

The Hemophore HasA from *Yersinia pestis* (HasA_{yp}) Coordinates Hemin with a Single Residue, Tyr75, and with Minimal Conformational Change

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Supporting Information

ABSTRACT: Hemophores from *Serratia marcescens* (HasA_{sm}) and *Pseudomonas aeruginosa* (HasA_p) bind hemin between two loops, which harbor the axial ligands H32 and Y75. Hemin binding to the Y75 loop triggers closing of the H32 loop and enables binding of H32. Because *Yersinia pestis* HasA (HasA_{yp}) presents a Gln at position 32, we determined the structures of apo- and holo-HasA_{yp}. Surprisingly, the Q32 loop in apo-HasA_{yp} is already in the closed conformation, but no residue from the Q32 loop binds hemin in holo-HasA_{yp}. In agreement with the minimal reorganization between the apo- and holo-structures, the hemin on-rate is too fast to detect by conventional stopped-flow measurements.

Although iron is important for many crucial biological functions, its chemical properties present unique challenges to living cells, which have to overcome the insolubility of Fe(III) and the toxicity of Fe(II) by sequestering iron in heme, iron–sulfur clusters, or iron binding proteins. The very low concentrations of free iron in host cells pose significant challenges to pathogenic bacteria, which have evolved efficient strategies to scavenge iron, including secretion of hemophores, siderophores, hemolysins, proteases, and cytotoxins.^{1,2} Given that ~70% of total iron is bound to hemoglobin, heme is an important iron source. Thus, a strategy used by bacteria to acquire heme is the deployment of hemophores, which are outer membrane-exposed, or secreted proteins involved in the path of heme transfer from their location in the host to the bacterial cytosol,^{2,3} where the macrocycle is degraded by heme-degrading enzymes to release the iron.^{1,4,5} The HasA-type hemophore was first identified in *Serratia marcescens*⁶ and then shown to be conserved in several Gram-negative pathogens, including *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Yersinia pestis*, *Yersinia pseudotuberculosis*, *Erwinia carotovora*, and *Pectobacterium carotovorum*.^{7–11} Hemophores from *S. marcescens*

(HasA_{sm})^{12,13} and *P. aeruginosa* (HasA_p)^{7,14} have been structurally characterized in their apo- and heme-bound (holo) forms and found to be nearly identical. The hemin iron in holo-HasA_p (and HasA_{sm}) is coordinated by H32 and Y75. Each axial ligand is harbored in a loop, termed either the H32 or Y75 loop. The main difference between the apo- and holo-structures is a large rearrangement of the H32 loop, which relocates H32 ~30 Å (Figure 1). Structural and spectroscopic studies carried out with

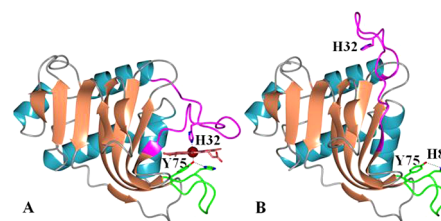


Figure 1. Structure of (A) holo-HasA_p (PDB: 3ELL) and (B) apo-HasA_p (PDB: 3MOK) showing the proximal (Y75) and distal (H32) ligands. The Y75 loop is shown in green, the H32 loop is in magenta and the heme is in red. Heme loads onto the Y75 loop, triggers closure of the H32 loop, and enables H32 to coordinate the heme iron.

WT and H32A HasA_p^{7,15} showed that hemin loads onto the Y75 loop within a few milliseconds. Once hemin is loaded, its proximal side is likely rapidly coordinated by Y75, while coordination by H32 is significantly slower (hundreds of milliseconds to second scale). In the structure of H32A holo-HasA_p in complex with imidazole, hemin-iron is coordinated by Y75 and imidazole, and the H32 loop is near the distal heme face and adopts a conformation very similar to that of WT holo-HasA_p; NMR studies suggest that this conformation is maintained in solution.⁷ These findings led to the conclusion

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that heme loading onto the Y75 loop triggers closing of the H32 loop. Results from targeted molecular dynamic simulations allowed identification of motions that are likely important for transmitting the presence of heme in the Y75 loop to the H32 loop in order to initiate its closing.⁷

Given the unusual His/Tyr coordination of the heme-iron and the induced fit closing of the H32 loop upon heme loading onto the Y75 loop of HasA_p or HasA_{sm}, it is intriguing that H32 is not conserved among HasA proteins. Hemophore sequences from *Yersinia* species (Figure S1, Supporting Information) contain a Gln at position 32 and do not have a His residue close in the sequence that could coordinate the heme iron. These observations suggest that if the hemophore structures of *Yersinia* species are similar to HasA_p and HasA_{sm}, the heme iron would be axially coordinated by an unprecedented set of ligands, Q32 and Y75. In order to understand how the structures of hemophores compensate for the absence of H32, we carried out the structural characterization of the hemophore from *Yersinia pestis* KIM10+ (HasA_{yp}) in its heme-free (apo-) and heme-bound (holo-) forms. As will be shown below, unlike HasA_p and HasA_{sm}, the structure of apo-HasA_{yp} is in a closed conformation and undergoes minimal rearrangement upon binding heme.

