Purification and Characterization of an Extracellular Heme-Binding Protein, HasA, Involved in Heme Iron Acquisition†

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ABSTRACT: Many bacterial hemoproteins involved in heme acquisition have been isolated recently, comprising outer membrane receptors and extracellular heme-binding protein. The mechanisms by which these proteins extract heme have not been described up to now. One such protein, HasA, which can bind free heme as well as capture it from hemoglobin, is secreted by the Gram-negative bacteria *Serratia marcescens* under iron deficiency conditions. The fact that HasA does not present sequence similarities with other known hemoproteins suggests that it posseses a new type of heme binding site. This work describes the main physicochemical properties of HasA, essential for understanding its function. HasA is a monomer of 19 kDa that binds one *b* heme per molecule with high affinity. The electron paramagnetic resonance spectra indicate that the heme iron is in a low-spin ferric state and that the two iron axial ligands are His and His^-. The low oxidation-reduction potential value (−550 mV vs standard hydrogen electrode) of the heme bound to HasA suggests that heme could be exposed to the solvent. According to circular dichroism data, the binding of heme does not seem to modify the conformation of HasA.

Free soluble iron, an essential nutrient for microorganisms, is not readily available under biological conditions (Weinberg, 1978). Gram-negative bacteria have therefore developed various strategies to acquire iron. Most of them secrete low molecular weight molecules called siderophores which bind poorly soluble ferric iron with a very high affinity (Neilands, 1981).

Other mechanisms involved in iron uptake utilize host iron compounds such as free heme, hemoglobin, or hemopexin. These heme utilization systems require, in most cases, direct recognition of free heme or of hemoproteins by an outer membrane receptor allowing heme transport through the outer membrane and then uptake across the inner membrane involving a specific heme permease (Stojiljkovic & Hantke, 1992, 1994; Stojiljkovic *et al*., 1995; Stevens *et al*., 1996; Hornung *et al*., 1996; Bramanti & Holt, 1993; O'Connell *et al*., 1996; Henderson & Payne, 1994). Alternatively, in two systems, an extracellular heme binding protein is required to shuttle free heme or heme from hemoproteins to a specific outer membrane receptor (Létoffé *et al*., 1994a; Cope *et al*., 1995). *Haemophilus influenzae* (Cope *et al*., 1995) secretes HxuA, a protein involved in heme uptake. This protein can bind the heme−hemopexin complex and allows bacteria to grow in the absence of any other source of heme. The other known extracellular heme binding protein, HasA, which we will name hemophore, is secreted by *Serratia marcescens* (Létoffé *et al*., 1994a).

Hence, during the last years, many new heme and hemoprotein binding proteins have been isolated, comprising outer membrane receptors and extracellular proteins. In many cases the corresponding genes have been identified, and biochemical techniques, such as affinity chromatography and ligand binding assay, have demonstrated that they could bind heme or hemoproteins. However, their physicochemical properties have not been studied up to now. Nothing is known about the structure of their binding sites and the mechanisms by which they extract heme are not yet elucidated.

HasA is a 19 kDa protein that can bind free heme and acquire it from hemoglobin. It is necessary for the utilization of heme iron by bacteria and it acts as a heme carrier. HasA belongs to the family of secreted proteins lacking a N-terminal signal peptide. It has a C-terminal targeting sequence and is secreted by a specific ATP binding cassette (ABC) transporter, composed of three envelope proteins: an ABC protein, a membrane fusion protein located in the inner membrane, and an outer membrane protein (Létoffé *et al*., 1994b). HasA does not present sequence homology with other known proteins, especially with other hemoproteins. The only sequence similarity between HasA and other proteins was found with proteins secreted *via* an ABC transporter. It consists of a short motif (ELLAA in HasA) located at the C-terminal extremity, composed of a negatively charged residue followed by three or four hydrophobic residues and is involved in the secretion process (Wolff *et al*., 1997).

There is no information about the heme binding site of HasA, and the way by which HasA takes up and releases

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heme is entirely new. In the present paper, we describe the main physicochemical properties of HasA, essential for understanding its function.

MATERIALS AND METHODS

Expression and Purification of HasA. *Escherichia coli* strain TG1 (Δ*lac proAB thi supE*/F′ *traD36 proAB lacI*^{q} *lacZ*ΔM15) was transformed as described by Létoffé *et al*. (1994a) with plasmid pSYC34. Bacteria were grown in a 1 L bioreactor, in Terrific Broth medium (Miller, 1992) supplemented with 25 μg/mL kanamycin and 2% glycerol, for 7 h at 37 °C. Final A_{600} was 29. The culture supernatant was precipitated with ammonium sulfate at 60% saturation. The precipitate was centrifuged for 45 min at 10000*g* and the pellet was dissolved in a minimal volume of 10 mM Tris-HCl buffer, pH 7.5. The resulting solution was extensively dialyzed against the same buffer. Aliquots of the dialysate were loaded on a Sephacryl S-200 HR (Pharmacia) column (70 × 1.6 cm) equilibrated with 0.1 M Tris-HCl and 0.1 M NaCl buffer, pH 7.5. Fractions were collected and their HasA content was evaluated from their UV−visible absorption spectra. Fractions that contained HasA but were not homogeneous, as determined by SDS−polyacrylamide gel electrophoresis (SDS−PAGE), were pooled, centrifuged on Centricon concentrators (Amicon, 10 kDa cutoff), and subjected to a second gel filtration. All the purification steps were performed at +4 °C. The pool of the homogeneous fraction, which we will call the purified HasA sample, was stored at −20 °C.

