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The crystal structure of the secreted dimeric form of the hemophore HasA reveals a domain swapping with an exchanged heme ligand.

Mirjam Czjzek², Sylvie Létoffé³, Cécile Wandersman³, Muriel Delepierre¹, Anne Lecroisey¹ & Nadia Izadi-Pruneyre^{1*}

1:Unité de Résonance Magnétique Nucléaire des Biomolécules CNRS URA 2185 Département de Biologie Structurale et de la Chimie, Institut Pasteur, 75724 Paris Cedex 15 France. 2:Present address: CNRS; Université Pierre et Marie Curie-Paris6, Laboratoire International Associé-Dispersal and Adaptation in Marine Species, Unité Mixte de Recherche 7139, Station Biologique, F-29682 Roscoff Cedex, France. 3: Unité des Membranes Bactériennes, CNRS URA 2172 Département de Microbiologie Fondamentale et Médicale, Institut Pasteur, 75724 Paris Cedex 15 France.

running title: domain swapped dimeric hemophore

To whom correspondence should be addressed to:

Nadia Izadi-Pruneyre, Unité de Résonance Magnétique Nucléaire des Biomolécules CNRS URA 2185 Département de Biologie Structurale et de la Chimie, Institut Pasteur, 28, rue du Dr. Roux, 75724 Paris Cedex 15 France. tel: 33 1 40 61 36 66 fax: 33 1 45 68 89 29 e-mail: nizadi@pasteur.fr

The atomic coordinates and structure factor (codes 2CN4 and r2cn4sf) have been deposited in the Protein Data Bank.

Abbreviations used: GdnHCl, guanidine HCl ;OD, optical density; PDB, Protein Data Bank; rmsd, root mean square deviation.

ABSTRACT

To satisfy their iron needs, several gram-negative bacteria use a heme uptake system involving an extracellular heme-binding protein called hemophore. The function of the hemophore is to acquire free or hemoprotein-bound heme and to transfer it to HasR, its specific outer membrane receptor, by protein-protein interaction. The hemophore HasA secreted by *Serratia marcescens*, an opportunistic pathogen, was the first to be identified and is now very well characterized. HasA is a monomer that binds one *b* heme with strong affinity. The heme in HasA is highly exposed to solvent and coordinated by an unusual pair of ligands, a histidine and a tyrosine.

In the present work, we report the identification, the characterization and the X-ray structure of a dimeric form of HasA from *Serratia marcescens*: DHasA. We show that both monomeric and dimeric forms are secreted in iron deficient conditions by *Serratia marcescens*. The crystal structure of DHasA reveals that it is a domain swapped dimer. The overall structure of each monomeric subunit of DHasA is very similar to that of HasA but formed by parts coming from the two different polypeptide chains, involving one of the heme ligands. Consequently DHasA binds two heme molecules by residues coming from both polypeptide chains. We show here that, while DHasA can bind two heme molecules, it is not able to deliver them to the receptor HasR. However, DHasA can efficiently transfer its heme to the monomeric form that, in turn, delivers it to HasR. We assume that DHasA can function as a heme reservoir in the hemophore system.

INTRODUCTION

Iron is essential for many metabolic pathways. However despite its abundance, it is practically insoluble in the presence of oxygen at a physiological pH and is thus scarcely available. Heme-containing compounds, which are widespread among vertebrate hosts, are advantageous iron reservoirs for bacterial pathogens. Therefore several Gram-negative bacteria use a heme uptake system involving an extracellular heme-binding protein called hemophore. The function of hemophores is to acquire free or hemoprotein-bound heme and to deliver it to a specific receptor at the cell surface whereby the heme is internalized and then used as iron source.

The hemophore secreted by the opportunistic pathogen *Serratia marcescens*, HasA, was the first to be identified (1) and is now very well characterized. It is secreted under iron deficiency condition by an ABC- type pathway *via* a C-terminal secretion signal. This 19kDa protein binds one *b* heme molecule with an affinity of $5.3 \cdot 10^{10} \text{M}^{-1}$ (2,3). HasA is able to bind free heme or to capture it from other hemoproteins, like hemoglobin. The heme transfer from HasA to its receptor HasR which has a much lower affinity for heme ($K_a 5 \cdot 10^6 \text{M}^{-1}$) occurs by protein-protein interaction (4). The next steps of heme internalization, including the heme transfer from the receptor to the periplasm and the recycling and/or ejection of the empty hemophore from the receptor are dependent on the energy given by an inner membrane complex composed of three proteins TonB-ExbB-ExbD (5).

