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Heme-bound SiaA from *Streptococcus pyogenes*: Effects of Mutations and Oxidation State on Protein Stability

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Abstract

The protein SiaA (HtsA) is part of a heme uptake pathway in *Streptococcus pyogenes*. In this report, we present the heme binding of the alanine mutants of the axial histidine (H229A) and methionine (M79A) ligands, as well as a lysine (K61A) and cysteine (C58A) located near the heme propionates (based on homology modeling) and a control mutant (C47A). pH titrations gave pK_a values ranging from 9.0 to 9.5, close to the value of 9.7 for WT SiaA. Resonance Raman spectra of the mutants suggested that the ferric heme environment may be distinct from the wild-type; spectra of the ferrous states were similar. The midpoint reduction potential of the K61A mutant was determined by spectroelectrochemical titration to be 61 ± 3 mV vs. SHE, similar to the wild-type protein (68 ± 3 mV). The addition of guanidine hydrochloride showed two processes for protein denaturation, consistent with heme loss from protein forms differing by the orientation of the heme in the binding pocket (the half-life for the slower process was one to three days). The ease of protein unfolding was related to the strength of interaction of the residues with the heme. We hypothesize that kinetically facile but only partial unfolding, followed by a very slow approach to the completely unfolded state, may be a fundamental attribute of heme trafficking proteins. Small motions to release/transfer the heme accompanied by resistance to extensive unfolding may preserve the three dimensional form of the protein for further uptake and release.

Keywords

Streptococcus pyogenes; heme; uptake; resonance Raman; denaturation; guanidine; kinetics; unfolding

1. Introduction

Iron is a key nutrient for many bacteria [1]. Iron(III), however, has very low aqueous solubility [2,3], making strategies that do not involve direct uptake of uncomplexed iron

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imperative. In the human body, about 95% of the iron occurs in the heme cofactor [4,5], which serves as a significant source of iron for bacterial pathogens. The most common protein source of heme iron in human bacterial infections is methemoglobin [6].

Heme uptake is often key in the virulence of pathogenic bacteria [7]. Gram-positive and Gram-negative bacteria have evolved similar strategies for acquiring heme [1,8–17]. All pathways studied to date involve a series of proteins that transfer the heme from the exterior milieu into the cell interior. Recent reviews have focused on the structures of heme transport proteins [5,18,19] and pathways in specific bacteria including *Bacillus anthracis* [20] and *Staphylococcus aureus* [21–24].

A large number of bacteria have been studied to determine their method of obtaining heme; some acquire heme from their environment, others have a biosynthetic pathway to produce the heme, and some use both approaches [25]. Using bioinformatics techniques, Cavallaro et al. found that approximately 5% of the species rely only on heme uptake, 30% use biosynthesis only, 50% use both pathways, and 15% use neither. Those which use only heme uptake are of particular interest because absent a biosynthetic pathway, they may be susceptible to iron starvation via interruptions in external heme uptake. Interference with heme uptake might reduce the virulence of infections with these organisms. Cavallaro et al. have noted that heme uptake seems to be related to pathogenicity in Gram-positive bacteria, with approximately 80% of bacteria that acquire heme being pathogenic [25].

Streptococcus pyogenes is one of the few species of bacteria that can only obtain heme from its environment. *S. pyogenes*, also known as Group A streptococcus (GAS), is a pathogenic Gram-positive bacterium that causes a variety of infections [26,27]. Iron is an essential nutrient for *S. pyogenes* and a number of heme-containing sources can support *in vitro* growth of this organism, including hemoglobin, the haptoglobin-hemoglobin complex, myoglobin, heme-albumin, and catalase [28]. The ability to obtain iron has been shown to affect the virulence of this pathogen as observed in mutants of *S. pyogenes* which are defective in heme uptake [29]; mutations resulted in attenuated virulence in animal models. *S. pyogenes* is increasingly resistant to macrolide antibiotics [30–33], potentially posing significant risks for infected populations.

