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Spectroscopic Identification of Heme Axial Ligands in HtsA That Are Involved in Heme Acquisition by *Streptococcus pyogenes*[†]

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ABSTRACT: The heme-binding proteins Shp and HtsA of *Streptococcus pyogenes* are part of the heme acquisition machinery in which Shp directly transfers its heme to HtsA. Mutagenesis and spectroscopic analyses were performed to identify the heme axial ligands in HtsA and to characterize axial mutants of HtsA. Replacements of the M79 and H229 residues, not the other methionine and histidine residues, with alanine convert UV−vis spectra of HtsA with a low-spin, hexacoordinate heme iron into spectra of high-spin heme complexes. Ferrous M79A and H229A HtsA mutants possess magnetic circular dichroism (MCD) spectra that are similar with those of proteins with pentacoordinate heme iron. Ferric M79A HtsA displays UV−vis, MCD, and resonance Raman (RR) spectra that are typical of a hexacoordinate heme iron with histidine and water ligands. In contrast, ferric H229A HtsA has UV−vis, MCD, and RR spectra that represent a pentacoordinate heme iron complex with a methionine axial ligand. Imidazole readily forms a low-spin hexacoordinate adduct with M79A HtsA with a K_d of 40.9 μM but not with H229A HtsA, and cyanide binds to M79A and H229A with K_d of 0.5 and 19.1 μM, respectively. The ferrous mutants rapidly bind CO and form simple CO complexes. These results establish the H229 and M79 residues as the axial ligands of the HtsA heme iron, indicate that the M79 side is more accessible to the solvent than the H229 side of the bound heme in HtsA, and provide unique spectral features for a protein with pentacoordinate, methionine-ligated heme iron. These findings will facilitate elucidation of the molecular mechanism and structural basis for rapid and direct heme transfer from Shp to HtsA.

Heme is a major source of essential iron for bacterial pathogens (*1*, *2*). Some Gram-positive pathogens such as *Streptococcus pyogenes* and *Staphylococcus aureus* produce cell surface proteins to acquire heme from host hemoproteins and relay it through the bacterial envelope to a heme-specific ATP-binding cassette (ABC) transporter, which transports heme across the cytoplasmic membrane. The *S. pyogenes* heme acquisition system consists of the surface proteins Shr and Shp and the ABC transporter HtsABC (also called SiaABC) (*3*−*5*), and the surface proteins IsdA, IsdB, IsdC, and IsdH and the ABC transporter IsdDEF constitute the heme acquisition machinery in *S. aureus* (*6*). Shr efficiently transfers its heme to Shp but not to HtsA[1] (*7*), and Shp directly donates its heme to HtsA (*8*), supporting a pathway of heme uptake in which Shr extracts heme from methemoglobin and Shp relays heme from Shr to HtsA, the lipoprotein component of the HtsABC transporter. A similar pathway has been elucidated for the heme acquisition process in *S. aureus* in which IsdB acquires heme from methemoglobin and transfers it directly or through IsdA to IsdC, which then donates its heme to IsdE, the lipoprotein component of the IsdDEF transporter (*9*−*11*). Despite these advancements, the chemical mechanisms of these heme transfer reactions largely remain unknown.

The proteins involved in heme acquisition in bacteria usually bind heme. Heme iron in hemoproteins is either in a pentacoordinate or hexacoordinate form, with four ligands from protoporphyrin IX and one or two axial ligands from the side chains of histidine, lysine, tyrosine, methionine, and/or cysteine residues of proteins. Combinations of the strong ligands, histidine, lysine, methionine, and cysteine, in the hexacoordinate binding result in the low-spin Fe(III) and Fe(II) states with strong absorption Soret peak and two Q_{ov} or $\alpha\beta$ bands, whereas pentacoordinate heme iron is in the high-spin state and lacks the dominant α band at the reduced state (*12*). The heme iron in Shp and HtsA is hexacoordinate and in the low-spin state (*8*), and the Shr heme iron is also hexacoordinate (*7*). According to the crystal structure and protein engineering experiments, the heme iron axial ligands of the Shp heme iron are methionine 66 and methionine 153 (*13*, *14*). In contrast, the IsdA, IsdC, and IsdE proteins of the *S. aureus* system utilize tyrosine, tyrosine, and methionine/histidine residues as their axial ligand(s), respectively (*15*−*17*). Spectroscopic analyses indicate that the HtsA heme iron has a low-spin hexacoordination with methionine and histidine axial ligands (*18*). However, the identities of these axial ligands have not been experimentally established.

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[1]Abbreviations: HtsA, lipoprotein component of the heme-specific ATP-binding cassette transporter; H229, histidine residue at position 229 of the HtsA protein; M79, methionine residue at position 79 of the HtsA protein; H229A, HtsA mutant carrying alanine replacement of H229; M79A, HtsA mutant carrying alanine replacement of M79.

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The Shp-to-HtsA heme transfer reaction follows a concerted two-step kinetic mechanism in which Shp first forms a complex with heme-free HtsA (apoHtsA) and transfers its heme to apoHtsA in a single kinetic phase with a rate constant of 43 s^{-1}, which is ~40000 times greater than the rate of simple heme dissociation from oxidized Shp (*8*). This kinetic mechanism implies that the reaction follows either a sequential axial displacement mechanism where the first axial displacement is rate-limiting or a simultaneous axial displacement mechanism in which the two axial bonds of the heme iron in the holoHtsA product are formed at about the same time. Alanine replacements of the methionine 66 and methionine 153 axial ligands of the Shp heme iron cause similar alteration of the kinetic mechanism of the Shp/apoHtsA reaction, supporting the simultaneous axial displacement mechanism (*14*). Heme axial mutants of HtsA and their properties in coordination, spin state, various spectra, and accessibility on the axial sides will be valuable to vigorously establish the axial displacement mechanism of the Shp/HtsA reaction. Thus, each of the histidine or methionine residues of HtsA was replaced with alanine by site-directed mutagenesis, and resulting HtsA mutants were characterized by UV−vis, MCD, EPR, and resonance Raman spectroscopies, accessibility to exogenous ligands, and relative affinity for heme. The results demonstrate that the heme iron of H229A mutant protein is pentacoordinate and inaccessible to imidazole, and the M79A mutant protein has a hexacoordination with water and His ligands, indicating that the M79 and H229 residues are the axial ligands of heme in HtsA and that the M79 side of the heme pocket is more accessible than the H229 side.