The structure of HasA shows an original $\alpha+\beta$ fold, with an unusual arrangement of the heme-binding site. The α helices are all packed on one side of the molecule and the β strands on the other side. The heme is held by two loops at the interface of the α and β parts of the molecule and is highly exposed to solvent. The iron atom is ferric and coordinated by the N ϵ of a

histidine residue on one side (His32) and to the O η of a tyrosine residue (Tyr75) on the other (6).

We report here the identification, the characterization and the X-ray structure of a dimeric form of the hemophore present in the culture medium: DHasA. Our results reveal that DHasA is a domain-swapped dimer in which the same structural element is exchanged between the two subunits. It is noteworthy that the exchanged part contains one of the heme ligands, His32.

Domain swapping was first described at the atomic level by Eisenberg and coworkers in 1994 in the crystal structure of diphtheria toxin (7). It is a mechanism by which one protein molecule exchanges a structural “domain” with an identical partner (“domain” is not used in a strict sense). The ‘domain’ can be as large as an entire tertiary globular domain, or as small as a single element of secondary structure. Currently more than 60 proteins have been identified as domain-swapped protein (8). It has been advanced that the domain swapping plays a role in the regulation of function, in an evolutionary strategy to create protein complexes, or in misfolding and aggregation, but its biological relevance is still unclear (9). In this work, we show that DHasA could function as an efficient heme reservoir in the hemophore system.

MATERIAL AND METHODS

Expression and purification of the HasA proteins

ApoHasA proteins, HasA and DHasA, were obtained from the culture supernatant of *Escherichia coli* strain POP3 (pSYC34PAM) grown at 30°C in M9 glycerol medium and purified as described previously (10). *Serratia marcescens* SM365 was also grown at 30°C in M9 glycerol supplemented with 0.2 mM 2,2'-dipyridyl to reduce available iron.

The holoforms were prepared by adding a freshly prepared hemin solution to the apo-proteins at a ratio of about 120%. The excess of heme was eliminated by gel filtration. All of the samples were in 50 mM sodium phosphate buffer, pH 7 otherwise mentioned.

The HasA concentration was calculated using the previously determined $\epsilon_{277\text{nm}}$ value of 19500 $\text{M}^{-1}\text{cm}^{-1}$ (11). The same $\epsilon_{277\text{nm}}$ value was used to determine the concentration of DHasA sample in monomeric protein. All absorption measurements were done in a Perkin-Elmer Lambda 2 spectrophotometer using 1 or 0.2 cm path length cuvettes.

Hemin solution

Hemin solutions were prepared just before use to prevent heme aggregation. Bovine hemin (Sigma) was dissolved in 150 μl of 0.1 N NaOH, diluted to volume desired by the adequate buffer, and centrifuged. The concentrations of the hemin solutions were measured in 0.1 N NaOH using the $\epsilon_{385\text{ (0.1N NaOH)}}$ value of 58440 $\text{M}^{-1}\text{cm}^{-1}$ (12)

Analytical Ultracentrifugation

Ultracentrifugation studies were performed in an XLA (Beckmann) analytical ultracentrifuge using 12mm path length cells. Purified DHasA samples were $2 \cdot 10^{-5}$ M (monomer concentration) in 20 mM sodium phosphate, pH 7. The sedimentation/diffusion equilibrium experiments were performed as previously described (2).

Dimer/monomer formation

The presence of dimeric and monomeric proteins in various culture supernatant was tested by native-PAGE, followed by immunodetection with anti-HasA antibodies and revealed by ECL+ (Amersham) as described previously by Létoffé *et al* (13). The immunodetection was used to increase the detection threshold of the samples concentration. Purified monomeric and dimeric HasA of known concentrations of 10^{-5} , 10^{-6} and 10^{-7} M were used as standard in order to estimate the concentration of secreted protein.

Heme binding

Heme binding experiments were performed by UV-visible spectroscopy by following the Soret band at 407 nm. The concentrations of DHasA and of the hemin solution were respectively $1.8 \cdot 10^{-6}$ M (monomer concentration) and $2.25 \cdot 10^{-5}$ M.

Isothermal titration calorimetry (ITC)

Titration were performed at 25°C using a MicroCal MCS titration calorimeter (MicroCal Inc., Northampton, MA). Proteins and hemin were in 20 mM sodium phosphate, pH 7. Solutions were thoroughly degassed by stirring under vacuum before use. Due to heme adsorption, the calorimeter cell and the microsyringe used for injections were extensively washed after each experiment. For all titrations, the heat of dilution of the ligand was measured by injecting the ligand into the buffer alone or by injecting an excess of ligand into the cell after saturation. The value obtained was subtracted from the heat of reaction to obtain the effective heat of binding. All of the injections were carried out with 3 min intervals.

