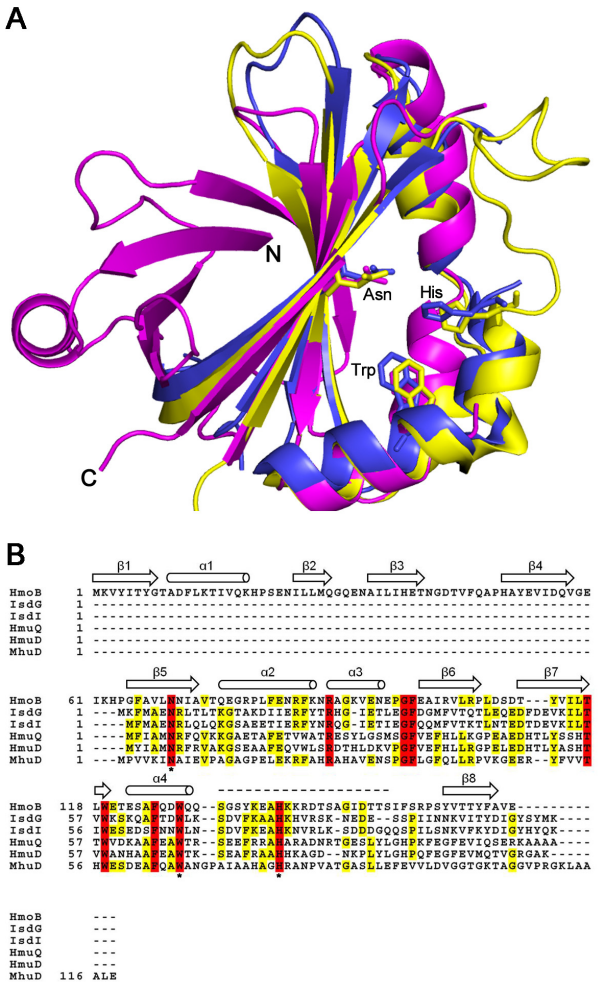

Fig. 2. Comparison of HmoB and heme oxygenases (A) Superposition of HmoB (magenta) with IsdI (yellow, PDB ID: 2ZDP) and IsdG (blue, PDB ID: 1XBW). The three conserved residues (Asn, Trp and His) that are critical for heme binding and degradation are shown in ball-and-stick model. The N- and C-terminus of HmoB are labeled as N and C, respectively. (B) Multiple sequence alignment of *B. subtilis* HmoB, *S aureus* IsdG (accession code : Q8NX62), *S. aureus* IsdI (Q-2G1J2), *Bradyrhizobium Japonicum* HmuQ (BAC52340), *B. Japonicum* HmuD (BAC52688) and *Mycobacterium tuberculosis* MhuD (PDB ID: 3HX9). Completely conserved residues are colored in red and highly conserved residues in yellow. The secondary structures of HmoB are shown above the sequence. The dotted line indicates the disordered region of HmoB. The three critical residues (Asn, Trp and His) are marked with asterisks.

Å (A-C) and 0.234 Å (B-C). The buried surface area between the putative trimer interfaces was 648 Å^2, which constituted about 8.6% of the total surface area of each subunit when calculated by the ProtorP server (7). This surface area is rather small compared to the typical buried surface area of protein-protein interfaces of about 1,300-1500 Å^2 (8). HmoB eluted as two peaks in a size exclusion column indicating a possible equilibrium between a trimer and a monomer in solution (Supplement 1).

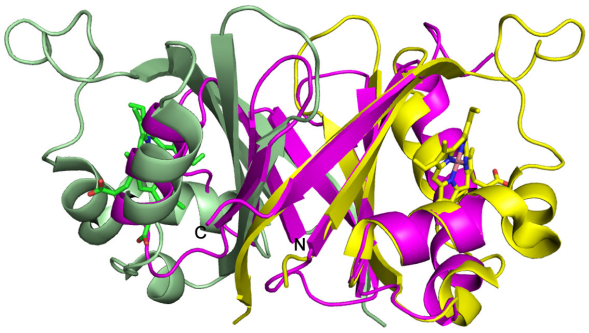

Fig. 3. Structural superposition of *B. subtilis* HmoB monomer (magenta) and *S. aureus* IsdI homodimer (yellow and lime). The bound heme groups in IsdI are drawn in ball-and-stick model. The N- and C-terminus of HmoB are labeled as N and C, respectively.