Polyacrylamide Gel Electrophoresis. SDS−PAGE was performed according to Laemmli (1970) using a 15% polyacrylamide gel. Gels were stained with Coomassie blue or tetramethylbenzidine, a specific marker of hemoproteins (Thomas *et al.*, 1976).

Estimation of Protein, Hemin, and Heme Content. Protein content of unpurified samples was determined by the method of Bradford (1976), using purified HasA as standard.

Concentration of purified HasA solutions was calculated from their absorbance at 277 nm assuming an ϵ_{277} of 25 100 $M^{-1}\cdot cm^{-1}$. The value of ϵ was determined by amino acid analysis of aliquots of a purified HasA solution of known absorbance, after 6 N HCl hydrolysis for 24 h at 110 °C.

Hemin (bovine hemin from Sigma) was initially dissolved in a minimal volume of 0.1 M NaOH and diluted in the appropriate buffer. Solutions were prepared just before use. Hemin concentration was calculated from the absorbance at 385 nm using the previously published ϵ_{385} of 58 400 $M^{-1}\cdot cm^{-1}$ (Dawson *et al*., 1986).

Heme content in HasA solutions containing both the apo- and the hemoprotein was calculated from the absorbance at 407 nm (Soret region), assuming an ϵ_{407} of 78 000 $M^{-1}\cdot cm^{-1}$ for the hemoprotein. This value of ϵ was determined by the pyridine hemochrome method. The absorbance of a purified HasA sample was measured at 407 nm. Sample (1 vol) was diluted with alkaline pyridine (2 vol) and an excess of solid sodium dithionite was added to the solution. The heme was dissociated from the protein and the concentration of the pyridine hemochrome that was formed was determined from the absorbance at 557 nm, using the ϵ_{557} value of 34 530 $M^{-1}\cdot cm^{-1}$ (Berry *et al*., 1987).

All absorption measurements were done in a Perkin-Elmer Lambda 2 spectrophotometer thermostated at 25 °C using 1 or 0.2 cm path length cuvettes.