A synthetic gene coding for HasA_{yp} was expressed in *Escherichia coli* BL21-GOLD (DE3) cells. Purification yields a mixture of apo- and holo-proteins truncated at the C-terminus (Supporting Information). C-terminal cleavage of hemophores is common^{11,17} and may have functional relevance: The most abundant form of HasA_p secreted by *P. aeruginosa* is the truncated protein missing 15–21 C-terminal residues.¹⁸ In contrast, the most abundant form of HasA_p secreted by quorum sensing-impaired mutants of *P. aeruginosa* is full-length HasA_p.¹⁸ Consequently, the structural characterization of HasA_p was carried out with truncated protein.¹⁴ Herein we report the structural characterization of C-terminus truncated HasA_{yp}.

The Q32 Bearing Loop in apo-HasA_{yp} is in the Closed Conformation. Tetragonal and hexagonal crystal forms were obtained from apo-HasA_{yp}. The tetragonal crystal form (apo-HasA_{yp}^{tet}), which has one molecule in the asymmetric unit, was refined to a resolution of 1.1 Å (Table S1 and Figure 2A). Electron density was observed from S2 to M184, except for T48 and L49, which were not modeled. Apo-HasA_{yp} exhibits the $\alpha + \beta$ fold characteristic of HasA_p and HasA_{sm},^{12,14} which is composed of a “ β -sheet wall” of nine antiparallel β -strands connected by hairpins and an “ α -helix wall” composed of three α -helices and a 3_{10} -helix. The structure of the Y75 loop, extending from Y75 to F83, is identical to the Y75 loops in HasA_p and HasA_{sm}. Interestingly, the loop bearing Q32, extending from N26 to S42, adopts a different conformation from that seen in apo-HasA_p and apo-HasA_{sm} (Figure 2B). Its conformation is very similar to the “closed” conformation seen in the H32 loop of holo-HasA_p and holo-HasA_{sm}. The closed loop conformation of the Q32 loop in apo-HasA_{yp}^{tet} is also observed in the structure obtained from the hexagonal crystal form (HasA_{yp}^{hex}), which exhibits two molecules in the asymmetric unit and was refined to 1.6 Å resolution (Table S1, Figure S2-A). Both molecules in the asymmetric unit are nearly identical (C_α -RMSD = 0.76 Å), exhibit well-defined electron density for residues S2 to D180, and contain a PEG molecule between the Q32 and Y75 loops. Superposing the HasA_{yp}^{tet} structure with the structures of molecules A and B of HasA_{yp}^{hex} reveals near identical architectures (see Figure S2-B), except for the apparent absence of PEG or other exogenous molecules between the Q32 and Y75 loops of apo-HasA_{yp}^{tet}.

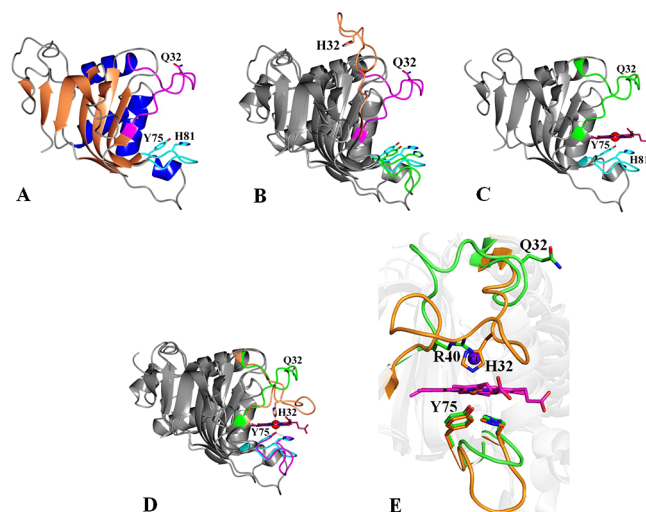


Figure 2. (A) Structure of apo-HasA_{yp}^{tet} with the Q32 loop shown in magenta and the Y75 loop in cyan; Q32, Y75, and H81 are shown in sticks. (B) Superimposed structures of apo-HasA_p (PDB ID: 3MOK) and apo-HasA_{yp}^{tet} where the H32 loop in apo-HasA_p is shown in coral and the Q32 loop in apo-HasA_{yp}^{tet} in magenta. (C) The structure of holo-HasA_{yp} is very similar to that of apo-HasA_{yp}; the Q32 loop is in green and the Y75 loop in cyan. (D) Superposition of holo-HasA_{yp} and holo-HasA_p (PDB ID: 3ELL) structures. The heme iron in HasA_p is coordinated by Y75 and H32, whereas in holo-HasA_{yp} it is coordinated by Y75 (also see Figure 3). (E) Zoomed-in view comparing the heme-binding pockets of holo-HasA_{yp} (green) and holo-HasA_p (orange); a chloride ion (purple sphere) in the distal pocket of HasA_{yp} is in the position occupied by the side chain of H32 in holo-HasA_p and holo-HasA_{sm}. Structures were superimposed using the program Superpose.¹⁶

