An Induced Fit Conformational Change Underlies the Binding Mechanism of the Heme Transport Proteobacteria-Protein HemS*[

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Bacteria rely on their environment and/or host to acquire iron and have evolved specialized systems to sequester and transport heme. The heme uptake system HemRSTUV is common to proteobacteria, and a major challenge is to understand the molecular mechanism of heme binding and transfer between the protein molecules that underlie this heme transport relay process. In the Gram-negative pathogen Yersinia enterocolitica, the HemRSTUV system culminates with the cytoplasmic recipient HemS, which stores and delivers heme for cellular needs. HemS belongs to a family of proteins essential and unique to proteobacteria. Here we report on the binding mechanism of HemS based on structural data from its apo- and ligand-loaded forms. This heme carrier protein associates with its cargo through a novel, partly preformed binding pocket, formed between a large, rigid body-like domain movements, and together, these bring about a switch from an open, apo-form to a closed, bound state. This is the first report in which both liganded and unliganded forms of a heme transport protein are described, thus providing penetrating insights into its mechanism of heme binding and release.

Iron plays a key role in many biological processes and is therefore an essential nutrient for living organisms. Iron acquisition is challenging for pathogenic bacteria given that in the host, free iron and iron in the form of heme is usually sequestered by high affinity binding proteins such as lactoferrin, transferrin, hemopexin, or serum albumin. The resulting low levels of free iron in tissues and in the blood serum make it difficult for microbes to grow and proliferate, thus contributing to the nonspecific host defense against pathogens. To overcome their iron dependence in this iron-limited environment, pathogenic bacteria have evolved specialized proteins to “steal” heme from the heme proteins of the host and use it as an iron source (1–6).

Common to many Gram-negative species, the heme-scavenging HemRSTUV system from Yersinia enterocolitica consists of four interlinked but structurally distinct components culminating with the cytosolic recipient HemS (1, 3, 7). Although the names of homologous proteins in distinct species differ, their functions are likely to be the same given the relatively high levels of sequence conservation (3, 8). Genetic studies on this heme transport system in Y. enterocolitica and other species revealed the common organization of the components in a single operon, whose expression is regulated through the abundance of iron and heme (9–16). However, the mechanisms for heme transport, binding, and release by any of the components of the uptake system are still unexplored.

HemS is presently the best studied component of the HemRSTUV system. Essential for Yersinia, HemS was originally thought to be a heme oxygenase and was also presumed to protect the cellular environment from the toxic effects of free heme (7). Work on the homologous protein ShuS from Shigella dysenteriae, which shares 64% sequence identity with HemS, showed that ShuS binds one heme per molecule with an affinity in the micromolar range (17). The determination of the apo-structure of the homologous protein ChuS from Escherichia coli revealed a novel fold composed of two similar domains likely to have evolved through gene duplication and fusion (18).

Despite the structural data, no binding sites were identified. In contrast to ShuS, ChuS was argued to be a heme oxygenase and to bind two heme molecules per protein molecule. Recent work on PhuS from Pseudomonas aeruginosa showed that this molecule binds one heme per monomer and is not a heme oxygenase but is responsible for passing heme to a heme oxygenase (19). It is therefore most likely that HemS and its homologues are heme transporters facilitating transfer of heme from the uptake machinery to enzymes that either utilize heme per se or break heme down. Here we report the crystal structures of apo- and heme-bound HemS.
**Experimental Procedures**

Cloning, Expression, Purification, and Crystallization—HemS from *Y. enterocolitica* was cloned and crystallized as described previously (20). Care was taken to cleave the His tag off the protein and ensure that no tagged protein was used in crystallization experiments. To crystallize the apo-form, heme-HemS was separated from apo-HemS by ion-exchange chromatography (MonoQ, Amersham Biosciences) equilibrated with 50 mM BisTris\(^{-}\)-propane, pH 6.5. Apo-HemS crystals were obtained in 100 mM Bis, pH 9, 2.45 M ammonium sulfate, and 5% polyethylene glycol 400, with a protein concentration of 30 mg/ml. Purified samples of HemS reconstituted with heme showed a Soret peak at 411 nm, in agreement with the Soret bands previously reported for ShuS at 410 nm (17), ChuS at 408 nm (18), and PhuS at 410 nm (19).

Collection and Processing of X-ray Diffraction Data, Phase Determination, and Model Refinement—Diffraction data were measured at the synchrotron radiation source European Synchrotron Radiation Facility (ESRF), Grenoble, France (heme-HemS, wavelength 0.984 Å, BM14; apo-HemS, wavelength 0.933 Å, ID14-2) and processed with MOSFLM (21) and SCALA (22). The structure of the heme-HemS complex was solved by molecular replacement using PHASER (23) with the apo-ChuS structure, Protein Data Bank code 1U9T (18). To solve the apo-HemS structure, the coordinates of heme-HemS were used as a search model. Model building was carried out in COOT (24), and restrained refinement was carried out with REFMAC5 (25) and TLS (26). An optimal number of 10 TLS groups (11 for apo-HemS) was determined using the TLSMD web server for the generation of multigroup TLS models (27). Data processing and model refinement statistics are summarized in supplemental Table S1. The refined models have excellent geometry and no Ramachandran outliers. Surface calculations were carried out using the CCP4 program AREAIMOL (28). Structural superpositions were carried out with the CCP4 program LSQKAB (28). All structural figures were prepared using PyMOL (Delano Scientific) unless otherwise stated.