Comparison with IsdG family heme oxygenases

Sequence comparison showed that the C-terminal region (residues 64-166) of HmoB aligns with other IsdG family heme oxygenases with sequence identities of 28.4, 30.0, 21.2, 23.2 and 27.1% with *S. aureus* IsdG, *S. aureus* IsdI, *B. Japonicum* HmuQ, *B. Japonicum* HmuD and *M. tuberculosis* MhuD, respectively (Fig. 2B). The monomer of dimeric IsdG and IsdI shared a common ferredoxin-like βαβ-fold with the C-terminal region (β5-β8) of HmoB with root mean square deviations of 1.33 Å and 1.07 Å, respectively (Fig 2A). The C-terminal region of HmoB also contains critical residues for heme binding and degradation such as N70, W128 and H138 that are completely conserved among IsdG family heme oxygenases (shown in ball-and-stick model in Fig. 2A and indicated with asterisk in Fig. 2B). H138 of HmoB is not shown in Fig. 2A because residues 131-151 are not included in the model due to disorder. All of the previously characterized IsdG family heme oxygenases form homodimers with two heme binding sites (Fig. 3). These homodimers include a central eight stranded antiparallel β-barrel surrounded by α-helices on opposite sides of the β-barrel. As shown in Fig. 3, the C-terminal region (β5-β8) of HmoB superposes well with one monomer (yellow) of IsdI homodimer. Interestingly, the N-terminal region of HmoB is similar to the other monomer (lime) of IsdI homodimer. Especially, the N-terminal β-strands (β1, β2, β3 and β4) of HmoB aligns well with the β-strands of the other monomer (lime). HmoB is distinct from previously studied IsdG family heme oxygenases in that HmoB does not form a homodimer and represent a novel heme oxygenase structure. In conclusion, structural and sequence comparison with IsdG family heme oxygenases suggests that *B. subtilis* HmoB is a heme oxygenase with novel structure and oligomerization state.

MATERIALS AND METHODS

Cloning, protein preparation, and purification

The *hmoB* gene was amplified from the *B. subtilis* genomic DNA by polymerase chain reaction (PCR) using primers (5′-GCTAGCATGAAGGTTTATATTACATATGGG-3′, 5′-GAATTCCTATTCGACAGCGAAATATGTGG-3′). The purified PCR product was cloned into the pET28b vector using *Nhe*I and *Eco*RI enzymes with an N-terminal His6-tag and thrombin-cut site. After thrombin cut, 6 amino acids (GSHMAS) from the vector sequence are left on the N-terminus of HmoB protein. The construct was transformed into BL21(DE3) *E. coli* strain (Novagen). The cells were grown in M9 medium containing 30 μg/ml kanamycin at 37°C until the OD_{600nm} reached 0.6, and then an amino acid mixture (100 mg/L of Lys, Phe, Thr, 50 mg/L of Ile, Leu, Val, Sel-Met) was added 30 min before induction with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Growth was continued for 4 hrs and the cells were harvested by centrifugation and lysed by sonication in 20 mM Tris-HCl pH 7.5 and 250 mM NaCl buffer (lysis buffer). The lysate was cleared by centrifugation and the supernatant was loaded onto a Ni-Sepharose 6 affinity column and eluted with stepwise gradient of 50-400 mM imidazole in lysis buffer. After the N-terminal His6-tag from the vector was cut by thrombin at 4°C, HmoB was further purified using a Superdex75 size-exclusion column (GE Healthcare) equilibrated with a buffer composed of 20 mM Tris-HCl, 200 mM NaCl, 2 mM dithiothreitol, and 2 mM EDTA. The purity of the protein was analyzed by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Crystallization, data collection, and structure determination

The purified HmoB was concentrated to 16.7 mg/ml by centrifugal ultrafiltration (Amicon). Crystals of HmoB were obtained by a hanging-drop vapor-diffusion method at 20°C using a well solution composed of 28% polyethylene glycol (PEG) 4,000, 0.2 M magnesium chloride, and 0.1M Tris-HCl pH 8.5. Crystals were transferred into 28% PEG 4000, 0.2 M magnesium chloride, 0.1M Tris-HCl pH 8.5 and 15% glycerol as a cryoprotectant solution and flash-frozen in liquid nitrogen. X-ray diffraction data were collected to 2.0 Å resolution at the SPring-8 beamline BL41XU (Japan). The data were processed with HKL2000 (9) and an initial model of HmoB was obtained by the Phenix program (10) using the peak and edge dataset. The space group was $P6_122$ and the asymmetric unit contained three subunits. The Matthews coefficient (Vm) was 1.87 $Å^3$/Da, and the estimated solvent content of the crystal was 34.2%. The model was refined with REFMAC (11) and manual model building was performed using the COOT program (12). Seventy residues out of 498 were not observed in the electron density and were not included in the final model. The Ramachandran plot produced by PROCHECK showed that 100% of residues are in the most favored or favored region (13). The coordinate and structure factors for *B. subtilis* HmoB have been deposited in the RCSB Protein Data Bank with accession code 3TVZ.

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