MATERIALS AND METHODS

Materials. The QuickChange site-directed mutagenesis kit was purchased from Stratagene (La Jolla, CA). Resins for protein purification were from GE Healthcare Biosciences (Piscataway, NJ). Bovine hemin chloride was from Sigma. Imidazole, sodium fluoride, and potassium cyanide were purchased from ACROS Organics.

Gene Cloning and Site-Directed Mutagenesis. A histidine residue in the 6×His tag of 6×His-tagged M79A HtsA at high concentrations could ligate to the heme iron in this mutant. To avoid the 6×His tag in recombinant HtsA, the *htsA* gene in pLP1795 (*4*) was PCR cloned into pET-21d at the *Nco*I and *Eco*RI sites using primers 5′-ACCATGGGTAGTGCTGAAACTGGTGTGAATCAGCACCCTAAAAC-3′ and 5′-CGAATTCTTAGTTTTCACTTGATAAGATTG-3′, yielding pLP1795-2. The nucleotides underlined were included in the primer to introduce a 7 amino acid residue tag (MGSAETG) that is fused to the second residue, asparagine 21, of mature HtsA. This peptide fragment was included to achieve overexpression of HtsA because, for unknown reason, there was no overexpression of mature HtsA without any tag. Each histidine or methionine residue of HtsA was replaced with alanine using the QuickChange XL site-directed mutagenesis kit, plasmid pLP1795-2, and paired primers with an alanine codon replacing the corresponding histidine or methionine codon according to manufacturer's protocol. The cloned *htsA* and mutant genes were entirely sequenced to rule out spurious mutations and to confirm desired mutations.

Protein Purification. Recombinant HtsA and its mutant proteins were expressed in *Escherichia coli* BL21(DE3) containing corresponding plasmids by IPTG induction. The cell pellet from a 6 L culture was resuspended in 50 mL of Tris-HCl, sonicated on ice for 15 min, and centrifuged. The supernatant obtained was loaded onto a DEAE column (2.5 × 10 cm), and the column was washed with 200 mL of Tris-HCl, pH 8.0, and eluted with a 150 mL linear gradient of 0−0.25 M NaCl. Fractions containing HtsA were pooled, and the sample was adjusted to 1.3 M $(NH_4)_2SO_4$ and loaded onto a phenyl-Sepharose column (1.5 × 5 cm). The column was washed with 100 mL of 1.3 M $(NH_4)_2SO_4$ and eluted with a 100 mL linear gradient of 1.3−0 M $(NH_4)_2SO_4$. Fractions containing >95% HtsA, as assessed by SDS−PAGE, were pooled and dialyzed against 20 mM Tris-HCl.

Preparation of HoloHtsA Proteins. Purified HtsA proteins were a mixture of holo and apo forms. Homogeneous holoHtsA proteins were obtained by reconstitution with hemin, as previously described (*4*). Protein concentrations were determined using the modified Lowry protein assay kit from Pierce with bovine serum albumin as a standard. Heme contents of holoHtsA proteins were measured with the pyridine hemochrome assay using $\varepsilon_{418} = 191.5\ mM^{-1}\ cm^{-1}$ (*19*).

Magnetic Circular Dichroism (MCD) Measurements. MCD spectra of oxidized and reduced holoHtsA proteins in 50 mM phosphate buffer at indicated pH were recorded using JASCO J-710 spectropolarimeter equipped with an Alpha Scientific 3002-1 electromagnet under the following conditions: bandwidth, 1 nm; accumulation, 3 scans; scan rate, 100 nm/min; resolution, 0.5 nm; magnetic field, 12.9 kG (1.29 T); and temperature, 25 °C. The spectra of the reduced proteins were taken in the presence of excess dithionite. Buffer blank-corrected CD spectra obtained without magnetic field were subtracted from corresponding buffer-corrected MCD spectra using the Jasco software. These processed MCD data were then used to calculate the final MCD data in units of $\Delta\varepsilon_M\ (M\ cm\ T)^{-1}$ based on protein concentration, light path, magnetic field, and molar ellipticity $\Theta_M\ (deg\ cm^2\ dmol^{-1}\ T^{-1}) = 3300\ \Delta\varepsilon_M$.

Resonance Raman (RR) spectroscopy. RR spectra of HtsA proteins in 20 mM Tris-HCl buffer were recorded with a krypton ion laser (I-302-Coherent) equipped with a Spex Triplemate Raman spectrophotometer and a liquid nitrogen-cooled CCD detector using the following conditions: laser excitation wavelength, 413.1 nm; number of scans, 3−25; integration time per scan, 25 s; and power, 10−20 mW. Raman frequencies were calibrated using aspirin.

Electron Paramagnetic Resonance (EPR) Measurement. EPR spectra of HtsA proteins were measured with a Bruker EMX spectrometer using the following conditions: frequency, 9.6 GHz; power, 3 mW; modulation amplitude, 10 G; modulation frequency, 100 kHz; and temperature, 10 K. Estimation of the low-spin signals was based on comparison of the area of the $g = 3$ absorption-like signal with the analogous low-spin signal of metmyogolbin at pH 9.5 (Mb(Fe(III)OH)).

