Deciphering the Structural Role of Histidine 83 for Heme Binding in Hemophore HasA*15

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Heme carrier HasA has a unique type of histidine/tyrosine heme iron ligation in which the iron ion is in a thermally driven two spin states equilibrium. We recently suggested that the H-bonding between Tyr75 and the invariantly conserved residue His83 modulates the strength of the iron–Tyr75 bond. To unravel the role of His83, we characterize the iron ligation and the electronic properties of both wild type and H83A mutant by a variety of spectroscopic techniques. Although His83 in wild type modulates the strength of the Tyr–iron bond, its removal causes detachment of the tyrosine ligand, thus giving rise to a series of pH-dependent equilibria among species with different axial ligation. The five coordinated species detected at physiological pH may represent a possible intermediate of the heme transfer mechanism to the receptor.

The ability of bacteria to cause diseases depends on many parameters allowing the pathogen to invade the vertebrate host. One of them is its skill to scavenge the host iron ion. Indeed, iron is an essential metal for nearly all living organisms. However, its oxidized iron(III) form is hardly soluble, and its reduced iron(II) form is highly toxic. Therefore, in biological fluids, iron mostly exists as a complex with iron-binding proteins or in heme carrier proteins and is scarcely available. Hence, Gram-negative bacteria have developed multiple iron/heme acquisition systems to survive. Most of them rely on outer membrane receptors either specific for exogenous iron/heme sources present in the biotope or for molecules called siderophores or hemophores. These molecules are synthesized and released by bacteria into the extracellular medium (1). Their function is to capture iron or heme from the host and to return it to the specific receptor.

The first hemophore was discovered in Serratia marcescens (2). Since then, several orthologs have been found in Pseudomonas aeruginosa (3), Pseudomonas fluorescens (4), Yersinia pestis (5), Yersinia enterocolitica (Sanger Institute) and Yersinia pseudotuberculosis (6). HasA (heme acquisition system) hemophores form an independent family of highly conserved heme-carrier proteins that are not homologous to any other known proteins. They bind free heme, or extract heme from proteins such as hemoglobin and hemopexin, and deliver it to heme-specific outer membrane receptors, termed HasR (1), which in turn release it into the bacteria (2, 7).

S. marcescens wild-type HasA (HasAWT) is a 19-kDa monomeric protein that binds b-heme with a very high affinity (Kd = 1.9 × 10⁻¹¹ M) and a 1:1 stoichiometry (8, 9). In the heme-loaded hemophore holoHasAWT, the iron ion is in the oxidized form with the lowest redox potential (−550 mV) reported so far for heme-binding proteins (9). The x-ray structure of holo-HasAWT shows an unprecedented heme-binding site (10). Heme is held by two extended loops that connect the α and β faces of the protein and is rather exposed to solvent (186 Å²) (Fig. 1). The heme iron ion is bound by an unusual pair of ligands: a tyrosine (Tyr75) and a histidine (His83) (10). This ligand pair has only been observed in a very few proteins, e.g. some hemoglobins from invertebrates (11–13), the reduced cytochrome c maturation protein CcmE (14), and the oxidized cytochrome cd₁ nitrite reductase (15). Some abnormal human hemoglobins (16) and mutated heme proteins also present this type of coordination (17). X-ray and NMR data previously showed that, in holoHasAWT, the Tyr75–iron forms a tight hydrogen bond with the N61 of a neighboring histidine, His83, that increases the nucleophilic character of Tyr75 and strengthens the Tyr75–iron coordination bond (18, 19). The role of His83 in heme binding was highlighted by alanine mutagenesis of the two iron axial ligands and of His83, followed by the determination of the Kd for heme in the mutant proteins. Consistently, heme affinity in H83A is 265 times smaller (Kd = 5.0 × 10⁻⁹ M) than in HasAWT, whereas the mutation of the axial ligand induces a 400-fold loss of affinity for Y75A and only a 5-fold loss for H32A. Moreover, His83 has been proposed as an alternative iron ligand in the absence of Tyr75 or of both His32 and Tyr75 (20). Noteworthy, in contrast to His32, the Tyr75–His83 pair is conserved in all the hemophores, which suggests that the Tyr75–His83 hydrogen bond plays a central and recurrent role for the activity of these heme carrier proteins.

To decipher the structural role of His83 in HasA, we characterized the electronic properties and the iron coordination of holoHasAWT and holoH83A in the oxidized form as a function of pH, using a panel of techniques such as NMR, EPR, x-ray crystallography, resonance Raman, and UV-visible spectroscopy.
pies. Although holoHasAWT possesses a unique coordination as a function of pH, substitution of His83 with an alanine residue gives rise to pH-dependent coordination forms with different axial ligation. The role of the Tyr15–His83 hydrogen bond for the stabilization of the Tyr15-iron axial coordination was unraveled. Disruption of this hydrogen bond at physiological pH results in a five-coordinate species that most probably represents an intermediate of the heme transfer mechanism to the receptor.

**EXPERIMENTAL PROCEDURES**

**Preparation of HasA Proteins**—Wild-type hemophore HasA WT from *S. marcescens* and mutant H83A were obtained and cloned as previously reported (2, 20). Uniformly 15N- and 13C-labeled proteins were produced at 303 K in M9 minimal medium containing 15NH4Cl and 13C-glycerol as the sole nitrogen and carbon sources and were purified as described previously (9, 21). The purity of the proteins was checked by SDS-PAGE. The protein concentrations were calculated previously (9, 21). The purity of the proteins was checked by SDS-PAGE. The protein concentrations were calculated previously (9, 21). The purity of the proteins was checked by SDS-PAGE. The protein concentrations were calculated previously (9, 21). The purity of the proteins was checked by SDS-PAGE. The protein concentrations were calculated previously (9, 21). The purity of the proteins was checked by SDS-PAGE. The protein concentrations were calculated previously (9, 21).