In *S. pyogenes*, one pathway for heme import employs a dedicated ATP binding cassette (Figure 1). This cassette has been termed the streptococcal iron acquisition or SiaABC system [34] and is also known as HtsABC (heme transport system) [35]. In this ABC transporter, SiaA is the membrane-anchored heme binding protein that acquires heme and transfers it to SiaB which in turn carries the heme across the lipid bilayer. In this process, energy is provided from ATP hydrolysis by SiaC, which is located on the inner side of the membrane. The SiaABC heme transport system is part of a conserved ten-gene cluster [34]. The two genes upstream express Shr [34,36] and Shp [37,38]. Shr receives heme from hemoglobin [39,40] and transfers it to Shp [36]. Shp, which has two axial methionine ligands [37], transfers heme to SiaA [41] with rate constants that are similar in the oxidized and reduced forms [42–44]. Our previous biophysical studies on wild-type (WT) SiaA showed the heme was six-coordinate (6c) and low-spin (LS) in both the ferric and ferrous oxidation states of the protein with methionine and histidine as the axial ligands [45]; further

spectroscopic analyses have confirmed these findings [46]. Homology modeling (Figure 2), with IsdE from *Staphylococcus aureus* [48] as the closest homologous protein, indicated that the specific axial ligand residues were likely to be M79 and H229; site-directed mutagenesis studies have verified this [46,47].

Elucidation of the factors controlling heme binding and release steps along heme acquisition pathways are benefiting from biophysical studies of the proteins. The main controlling factors emerging from this work are the nature of the axial ligand(s), electrostatic interactions between the protein and the heme, through its propionates and the iron center, and hydrophobic interactions between the protein and the porphyrin face [5,49–53]. Herein, we describe the factors affecting the stabilities of heme-bound states of SiaA and selected mutants. We report results on two new mutants, C58A and K61A, as well as a control mutant, C47A, predicted to be at some distance from the heme. Homology modeling suggests that C58 is near the heme propionates and K61 is close to the propionate that extends from the heme binding pocket. In addition, we expand on recent studies of M79A and H229A [46,47]. The reduction potentials of the mutants of SiaA have been determined by spectroelectrochemical titration and compared with that of WT SiaA. Structural aspects of the heme pocket, states of the bound heme, and heme protein interactions have been probed by resonance Raman (rR) spectroscopy [16,54–56].

Unfolding studies, using guanidine hydrochloride (GdnHCl) as a denaturant, have been used here to probe the influence of residues near the heme on the relative stabilities of the heme-bound states of WT SiaA and its aforementioned mutants. Unfolding studies of heme protein mutants have been used to gain information on the contributions of specific residues to heme binding in other *b*-type heme proteins. For example, the roles of various residues in myoglobin were probed by unfolding specific mutants [57]. Similar studies have also been performed on cytochrome *b*₅₆₂ [58,59] and cytochrome *b*₅ [60,61]. These types of studies provide insight into the overall stability of the protein in which specific heme pocket residues, predicted to interact with the heme, are probed. In the case of heme binding and transport proteins, we anticipate that disrupting these interactions by unfolding can lead to a better understanding of the important residues involved in the heme uptake and release mechanism of the protein.

2. Experimental

2.1. Homology modeling

The homology model of SiaA was built by using I-TASSER, a secondary structure prediction program [62]. IsdE from *Staphylococcus aureus* [48] was the closest homologous protein (PSI-BLAST results for SiaA show 45% identity to IsdE with 69% positives); the root mean square difference (RMSD) between the model and IsdE was 1.35 Å. The model was visualized using PyMOL [63].

2.2. Materials

E. coli strain Top10 competent cells, ShuAf, ShuAr primers and Top10/pSiaA-His cells were made as described previously [45]. The QuikChange II Site-Directed Mutagenesis Kit was

from Stratagene (La Jolla, CA). The Plasmid Mini Kit, Taq PCR Master Mix Kit, and QIAquick® Gel Extraction Kit were from QIAGEN (Valencia, CA). Oligonucleotides for site-directed mutagenesis were synthesized by Invitrogen (Carlsbad, CA). L-arabinose was manufactured by Acros Organic (Gell, Belgium).

2.3. Preparation of plasmids

Site-directed mutagenesis was used to construct recombinant SiaA proteins with C47A, C58A, K61A, M79A or H229A amino acid substitutions. A QuikChange II kit was used to prepare the mutants essentially according to the manufacturer's instructions using the pSiaA-His plasmid as a template [34]. The forward and reverse primers (underlined letters indicate the mismatches) for each mutant are shown in Table 1. The constructed plasmids were introduced into *E. coli* Top10 competent cells by chemical transformation and clones were selected on Luria-Bertani (LB) plates containing 100 µg/mL ampicillin. The resulting plasmids express the corresponding SiaA mutant as an N-terminal fusion to His-Xpress epitope from the arabinose-regulated promoter, P_{BAD} as described previously [34]. Taq PCR Master Mix Kit was used to amplify SiaA DNA segments, and the sequence of the wild type and mutant proteins was determined by Applied Biosystems model ABI 377 DNA sequencer at the DNA Core Facility at Georgia State University. Sequencing confirmed the presence of the mutant gene in the correct orientation in each of the plasmids.