The theoretical titration curves were fitted to the experimental data using the ORIGIN software (Micorcal). The molar binding stoichiometry and molar binding enthalpy (ΔH) were determined directly from the fitted data. The affinity constant of DHasA for heme, which is higher than 10^8 M, was too large to be measured by ITC direct titration. Therefore, it was determined by the displacement method (14) using the H32A-H83Q double mutant of the heme binding pocket with $K_a = 2.6 \times 10^7 \text{ M}^{-1}$ and $\Delta H = -29.2 \text{ kJ.mol}^{-1}$ as previously described by Deniau *et al* (3). The Gibbs (binding) free energy (ΔG) and molar entropy of binding (ΔS) were calculated using the equation: $\Delta G = -RT \ln K_a = \Delta H - T\Delta S$, where R is the gaz constant and T is the temperature in Kelvin.

E.coli growth assays

Growth stimulation of C600 $\Delta\text{hemA}::\text{km att } \lambda::\text{hasR}$ by exogenously supplied purified protein was tested as follow. Cells expressing HasR were grown in LB medium supplemented with

20 μ g/ml of delta aminolevulinic acid (LBD) and appropriate antibiotics to an OD₆₀₀ of 1 and 100 μ l of the resulting culture was mixed with 3 ml of top agar and plated on LBD 0.02 % arabinose. Aliquots of 50 μ l of serial dilutions of various apo/holo monomer/DHasA were added to wells punched in the solidified agar. Growth around the wells was noted after overnight incubation at 37°C.

NMR spectroscopy

NMR samples contained about 1.5 mM HasA or DHasA (monomer concentration) in 50 mM Sodium phosphate buffer, pH 5.6. NMR spectra were recorded on Varian Inova 500 or 600 MHz spectrometers equipped with the VNMR 6.1 software and with Sun Sparc Ultra 2 and Ultra 5 respectively. ¹H-¹⁵N HSQC experiments (15) were recorded at 30°C using 1024 x 128 data points and 64 scans. The one-dimensional ¹H NMR spectra were acquired at 50°C with a spectral width of 200 ppm, 8k data points and a recycle delay of 40 ms. Water suppression was achieved by presaturation.

Protein crystallization

The protein concentration was about 4.0 mg/ml in 50 mM phosphate buffer at pH 7 and 0.02% NaN₃. Initial crystallization trials were performed with MDL (Molecular Dimensions Limited) and Decode Genetics ("Wizard" 1&2, Emerald Biostructures), that is, a total of 192 trials in two 96 multi-well crystallization plates from Greiner. These trials were set up using a Cartesian crystallization robot, mixing 200 μ l of protein with 100 μ l of reservoir solution.

Crystals were grown by the hanging-drop vapor-diffusion method using 24-welled Linbro plates. Hanging drops on siliconized cover slips were prepared by mixing 2 μ l of the protein solution with 1 μ l of the reservoir solution that contained 100 mM Tris buffer at pH 8.5, 2 M NaH₂PO₄ and 2 M K₂HPO₄. They were then placed over 0.5 ml of the reservoir solution. All crystallization trials were carried out at 20° Celsius.

Data collection and processing

X-ray diffraction data of a DHasA crystal were collected at 100 K at the European Synchrotron Research Facilities (ESRF, Grenoble, France) beamline ID29 using an ADSC Quantum 4R CCD detector. The crystal was flash-cooled in a liquid-nitrogen stream with 10 %(v/v) glycerol as cryoprotectant. The wavelength of the synchrotron X-rays was 0.979 Å. The crystal was rotated through 100° with a 1.0° oscillation range per frame. The raw data were processed using the program MOSFLM (16) and then merged and scaled using the program SCALA (17).

Structure determination and refinement

The molecular replacement with the program AMoRe (18) in the resolution range of 9 to 3.1 Å with the model of monomeric HasA (PDB ID: 1B2V) gave two solutions corresponding to the two monomeric subunits that form the dimer, with an overall correlation coefficient of 0.59 and an R-factor of 37.4 %. The refinement was performed using REFMAC5, part of the CCP4-package (17). Water molecules were added using CCP4/warp (19). The stereochemistry of the final structures was evaluated using PROCHECK (20). All refinement statistics are summarized in Table 1.

RESULTS

Identification of a dimeric form of HasA in solution -DHasA-

Gel filtration chromatography of the supernatant of POP3 (pSYC34PAM) over-expressing HasA in minimum medium at 30°C, gives two peaks (Figure 1). Their ratio varies depending on the culture from 10 to 50%. Analysis of the fractions corresponding to these peaks by SDS-PAGE shows that they both contain one protein that migrates like HasA, HasA being the unique protein secreted in *E. coli* overexpressing HasA. The slower migrating peak corresponds to the monomeric form of HasA, previously characterized and studied (2). The

faster migrating peak has an apparent molecular weight corresponding to twice that of HasA, it will be called DHasA hereafter.