Table 1: Amino Acid Sequence of HasA

```
          10         20         30         40
MAFSVNYDSS FGGYSIHDYL GQWASTFGDV NHTNGNVTDA

          50         60         70         80
NSGGFYGGSL SGSQYAISST ANQVTAFVAG GNLTYTLFNE

          90        100        110        120
PAHTLYGQLD SLSFGDGLSG GDTSPYSIQV PDVSFGGLNL

         130        140        150        160
SSLQAQGHDG VVHQVVYGLM SGDTGALETA LNGILDDYGL

         170        180        188
SVNSTFDQVQ AATAVGVQHA DSPELLAA
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Preparation of the Apoprotein. Heme was extracted from the hemoprotein by cold acid−acetone treatment (Di Ioro, 1981). One milliliter of chilled HasA solution was added drop by drop with stirring into 100 mL of acid−acetone [acetone containing 0.2% (v/v) 12 N HCl] at −20 °C. The addition was completed in about 10 min. The solution was centrifuged at 10000*g* for 30 min at −20 °C. The heme containing supernatant was discarded. If the precipitate was red-colored, this operation was done once more. The final precipitate was dissolved in a solution of 7 M urea and 0.1 M Tris-HCl, pH 7.5, in order to obtain 2−5 μM apoprotein. The solution was centrifuged at 10000*g* for 10 min and the supernatant was extensively dialyzed against 0.1 M Tris-HCl and 0.1 M NaCl buffer, pH 7.5, at 4 °C. The apoprotein was stored at −20 °C.

Binding Studies. Heme binding studies were carried out by difference absorption spectroscopy in the Soret region. Aliquots of hemin solution were successively added in both the apoprotein and the reference cuvettes thermostated at 25 °C. Spectra were recorded 5 min after the heme addition on a Perkin-Elmer Lambda 2 spectrophotometer.

The heme binding was also monitored by fluorescence quenching measurements of the one tryptophan residue of HasA (Table 1) after addition of different amounts of hemin to the apoprotein. ApoHasA concentration and final sample volume (0.5 mL) were kept constant. The excitation wavelength was 290 nm and the emission wavelength determined from the maximum of the emission spectrum (data not shown) was 330 nm. Experiments were performed at 25 °C with a Perkin-Elmer LS5B spectrofluorometer. Each emission value was the average of 3 consecutive measurements. All fluorescence intensity measurements were corrected for the inner-filter effect due to the absorbance of the heme according to

$$F_c = F_m \times 10^{(A_1+A_2)/2}$$

where F_c is the corrected fluorescence intensity, F_m is the measured fluorescence intensity, and A_1 and A_2 are the respective hemin absorbances at the excitation and emission wavelengths.

Circular Dichroism. CD spectra were recorded at 25 °C in a Jobin-Yvon (Longjumeau, France) CD6 spectrodichrograph using 0.2 mm path length cells. Each spectrum was the average of five consecutive scans from 180 to 260 nm with 2 s of integration time/nm. The spectral bandwidth was kept at 2 nm and the wavelength increment was 1 nm/step. Measurements were made on purified HasA solutions (2.1×10^{-5} M) and on apoHasA solutions (9.6×10^{-6} M) to which different aliquots of hemin had been added [25%, 50%, 70%, and 90% (mol/mol)]. Spectrum of the buffer

alone (10 mM sodium phosphate, pH 7.5) recorded under identical conditions was substracted from the sample spectra. Ellipticity was expressed as mean residue molar ellipticity [Θ]. The secondary structure content was estimated according to the method of Manavalan and Johnson (1987).

Determination of the Heme Class. The type of heme bound to HasA was determined from the absorption spectra of the pyridine hemochrome dissociated from the protein, as previously described.

Analytical Ultracentrifugation. Ultracentrifugation studies were performed in an XLA (Beckmann) analytical ultracentrifuge using 12 mm path length cells. Purified HasA samples were either 0.02 or 0.1 mM in 0.1 M Tris-HCl and 0.1 M NaCl buffer, pH 7.5.

The sedimentation/diffusion equilibrium was achieved after 22 h at 30 000 rpm. The sedimentation velocity experiment was carried out for 3.5 h at 58 000 rpm. The absorbance in the cell was scanned as a function of the distance to the rotation axis, at 277 nm for the 0.02 mM sample and at 430 nm for the 0.1 mM sample. The buffer was 0.1 M Tris-HCl and 0.1 M NaCl, pH 7.5. Experiments were performed at 20 °C. Data were analyzed by means of the DATA-RED software provided with the XLA centrifuge. The partial specific volume of HasA (0.715 mL/g) was calculated from the amino acid composition of the protein (Laue *et al.*, 1992) and the solvent density (1.02 g/cm^3) was measured by weighing.