**Absorption Spectroscopy**—The absorption measurements were achieved in a Perkin-Elmer Lambda 2 spectrophotometer at ambient temperature using 1- or 0.2-cm path length cells.

**EPR Spectroscopy**—EPR spectra were recorded with a Bruker ESP300E spectrometer fitted to an Oxford Instrument ESR900 helium flow cryostat. The experiments were carried out on protein samples with concentration ranging from 0.1 to 0.5 mM in 50 mM sodium acetate buffer, pH 5.5, 50 mM phosphate buffer, pH 7, and 50 mM Tricine, pH 9. Spin quantitations of the low and high spin heme signals were performed in nonsaturating conditions as previously described (23).

**X-ray Crystallography**—The crystallization experiments were performed using the hanging drop vapor diffusion method. The drops were prepared by mixing 2 µl of the protein solution (4.0 mg/ml in 50 mM phosphate buffer, pH 7, 0.02% propanesulfonic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; HS, high spin; LS, low spin.

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2 The abbreviations used are: rR, resonance Raman; CAPS, 3-(cyclohexylamino)propanesulfonic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; HS, high spin; LS, low spin.
Structural Role of His<sup>83</sup> in HasA

FIGURE 2. Final electron density of the heme region of H83A (Protein Data Bank code 2UYD) surrounded at a 1 σ level, showing the two axial ligands Tyr<sup>75</sup> and His<sup>32</sup>. The diffraction data have been refined with the two heme group orientations related by 180° rotation along the α-γ meso axis as identified in holoHasAWT (19, 26). The low resolution of the electron density might be due to high flexibility and/or the presence of several conformations.

Na<sub>N</sub>) with 1 μl of the reservoir solution (100 mM sodium cacodylate, pH 6.5, 200 mM Zn(OAc)<sub>2</sub>, 10% (v/v) 2-propanol). The brownish crystals grew to 0.2 mm over a period of 2 months at 20 °C.

X-ray diffraction data were collected at 100 K at the ESRF (Grenoble, France) beamline ID14-EH1 using an ADSC Quantum 4R CCD detector. The wavelength of the synchrotron x-rays was 0.933 Å. The crystal was rotated through 90° with a 1.0° oscillation range/frame. The raw data were processed and merged with MOSFLM (24) and SCALA (25). The crystals of holoH83A (space group P3<sub>2</sub>2<sub>1</sub>) diffracted to a maximum resolution of 2.6 Å. Molecular replacement with holoHasAWT (Protein Data Bank code 1B2V) as model gave a unique solution related by 180° rotation along the α-γ meso axis as identified in holoHasAWT (19, 26). Water molecules were added with CCP4/wARP (27). The stereochemistry of the final structure was evaluated using PROCHECK (28). The refinement statistics and final R factors are listed in Table 1.

RESULTS

Visible Absorbance Spectra of Holoproteins

Fig. 3 shows the absorbance spectra of holoHasAWT and holoH83A. The spectrum of holoHasAWT at pH 5.6 (Fig. 3A) shows maxima at 406 nm (Soret band), 494, 537, 568, and 618 nm that are consistent with a mixture of two species, one high spin (HS) and one low spin (LS). Indeed, whereas the charge transfer band at 618 nm is characteristic of a HS species, the presence of two Q-bands at 537 and 568 nm indicates the presence of a LS species. The absorbance spectrum did not change upon titration from pH 4.4 to 10.4, showing that the population of the two species is pH-insensitive. It is noteworthy that the addition of KCN to the protein at a 15-fold excess did not produce any spectroscopic changes, either at pH 7.3 or at pH 9.4 (data not shown).

In contrast to holoHasAWT, the absorbance spectrum of holoH83A is sensitive to pH. At acidic and neutral pH, the spectra exhibit a Soret maximum at 404 nm, Q-bands at 502 and 536 nm, and a CT band at 630 nm (Fig. 3B). This pattern is consistent with the presence of a HS species. When pH increases, bands at 502, 536, and 630 nm decrease, whereas new bands emerge at 490, 543, and 610 nm. The disappearance of the charge transfer band at 630 nm at the advantage of the 610-nm band indicates that the HS species observed at acidic and neutral pH progressively disappears when pH increases for the benefit of a new HS species. Moreover, a LS species appears, as shown by a new band at 576 nm. Thus, at alkaline pH, holoH83A exists as a mixture of a HS and a LS species, the HS species being different from the one observed at acidic pH. Like holoHasAWT, no detectable change was observed after the addition of a 15-fold excess of a KCN solution neither at pH 7.3, where a HS species is dominant, nor at pH 9.4, where two HS species and one LS species are present.

Resonance Raman Spectra of holoHasAWT and holoH83A Mutant

Consistent with previously reported NMR data, the rR spectra of holoHasAWT show a mixture of LS and HS species. The rR data further indicate that the HS component of holoHasAWT is a hexacoordinate species. The 1300–1700 cm<sup>-1</sup> region of heme rR spectra comprises bands corresponding to porphyrin in-plane vibrational modes. They are diagnostic for oxidation