Binding of Imidazole and Cyanide to HtsA Proteins. One milliliter of 7 μM wild-type, M79A, or H229A HtsA protein was mixed with imidazole or potassium cyanide at 5−500 μM and incubated at 20 °C for at least 10 min, and UV−vis and MCD spectra were recorded. The dissociation constants (K_d) of the bindings were calculated by analyzing absorbance change (ΔA) using the method of Wu and Hammes (*20*) (eq 1)

$$K_d = ([HtsA]_o - [P])([ligand]_o - n[P])/[P] \quad (1)$$

where $[HtsA]_o$ and $[ligand]_o$ are the total molar concentrations of wild-type or mutant HtsA and imidazole or cyanide, respectively,

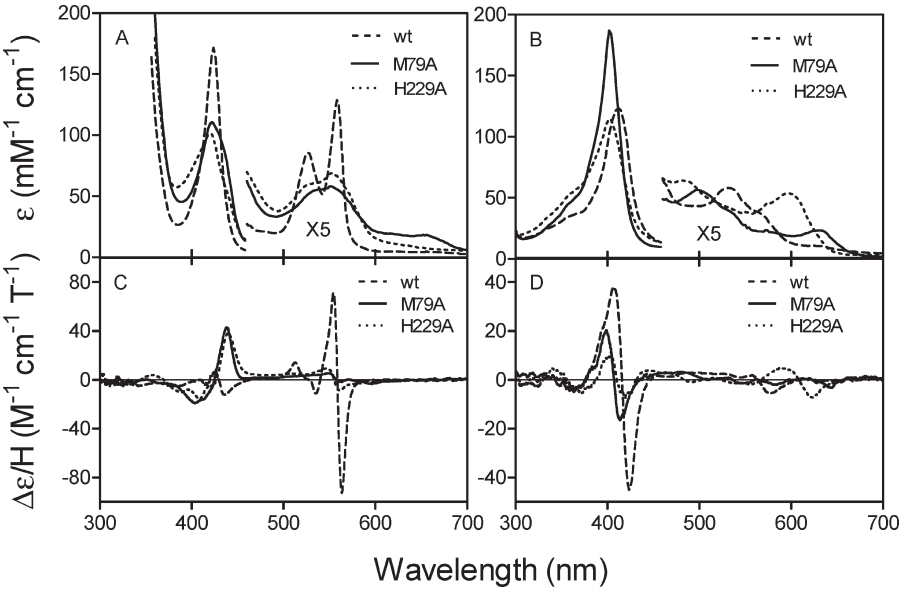

FIGURE 1: UV−vis absorption and MCD spectra of ferrous (A and C) and ferric (B and D) wild-type (wt), M79A, and H229A HtsA in 20 mM Tris-HCl, pH 8.0. The reduced spectra were recorded in the presence of excess dithionite.

and n and [P] are the number of imidazole or CN^{-1} binding sites per HtsA molecule and the concentration of the HtsA/ligand complex, respectively. [P] at different $[ligand]_o$ was calculated using the equation $P = (\Delta A/\Delta A_{max})[HtsA]_o$, where ΔA_{max} was ΔA when HtsA was saturated with the ligand and determined from the intercept on the Y axis in the double reciprocal plotting of ΔA and $[ligand]_o$.

Binding and Rebinding of Carbon Monoxide (CO) to HtsA Proteins. To examine CO binding, wild-type (3.3 μM), H229A (3.8 μM), and M79A (2.2 μM) HtsA proteins were reduced by dithionite and equilibrated with 1 atm of 100% CO (1 mM CO), and the spectra of the samples were recorded. Rapid CO association kinetics were measured by photolysis of the preformed complexes of reduced HtsA H229A and M79A with CO each at 2 μM using a 0.5 μs excitation pulse (577 nm, phase-R 2100 dye laser) in the presence of 1 mM CO. Time courses of CO rebinding were monitored at 420 nm.

Reactions of HoloHtsA Proteins with H64Y/V68F Apomyoglobin. Whether the HtsA proteins significantly lose heme to H64Y/V68F apomyoglobin, a heme scavenger (*21*), was estimated by comparing the optical absorption spectra of holoHtsA and H64Y/V68F holomyoglobin with those of the mixtures of 2 μM holoHtsA protein/40 μM H64Y/V68F apomyoglobin. Spectral changes of the reactions were continuously monitored for 24 h and decreased to zero by 6 h after the start of the reactions. Thus, the spectra of the mixtures were taken using a SPECTRA^{Max} 384 Plus spectrophotometer after 6 h incubation in 20 mM Tris-HCl, pH 8.0, for determining relative heme affinity of HtsA proteins.

RESULTS

Alterations of the HtsA Absorption Spectra Caused by H229A and M79A Replacements. To find out which histidine residue is a heme axial ligand in HtsA, each of the five histidine residues at positions 24, 229, 251, 289, and 293 was replaced with alanine by site-directed mutagenesis. The optical absorption spectra of the H24A, H251A, H289A, and H293A mutant proteins at both ferric and ferrous states are very similar to those of wild-type HtsA (data not shown). However, reduced H229A has reduced intensity of the Soret peak and no longer displays the dominant α band at 560 nm (Figure 1A), indicating that the heme iron in H229A is not in the hexacoordinate complex with two strong axial ligands. The Soret absorption peak of oxidized H229A shifts to 402 nm from 412 nm in wild-type HtsA (Figure 1B). In addition, the 532 and 560 nm peaks in wild-type HtsA are replaced with peaks at 482 and 600 nm in H229A (Table 1). These observations suggest that His229 is one of the two axial ligands of the heme iron in HtsA.

To determine which methionine residue is the other axial ligand, the six internal methionine residues at positions 79, 126, 173, 216, 238, and 270 were individually replaced with alanine by site-directed mutagenesis. Among the six mutant proteins, only M79A shows dramatically different absorption spectra from the wild-type protein. Like reduced H229A, ferrous M79A has a reduced intensity of the Soret peak and lacks the resolved α and β bands (Figure 1A), suggesting that reduced M79A also has a pentacoordinate heme iron. Ferric M79A has a spectrum with peaks at 402, 498, 536, and 630 nm with a greater extinction coefficient of the Soret peak than that of the wild-type protein (Figure 1B), and the profile in the region of 460−700 nm is similar to that of aquometHb (*22*), suggesting that oxidized M79A has a hexacoordinate heme iron with histidine and water axial ligands. These results strongly suggest that M79 is the other axial ligand of the heme iron in HtsA.