**Results and Discussion**

Structure of the Heme-HemS Complex—The heme-HemS complex was solved by molecular replacement using the atomic coordinates of apo-ChuS (1U9T) (18) and refined against data to 1.7 Å spacing. The porphyrin could be oriented with no ambiguity in well defined density peaks. Although His-196 could be clearly identified as a ligand to the iron, only very weak density at the sixth coordination position was observed. Despite the excellent definition of the map for neighboring side chains such as Arg-102, Met-244, and Ile-255 (supplemental Fig. S1), coordinates for a water or hydroxide could only be refined in the distal pocket at partial occupancy (see the Supplemental Experimental Procedures). We presume that the iron in HemS has a sixth-coordinate water (or hydroxide); the structure shows that as the protein tightly clamps the heme between His-196 and Phe-199 on the proximal side and Arg-102, Phe-246, and Leu-92 on the distal side, the porphyrin is bent, resulting in a marked buckling of the four pyrroles away from planarity, which would make an octahedral, hexa-coordinate geometry highly distorted. Thus, the affinity for the bound water might be low, and an association/dissociation equilibrium in the crystal may cause the disorder indicated by the peaks of very weak difference density.

One heme ligand is bound to HemS in the structure of the complex, showing a 1:1 stoichiometry consistent with the biochemical and spectroscopic data on the homologous proteins ShuS (17) and PhuS (19). On the other hand, data on the protein ChuS from *E. coli* were interpreted as to indicate that two heme molecules bind to one protein molecule (18). In the case of HemS, no heme binding through dimerization or oligomerization is expected to occur since gel filtration analysis with different protein and salt concentrations showed that HemS is a monomer (supplemental Fig. S2) and does not form dimers, as for PhuS from *P. aeruginosa* (19), or oligomers, as for ShuS from *S. dysenteriae* (17).

The molecular architecture of HemS is characterized by two topologically homologous domains that join, forming a pair of large, stacked central β-sheets (Fig. 1A). The twist of these sheets and the helices that pack at each end of the double sheet create two distinctive pockets (Fig. 1, A and B). Heme is bound at the deeper, more pronounced pocket, and its tetrapyrrrole ring is buckled and distorted. The proximal side of the heme, which is the side with the coordinating histidine, is embraced by one α-helix (α7) from a three-helix subdomain (α6, α7, and α8); His-196 stems from the beginning of this helix. Both the distal side and the heme propionate groups are flanked by the extensive, convex face of the twisted β-sheet.

**Molecular Recognition of Heme by HemS**—The structure of the heme-binding pocket is dominated by the large, twisted β-sheet that curves around the heme ligand. All the distal residues and the groups interacting with the propionates are provided by the sheet dome (Fig. 2A). Arg-102 lies on the distal side and packs over the porphyrin plane; its guanidinium group is placed about 4 Å from the iron and makes a salt-bridging interaction with Asp-100. This electrostatic contact is sealed away from the solvent by distal residues Leu-94 and Leu-92, which, together with Ala-90, align to form a barrier burying Arg-102 almost completely in the binding site. Arg-102 is sandwiched between Leu-92 and Ile-255. Phe-246 packs over the propionate-bearing pyrroles and completes a line of four distal residues that starts with Leu-92, Arg-102, and Ile-255. Further hydrophobic packing is provided by Met-244 and Val-253, which slot along the sides of Phe-246 (Fig. 2A). This non-polar cap gives way to a solvent-inaccessible polar and electrostatic region involving the side chains of Arg-209, Gln-316, Tyr-318, Lys-294, and Arg-321. These residues, together with two structural water molecules, engage in a series of interactions that firmly anchor the propionate groups to the interior of the protein (Fig. 2B). On the proximal side, His-196 coordinates the iron and interacts with Asp-194. Phe-199 and Val-195 pack in the vicinity of the imidazole of His-196 and make van der Waals contacts with the heme.

HemS homologues, unique to the groups of α-, β-, and γ-proteobacteria, share sequence identity and similarity greater than 30 and 40%, respectively (supplemental Figs. S3 and S4).
Residues that form the heme pocket clearly map onto conserved regions of the sequence (Fig. 1B).