The molecular weight of DHasA was determined by analytical ultracentrifugation with the sedimentation/diffusion equilibrium experiment. The experimental data fit in a satisfactory manner using a one-solute ideal solution model, with a molecule of molecular weight 36900 ± 412 (data not shown). This value is in agreement with that of 38302 for a dimeric form of HasA, the variation remaining within the limits of methods.

DHasA is formed *in vivo*

DHasA is secreted by *Serratia marcescens* in comparable proportions to that observed in *E.coli*. This observation proves that it is not an artefact of over-expression in *E.coli*.

DHasA is observed by native-PAGE in the culture supernatant. The formation of DHasA is thus not dependent on purification conditions, especially not on the Ammonium Sulfate precipitation leading to high protein concentration. As observed in Figure 2, DHasA and HasA secreted by *S. marcescens* exist both in cleaved and uncleaved forms. The conditions of cleavage have already been studied for HasA and the study revealed that cleavage is performed by *S. marcescens* major protease, PrtSM, and concerns the twelve C-terminal residues of HasA (21).

The presence of DHasA is not the result of an accumulation of the monomeric form during the culture. Indeed, it is detected during the exponential phase of culture when cell density reaches an OD₆₀₀ of 1 and when the total HasA protein concentration is estimated to be 10^{-6} M and not only during the stationary phase.

DHasA, like HasA, is secreted in minimum medium in the apoform as shown by the absence of the Soret band at 407 nm, characteristic of the holoform, in the UV-visible spectrum. The holoform can be obtained by adding a solution of hemin to the culture medium. The amount

of dimeric form obtained is comparable to that obtained when the medium is not supplemented with hemin. The presence (or absence) of heme in the culture medium has thus no influence on DHasA formation.

Structural comparison of DHasA and another dimeric form obtained *in vitro*

Three different crystal forms of HasA were obtained under different crystallization conditions (22). The overall structure of HasA in the three crystals is the same. Nevertheless, even though all were obtained from a purified monomeric sample, HasA is in a dimeric form in one of the crystal (PDB ID: 1DK0). Therefore the question arises: are the two dimeric proteins obtained *in vitro* and *in vivo* identical? In order to answer this question we have analyzed the structure of DHasA by NMR to determine if it is equivalent to that of 1DK0.

The ^{15}N - ^1H HSQC spectrum is a fingerprint of the backbone conformation of a protein. Each ^{15}N - ^1H cross-peak reflects amino acid local environment in protein. The comparison of ^{15}N - ^1H HSQC spectrum of ApoDHasA with that of ApoHasA for which the complete assignment is known (10) allowed us to obtain structural informations. This spectral comparison was done at two concentrations, namely 0.4 and 1.5 mM. At both concentrations, the number of ^{15}N - ^1H amide connectivities present in the ApoDHasA spectrum is comparable to that usually found in the ApoHasA spectrum at the limit of resolution (169 vs 172 respectively). The high similarity between both spectra suggests a high structural homology between the two protein forms.

Most of the residues could be easily assigned by homology. Residues 48-58, 71, 72, 73, 75 and 77 could not be assigned in DHasA spectra. This observation indicates that while the global structure of the protein is conserved, the environment and/or the structure of these residues are changed indicating that they are involved in dimerisation. Residues 42, 43, 45,

67, 69, 82, 83, 86, 99 which display slight differences in their chemical shifts as compared to the monomeric form (up to 1 ppm in ^{15}N and 0.2 ppm in ^1H dimension) may be located at the immediate sequential and/or spatial vicinity (backbone and side-chain) of residues at interface. (Figure 3)

The crystal structure of the dimeric form of HasA, 1DK0, could not explain all the differences between the ApoHasA and ApoDHasA spectra. Conversely, residues 88-107 located at the interface of the monomeric subunits of HasA in 1DK0 do not show significant variation of chemical shifts. These observations are indicative of substantial differences in the quaternary structure of DHasA with respect to that of 1DK0. Thus, the dimeric form observed in the crystal structure of 1DK0 might be an intermediate of aggregation, stabilized in the crystal. Indeed, at high ionic strength (*i.e* 150mM NaCl) the presence of some aggregated HasA has been detected.