EPR Spectroscopy. EPR spectra were recorded at 77 K using a Varian E109 spectrometer. The microwave power was 10 mW, the klystron was frequency 9.18 GHz, the modulation amplitude was 10 G, and the time constant was 1 s. The magnetic field was calibrated with the stable radical 1,1-diphenyl-2-picrylhydrazyl used as a marker ($g = 2.0036$). Experiments were carried out on purified HasA samples containing 15% heme (mol/mol) concentrated to 1 mM in 10 mM sodium phosphate buffer, pH 7.5 or 5.5. For some experiments hemin was added to the protein up to 90% (mol/mol).

Isoelectric Focusing. Isoelectric focusing was performed by PhastSystem using a Phastgel IEF 3−9 (Pharmacia) and the broad p*I* calibration kit (pH 3−10).

Electrochemistry. Cyclic voltammetry (CV) and square-wave voltammetry (SWV) experiments were carried out as previously reported (Dolla *et al.*, 1994) using as working electrode a pyrolytic graphite permselective-membrane electrode (Haladjian *et al.*, 1996). All measurements were performed at room temperature. The supporting electrolyte was 0.5 M Tris-HCl buffer, pH 7.6. Solutions were deoxygenated by bubbling with nitrogen. The scan rate was 20 mV·s^{-1} (CV) or 5 mV·s^{-1} (SWV). Unless otherwise specified, all reported potentials are referred to the Ag/AgCl (saturated NaCl) reference electrode. Potentials versus the standard hydrogen electrode (SHE) can be obtained by adding 210 mV.

RESULTS

Purification of HasA. HasA was the main protein secreted into the culture medium under our growth conditions. Its purification was achieved in two steps. Addition of ammonium sulfate to 60% saturation was sufficient to precipitate all HasA and to eliminate most of the pigments remaining in the supernatant. Other contaminants and pigments were eliminated by gel filtration on the Sephacryl column (Figure 1). Pure fractions gave the same single band on SDS gels with both Coomassie blue and tetramethylbenzidine staining, at approximatively 19 kDa (data not presented). The absorption spectra of the fractions containing HasA showed a pattern typical of a hemoprotein with a peak at 277 nm and another one in the Soret region, at 407 nm (Figure 2, trace A). However, pure fractions presented abnormally high A_{277}/A_{407} ratios, ranging from 2.1 to 2.5, instead of less than 1, the value usually observed for hemoproteins (Di Iorio, 1981; Lee *et al.*, 1991; Brunt *et al.*, 1992; Modi *et al.*, 1995). This result suggested that our sample did not contain a 1/1 molar ratio of heme/protein. Therefore, the sample could either be a multimer binding less than one heme molecule per monomer or it could be composed of both an apo- and a hemoprotein. Purification yields are given in Table 2.

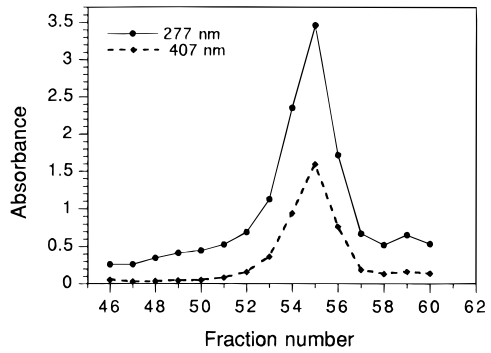

FIGURE 1: Gel filtration on a Sephacryl 200 HR (Pharmacia) column (70 × 1.6) equilibrated with 0.1 M Tris-HCl and 0.1 M NaCl buffer, pH 7.5. The flow rate was 7.5 mL/h. (—, 277 nm) Absorbance maximum of HasA; (- - -, 407 nm) absorbance maximum of heme-bound HasA. The volume of each fraction was 1.5 mL.

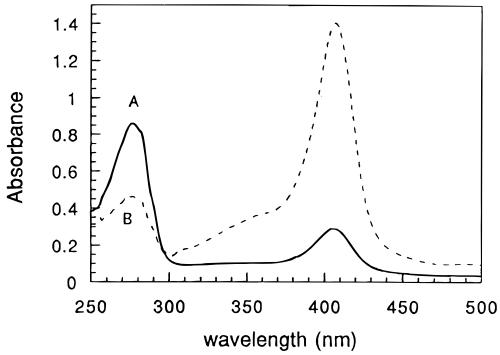

FIGURE 2: UV−visible absorption spectra of HasA in 0.1 M Tris-HCl and 0.1 M NaCl buffer, pH 7.5. (A) Purified HasA, 3.4 × 10^{-5} M. (B) ApoHasA, 1.8 × 10^{-5} M, saturated with hemin in a 1/1 ratio.

Table 2: Purification Data[a]

purification step	total protein (mg)	HasA (mg)	recovery (%)
culture supernatant	620	500	100
precipitation and dialysis	550	475	95
first S-200 column	356	285	57
second S-200 column	300	228	46

[a] A detailed description of the procedure is given in the Materials and Methods section. Values are based on a preparation from 1 L of culture.

Analytical Ultracentrifugation. The molecular weight distribution of the purified HasA sample was determined in the sedimentation/diffusion equilibrium experiment. Scan-

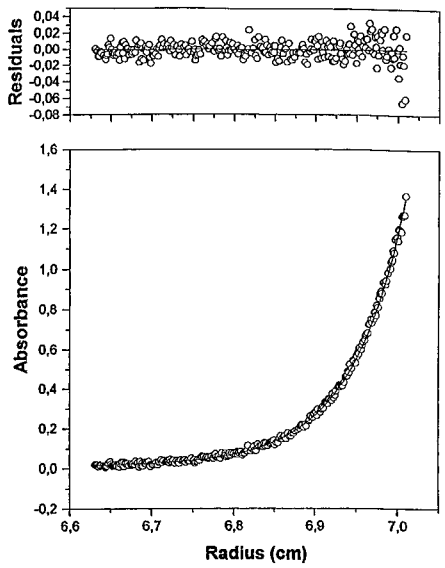

FIGURE 3: Sedimentation/diffusion equilibrium of HasA (2×10^{-5} M). The absorbance at 277 nm was scanned as a function of the distance to the rotation axis. Ten scans were accumulated. Lower graph: The experimental points (open circles) were used to generate a fitted curve (continuous line) using as a model an ideal solution with one solute molecule. Upper graph: the residuals in term of standard deviations from the weighted fit.