MCD Spectra of the HtsA Proteins. As Sook et al. described (*18*), the MCD spectrum of ferrous HtsA is similar to ferrous cytochrome *c*, which contains a low-spin, hexacoordinate heme iron with the Met and His axial coordination (*23*, *24*). Replacements of H229 and M79 with alanine dramatically reduce and increase the intensities of the Q and Soret bands, respectively, and eliminate the trough of the derivative feature of the Soret band (Figure 1C). The MCD spectrum of ferrous M79A is similar to that of ferrous horseradish peroxidase (*25*), which is known to contain pentacoordinate heme iron with a histidine ligand (*26*). The intensities of the ferrous H229A MCD spectrum are similar to those of the M79A MCD spectrum, suggesting that ferrous heme iron in H229A is also pentacoordinate. However, the MCD profiles of ferrous M79A and H229A

Table 1: Heme Coordination and Spectral Features of Wild-Type and Mutant HtsA Proteins

		absorption peaks			major MCD features			
protein[a]	known/proposed axial ligand(s)	Soret (nm)	ε (mM^{-1} cm^{-1})	visible (nm)	Soret (nm)	$\Delta\varepsilon$/H (M cm T)$^{-1}$	visible (nm)	$\Delta\varepsilon$/H (M cm T)$^{-1}$
wt HtsA	Met/His	412	123	532	407	38	557	1.9
				562[b]	416	0	564	0
					423	−45	578	−5.6
M79A HtsA	His/H_2O	403	187	498	399	20	548	−1.5
				536[b]	407	0	580	−2
				630	414	−16.5	643	−3.3
H229A HtsA	Met	402	114	482	404	11	517	0.8
				524	410	0	555	−2
				600	419	−7.5	589	4.9
							608	0
							624	−7.2

[a]All spectra were measured at pH 8.0 unless specified. [b]Estimated from shoulders.

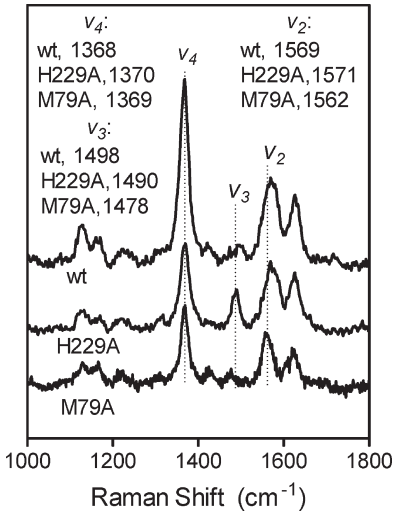

FIGURE 2: Raman spectra with excitation at 413.1 nm for wt, M79A, and H229A HtsA proteins each at 200 μM in 20 mM Tris-HCl, pH 8.0.

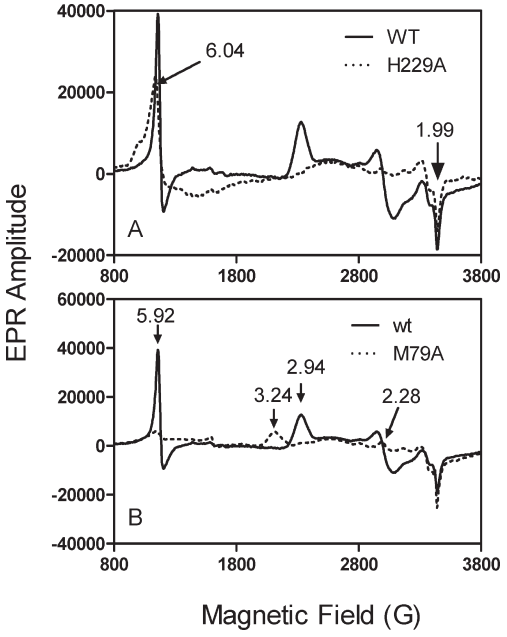

FIGURE 3: EPR spectra of wt, M79A, and H229A HtsA proteins at 100 μM in 20 mM Tris-HCl, pH 8.0.

are obviously different, apparently reflecting the fact that the remaining axial ligand residue in the two mutant proteins is different. These results support that H229 and M79 are the axial ligands of the HtsA heme iron.

Ferric wild-type HtsA has a MCD spectrum typical of a low-spin, hexacoordinate heme iron with histidine and methionine axial ligands (Figure 1D), confirming the previous finding (*18*). As expected, the MCD spectrum of ferric M79A is high spin (Figure 1D), and the profile of the M79A spectrum in the charge transfer region is similar to that of aqueous metMb (*27*), suggesting again that ferric M79A has a water- and histidine-ligated hexacoordinate heme iron.

The H229A replacement caused more profound change in the MCD spectrum than the M79A mutation (Figure 1D). Notably, the intensities of the peak and trough of the Soret band of ferric H229A are small and similar to those of ferric horseradish peroxidase, Aplysia myoglobin, cyanogen bromide-modified myoglobin, and H64L and H64V myoglobin whose heme irons have a pentacoordination with a histidine axial ligand (*26*, *28*), suggesting that ferric H229A has a pentacoordination. However, the MCD spectrum profile of ferric H229A is obviously different from those of these proteins with histidine-ligated, pentacoordinate heme iron, particularly possessing a band with a derivative feature around 608 nm. The different MCD profile of H229A HtsA from the proteins with histidine-ligated, pentacoordinate heme iron is most likely because H229A HtsA has a methionine ligand.