No dissociation of DHasA into monomers was observed by NMR at 30°C at a protein concentration of 1.5 mM during one week and inversely no dimerisation of the monomeric sample was observed at the same or higher concentrations (up to 1.8 mM). The lack of dissociation and the very high stability of DHasA, generally observed in domain-swapped dimers, suggest that DHasA might be this type of dimer. In order to check this hypothesis, we have thus determined the crystal structure of DHasA and studied properties and activity of this new HasA dimeric form by different methods.

DHasA crystallizes as a domain swapped dimer

DHasA has successfully been crystallized. The optimized reservoir conditions are 100 mM Tris buffer at pH 8.5, 2 M K_2HPO_4 and 2 M NaH_2PO_4 . The brownish crystals belonged to space group P2_1 , with unit parameters highly resembling those of 1DK0 (Table 1), and diffracted to a maximum resolution of 2.3 Å. Two molecules are present in the asymmetric

unit, giving a crystal volume per protein mass (V_M) of $2.17 \text{ \AA}^3 \text{ Da}^{-1}$ and a solvent content of 43% by volume (23). The coordinates describing two protein molecules with two heme groups per asymmetric unit were refined to a final R-factor and R_{free} of 16.9% and 22.3%.

The overall fold of each monomeric subunit of DHasA is very similar to that of HasA but formed by parts coming from the two different polypeptide chains present in the asymmetric unit. Each monomeric subunit is composed of seven beta strands packed on one side and four α -helices on the other side. But for each subunit residues 2-49 does not belong to the same polypeptide chain than other residues (Figure 4a-c). This crossing over of polypeptide chains to form a multimer assemblage is frequently observed in the so-called domain-swapped dimers. Given the fact that both the monomer and the domain swapped dimer of HasA exist in solution and in stable form, DHasA can thus be considered to be a *bona fide* example of 3D swapping as defined by Liu & Eisenberg (7). The backbone superimposition of one subunit of DHasA and the monomeric form of HasA shows a rmsd of 0.93. Significant structural changes occur at the dimer interface of the swapped domain, as compared to the monomeric form. As expected, the highest rmsd value is observed in the hinge loop (43-48) linking the swapped domain to the rest of its subunit. Besides to this, two residues, Asn34 and Gly35 also show high rmsd values, compared to the overall mean value, this being valid for the two subunits composing the dimer as shown in Figure 5a. The overall average B-factor, however shows that these regions do not display particular high B-factors as compared to monomeric HasA (Figure 5b). On the contrary, dimeric form of HasA appears to be less flexible, especially in the region of the heme binding pocket.

The heme binding sites are similar to that of the monomeric form, and are composed of two loops positioned at the interface of the α and β side that fix the heme in a relatively solvent exposed environment. The swapping makes both heme sites less accessible than that of the monomeric form. The resolution of the data unambiguously revealed that the heme can take

two orientations, related by a 180° rotation about the α , γ -meso axis, as has already been observed in the previously determined HasA structures (22). The heme iron of each monomeric part is coordinated by the O η of a tyrosine (Tyr75) and to the N ϵ of a histidine (His32) with the difference that the latter belongs to the swapped region, and the heme molecule of each subunit is actually bound by residues coming from the two polypeptide chains.

Heme environment of DHasA characterization by NMR

The ^1H NMR spectra of HoloDHasA shows the presence of two sets of resonances with relative intensity of 70:30 corresponding to the two possible orientations of the heme observed in the crystal structure (Figure 6). This ratio is well visible in a couple of isolated peaks at 72.5 and 75.7 ppm. The same ratio was equally found for the monomeric form as reported by Caillet-Saguy *et al* (24). The methyl resonances of the two heme molecules are observed between 25 and 50 ppm. These chemical shifts are intermediate between those typical for a high spin $S=5/2$ iron (III) heme and those for a low spin $S=1/2$ iron (III) heme. This presumes the existence of a high spin / low spin equilibrium as that observed for the monomeric form (24). The chemical shifts are however slightly different to that of the monomeric form at the same temperature and pH, indicating that the heme iron properties and environment in solution and/or the high spin/low spin species ratio are not exactly the same for the two protein forms.

Activity of DHasA

The function of HasA is to take up heme and to shuttle it to a specific receptor. To determine the implication of DHasA in the heme acquisition pathway mediated by the hemophore, if any, we have studied the heme binding properties and its delivery to the receptor HasR.

Heme binding: To study the heme binding, we carried out heme titration experiments by UV-visible spectroscopy and by ITC microcalorimetry as described in our previous works (2,3,11). The titration curve obtained by UV-visible spectroscopy shows that DHasA binds one molecule of heme/monomer (data not shown). The ITC titration data can be fitted without any constraint to a single set of sites model with a stoichiometry of 1 mol /mol of monomer and an affinity constant higher than 10^8 M^{-1} for each site. However, at the minimal concentration that could be used in the calorimetric cell to obtain a reliable heat emission signal, 10^{-5} M , the curve was too steep at the equivalence point to determine precisely the affinity constants (Figure 7). Hence, we determined their values by displacement titration using a low affinity mutant the H32A-H83Q: $9 \cdot 10^8 \text{ M}^{-1}$ for each site.