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**TABLE 1**

| Data collection and refinement statistics | HasA-H83A |
|-----------------------------------------|-----------|
| ESRF beamline                           | ID14-EH1  |
| Wavelength (Å)                          | 0.933     |
| Space group                             | P3221     |
| Unit cell parameters (Å and °)          | a = b = 110.7, α = β = 90°, c = 123.4 |
| Resolution (Å)                          | 23.9-2.6  |
| No. observations                        | 75944     |
| No. unique reflections                  | 21699     |
| R<sub>f</sub> (%)                       | 6.3 (43.3)|
| (I<sub>obs</sub>/I<sub>calc</sub>)       | 10.4 (2.8)|
| Rindex (%)                              | 0.014     |
| Bond lengths (Å)                        | 1.53      |
| Root mean square deviation              |           |
| Root mean square deviation              |           |
| Bond angles (°)                         |           |
| Bond lengths (Å)                        | 1.53      |
| Root mean square deviation              |           |
| Bond angles (°)                         |           |
| B factor (Å<sup>2</sup>)                |           |
| Mean overall                            | 44.3      |
| Main chain                              | 43.9      |
| Side chain                              | 44.7      |
| Heme atoms                              | 52.9/54.6 |
| Iron atom                               | 52.6      |
| Water atoms                             | 60.1      |

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* Root mean square deviation for anomalous differences, where I<sub>obs</sub> is the average of Friedel amplitudes at a single wavelength.
* The values in parentheses correspond to the highest resolution shell.
state, coordination number, and spin state of the heme iron atom (44, 45). The spectrum of holoHasA<sub>WT</sub> (Fig. 4A) is indicative of a ferric species with its characteristic ν<sub>3</sub> band at 1372 cm<sup>-1</sup>. The spin state marker bands indicate an equilibrium mixture of six coordinate high spin (6c HS) (ν<sub>2</sub>, 1561 cm<sup>-1</sup>; ν<sub>3</sub>, 1476 cm<sup>-1</sup>; ν<sub>10</sub> under the 1624 cm<sup>-1</sup> band) and six coordinate low spin (6c LS) (ν<sub>2</sub>, 1580 cm<sup>-1</sup>; ν<sub>3</sub>, 1503 cm<sup>-1</sup>; ν<sub>10</sub>, 1633 cm<sup>-1</sup>) states. The ratio of the two ν<sub>3</sub> and two ν<sub>10</sub> band intensities does not change between pH 5.2 and 9.2, whereas the LS bands increase in intensity at the expense of their HS counterparts as the temperature is lowered (data not shown). These observations suggest that the mixture of 6c HS and 6c LS hemes arises from either two pH independent axial ligand sets or a single axial ligand set characterized by a pH-insensitive spin state equilibrium.

For hemoproteins, excitation into the tyrosinate-Fe(III) CT band (near 500 nm) can provide resonance enhancement of Raman scattering by vibrations characteristic of bound phenolate. No bands attributable to bound tyrosine vibrations were identified in the 514.5-nm excited rR spectra of HasAWT. How-

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ture of the WT heme pocket has been modified. At low pH, a pronounced band is observed at 196 cm\(^{-1}\) (Fig. 4B). Although this is in a region often associated with modes having axial iron--His stretching character, the only such modes that have been identified in ferric hemes are in those having the bis-His axial ligand set. Because this set of ligands is inaccessible in holoH83A, the 196 cm\(^{-1}\) band is concluded to arise from a porphyrin mode. Based on its greater intensity at low pH, where HS pentacoordinate iron dominates the heme speciation, the 196 cm\(^{-1}\) band is attributed to a mode having porphyrin out-of-plane character.

Characterization of holoHasAWT and holoH83A Mutant by NMR

\(^1\)H NMR Spectroscopy—The analysis of \(^1\)H-\(^{15}\)N heteronuclear single-quantum correlation spectra of holoHasAWT and holoH83A indicate that for both systems the secondary structure and protein folding are maintained over the investigated pH range (supplemental Fig. S1 and Ref. 19).

One-dimensional experiments over large spectral windows were used to monitor the heme coordination sphere. We previously showed that, in HasAWT, HS and a LS species were in fast exchange on the NMR time scale (19). Indeed, the one-dimensional \(^1\)H NMR spectrum of the protein shows heme methyl resonances with chemical shift values intermediate
between those expected for a purely HS $S = 5/2$ heme iron(III) and those for a purely LS $S = 1/2$ heme iron(III) (Fig. 6A). One major and one minor set of resonances are visible on the spectrum, corresponding to the two possible orientations of the heme along the $\alpha$-$\gamma$ meso axis (10, 19). The chemical shifts and the nonselective $T_1$ and $T_2$ values for the hyperfine-shifted signals are in the $10$- to $40$-ms range, and the line widths are $\sim 400$ Hz at $16.4$ T (supplemental Table S1). The relaxation times are close to those expected for a HS species (39). Two exchangeable, broad and fast relaxing signals with a $70$-$30$ times are close to those expected for a HS species (39, 47). The $^1$H NMR spectrum of holoH83A clearly locates the $\text{His32 N}^1$H of iron coordinated histidine (39–42), corresponding to the high spin heme along the spectrum, and those for a purely LS $S$ are visible on the spectrum at $303$ K and pH $5.6$, with a $T_1 < 1$ ms and a line width of $\sim 1000$ Hz. It is characteristic of the N$\delta$1H of an axial histidine for a HS species (39, 46). No other histidine can coordinate the iron in holoH83A, it corresponds to His$^{32}$ N$\delta$1H. Longitudinal relaxation times are less than $1$ ms for the four heme methyls and $\sim 4$–$5$ ms for all the other downfield shifted signals. As illustrated in supplemental Fig. S2B, hyperfine-shifted resonances exhibit a Curie behavior, indicative of a pure HS state (39). The meso $^1$H shifts are characteristically downfield ($\sim 40$ ppm) for the hexacoordinate HS ferric complexes and upfield ($\sim 20$ to $70$ ppm) for the pentacoordinate high spin ferric complexes (39, 47). The $^1$H NMR spectrum of holoH83A clearly locates the $\text{His32 N}^1$H peak at $\sim 25$ ppm. Therefore, at acidic pH values holoH83A is a pentacoordinate HS species. In addition to the two sets of resonances described above, the spectrum of holoH83A at acidic and neutral pH exhibits unresolved overlapping signals between 20 and 40 ppm (Fig. 6, B and C). With increasing pH, the acidic pentacoordinate HS species decreases in intensity, whereas the overlapping signals at 20–40 ppm increase. Additionally, a minor set of signals ($\sim 10\%$) appears in the region 40–60 ppm. They are overwhelmed by the acidic HS form and can be unambiguously identified only at pH $> 9$ (Fig. 6D). The two alkaline species undergo line narrowing at increasing pH, indicative of an exchange process in the quasistatic regime. Line narrowing at higher pH allows the identification of several well resolved peaks in the 20–40-ppm region with $T_1$ of $\sim 3$ ms and line widths of $\sim 500$ Hz. The temperature dependence of these signals is nonlinear (supplemental Fig. S2C). All of these findings are consistent with an hydroxide-bound form (36, 61–63). Two broad and exchangeable signals, missing at acidic pH, are observable at alkaline pH (a major one at $64.7$ ppm and a minor one at $70.5$ ppm at pH $9.9$ and $303$ K) (Fig. 6D, inset). These resonances, characteristics of the imidazole N$\delta$1H of a heme iron-coordinated histidine (39–42), correspond to His$^{32}$ N$\delta$1H in the two heme orientations. Thus, His$^{32}$ is a heme iron ligand in the hydroxide-bound species. The minor alkaline form ($10\%$) is characterized by larger shifts with respect to the hydroxide form, accounting for a predominantly HS species.