ning was performed at two wavelengths, one characteristic of the protein (277 nm) and the other characteristic of bound heme (430 nm). As determined from heme titration (see below), the HasA sample used for ultracentrifugation experiments contained 85% apoprotein and 15% hemoprotein. The scan at 277 nm reflected essentially the distribution of the apoprotein, whereas that at 430 nm corresponded to the hemoprotein alone. In order to obtain the initial absorbance values of about 0.5 optimal for the experiments, 0.02 and 0.1 mM samples were used for scannings at 277 and at 430 nm, respectively. At both wavelengths a satisfactory fit to the observed distribution was obtained using a one-solute ideal solution model with a molecule of molecular weight 20 700 ± 800 at 277 nm and 20 400 ± 200 at 430 nm (Figure 3). These values are in agreement with those of 19 271 Da for the apoprotein (calculated from the sequence) and of 19 923 for the hemoprotein (the sum of the moleculars weights of apoprotein and heme), the variations remaining within the limits of the methods. This result clearly indicates that purified HasA contains essentially monomers, since no trace of multimers was detected. As suggested from its A_{277}/A_{407} ratio, the purified sample of HasA was thus a mixture of an apo- and a hemoprotein.

Sedimentation velocity experiments (Harding *et al*., 1992) allowed to determine HasA sedimentation (2.26 S) and diffusion (1.03×10^{-6} cm^2•s^{-1}) coefficients (Figure 4). From these values, a Stokes radius of 21 Å was calculated according to the Svedberg relation. The frictional coefficient f of HasA, determined from the diffusion coefficient value, was equal to 3.92×10^{-8} g•s^{-1}. As the frictional coefficient f_0 of a spherical particle with the same molecular weight and partial specific volume as HasA would be equal to 3.31×10^{-8} g•s^{-1}, it appears that HasA having a f/f_0 ratio of 1.18 is a globular protein.

Circular Dichroism. Renaturation of the apoHasA sample prepared by acid−acetone treatment was checked by far-UV CD before the protein was used in binding studies. CD

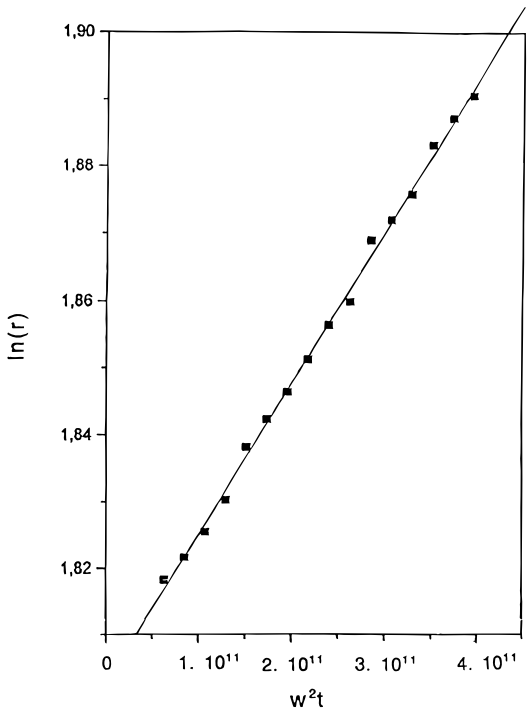

FIGURE 4: Determination of the sedimentation coefficient of HasA. A sample of HasA (2×10^{-5} M) was centrifuged at 58 000 rpm and 20 °C. The sedimentation coefficient was determined from the slope of a graph of ln (r) vs $\omega^2 t$, where r is the distance from the center of rotation, ω is the angular velocity, and t is the time after the rotor reached maximum velocity.

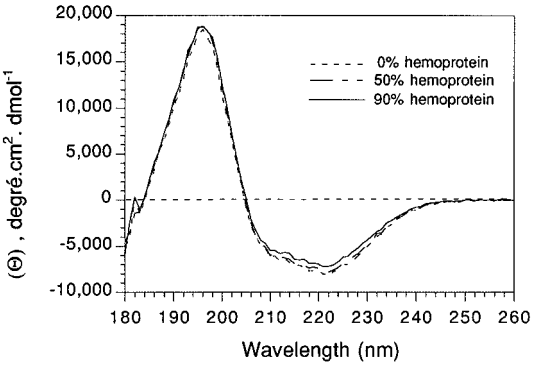

FIGURE 5: Far-UV circular dichroism spectra of apoHasA (0% hemoprotein) and purified HasA samples with 50% and 90% hemoprotein in 10 mM sodium phosphate buffer, pH 7.5.

spectra recorded for apoHasA and for HasA samples with heme content ranging from 15% up to 90% (mol/mol) were practically indistinguishable (Figure 5). This similarity of spectra shows that unfolding, which occurred during the acid-acetone treatment, did not alter the final overall secondary structure content of the apoprotein. Moreover, the fact that addition of heme did not produce any measurable effect indicates that no appreciable change of conformation occurs upon complex formation.

Decomposition of the CD spectra using the method of Hennessey and Johnson (1981) with variable selection of protein spectra (Manavalan & Johnson 1987) indicated 29% α-helix, 23% β-structure, 13% turns, and 30% unordered structures.

Binding Studies: (A) Heme Titration. Addition of small amounts of hemin stock solution (7 × 10−4 M) to the apoprotein (1.3×10^{-5} M) produced a UV−visible absorption spectrum similar to that of the purified HasA sample

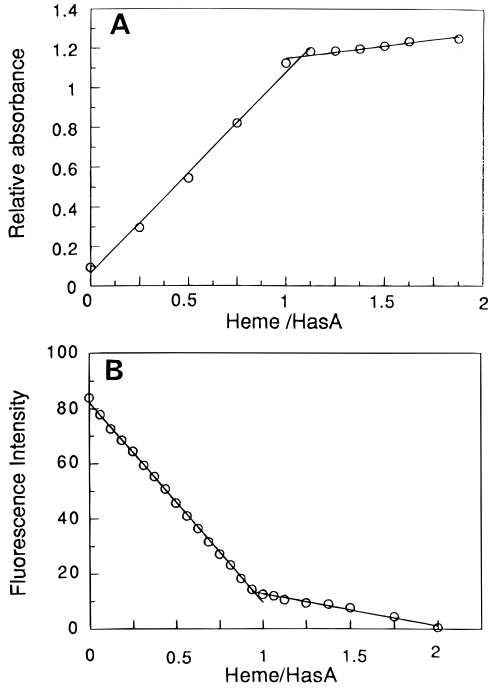

FIGURE 6: Heme titration. (A) Difference absorption spectroscopy. Effect of increases of heme on the association with HasA monitored at 407 nm, using 1.3×10^{-5} M apoHasA in 0.1 M Tris-HCl and 0.1 M NaCl buffer, pH 7.5. (B) Fluorescence quenching of heme binding to HasA. Fluorescence emission of several samples containing 1 μM HasA in 0.1 M Tris-HCl and 0.1 M NaCl buffer, pH 7.5, and different concentrations of heme was measured at 330 nm. The excitation wavelength was 290 nm.