From the results of the ITC titration experiments, we could deduce an enthalpy value of $-110.5 \pm 2 \text{ kJ.mol}^{-1}$ and an entropy value of $-59.4 \text{ kJ.mol}^{-1}$ for the heme binding. The interaction of heme with DHasA is thus strongly enthalpy driven as observed in our previous work (3) for the monomeric form of HasA ($-105.4 \pm 3.8 \text{ kJ.mol}^{-1}$). The decrease in binding entropy in DHasA ($T\Delta S = -59.4 \text{ kJ.mol}^{-1}$) compared to HasA ($T\Delta S = -44.3 \text{ kJ.mol}^{-1}$) is consistent with a decrease of the solvent exposed hydrophobic surface area around the heme from 14 \AA^2 to 10 \AA^2 .

The difference of heme iron properties in solution deduced from NMR analysis and the slight difference of some residues position in the loop containing His32, in particular Asn34 and Gly35, linked to the heme propionate groups by hydrogen bonds, can explain the lower affinity of DHasA for the heme molecule. Indeed, besides the residues of the hinge loop, these are the residues that display the highest rmsd values in the superposition of each monomer of DHasA with the crystal structure of monomeric HoloHasA (1B2V). The difference in the positions of Asn34 and Gly35 is only seen in the presence of heme (Figure 5a).

Heme delivery of DHasA to HasR: Added exogenously, the HoloDHasA does not allow heme acquisition of C600 *ΔhemA::km at λ::hasR*. It has recently been shown that the heme of HasA is transferred to the receptor HasR by protein-protein interaction (4). The inability of HoloDHasA to transfer directly its heme to HasR can be due either to an absence of interaction between these two proteins or to an inability of HoloDHasA to deliver its heme to HasR. The first hypothesis was tested using the solid-phase binding assay. Aliquots of 10^7 CFU of POP3 (pam238-hasR) HasR producing bacteria and of the control strain POP3 (pam238) were spotted onto nitrocellulose membranes and incubated with serial dilutions of apo or HoloDHasA protein. The interaction was probed with a polyclonal antiHasA serum (25). As shown in Figure 8, no binding affinity above background was observed with samples incubated with 10^{-6} , 10^{-7} or 10^{-8} M apo or HoloDHasA indicating that the DHasA is unable to bind to the receptor HasR.

Nevertheless in our growth test, HoloDHasA mixed with ApoHasA allows the heme acquisition by bacteria and become thus a source of heme for bacteria. This result indicates that the heme of DHasA is transferred to HasA who delivers it, in turn, to the specific receptor (Table 2).

DISCUSSION

In this work we have identified a secreted dimeric form of HasA, DHasA. Its structure reveals that DHasA is a domain-swapped dimer. Three-dimensional domain swapping is an event by which a monomer exchanges part of its structure with an identical monomer to form an oligomer, where each subunit has a similar structure to the monomer. While each subunit structure of DHasA compared to that of HasA monomeric form is entirely conserved significant structural changes occur at the dimer interface of the domain-swapping. As expected, the highest rmsd value is observed in the hinge loop (43-48), composed of

GGFYGG. Although the relation between the hinge loop sequence and swapping phenomenon is not actually demonstrated, the presence of prolines and residues generating some flexibility have been put forward in facilitating the domain swapping process. Here, the presence of 4 glycine residues in the hinge loop ensures the flexibility needed to perform the swapping. Furthermore, the presence of two hydrophobic residues (FY) constrained at the dimer interface due to both steric hindrance and their hydrophobic character may be a factor that drive the hinge loop to swap or not. The hinge loop sequence is strictly conserved in *Erwinia caratovora* hemophore (http://www.sanger.ac.uk/Projects/E_caratovora) which is not yet characterized. It is conserved in the form of GXFXXG in all of the other hemophores found in *Pseudomonas aeruginosa*, *P. fluorescence*, *Yersinia pestis*, and *Y. enterocolitica*. The swapping brings residues Phe45 and Tyr46 close to the 75-85 loop of the same polypeptide chain, creating supplementary hydrophobic interactions among residues of the same chain. As a consequence, Phe78 is in a more hydrophobic environment than that observed in the monomeric form where it is exposed to the solvent.