No spectra changes were observed upon the addition of a 15-fold buffered KCN to holoH83A at pH $7.3$ or $9.4$. Thus, whatever the dominant species in solution, holoH83A does not bind cyanide at any pH.

$^1$H NMR Spectroscopy—holoH83A spectra were recorded at various pH values at $303$ K. The histidine resonances were assigned by comparison with the already available assignment of holoHasAWT and holoH83A at pH $5.5$ and $7.0$, at $317$ K (19). The His$^{32}$ N$\delta$1 resonance is observed at $194.2$ ppm, at pH $7$ (Fig. 7). The His$^{32}$ Ne2 signal is undetectable as in holoHasAWT, in agreement with its coordination to the heme iron. The spectrum recorded at pH $9$ shows dramatic broadening of all reso-

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**FIGURE 6.** $^1$H NMR spectra, reported in the region 105–40 ppm, of holoHasAWT at pH 5.6 (A) and holoH83A at pH 5.2 (B), pH 7.1 (C), and pH 9.9 (D). The spectra were recorded at $11.7$ T at $303$ K in $20$ mM phosphate buffer. The signals of the minor alkaline form are marked by asterisks. The inset in D shows the His$^{32}$ N$\delta$1H signals.  

The holoH83A $^1$H NMR spectrum obtained at acidic pH displays two sets of well resolved peaks in a 3:1 ratio with four distinct heme-methyl resonances between 60 and 80 ppm, typical of HS species (Fig. 6B) (39). They correspond to the two different heme orientations in the heme-binding pocket. A very broad, exchangeable, resonance associated with the high spin species is present at $102.3$ ppm, in the spectra at $303$ K and pH $5.6$, with a $T_1 < 1$ ms and a line width of $\sim 1000$ Hz. It is characteristic of the N$\delta$1H of an axial histidine for a HS species (39, 46). No other histidine can coordinate the iron in holoH83A, it corresponds to His$^{32}$ N$\delta$1H. Longitudinal relaxation times are less than $1$ ms for the four heme methyls and $\sim 4$–$5$ ms for all the other downfield shifted signals. As illustrated in supplemental Fig. S2B, hyperfine-shifted resonances exhibit a Curie behavior, indicative of a pure HS state (39). The meso $^1$H shifts are characteristically downfield ($\sim 40$ ppm) for the hexacoordinate HS ferric complexes and upfield ($\sim 20$ to $70$ ppm) for the pentacoordinate high spin ferric complexes (39, 47). The $^1$H NMR spectrum of holoH83A clearly locates the meso-H-peak at $\sim 25$ ppm. Therefore, at acidic pH values holoH83A is a pentacoordinate HS species. In addition to the two sets of resonances described above, the spectrum of holoH83A at acidic and neutral pH exhibits unresolved overlapping signals between 20 and 40 ppm (Fig. 6, B and C). With increasing pH, the acidic pentacoordinate HS species decreases in intensity, whereas the overlapping signals at 20–40 ppm increase. Additionally, a minor set of signals ($\sim 10\%$) appears in the region 40–60 ppm. They are overwhelmed by the acidic HS form and can be unambiguously identified only at pH $> 9$ (Fig. 6D). The two alkaline species undergo line narrowing at increasing pH, indicative of an exchange process in the quasistatic regime. Line narrowing at higher pH allows the identification of several well resolved peaks in the 20–40-ppm region with $T_1$ of $\sim 3$ ms and line widths of $\sim 500$ Hz. The temperature dependence of these signals is nonlinear (supplemental Fig. S2C). All of these findings are consistent with an hydroxide-bound form (36, 61–63). Two broad and exchangeable signals, missing at acidic pH, are observable at alkaline pH (a major one at $64.7$ ppm and a minor one at $70.5$ ppm at pH $9.9$ and $303$ K) (Fig. 6D, inset). These resonances, characteristics of the imidazole N$\delta$1H of a heme iron-coordinated histidine (39–42), correspond to His$^{32}$ N$\delta$1H in the two heme orientations. Thus, His$^{32}$ is a heme iron ligand in the hydroxide-bound species. The minor alkaline form ($10\%$) is characterized by larger shifts with respect to the hydroxide form, accounting for a predominantly HS species.
Structural Role of His\textsuperscript{83} in HasA