Resonance Raman Spectra. The high-frequency region of the RR spectra of the HtsA proteins in Figure 2 reveals bands characteristic of the oxidation (ν_4) and spin and coordination (ν_2, ν_3) states of the heme iron atom (*29*). M79A protein had the ν_4, ν_3, and ν_2 peaks at 1369, 1478, and 1562 cm^{-1}, respectively, which are close to the ν_4 (1370 cm^{-1}), ν_3 (1480 cm^{-1}), and ν_2 (1559 cm^{-1}) peaks of $(Me_2SO)_2Fe^{III}PP^+$, a high-spin hexacoordinate model compound (*30*), and the ν_3 (1481 cm^{-1}) and ν_2 (1561 cm^{-1}) peaks of the aquometHb protein in which the heme iron has a high-spin hexacoordination with water and histidine ligands (*22*). The ν_4, ν_3, and ν_2 peaks of the H229A mutant were at 1370, 1490, and 1571 cm^{-1}, respectively, which are close to the 1374 cm^{-1} (ν_4), 1491 cm^{-1} (ν_3), and 1572 cm^{-1} (ν_2), respectively, of the His(F8)Tyr Mb mutant, which has five-coordinate, high-spin iron (*31*). Since previous studies have established that protein-induced shifts of the heme frequencies above 1300 cm^{-1} are much

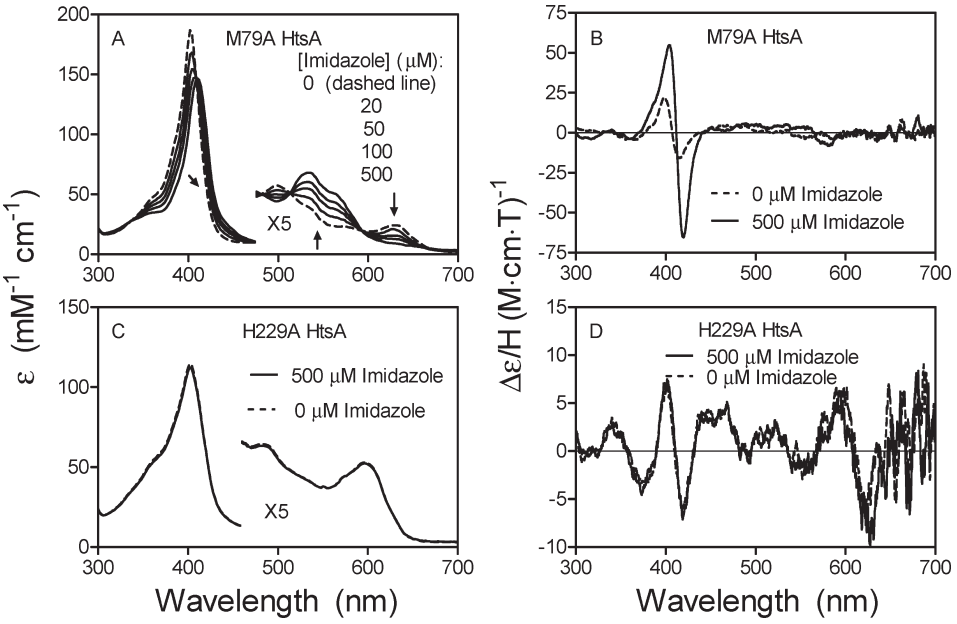

FIGURE 4: Effects of imidazole on UV−vis (A and C) and MCD (B and D) spectra of M79A (A and B) and H229A (C and D) HtsA. The UV−vis and MCD spectra of each HtsA protein in 20 mM Tris-HCl (for UV−vis) or 50 mM potassium phosphate (for MCD), pH 8.0, at the indicated imidazole concentrations are shown. The arrows in panel A indicate the directions of the spectral shifts as imidazole concentration increased.

Table 2: Dissociation Constants for Imidazole and Cyanide Binding to the HtsA Proteins

	K_d (μM)	
protein	for imidazole binding	for cyanide binding
wt HtsA	no binding	55
M79A HtsA	40.9	0.5
H229A HtsA	no binding	19.1

smaller than those induced by changes in spin and ligation state (*22*, *30*), our results indicate that the heme irons in H229A and M79A are high-spin, pentacoordinate and high-spin, hexacoordinate, respectively.

EPR Results. As expected, >90% of ferric wild-type HtsA heme iron has a rhombic low-spin signal with $g_z = 2.94$ and $g_y = 2.28$ (Figure 3), which are identical with or close to $g_z = 2.94$ and $g_y = 2.29$ of 6×His-tagged HtsA (*8*), and no low-spin signal was detected in ferric H229A HtsA (Figure 3A). Surprisingly, ~70% of the ferric M79A HtsA heme iron had a rhombic low-spin signal with $g_z = 3.24$ (Figure 3B). This unexpected result was not consistent with the high-spin features of the UV−vis absorption, MCD, and RR spectra at room temperature, suggesting that low temperature might have caused a coordination change.

Accessibility of the Heme Iron in HtsA Axial Mutants to Imidazole. To examine the accessibility for exogenous iron ligands of the H229 and M79 sides of the bound heme in HtsA, the binding of imidazole, which has the same structure with the side chain of histidine, to H229A and M79A was examined by UV−vis and MCD spectroscopy. Addition of imidazole causes a dose-dependent shift of the M79A absorption spectrum to one that is typical of a hexacoordinate low-spin heme iron (Figure 4A), and the analysis of the spectral change data using eq 1 found that the mutant protein binds one imidazole per protein molecule with a K_d of 40.9 μM (Table 2). These results indicate that imidazole ligates to the M79A heme iron. The MCD spectrum of M79A in the presence of 0.5 mM imidazole is consistent with the imidazole binding (Figure 4B). In contrast, imidazole at 0.5 mM did not alter the absorption and MCD spectra of H229A HtsA (Figure 4C,D), indicating that imidazole cannot bind to H229A under these conditions. These results suggest that imidazole can access the alanine 79 position of M79A HtsA but not the alanine 229 position of H229A HtsA.