The swapping in DHasA conserves the overall structure of the heme binding site as well as the ligands of the heme group, although it is noteworthy that one of them, His 32, is provided by the swapped domain. Nevertheless, some differences are observed in the heme binding pocket of DHasA as compared to that of the monomeric form: i) both heme sites of DHasA are less accessible to solvent because of the swapping and the heme binding pocket appears to be less flexible by the protein-protein interface (lower B-factors in Figure 5b) ii) residues Asn34 and Gly35, which belong to the swapped domain and are hydrogen bonded to the heme propionate groups, show higher rmsd than that of the rest of protein (except for the hinge loop, Figure 5a). These differences can explain the variations of heme chemical shifts observed in solution by NMR and the lower affinity of DHasA for heme compared to that of monomeric form ($9 \cdot 10^8 \text{ M}^{-1}$ vs $5,3 \cdot 10^{10} \text{ M}^{-1}$).

We have shown here that DHasA exists in the culture supernatant and its presence does not result from over-expression or purification conditions which might expose the protein to destabilizing condition at high concentration. This observation and the very high stability of HasA -T_m of 60°C and half unfolding concentration of GdnHCl of 2.7 M- (26), allow to assume that DHasA folds directly from an unfolded state and by an independent pathway, and not *via* the initial formation of the monomer. This model has also been proposed by Rousseau and co-workers for suc1, a cell cycle regulatory protein, with two native states, a monomer and a domain swapped dimer (27, 28). Assuming that a single polypeptide sequence gives access to the two forms of HasA under the same conditions, both in *E.coli* and in *S.marcescens* and this apparently independently of any particular bacterial signal, two questions arise: what are the folding pathways for the monomer and the domain swapped dimer and how are they connected to each other? The fact that HasA of *Serratia marcescens* is secreted *via* its C-terminal secretion signal means that the polypeptide needs to be fully synthesized before being secreted. Moreover, the cytoplasmic chaperone SecB has been shown to be essential for HasA secretion *in vivo* (29). In its absence, folded HasA accumulates in the cytoplasm and is unable to be secreted (30). Together, these data show that the folding of HasA should be achieved during or after secretion. Furthermore, *in vitro* study of refolding of denatured HasA has demonstrated that Sec B slows down HasA folding. In this study, refolding of denatured HasA has been analyzed by stopped-flow measurements of the fluorescence of its unique tryptophan (26). DHasA had not been characterized before and consequently the mono/oligomeric state of refolded HasA has not been checked in that study. Nevertheless, as the tryptophan, located in the swapped part of DHasA, has exactly the same environment in both monomeric HasA and in DHasA, the formation or the presence of DHasA, if any, could not be detected by the fluorescence signal. The folding/unfolding study of both forms of HasA at different concentrations, while checking the presence and the

relative amount of each form for example by analysis of the fluorescence anisotropy decay, will provide further information on the folding pathway and contribute to a better understanding of the domain swapping phenomenon.

Currently more than 60 proteins have been identified as domain-swapped oligomers with no apparent structural or functional connection between them. Despite the increasing number of examples, clear evidence for a functional role of domain swapping is lacking (28). Several examples exist for which only the domain swapped version of the protein has been described or only the swapped multimeric form is functional. In some cases, however, both swapped and unswapped forms of the protein have been characterized and domain swapping has been proposed to have a regulatory function. For example, Glyoxalase I from *Pseudomonas aeruginosa* exists both in an active domain-swapped dimeric state and as a metastable less active monomer (31). *In vitro* conversion from dimeric to metastable monomeric Glyoxalase I can be triggered upon addition of glutathation. After removal of glutathione, glyoxalase I slowly reverts to a more stable and active domain-swapped form. However, no evidence exists for such a mechanism *in vivo*. Another interesting finding is that formation of domain-swapped oligomers can introduce allosteric regulation of the protein activity already present in the monomer, as shown in bovine seminal Rnase (32). It is interesting to mention the example of Cyanovirin-N for which an identical activity and binding affinity towards gp120 was observed for both the pure monomeric or dimeric protein. Both forms are stable, however the elevated value of the dimerization constant (2.5mM at 50°C) suggests that the dimeric form does not exist under physiological conditions (33). In our case, both the monomeric and the domain swapped dimeric forms exist *in vivo* and are stable and active. The monomeric form of HasA binds one molecule of free or hemoprotein-bound heme and delivers it to a specific outer membrane receptor, HasR, by protein-protein interaction (4). The swapped dimeric form, DHasA, binds two molecules of heme with an affinity constant which remains