13C NMR Spectroscopy—Resonances of the iron-coordinated residues can be observed using 13C direct detection in one-dimensional experiments (19). The comparison of holoHasA\textsubscript{WT} and holoHasA spectrum at pH 5.6 is shown in Fig. 8. The holoHasA spectra show eight hyperfine-shifted well resolved peaks in the downfield region (Fig. 8B, peaks A–H). Two additional signals were observed at lower temperature (283 K) (data not shown). On the basis of the relative intensity, five pairs corresponding to the two heme orientations could be identified, a set of major signals labeled A, C, D, F, and H and a set of minor signals. Four pairs have \( T_1 \) values shorter than 1 ms, and one pair has \( T_1 \) in the 10–13-ms range (Table 2). \( T_1 \) values reported for holoHasA\textsubscript{WT} are of the same order of magnitude (19). Analogous with the findings for the 1H heme resonances, larger hyperfine shifts with respect to the wild-type and Curie behavior are observed in the 13C spectrum of the mutant. This behavior can be accounted for by the presence of a HS form. The number of hyperfine-shifted resonances is smaller than for holoHasA\textsubscript{WT}, consistent with the presence of a single protein ligand. We therefore assigned them to the axial ligand His\textsuperscript{32}.

At pH 8.3, a new set of weakly hyperfine-shifted signals is observed in the range -1000 to -50 ppm (Fig. 8C, inset). Their relative intensity with respect to that of the strongly hyperfine-shifted signals is the same as observed in the corresponding 1H spectrum between the 5c HS and hydroxide-bound forms. Resonances in a similar chemical shift range have been detected for the axial ligands in LS cytochrome c.\textsuperscript{3}

The paramagnetic tailored 13C-13C COSY spectra in the aromatic region at acidic pH of holoHasA\textsubscript{WT} and holoHasA are essentially superimposable. However, in the mutant, a new cross-peak appears in the region diagnostic for CB-C\( \gamma \) correlations of aromatic residues. This peak can only correspond to the Tyr\textsuperscript{75} CB-C\( \gamma \) correlation because all the other aromatic residues have been already assigned in both spectra, which are similar in this region (Fig. 9, A and B). In the holoHasA spectrum at pH 8.3, the latter correlation is still present. Moreover, an additional peak appears that corresponds to the Tyr\textsuperscript{75} C\( \beta \)-C\( \gamma \) correlation.

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\( ^{3}\) P. Turano, unpublished results.
relation of the low spin form (Fig. 9C). $^{13}$C-$^{13}$C COSY cross-peaks are detectable only if their line width is smaller than $\sim 300$ Hz (48). A coordination bond induces a strong electron spin delocalization from the metal to the ligand affecting both chemical shift and nuclear relaxation (49), thus making the signals of the ligands undetectable in the $^{13}$C-$^{13}$C COSY. This means that Tyr$^{75}$ is not a heme iron ligand in any of the two forms detected by NMR as a function of pH. In holoHasA$_{WT}$ $^{13}$C-$^{13}$C COSY, Tyr$^{75}$ $\beta$-$\gamma$ correlation was not observed (Fig. 9A).

**Electron Paramagnetic Resonance Spectroscopy of the Ferric Proteins**—At neutral pH, holoHasA$_{WT}$ exhibits a rhombic LS signal with $g$ values at 2.80, 2.20, and 1.71 (Fig. 10A). A weak HS $S = 5/2$ signal with a slight rhombic character is also visible at $g = 6.3$ and 5.8. It represents less than 10% of the total spin intensity. The spectrum does not change at pH 5.5 (not shown), but at pH 9.0 some spectral heterogeneities are revealed by the appearance of a second LS component at $g = 2.76$, 2.20, and 1.76. However, the ratio of the HS to LS signals remains essentially unchanged at this pH (Fig. 10B). Thus, the EPR results indicate that the coordination sphere of the iron ion is not modified between pH 5.5 and 9.0.

To compare the local environment of the heme Fe$^{3+}$ ion for the protein in solution and in the crystal, EPR experiments were also performed on a sample made of small HasA$_{WT}$ crystals spread in mother liquor at pH 8 and frozen. The spectrum of this polycrystalline sample shows a major LS signal at $g = 2.8$, 2.2, and 1.71 and a minor HS signal (Fig. 10C). Their close similarity with those given by the protein solution shows that the magnetic properties and the coordination of the heme Fe$^{3+}$ ion are the same.

The EPR spectrum of holoH83A variant at acidic pH shows a HS signal with axial symmetry (Fig. 10D) at $g_\perp = 5.8$ and $g_\parallel = 1.99$. At neutral and basic pH (Fig. 10, E and F), a LS signal appears as the major component with $g = 2.73$, 2.19, and 1.78, and a slightly rhombic HS signal at $g = 6.5$ and 5.4 is also present. The relative proportion of the HS signal appeared to be preparation-dependent but remains lower than 20%.

**X-ray Structure of Ferric holoH83A Mutant**—One molecule was present in the asymmetric unit, giving a crystal volume/protein mass ($V_{m}$) of 4.33 Å$^3$ Da$^{-1}$ and a solvent content of 72% by volume (50). The coordinates describing one protein molecule with one heme group per asymmetric unit were refined to a final $R$ factor and $R_{free}$ of 23.6% and 27.3%, respectively. As for the structure of holoHasA$_{WT}$, the last 14 C-terminal residues are undetectable.