Binding of Cyanide to HtsA. To determine whether the alanine 229 position of the H229A HtsA mutant is completely inaccessible to external ligands, the binding of cyanide, a small and strong external ligand, to the HtsA heme iron was examined. Cyanide binds to the heme iron in both M79A and H229A, causing shifts in the absorption and MCD spectra that are consistent with the formation of hexacoordinate heme iron complexes (Figure 5). M79A and H229A were found to bind one cyanide with K_d of 0.5 and 19.1 μM, respectively. Thus, the alanine 229 site of the H229A mutant is not completely inaccessible. However, the cyanide binding to the H229A heme iron is weaker than the binding to the M79A heme iron by about 40-fold, indicating that the H229 side of the HtsA heme is more sterically packed than the M79 side. It has been shown that wild-type HtsA binds cyanide (*18*). The absorption and MCD spectra of wild-type and M79A HtsA in the presence of 0.5 mM potassium cyanide were almost identical but are different from those of the H229A−cyanide mixture (Figure 5C,D), suggesting that cyanide replaces the M79 ligand in the cyanide binding to wild-type HtsA.

Binding and Rebinding of CO to HtsA. To analyze ferrous ligand binding, we examined the binding and rebinding of CO to ferrous wild-type and mutant HtsA proteins. The ferrous wild-type HtsA bound CO but not completely after incubation with 1 mM CO for 25 min, and the ferrous H229A and M79A mutants formed a simple CO complex (Figure 6). The apparent first-order rate constants of CO association after photolysis at 1 mM CO were 2.1×10^4 s^{-1} and 1.1×10^4 s^{-1} for the M79A and H229A mutants, respectively, suggesting that the mutants can bind CO rapidly. Absorbance change during photolysis of the wild-type

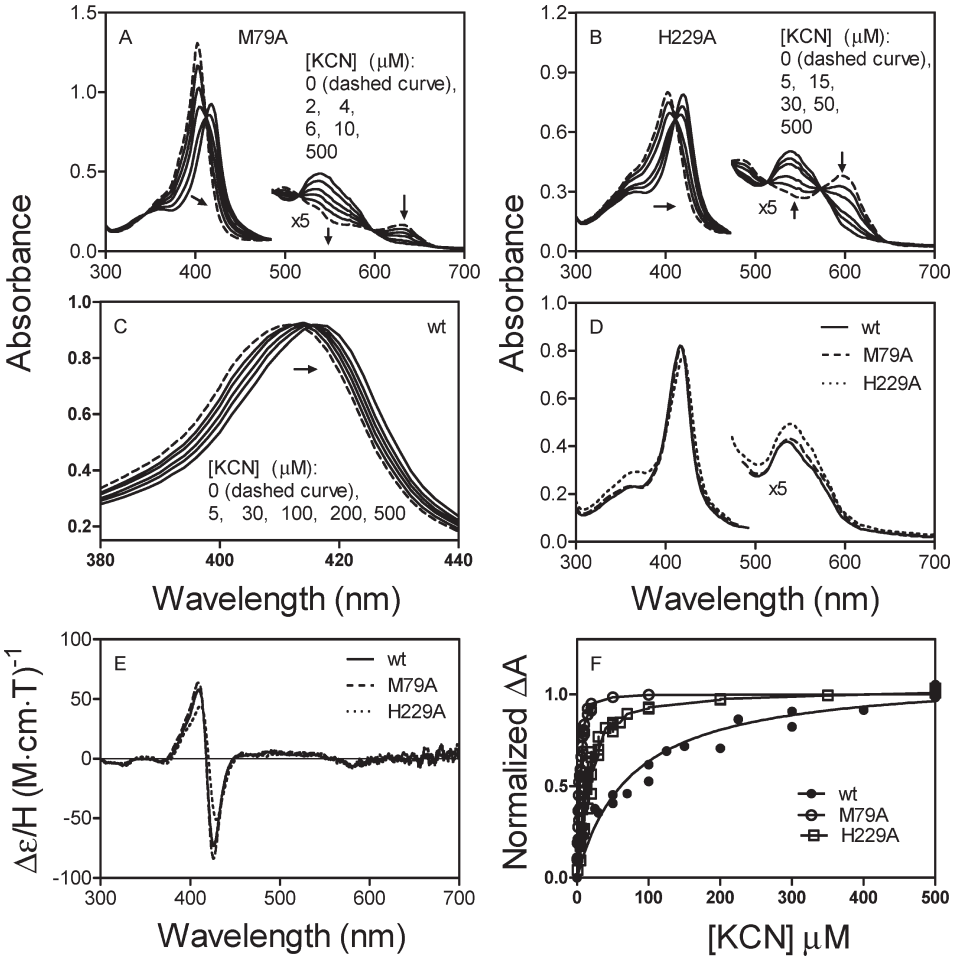

FIGURE 5: Binding of cyanide to wt, M79A, and H229A HtsA. (A−C) Cyanide concentration-dependent shifts of the UV−vis spectra of 7 μM M79A (A), H229A (B), and wt (C) HtsA in 20 mM Tris-HCl, pH 8.0. (D, E) Overlays of the UV−vis (D) or MCD (E) spectra of wt, M79A, and H229A HtsA in the presence of 500 μM KCN. (F) Cyanide titration of absorbance change during cyanide binding. The absorbance changes were normalized by setting the maximum absorbance changes at 1.