with a peak in the Soret region, at 407 nm, whereas maximum absorbance of free hemin under the same conditions is 392 nm (Figure 2B). The amount of heme capable of interacting with the protein was measured by difference absorption spectroscopy. Result of the titration is presented in Figure 6A. It shows that HasA interacts with heme in a 1/1 molar ratio of heme/protein. Therefore, from the absorbance at 407 nm, as described in Materials and Methods, the heme content in purified samples of HasA could be estimated at 10−20% (mol/mol) depending on the culture.

Heme binding experiments performed with a purified sample of HasA containing 15% heme (mol/mol) gave results similar to those obtained with apoHasA. Both the apoprotein in the purified sample of HasA and the apoprotein obtained by acid−acetone treatment bind heme with a 1/1 stoichiometry. These data agree with the results of CD experiments suggesting a proper renaturation of the apoprotein after acid−acetone precipitation.

(B) K_d Measurement. Fluorescence quenching experiments were performed at two concentrations of apoHasA, namely, 10^{-6} M and 10^{-7} M. The fluorescence signal, due to the single tryptophane residue of the protein, was too weak to give interpretable data at lower concentrations ($\leq 10^{-8}$ M). Equilibrium conditions, that is, the concentration in binding sites comparable in magnitude to the K_d of the heme−apoHasA complex, were not reached at these apoHasA concentrations, however, and fluorescence experiments only confirm the stoichiometry of the binding (Figure 6B).

Experiments of competition between heme and 8-anilino-1-naphthalenesulfhonic acid (ANS) to form a complex with apoHasA were done to determine the K_d. ANS is known to be a suitable and sensitive probe for nonpolar sites. When bound, its fluorescence changes markedly: the emission peak shifts to blue and the quantum yield increases greatly. Since hemin was shown to displace ANS from its complexes with apohemoglobin and apomyoglobin (Stryer, 1965), we could expect that it would simularly displace ANS from its complex with apoHasA. However, when ANS was added to the apoprotein, neither a significant increase in its fluorescence emission nor a shift of its emission peak to the blue was observed (data not shown). Either ANS did not bind to apoHasA or its environment in the complex, if any, was not hydrophobic enough to produce changes in its fluorescence.

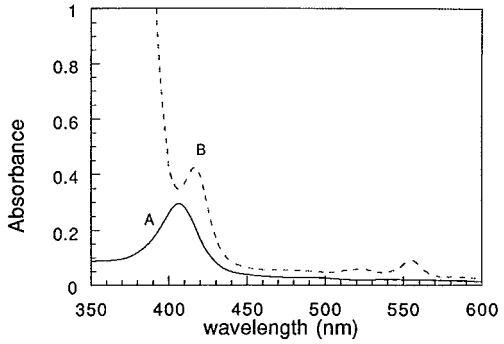

FIGURE 7: Visible absorption spectra of heme bound to purified HasA (1.2×10^{-5} M with 15% hemoprotein). Trace A, oxidized state (as isolated); trace B, reduced state with sodium dithionite in alkaline pyridine (sodium dithionite absorbs in the UV region).

Although they did not allow us to determine the exact value of the K_d of hemoHasA, the binding studies described above gave an estimation of this value. It is most likely less than to 10^{-8} M, since at a concentration of 10^{-7} M in protein we were in the titration conditions.

Determination of Heme Class Type. Absorption spectra of reduced pyridine hemochromes are specific for different types of heme (Yamanaka, 1992). As shown in Figure 7, the spectrum of reduced pyridine hemochrome dissociated from purified HasA sample showed a maximum at 556 nm, a wavelength characteristic of heme *b*. The heme class type was confirmed by EPR experiments (see below). When hemin was added up to 90% (mol/mol) to a purified HasA sample initially containing 15% heme (mol/mol), the only change observed in the EPR spectra was an amplification of signals (data not shown). No shift or shouldering of signals appeared and the *g* values, which depend on the local environment of the iron atom and therefore on the heme peripheral groups, were not changed. These results indicated that heme in the purified sample of HasA was of the same type as that of hemin, that is, type *b*.

EPR Spectra. The number and nature of heme ligand(s) of hemoHasA, as well as the state of the iron atom, were determined from the EPR spectra of the purified protein containing 15% and 90% heme (mol/mol) at pH 7.5 (Figure 8). The *g* values were $g_z = 2.855$, $g_y = 2.208$, and $g_x = 1.706$. They are specific for a low-spin ferric heme (Blumberg, 1981). The rhombicity $(V/\lambda) = 3.76$ and tetragonality $(\Delta/\lambda) = 4.16$ (V, rhombic crystal field parameter; Δ, axial crystal field parameter; λ, spin−orbit coupling parameter) were calculated according to Taylor (1977). They are typical of a His-His^- pair of axial ligands (Blumberg & Peisach, 1971; Yoshimura & Ozaki, 1984).