Although holoH83A only diffracted to medium resolution, and the B-factors are substantially larger than those of holoHasA$_{WT}$ (supplemental Fig. S3), comparison of both structures show that they are very similar (supplemental Fig. S4). Overall root mean square deviation is 0.51 Å. The most significant differences concern the two heme-binding loops, i.e. residues 32–44 and 73–81 that move away from the heme in holoH83A, with an apparent increase in the His$^{32}$ Ne2-Fe and Tyr$^{75}$ Oη-Fe bond lengths. However, the quality of the structure precludes to determine whether Tyr$^{75}$ is coordinated to the heme iron in the crystal of holoH83A or there is no more an axial ligand, as established for holoHasA$_{WT}$.
the tyrosinate form of Tyr\(^{75}\) is stabilized by a tight hydrogen bond between His\(^{83}\) N\(\delta1\) and Tyr\(^{75}\) O\(\eta\) (1.86 Å). In holoH83A, the only potential candidate to form a hydrogen bond with the hydroxyl proton of Tyr\(^{75}\) is the Ala\(^{83}\) carboxyl oxygen. Although the distance between the two atoms, ~4 Å, does not preclude the formation of a weak hydrogen bond, the tyrosinate character of Tyr\(^{75}\) would remain poor.

**DISCUSSION**

The heme iron spin state and the coordination sphere of both holoHasA\(_{WT}\) and holoH83A have been defined over a wide range of pH values. All of the spectroscopic techniques indicate that the heme iron coordination in holoHasA\(_{WT}\) does not undergo changes in the pH range 4.5–10.4. Resonance Raman shows that the sixth position in the high spin state is neither vacant nor occupied by a water molecule, supporting His\(^{32}\) and Tyr\(^{75}\) ligation. NMR data accounts for a thermal high spin–low spin equilibrium in fast exchange on the NMR time scale with a single set of axial ligands, His\(^{32}\) and Tyr\(^{75}\). The equilibrium is related to modulations in the coordination of the iron between the iron and Tyr\(^{75}\), which in turn depends on the strength of the hydrogen bond between Tyr\(^{75}\) O\(\eta\) and His\(^{83}\) N\(\delta1\) and on the tyrosine protonation state. For holoH83A, the different techniques monitor changes in iron coordination number and spin state as a function of pH that are summarized in Table 3.

In solution at acidic pH the major form of holoH83A is a high spin pentacoordinate species. The fifth ligand is unambiguously identified by NMR as His\(^{83}\) as His\(^{83}\). Resonance Raman reveals the presence of two additional six coordinated species with HS and LS state. Relative intensities of rR bands do not generally correlate with the relative populations because of differences in their resonance enhancement. NMR spectra at acidic pH show evidence of a minor species, characterized by broad features in the 20–40 ppm range, accounting for ~20% of total signal intensity and attributed to an OH\(^-\) bound form. Signal broadening arises from an exchange process, quasi-slow on the NMR time scale, with another protein form, whose intensity is much lower than 5 \(\times\) 10\(^4\) s\(^{-1}\) for the transition between the 6c LS form and the 6c LS form and much lower than 5 \(\times\) 10\(^4\) s\(^{-1}\) for the transition between five-coordinated to six-coordinated species. Although the various spectroscopic techniques we employed here probe a wide range of time scales, they converge to a consistent view of pH-dependent heme speciation in solution.

As outlined above, relaxation data and chemical shifts of the dominant alkaline species account for a His\(^{83}\)/OH\(^-\) axial coordination. As described in the literature for a variety of ferric heme proteins, this is not a purely low spin state as also confirmed by the temperature dependence of the chemical shifts (51–54).

Acid-base transitions taking place in ferric heme proteins with a H\(_2\)O/OH\(^-\) character are generally correlated to a change in the protonation status of a close residue in the heme pocket (55). This residue, which is a histidine in most cases, forms a hydrogen bond with the iron bound water (55). The histidine deprotonation causes ionization of the bound water, resulting in a predominantly low spin hydroxide form. In holoH83A, the most likely residue for deprotonation in close proximity to the bound water is Tyr\(^{75}\). Thus, we propose that, at increasing pH, the phenolic oxygen atom of Tyr\(^{75}\) forms a hydrogen bond with the water ligand, thereby stabilizing the hydroxide complex, as summarized in Scheme 1 and Table 3. The modulation of the H-bond strength involving H\(_2\)O/OH\(^-\) may broaden the HS and LS Fe–OH modes, such that they are not observed in the low frequency EPR spectrum. The pK\(_a\) of the transition from the five-coordinated form and the six-coordinated form is ~8.4 (Scheme 1). Transitions involving deprotonation of bound water have been reported to occur with a wide range of pK\(_a\) values (56, 57). In the present case, exchange broadening prevents us from an accurate estimate of the pK\(_a\) for the transition between the 6c HS and the 6c LS form (Scheme 1). However, their relative intensity does not vary at pH > 8. Estimates of rate constants for the two equilibria depicted in Scheme 1 are ~6 × 10\(^5\) s\(^{-1}\) for the transition between the 6c HS and the 6c LS form and much lower than 5 \(\times\) 10\(^4\) s\(^{-1}\) for the transition between five-coordinated to six-coordinated species. Although the various spectroscopic techniques we employed here probe a wide range of time scales, they converge to a consistent view of pH-dependent heme speciation in solution.

Accessibility of heme to anionic ligands seems restricted in holoHasA\(_{WT}\) and holoH83A, which do not bind cyanide, either at neutral or at basic pH, despite their spin state. Cyanide is usually considered to be a strong ligand for ferric heme. How-
ever, the nature of the residues in the heme active site heavily affects the binding properties of the cyanide anion. Reduced or even loss of affinity toward HS heme iron (III) has been found in the case of heme hydrophobic environment (58–61). In holo-HasA<sub>WT</sub> and holoH83A, the heme is surrounded by residues involved in both hydrophobic and stacking interactions. The only three polar residues in the vicinity of the heme are all on the His<sup>32</sup> side. In holoH83A, the replacement of a histidine with an alanine mutant makes the Tyr<sup>75</sup> side of the heme more accessible but also more hydrophobic.