high, even if it is lower than that of the monomeric form. While it is not capable to deliver its hemes directly to the extracellular receptor HasR, since it does not interact with this receptor, it can, however, transfer them to the monomeric form of HasA that, in turn, will interact with HasR to deliver heme. Létoffé *et al* have shown that two regions of the hemophore are involved in the interaction with HasR, namely the beta strand composed of residues 51 to 60 and the region consisting of residues 95 to 107 (13). These two regions in DHasA become partially inaccessible by dimerization. Furthermore, steric hindrance due to the bigger size of DHasA can be an obstacle for recognition and interaction with HasR. Therefore, we propose that DHasA could serve as a heme reservoir, stocking it and keeping it more accessible for bacteria by the Has system. The efficiency and advantage of DHasA for the Has system would result in its ability to bind two heme molecules with an affinity lower than that of the monomeric form but still high compared to other heme binding proteins in serum, like albumin (the two highest affinities determined for heme-albumine are $7.8 \cdot 10^7 \text{ M}^{-1}$ and $2.3 \cdot 10^8 \text{ M}^{-1}$ (34). Furthermore, as the K_d for heme binding of heme receptors from other bacteria is estimated to be in the order of μM , the capacity of DHasA to serve as a heme reservoir for the Has system makes the heme acquisition *via* hemophore efficient and advantageous for *Serratia marcescens* in the presence of other bacteria sharing the same space.

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Figure Legends:

Figure 1: Gel filtration on a HiPrep 16/60 Sephacryl S-100 High resolution equilibrated with 50 mM sodium phosphate buffer, pH 7. The flow rate was 30 mL/h. The volume of each fraction was 1.5 mL.

Figure 2: DHasA is directly observed in the culture supernatant of *S. marcescens* in iron limiting condition. Native-PAGE followed by immunodetection with anti-HasA antibody (1/2000) was used for its observation. Lane 1: monomeric purified HasA from *E. coli* as standard (HasA_{Ec}); lane 2: Purified DHasA from *E. coli* as standard (DHasA_{Ec}); Lanes 3 and 4: 0,2 and 0,6 OD₆₀₀ equivalent from the supernatant of SM365 grown in minimal medium supplemented with 0,4 mM 2,2'-dipyridyl (iron-limiting conditions). HasA_{Sm} and DHasA_{Sm} are secreted by *S. marcescens*.

Figure 3: Superimposition of 600 MHz ¹H-¹⁵N HSQC spectra of HasA (in black) and DHasA (in red) (1.5 mM in 20 mM sodium phosphate buffer, pH 5.6, 30°C). Both spectra were obtained using the same acquisition and processing parameters.

Figure 4: (A) Ribbon representation of the backbone of domain swapped dimeric DHasA (PDB ID: 2CN4). The two chains A and B are colored in red and blue, heme is in yellow. (B) One half DHasA composed of two polypeptide chains (blue/red). (C) Final refined electron density map in the region of the domain swapping, contoured at a 1 σ level. Chain A is colored in red and chain B is colored in yellow.

Figure 5: (A): R.m.s. deviation calculated by superimposing the backbone atoms of the monomeric form (1B2V) and the individual subunits of DHasA. The highest r.m.s deviation values observed for residues marked with a star are discussed in the text. The continuous and broken lines are related to molecule A and B of DHasA respectively. (B) Average B factor of all atoms as a function of residue number for DHasA molecule A (solid line), molecule B (grey squares) and compared to those of monomeric HasA (dashed line). The residues in the hinge region (43–48) of the swapping domain show comparable B-factors in both forms of protein.

Figure 6: ^1H NMR spectra of HoloHasA (A) and HoloDHasA (B) recorded at 500 MHz and at 50°C in 20mM sodium phosphate buffer, pH 5.6.

Figure 7: Isothermal Titration Calorimetry (ITC) of the interaction of DHasA with hemin in 50mM sodium phosphate buffer pH 7 at 25°C. The heat signal is shown (upper) together with the binding isotherm derived from this signal (lower). Protein concentration was 5.6×10^{-6} M (monomer concentration). Injections of 10 μl of a hemin solution at 5.9×10^{-5} M were performed with an interval of 3 min. The ITC titration data were fitted without any constraint to a single set (or class) of sites model with a stoichiometry of 1.024 ± 0.005 (mol/mol of monomer), an affinity constant higher than $> 10^8 \text{ M}^{-1}$ and a ΔH observed of $-108.8 (\pm 1.3) \text{ kJ.mol}^{-1}$ (or $-26.1 \text{ Kcal.mol}^{-1} \pm 0.3$) for each site.

Figure 8: Solid phase binding assays. Each sample was tested in triplicate at concentration indicated on the figure. The dot blots were probed with polyclonal anti-HasA serum at a 1/2000 dilution, revealed by ECL + (1/30000) and scanned with storm imager. Same results were obtained with holoproteins.