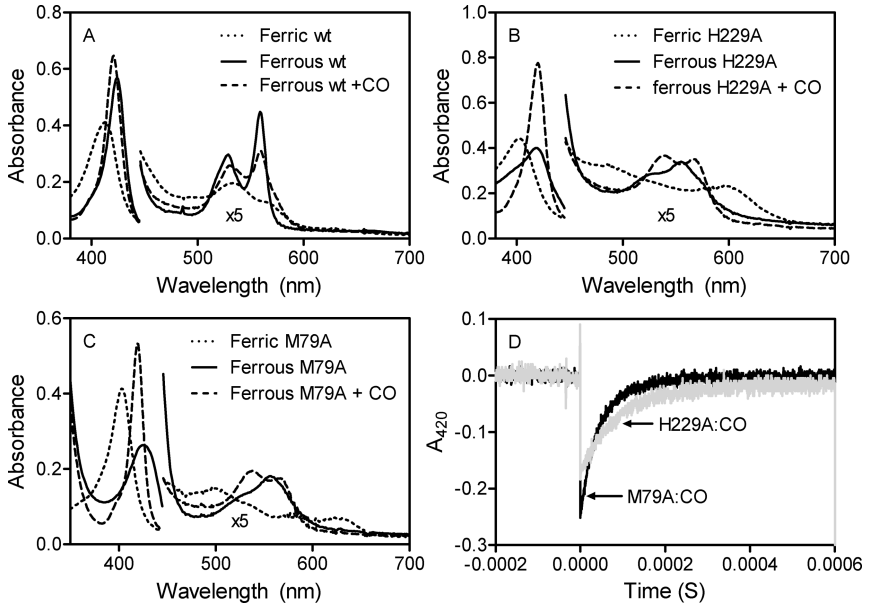

FIGURE 6: Binding of CO to HtsA proteins. (A−C) Spectra of ferric and ferrous wt (3.3 μM) (A), H229A (3.8 μM) (B), and M79A (2.2 μM) (C) HtsA proteins and their mixtures with 1 mM CO in 100 mM potassium phosphate, pH 7.0. (D) Rapid CO association kinetics after photolysis. A_{420} was measured using 2 μM M79A (black) or H229A (gray) protein and 1 mM CO in potassium phosphate, pH 7.0.

HtsA−CO complex was very small, and formation of the wild-type HtsA−CO complex took more than 10 min (data not shown). These results support the conclusion that the H229 and M79 residues are the axial ligands of the HtsA heme iron.

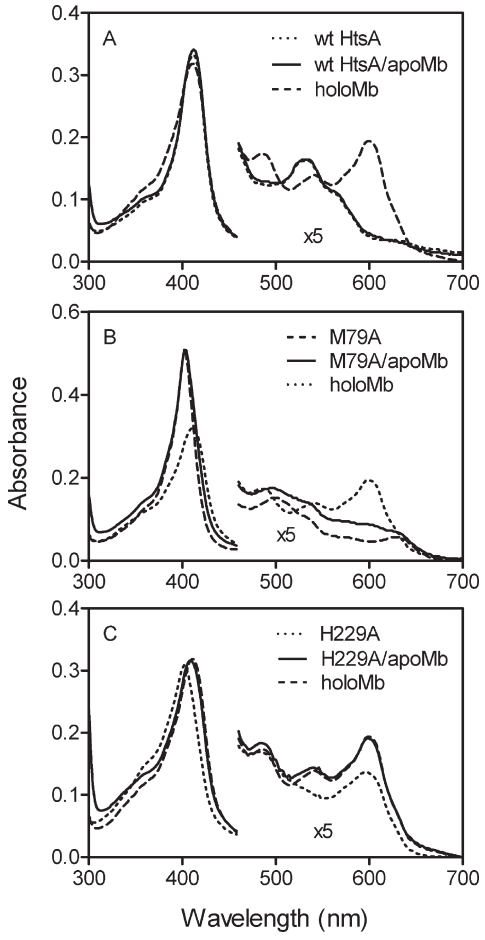

FIGURE 7: Reactions of H64Y/V68F apomyoglobin with the holoHtsA proteins demonstrating the relative heme affinities of HtsA proteins. Presented are the absorption spectra of the mixtures of 50 μM H64Y/V68F apoMb with 2.5 μM wt (A), M79A (B), and H229A (C) holoHtsA after 6 h incubation. The spectra of holo-Mb and corresponding holoHtsA protein are included for comparison.

Relative Affinities of wt and Mutant HtsA for Hemin. Affinities of hemoproteins for heme can be estimated by measuring the rates of heme association to apoproteins and dissociation from holoproteins. This method cannot be used to determine the affinity of the HtsA proteins because of their incomplete or no heme loss to H64Y/V68F apomyoglobin, the heme scavenger in measurement of heme dissociation. Thus, we could only estimate the relative heme affinity of the HtsA proteins based on their reactions with H64Y/V68F apomyoglobin. According to the spectra of 20:1 apoMb:holoHtsA mixtures (Figure 7), wild-type, M79A, and H229A HtsA proteins lost little, a small fraction, and most of their heme, respectively, to H64Y/V68F apomyoglobin, indicating an order of heme affinity for these proteins of wt > M79A > H229A. These results indicate that H229 is more important than M79 for the affinity of HtsA for heme, though the M79 ligand also contributes to the heme affinity.

DISCUSSION

Site-directed mutagenesis and spectroscopic analyses have been used to identify the axial ligand residues of the HtsA heme iron and to characterize the axial mutants of HtsA. The H229 and M79 residues were found to be the heme axial ligands in HtsA. The ferrous heme irons of H229A and M79A HtsA mutant proteins are pentacoordinate and in high-spin state and so is the heme iron of ferric H229A HtsA, whereas the ferric M79A heme iron appears to have hexacoordination with water and histidine axial ligands. The H229 side of the HtsA heme is more sterically limited and more important to the heme affinity than the M79 side of the HtsA heme. These findings will be important in rationally designing experiments to elucidate the molecular mechanism and structural basis for the rapid, direct heme transfer from Shp to HtsA. In addition, this study provides spectroscopic features of a protein with methionine-ligated, pentacoordinate heme iron.