His<sup>83</sup> modulates the stability of hexacoordinate heme in holoHasA<sub>WT</sub> through hydrogen bonding with the coordinated side chain of Tyr<sup>75</sup>. The purified hemophore, HasA<sub>WT</sub>, has a His83-Tyr75 H-bond resulting in a 5c heme. Because transfer of heme to the heme-binding loop bearing Tyr75, thereby triggering the elongation and breaking of the His<sup>83</sup>-Tyr<sup>75</sup> hydrogen bond.

The results presented herein show that disruption of the His<sup>83</sup>-Tyr<sup>75</sup> H-bond results in a 5c heme. Because transfer of heme to HasR requires scission of the Fe–HasA bonds, the present characterization of holoH83A at physiological pH provides a picture of a possible mechanistic intermediate in that heme transfer reaction.

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REFERENCES

1. Wandersman, C., and Delepelaire, P. (2004) Annu. Rev. Microbiol. 58, 611–647
2. Letoffé, S., Ghigo, J. M., and Wandersman, C. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9876–9880
3. Letoffé, S., Redeker, V., and Wandersman, C. (1998) Mol. Microbiol. 28, 1223–1234
4. Letoffé, S., Omori, K., and Wandersman, C. (2000) J. Bacteriol. 182, 4401–4405
5. Rossi, M. S., Fetherston, J. D., Letoffé, S., Carniel, E., Perry, R. D., and Ghigo, J. M. (2001) Infect. Immun. 69, 6707–6717
6. Chain, P. S., Carniel, E., Larimer, F. W., Lamerdin, J., Stoutland, P. O., Regala, W. M., Georgescu, A. M., Vergez, L. M., Land, M. L., Motin, V. L., Brubaker, R. R., Fowler, J., Hinnebusch, J., Marceau, M., Medigue, C., Simonet, M., Chenal-Francisque, V., Souza, B., Dacheux, D., Elliott, J. M., Derbis, A., Hauser, L. J., and Garcia, E. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 13826–13831
7. Ghigo, J. M., Letoffé, S., and Wandersman, C. (1997) J. Bacteriol. 179, 3572–3579
8. Deniau, C., Gilli, R., Izadi-Pruneyre, N., Letoffé, S., Delepierre, M., Wandersman, C., Briand, C., and Lecroisey, A. (2003) Biochemistry 42, 10627–10633
9. Izadi, N., Henry, Y., Haladjian, I., Goldberg, M. E., Wandersman, C., Delepierre, M., and Lecroisey, A. (1997) Biochemistry 36, 7050–7057
10. Arnoux, P., Haser, R., Izadi, N., Lecroisey, A., Delepierre, M., Wandersman, C., and Czjzek, M. (1999) Nat. Struct. Biol. 6, 516–520
11. Kraus, D. W., Wittenberg, J. L., J., F., and Peisach, J. (1990) J. Biol. Chem. 265, 16054–16059
12. Lecomte, J. T., Smit, J. D., Winterhalter, K. H., and La Mar, G. N. (1989) J. Mol. Biol. 209, 235–247
13. Das, T. K., Couture, M., Lee, H. C., Peisach, J., Rousseau, D. L., Wittenberg, B. A., Wittenberg, J. B., and Guertin, M. (1999) Biochemistry 38, 15360–15368
14. Uchida, T., Stevens, J. M., Daltrop, O., Harvat, E. M., Hong, L., Ferguson, S. J., and Kitagawa, T. (2004) J. Biol. Chem. 279, 51981–51988
15. Williams, P. A., Fulop, V., Garman, E. F., Saunders, N. F., Ferguson, S. J., and Haidu, J. (1997) Nature 389, 406–412
16. Nagatomo, S., Jin, Y., Nagai, M., Hori, H., and Kitagawa, T. (2002) Biophys. Chem. 98, 217–232
17. Maurus, R., Bogumil, R., Tuo, Y., Tang, H. L., Smith, M., Mauk, A. G., and Brayder, G. D. (1994) J. Biol. Chem. 269, 12606–12610
18. Wolf, N., Deniau, C., Letoffé, S., Simenel, C., Kumar, V., Stojilkovic, I., Wandersman, C., Delepierre, M., and Lecroisey, A. (2002) Protein Sci. 11, 757–765
19. Callet-Sagué, C., Delepierre, M., Lecroisey, A., Bertini, I., Piccioli, M., and Turano, P. (2006) J. Am. Chem. Soc. 128, 150–158
20. Letofe, S., Deniau, C., Wolf, N., Dassa, E., Delepelaire, P., Lecroisey, A., and Wandersman, C. (2001) Mol. Microbiol. 41, 439–450
21. Izadi-Pruneyre, N., Wolf, N., Castagne, C., Czisch, M., Wandersman, C., Delepierre, M., and Lecroisey, A. (1999) J. Biomol. NMR 14, 193–194
22. Izadi-Pruneyre, N., Wolf, N., Redeker, V., Wandersman, C., Delepierre, M., and Lecroisey, A. (1999) Eur. J. Biochem. 261, 562–568
23. Izadi-Pruneyre, N., Huche, F., Lukat-Rodgers, G. S., Lecroisey, A., Gilli, R., Rodgers, K. R., Wandersman, C., and Delepelaire, P. (2006) J. Biol. Chem. 281, 25541–25550
24. Leslie, A. G. W. (1990) in Crystallographic Computing, pp. 50–61, Oxford University Press, Oxford
25. Collaborative Computational Project, Number 4 (1994) Acta Crystallogr. D. Biol. Crystallogr. 50, 760–763