Electrochemistry. Purified HasA did not give any electrochemical signal with the graphite electrode at pH 7.6. Electrochemistry response of negatively charged proteins was

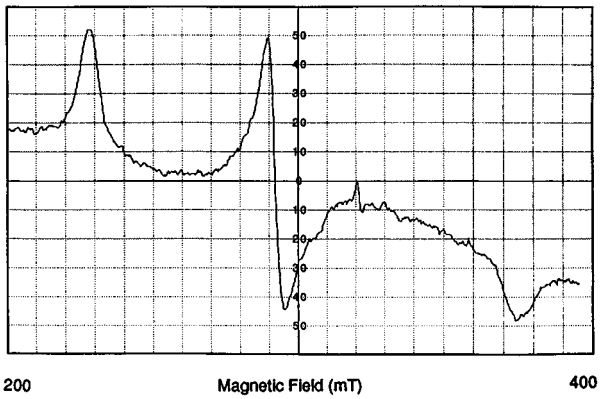

FIGURE 8: EPR absorption derivative spectra of HasA saturated with hemin (1 mM) in 10 mM sodium phosphate buffer, pH 7.5. Conditions: temperature, 77 K; microwave frequency, 9.18 GHz; modulation amplitude, 10 G; power, 10 mW.

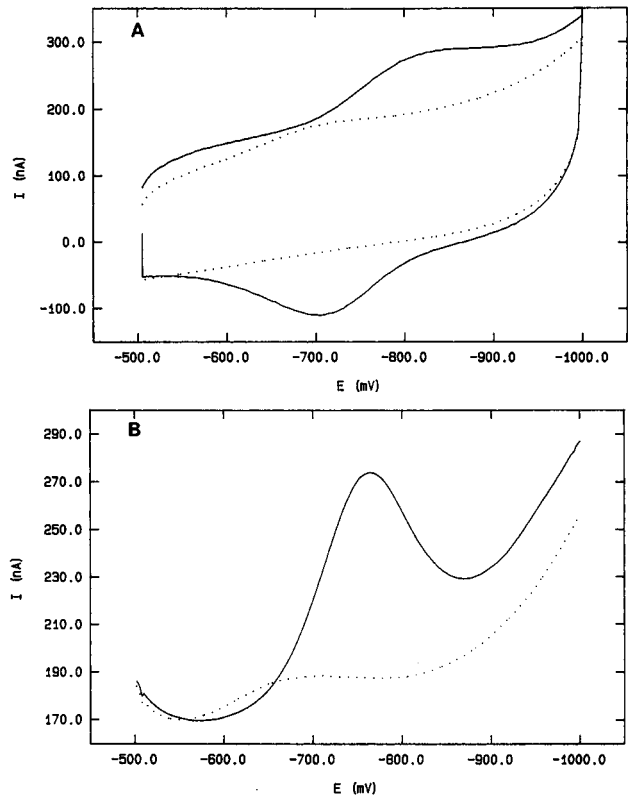

FIGURE 9: Cyclic (A) and square-wave (B) voltammograms at the permselective-membrane PG electrode using 1 μL of 77 μM polylysine solution + 2 μL of 68 μM HasA solution, in 0.5 M Tris-HCl buffer, pH 7.6. (Dotted line) HasA solution was replaced by 0.5 M Tris-HCl buffer, pH 7.6.

shown to be enhanced in the presence of cations or positively charged polypeptides at the surface of the electrode (Haladjian *et al*., 1996). HasA does not have any basic residue (see Table 1), and its isoelectric point calculated from its amino acid composition is 3.7. Electrofocusing on IEF 3−9 yielded a single band that migrated with a p*I* marker protein (p*I* = 3.5) near the anode wick, suggesting that the HasA p*I* is lower than 3.5. The electrode was therefore modified with a 25 μM solution of polylysine (MW 39 800, Sigma) in order to increase the electron transfer rate. CV and the SWV curves obtained with the modified electrode are shown in Figures 9. A single cathodic wave at −810 mV and a single anodic wave at −710 mV were observed. A single SWV peak was also detected at E_p = −760 mV. The mean of anodic and cathodic CV peak potentials and the SWV peak potential gave a common value of −760 mV (−550 mV if referred to the standard hydrogen electrode) for the oxidation−reduction potential. Complementary experiments using different polylysine concentrations from 15 to 40 μM yielded the same value.

DISCUSSION

To obtain sufficient iron for survival and replication, pathogenic bacteria must possess one or more efficient iron-scavenging systems capable of competing with or exploiting the iron transport and storage mechanisms of the host. Heme and some heme−protein complexes, including hemoglobin, can act as an alternative iron source for microorganisms (Martinez *et al*., 1990; Wooldridge & Williams, 1993). In addition to a siderophore-mediated iron uptake system, *Serratia marcescens* secretes a protein, HasA, under iron deficiency conditions that can bind free heme or acquire it from hemoglobin. HasA does not present any sequence homology with other hemoproteins. Its heme uptake and release mechanisms are unknown. This work presents the physicochemical properties of this new hemophore which is the only extracellular heme binding bacterial protein characterized to date.

We first show that this new hemophore possesses a single heme binding site per molecule and that it binds type *b* heme with high affinity. The affinity of apoproteins for heme is usually high and difficult to measure. The fraction of reversibly dissociated heme molecules is often too small to be detected by spectroscopic methods. Equilibrium dialysis, which is a very sensitive method to determine the equilibrium between free and bound ligands, cannot be carried out due to the stacking of hemin molecules unless drastic conditions are used (high pH or detergents). For all these reasons, the values of the K_d of heme−protein complexes that could be directly measured are at best close to 10^{-8} M, whereas in siderophores, affinity constants for iron as high as 10^{52} M can be measured (Harris *et al*., 1979). The strong heme−albumin binding site complex has a K_d of 5×10^{-7} M (Beaven *et al*., 1974), the liver fatty acid-binding protein and glutathione S-transferase have K_d values of $1-2 \times 10^{-7}$ M (Vincent & Müller-Eberhard, 1985; Müller-Eberhard & Nikkilä, 1989), and the heme-binding 23 kDa protein has a K_d of 5.5×10^{-8} M (Iwahara *et al*., 1995). For proteins with a higher affinity for heme, such as hemopexin, hemoglobin, and myoglobin, only K_d value estimations are available. They lie in the picomolar range (Hrkal *et al*., 1974; Gryczynski *et al*., 1995).