A previous spectroscopic analysis has unveiled that the hexacoordinate HtsA heme iron is ligated to histidine and methionine residues (*18*). The identities of these axial ligands have now been experimentally identified as the M79 and H229 residues in this study. This conclusion is first supported by the effects of alanine replacements of the HtsA histidine and methionine residues on UV−vis spectra of HtsA. The absorption spectrum of ferric H229A HtsA is similar to the spectra of ferric H102M and R98C/H102M cytochrome b_{562} mutants, which has a methionine axial ligand (*32*). This similarity supports that the H229A replacement results in a mutant protein with a methionine ligand. Conversely, the absorption spectrum of M79A is similar to that of aquometHb whose heme iron has a hexacoordination with water and histidine axial ligands (*22*), implying that the M79A replacement results in a mutant protein with a histidine axial ligand. Furthermore, both H229A and M79A at the ferrous state lose the features of hexacoordinate, low-spin heme iron with two strong ligands of wild-type HtsA, lacking the dominant α band in the absorption spectra, a feature possessed by deoxyHb whose ferrous heme iron is pentacoordinate. In addition, ferrous H229A and M79A, but not wild-type HtsA, rapidly form complexes with CO, which have absorption spectra similar with that of the HbCO complex.

MCD spectroscopy is particularly useful for providing information on coordination ligands and numbers of unknown hemoproteins by comparing their MCD spectra with those of hemoproteins with known heme binding properties (*33*, *34*). The MCD spectrum of ferrous M79A HtsA resembles the MCD spectrum of ferrous horseradish peroxidase (*25*), which is known to contain pentacoordinate heme iron with a histidine ligand (*26*), supporting that the ferrous M79A heme iron has a pentacoordination with a His axial ligand. The MCD spectrum of ferric M79A HtsA is similar to the MCD spectra of native metmyoglobin and a benzohydroxamic acid adduct of ferric horseradish peroxidase, which are high spin and have hexacoordination with water and histidine ligands (*27*), further supporting that the sixth coordination site of the heme iron in oxidized M79A HtsA is occupied by a water molecule. These MCD features of M79A HtsA further support the conclusion that the methionine 79 and histidine 229 are the axial ligands in HtsA. The RR spectrum of M79A supports this conclusion.

There had been no firmly established examples for hemoproteins with Met-ligated pentacoordination of heme iron. The heme iron of a H102M mutant of cytochrome b_{562} ligates to a methionine residue, and its other axial ligand cannot be identified (*32*); however, whether the heme iron in this protein is pentacoordinate has not been established. The RR analysis of H229A HtsA indicates that the heme iron in oxidized H229A HtsA is pentacoordinate. This conclusion on the coordination of the H229A heme iron is supported by the observations that H229A HtsA has a blue shift and smaller extinction coefficient for the Soret absorption peak compared with those of the

wild-type protein and small intensities of the MCD peaks in the Soret band, like those of known proteins with pentacoordinate heme iron, such as horseradish peroxidase, Aplysia myoglobin, cyanogen bromide-modified myoglobin, and H64L and H64V myoglobin (*27*, *28*). The axial ligand of the pentacoordinate heme iron in H229A HtsA is apparently methionine since the UV−vis spectrum of H229A HtsA is similar with that of the cytochrome b_{562} H102M mutant (*32*) but not with those of the pentacoordinate heme iron with a His axial ligand. In summary, ferric H229A HtsA has a pentacoordinate, methionine-ligated heme iron with novel MCD and UV−vis spectral features (Table 1), including particularly an absorption peak at ~600 nm.

Exogenous imidazole can readily ligate to the heme iron of M79A HtsA but not to H229A HtsA, indicating that the coordination site is sterically less accessible on the histidine 229 side of the bound heme in HtsA than the other side. This assertion is further supported by the observations that cyanide replaces M79 but not H229 in the wild-type HtsA protein, that the binding of cyanide to H229A HtsA is weaker than the cyanide binding to M79A HtsA, and that the CO binding to ferrous H229A HtsA is slower than the CO binding to ferrous M79A. Furthermore, H229 is more important than M79 for the affinity of HtsA for heme. These observations strongly suggest that the M79 side of the heme pocket in HtsA is more exposed to the solvent than the H229 site.

It is difficult to measure the heme affinity of hemoproteins with extremely high affinity. We have estimated the affinities of Shp and HtsA for heme by measuring rates of heme association to apoShp and apoHtsA and dissociation from their holo form (*8*). There was an error in the analysis for HtsA in which we mistakenly analyzed the partial reaction of holoHtsA with H64Y/V68F apoMb as a complete reaction (thus, the reported K_d for heme binding to HtsA in ref *8* was just a lower estimation). Although actual affinities cannot be determined in such cases, H64Y/V68F apomyoglobin is still valuable to estimate relative affinities of hemoproteins. The heme association constant for H64Y/V68F apomyoglobin is estimated to be 1×10^{12} M^{-1} at pH 7.0 based on a bimolecular rate constant of 1×10^{8} M^{-1} s^{-1} for hemin association to H64Y/V68F apomyoglobin (*35*) and a dissociation rate constant of 1.1×10^{-5} s^{-1} for this double mutant (*21*). Based on the spectra of their reaction mixtures with H64Y/V68F apomyoglobin, wild-type, M79A, and H229A HtsA proteins appear to have association constants for heme binding of $> 10^{12}$ M^{-1}.

The M79 and H229 axial residues of HtsA are corresponding to the M78 and H229 axial ligands of *S. aureus* IsdE (*16*, *36*). IsdE is also the lipoprotein component of the ABC transporter and shares 39% amino acid sequence identity with HtsA. HtsA directly and rapidly acquires heme from the surface protein Shp (*8*, *37*), and IsdE directly but slowly extracts heme from the surface protein IsdC (*10*, *11*). The H229 side of the IsdE heme pocket is also more buried than the M78 side (*16*). However, there are some differences between these two proteins. Prepared IsdE is a mixture of ferric and ferrous forms (*36*), whereas purified HtsA is in the ferric form. H229A IsdE is in a low-spin hexacoordinate complex, which was proposed to be a NO complex, and cannot interact with cyanide (*36*). H229A HtsA is in a high-spin state and can form a low-spin complex with cyanide. These differences might be due to the different preparation methods but more likely imply difference in heme binding in these proteins. Such implication is supported by an observation that IsdE cannot acquire heme from Shp nor does HtsA extract heme from IsdC (B. Lei, unpublished data).

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