Comparison with Other Heme Proteins—A striking feature of the HemS-binding site is Arg-102. Interestingly, a distal arginine is present in lignin peroxidase and other plant peroxidases (29, 30), although it is over 5 Å away from the iron, as opposed to Arg-102 in HemS, which is less than 4 Å from the metal and makes van der Waals contacts with the porphyrin plane. It appears that this arginine-heme interaction is unique to HemS. The extensive network of nine electrostatic contacts (seven direct and two water-mediated) that anchor the propionate groups is likely to provide a significant contribution to the association (Fig. 2B). A similarly large set of polar/electrostatic interactions has been seen in the structure of another heme transport protein, hemopexin (31), in which arginine, histidine, and tyrosine side chains lock the propionate groups into the complex. The only other bacterial heme carrier that has been structurally characterized is the hemophore HasA secreted by Serratia marcescens (32). HasA has a totally different fold, and its heme pocket has no features in common with HemS.
Conformational Changes upon Heme Binding

The structure of apo-HemS, refined against data to 1.9 Å spacing, reveals that conformational changes take place in response to ligand binding (Fig. 3). To check whether a relationship exists between structural changes and crystal packing, the lattice intermolecular contacts of the apo-HemS structure were carefully examined and compared with those in the heme-HemS complex. Even if the orthorhombic crystals of the apo- and heme-bound states of HemS do not have the same symmetry, their lattice contacts are very similar; in particular, a large area of contact, which appears to govern the packing, is in common between the two crystal forms. This therefore indicates that the structural changes observed in the apo-structure relative to the complex are highly unlikely to be an artifact of crystal packing.

Local changes include the side chains of Leu-94 (distal), Phe-104 (equatorial), and Gln-316 (near propionates) that switch to a different rotamer, thus becoming engaged in interactions with the ligand. Many other residues shift closer to the porphyrin (Fig. 3). Overall, it appears that the N- and C-terminal domains close into the binding pocket. Comparison of the apo- and bound forms using a difference distance matrix indeed shows that relatively few changes take place within the individual domains, whereas marked shifts occur in the position of the domains relative to each other. Domain movements can also be evaluated by measuring the distances between the centers of gravity of secondary structure elements (supplemental Table S2). If the molecule was separated into two distinct structural entities, movements up to 4 Å affect the domains almost as rigid-body shifts. Least-squares fitting of all atoms between the two structures gives an root mean square deviation of 1.4 Å and a maximal displacement of main chain atoms of more than 4 Å. Using the secondary structure elements only of the N-domain as a frame of reference (root mean square deviation of 0.5 Å), atomic superpositions show that the whole C-domain and particularly the α6, α7, and α8 helices move toward the N-terminal distal residues and the heme (Fig. 4).
Mechanism of Heme Binding by HemS

A Heme-induced Fit Accompanies the Switch from Open, Apo-State to Closed, Bound State—Upon heme binding, the N- and C-terminal domains pivot to close onto the ligand, effectively acting as a clamp. The HemS structure therefore switches between an apo, open form and a liganded, closed state (Fig. 4). Strikingly, the global shifts also affect β-strands, increasing the twist of the large β-sheet that forms the dome of the binding site. The ligand can bind to the apo-structure without the need of these conformational changes to remove steric barriers. Although part of the heme pocket is preformed, some side chains reshape the pocket to increase the fit of the heme. Most notably, Phe-104 swings close to the edge of the heme interlocking the ligand in the pocket by wedging a heme vinyl group against Ala-90 (Fig. 3).

The effect of ligand binding may be best described as an induced fit, as demonstrated for the glucose/hexokinase association almost three decades ago (33). In the case of HemS, the heme-induced fit brings about a cleft closure with maximal atomic main chain displacements of over 4 Å, accompanied by the burial of 350 Å² of solvent-accessible surface area in addition to the area buried by the heme. Analysis of temperature factors indicate that the α6, α7, and α8 elements have higher mobility than other parts of the structure (supplemental Fig. S5), thus defining the three-helix subdomain as a flexible region key for the induced-fit binding mechanism.

Structure-Function Relationships—In the heme-HemS complex, almost 60% of the solvent-accessible surface area of the ligand is buried (Fig. 1A). In HemS, the heme therefore remains significantly exposed to solvent relative to other heme proteins, which incorporate heme in such a way that 85–99.5% of the accessible area of the prosthetic group is excluded from the solvent. However, in HemS, the heme is a ligand that needs to be released as well as sequestered. The buried area of the heme in HemS is comparable with that in hemopexin (about 70%), and this may be related to the reversible binding required by the transport function of both these molecules.

To release its cargo, HemS must reverse the conformational changes observed upon heme binding. Switching from the closed, loaded state to the apo, open form may require the binding of an effector molecule since conditions of extreme pH and high salt are not sufficient to induce heme loss (data not shown). In fact, it has been established that PhuS from P. aeruginosa interacts with the heme oxygenase paHO (19), and it is possible that HemS also delivers its cargo to a heme oxygenase. It is proposed that a heme oxygenase enzyme or another effector molecule interacts with HemS and that formation of a complex would trigger the conformational switch required to unlock the heme and unload it to the oxygenase.

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