The Structures of apo- and holo-HasA_{yp} Are Nearly Identical. The crystal structure of holo-HasA_{yp} was solved to a resolution of 2.2 Å (Table S1 and Figure 2C). Well-defined electron density was traced from S2 to D180 for each of the 10 molecules in the asymmetric unit; superposing all 10 chains results in a C_α -RMSD = 0.50 Å. Figure 2 illustrates the surprising observation that, unlike HasA_p or HasA_{sm}, the Q32-loop in HasA_{yp} does not change conformation upon binding heme (C_α -RMSD = 0.56 Å). As in the previously characterized hemophores, the heme iron in HasA_{yp} is coordinated by Y75, which also forms a hydrogen bond (2.7 Å) with the N δ of H81 (H83 in HasA_{sm} and HasA_p). Despite the similar coordinating environment of the proximal binding site, the environment of the distal site in HasA_{yp} is distinct (Figure 2D,E) in that the sixth coordination site is not occupied by a protein-provided ligand. Note that Q32, which we hypothesized may coordinate the heme iron, is located approximately 14 Å away (C_α) from the heme iron, in a position very similar to the one it occupies in the apo-form. Q32 is clearly not part of the heme-binding pocket. The most significant change in the Q32 loop upon heme binding is the relocation of the R40 side chain (Figure 3). This small reorganization protects the heme distal face immediately after it binds to HasA_{yp}, a situation that contrasts with apo-HasA_p or apo-HasA_{sm}, where the heme distal face is exposed to the aqueous environment prior to closing of the H32 loop. The Q32-loop of HasA_{yp} is three residues shorter than the H32 loop of HasA_p and HasA_{sm} (Figures S1 and S3), which may decrease the conformational flexibility of the Q32 loop. This issue is currently under investigation in our laboratories.

Electron density immediately above the heme iron was best modeled as a chloride ion (Figure 3). The modeled Cl[−] is not

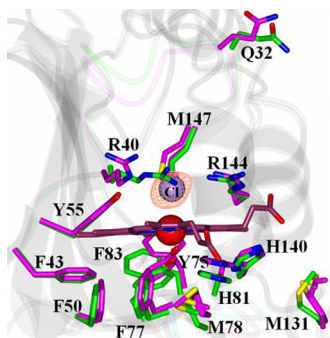


Figure 3. View of the heme-binding pocket in the superimposed structures of apo-HasA_{yp}^{tet} and holo-HasA_{yp} illustrate the minor structural differences between apo- (magenta) and holo- (green) HasA_{yp}. The distal site in the holo protein is coordinated by a chloride ion; the $F_o - F_c$ omit map contoured at 3σ is shown in mesh representation.

within binding distance of Fe(III) (Fe–Cl = 2.8 Å). In contrast, the resonance Raman and EPR spectra show that in solution the heme iron(III) in holo-HasA_{yp} adopts a six-coordinate high-spin configuration (Figure S4). Although attempts to detect Fe(III)–Cl and Fe(III)–OH stretching frequencies were unsuccessful, we presume that the sixth ligand might be a solvent molecule and/or a loosely bound chloride ion. Because HasA sequences with Q32 contain R40 but those with H32 do not (Figure S1), we hypothesize that R40 may stabilize the chloride ion observed in the holo-HasA_{yp} structure. In contrast to the large differences observed in the H32 loop, the structure of the Y75 loop is conserved among the three hemophores (Figure S5). As pointed out previously,⁷ the proximal loop forms a conserved hydrophobic surface for heme to interact with the hemophore. Accordingly, heme binding to HasA_{yp} causes minimal changes to the Y75 loop; the largest change is a rearrangement of the F83 side chain to accommodate the incoming heme (Figure 3).

HasA_{yp} Loads Hemin from Solution Faster than HasA_p. Stopped-flow experiments reveal that heme capture by apo-HasA_{yp} is complete within the millisecond deadline of the apparatus and is thus much faster than in apo-HasA_p (Figure S6). These observations support the idea that apo-HasA_{yp} adopts a closed configuration in solution and that minimal reorganization occurs upon binding heme. This distinctive behavior of HasA_{yp} is likely to also affect heme transfer to the receptor protein, which in *S. marcescens* is thought to modulate heme binding affinity to HasA_{sm} via interaction with the H32 loop.¹⁹

■ ASSOCIATED CONTENT

Supporting Information

Detailed experimental procedures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Accession Codes

PDB entries 4JER (apo-HasA_{yp}^{tet}), 4JES (apo-HasA_{yp}^{hex}), 4JET (holo-HasA_{yp})

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Notes

The authors declare no competing financial interest.

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