26. Arnoux, P., Haser, R., Izadi-Pruneyre, N., Lecroisey, A., and Czjzek, M. (2000) Proteins 41, 202–210
27. Perrakis, A., Sixma, T. K., Wilson, K. S., and Lamzin, V. S. (1997) Acta Crystallogr. D. Biol. Crystallogr. 53, 448–455
28. Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) J. Appl. Crystallogr. 26, 283–291
29. Jin, Y., Nagai, M., Nagai, T., Nagatomo, S., and Kitagawa, T. (2004) Biochemistry 43, 8517–8527
30. Pond, A. E., Roach, M. P., Sono, M., Rux, A. H., Franken, S., Xu, R., Thomas, M. R., Wilks, A., Dou, Y., Ikeda-Saito, M., Ortiz de Montellano, P. R., Woodruff, W. H., Boxer, S. G., and Dawson, J. H. (1999) Biochemistry 38, 7601–7608
31. Adachi, S., Nagano, S., Ishimori, K., Watanabe, Y., Morishima, I., Egawa, T., Kitagawa, T., and Makino, R. (1993) Biochemistry 32, 241–252
32. Sharma, K. D., Andersson, L. A., Terner, J., and Goff, H. M. (1989) J. Biol. Chem. 264, 12772–12779
33. Liu, Y., Moinen-Loccoz, P., Hildebrand, D. P., Wilks, A., Loehr, T. M., Mauk, A. G., and Ortiz de Montellano, P. R. (1999) Biochemistry 38, 3733–3743
34. Lukat-Rodgers, G. S., and Rodgers, K. R. (1998) J. Biol. Inorganic Chem. 3, 274–281
35. Song, S. H., Boffi, A., Chiancone, E., and Rousseau, D. L. (1993) Biochemistry 32, 6330–6336
36. Yeh, S. R., Couture, M., Ouellet, Y., Guertin, M., and Rousseau, D. L.
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37. Indiani, C., Feis, A., Howes, B. D., Marzocchi, M. P., and Smulevich, G. (2000) J. Am. Chem. Soc. 122, 7368–7376
38. Heering, H. a., Jansen, M. A. K., Thorneley, R. N. F., and Smulevich, G. (2001) Biochemistry 40, 10360–10370
39. Bertini, I., Turano, P., and Vila, A. J. (1993) Chem. Rev. 93, 2833–2932
40. Howes, B. D., Rodriguez-Lopez, J. N., Smith, A. T., and Smulevich, G. (1997) Biochemistry 36, 1532–1543
41. La Mar, G. N., Budd, D. L., Smith, K. M., and Langry, K. C. (1980) J. Am. Chem. Soc. 102, 1822–1827
42. La Mar, G. N., Frye, J. S., and Satterlee, J. D. (1976) Biochim. Biophys. Acta 428, 78–89
43. Wu, J. Z., La Mar, G. N., Yu, L. P., Lee, K. B., Walker, F. A., Chiu, M. L., and Sligar, S. G. (1991) Biochemistry 30, 2156–2165
44. Qin, J., La Mar, G. N., Dou, Y., Admiraal, S. J., and Ikeda-Saito, M. (1994) J. Biol. Chem. 269, 1083–1090
45. Bertini, I., and Luchinat, C. (1986) NMR of Paramagnetic Molecules in Biological Systems, Benjamin-Cummings Publishing Co., Menlo Park, CA
46. Chu, G. C., Tomita, T., Sonnichsen, F. D., Yoshida, T., and Ikeda-Saito, M. (1999) J. Biol. Chem. 274, 24490–24496
47. Pande, U., La Mar, G. N., Lecomte, J. T., Ascoli, F., Brunori, M., Smith, K. M., Pandey, R. K., Parish, D. W., and Thanabal, V. (1986) Biochemistry 25, 5638–5646
48. Bertini, I., Jimenez, B., Piccioli, M., and Poggi, L. (2005) J. Am. Chem. Soc. 127, 12216–12217
49. Arnesano, F., Banci, L., and Piccioli, M. (2005) Q. Rev. Biophys. 38, 167–219
50. Matthews, B. W. (1968) J. Mol. Biol. 33, 491–497
51. Beetlestone, J., and George, P. (1964) Biochemistry 3, 707–714
52. Yamamoto, Y., Suzuki, T., and Hori, H. (1993) Biochim. Biophys. Acta 1203, 267–275
53. Iizuka, T., and Kotani, M. (1969) Biochim. Biophys. Acta 194, 351–363
54. Iizuka, T., and Kotani, M. (1969) Biochim. Biophys. Acta 181, 275–286
55. Ishikawa, K., Takeuchi, N., Takahashi, S., Matera, K. M., Sato, M., Shibahara, S., Rousseau, D. L., Ikeda-Saito, M., and Yoshida, T. (1995) J. Biol. Chem. 270, 6345–6350
56. Antonini, E., Brunori, M., Greenwood, C., Malmstrom, B. G., and Rotilio, G. C. (1971) Eur. J. Biochem. 23, 396–400
57. Banci, L., Bertini, I., Bren, K. L., Gray, H. B., and Turano, P. (1995) Chem. Biol. 2, 377–383
58. Banci, L., Bertini, I., Eltis, L. D., and Pierattelli, R. (1993) Biophys. J. 65, 806–813
59. Keller, R. M., Wuthrich, K., and Debrunner, P. G. (1972) Proc. Natl. Acad. Sci. U. S. A. 69, 2073–2075
60. Bertini, I., Briganti, F., Monnanni, R., Scozzafava, A., Carlozzi, P., and Materassi, R. (1990) Arch. Biochem. Biophys. 282, 84–90
61. Motie, M., Kassner, R. J., Meyer, T. E., and Cusanovich, M. A. (1990) Biochemistry 29, 1932–1936
62. Letoffe, S., Debarbieux, L., Izadi, N., Delepelaire, P., and Wandersman, C. (2003) Mol. Microbiol. 50, 77–88