The K_d of the heme−HasA complex is lower than 10^{-8} M. This high affinity could account for the ability of HasA to take up heme from hemoglobin, as was shown for hemopexin and heme−albumin complex (Sleery & Muller-Eberhard, 1973). It is noteworthy that, under iron deficiency conditions, *S. marcescens* also secretes hemolysins (Braun *et al*., 1993) that can lyse the host erythrocytes and release hemoglobin, representing the most important alternative iron source in the host organism (74.3% in the mammalian host; Wooldridge *et al*., 1993).

As determined from EPR experiments, the heme iron in the heme apoHasA complex is in a low-spin ferric state and is coordinated to two axial ligands, His and His^-. The number and nature of axial ligands in hemoproteins are

variable and unrelated to the heme type or to the protein function. Histidine residues, however, provide one or both ligands in the majority of hemoproteins. Heme *c* in cytochrome *c*′, as well as heme *b* in hemoglobin and myoglobin, has a single histidine coordination (Finzel *et al*., 1985; Blumberg, 1981). Heme *b* in cytochromes usually has a bis(His) coordination (Mathews *et al*., 1972; Xia & Mathews, 1990). However, a single Cys^- is found in cyt P450 (Poulos *et al*., 1986), and His/Met and Met/Met couples are encountered in cytochromes b_{562} and b_1, respectively (Xavier, 1978; Mathews *et al*., 1979; Cheesman *et al*., 1990). The sixth ligand in the heme-apoHasA complex was shown to be an imidazolate at neutral and acidic pH. Indeed, the EPR spectrum recorded at pH 5.5 did not show any change from the spectrum obtained at pH 7.5 (data not presented) and the three *g* values were, respectively, identical at both pH values. Deprotonation of an imidazole axial ligand in hemoproteins involves hydrogen bonding of its N^δ with other residues in the polypeptide chain (Valentine *et al*., 1979; Landrum *et al*., 1980; Quinn *et al*., 1982, 1983). It is usually observed at basic pH. Hydrogen bonding then occurs between the $N^{\delta-}$ and hydrogen donors. On the other hand, the anionic character of the imidazolate observed in the heme−apoHasA complex at neutral and acidic pH must result from a strong interaction with hydrogen acceptors such as carboxylate groups of glutamate or aspartate residues.

The redox properties of hemoproteins depend on the number, nature, conformation, and H-bonding interaction of axial heme ligands. Hence, it has been shown that the bis(His) coordination is associated with low redox potentials. Histidine as sixth ligand is a factor of stabilization of the ferric porphyrin−imidazole complex and therefore a cause for the decreased redox potential (Raphael & Gray, 1989, 1991; Dolla *et al*., 1994). These effects, stabilization and decreased redox potential, are enhanced when the axial histidine is totally or partially deprotonated. Hemoproteins usually display redox potentials between −400 and +400 mV (Yamanaka, 1992). It has been proposed that the redox potential of hemoproteins was inversely related to the fraction of heme surface exposed to solvent on the basis of three-dimensional structures (Stellwagen, 1978) and of solvent perturbation studies (Schlauder & Kassner, 1979). The redox potential value of the heme apoHasA complex, −550 mV vs SHE, approaches those of ferrisiderophores, which are generally low (up to −750 mV for enterobactin; Hallé & Meyer, 1992). This highly negative value is not favorable for the reduction of the intact ferric porphyrin−imidazole complex in aerobic conditions. It also suggests that heme has a high degree of solvent exposure and that it is not buried deeply within the heme binding pocket.

The outer membrane receptor HasR of hemoHasA was recently identified by Ghigo and Wandersman (1997). The mechanism by which it recognizes the heme−protein complex and takes up heme is currently unknown. The high degree of heme solvent exposure could be in favor of a direct recognition of the accessible surface of heme in the complex. As shown by far-UV CD experiments, heme binding does not produce significant alteration of the secondary structure of HasA. Minor or compensating changes could, however, occur and not be detected. Whether the interaction of the heme−HasA complex and HasR produces a conformational change in HasA allowing the transfer of heme remains to be determined. NMR and microcalorimetry studies on both apo- and hemoHasA are under way to provide further information on the accommodation of heme in the heme binding pocket and to contribute to a better understanding of the heme uptake and release mechanisms.

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