2.4. Expression and purification of mutants

The proteins were expressed and purified from the appropriate plasmids as previously described [45] with small modifications. A representative description is given for the C58A mutant. C58A expression was induced with 0.02% arabinose for 4 h. The cell pellet was ruptured with two cycles of French press in 45 mL of buffer containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.1% Triton X-100, 10% v/v glycerol, and four tablets of protease inhibitor (Roche Complete Mini, EDTA-free). The solution was centrifuged for 20 min at 8,000 *g*, and the supernatant was syringe-filtered with a 0.45 µm filter (surfactant-free cellulose acetate membrane, Nalgene). All of the following purification steps were conducted at 4 °C using a GE Healthcare ÄKTA fast protein liquid chromatography instrument (FPLC, Amersham BioSciences), and all buffer solutions were pH 7.4 unless specified otherwise. The sample was loaded onto a GE Healthcare HisTrap™ HP column (5 mL, Amersham BioSciences) equilibrated with binding buffer (20 mM sodium phosphate, 500 mM NaCl, and 20 mM imidazole). Unbound material was washed out with 5 column volumes (CV) of binding buffer. C58A was eluted with buffer containing 20 mM sodium phosphate, 500 mM NaCl, and 500 mM imidazole via a 20 CV linear gradient. The purities of the fractions were evaluated using sodium dodecyl sulfate polyacrylamide gel electrophoresis. Fractions containing the individual SiaA mutants were combined, and imidazole and salts were removed by centrifugal filtration (Amicon Ultra-15, 5 kDa molecular weight cut-off, Millipore) using a buffer of 50 mM Tris-HCl, pH 7.0. The protocols for the other mutants were similar with the exceptions that M79A was purified using a HiTrap Q column (GE Healthcare) equilibrated with a buffer of 20 mM Tris-HCl and 10% v/v glycerol (designated as Buffer A). Unbound material was washed out with 8 CV of Buffer A. M79A was eluted with 10 CV of Buffer A containing 1 M NaCl via a linear gradient (0 – 100% NaCl).

2.5. Heme loading

Purified SiaA and mutants were a mixture of holo and apo forms. The holo protein was isolated in the ferric state. The extinction coefficients and heme loading of SiaA mutants were measured using the pyridine hemochrome assay [64]. The extinction coefficients are as follows: C47A $\epsilon_{414} = 145.3 \text{ mM}^{-1} \text{ cm}^{-1}$; K61A $\epsilon_{406} = 135.9 \text{ mM}^{-1} \text{ cm}^{-1}$. Total protein concentration was determined using the Bradford assay (Thermo Fisher Scientific, Inc.) with bovine serum albumin as a standard. The WT extinction coefficient was previously reported [35,46]. M79A and H229A were also previously reported [46,65].

2.6. SiaA heme extraction and refolding

Apo-SiaA was prepared using the Teale method [66]. A solution of SiaA (10 μM) in PBS buffer, pH 7.4, was reduced to pH 2 in a drop-wise fashion using 3 M HCl. The acidic SiaA solution was mixed with cold 2-butanone and placed on ice for 15 min. The heme layer was discarded.

To aid in protein refolding, urea was added to the apoprotein solution to give a final concentration of 8 M urea [67]. The solution sat at room temperature for 2 h. The apo-SiaA solution was dialyzed at 4°C against 4 M urea (in PBS) for 24 h, against 2 M urea (in PBS) for 2 h, and against 10 mM potassium phosphate, pH 7.0, for 24 h. The apoprotein solution was centrifuged at 4°C for 15 min at 5,000 *g* to remove any precipitate. The concentration of apo-SiaA was determined using the ExPASy $\epsilon_{280} = 37,360 \text{ M}^{-1} \text{ cm}^{-1}$ [68].

2.7. UV-visible spectroscopy

UV-visible spectra were recorded on a Varian 50 Bio spectrophotometer with a thermostated cell compartment. A TC125 temperature control unit (Quantum Northwest, Spokane, WA) was used to set the temperature of the cuvette compartment to 25°C. Quartz black-masked Suprasil cuvettes (Spectrocell, Inc.) with a 1 cm path length were used.

2.8. Circular dichroism spectroscopy

Circular dichroism (CD) spectra were recorded using a Jasco J-810 Spectropolarimeter. Quartz Suprasil cuvettes (Fisherbrand, Inc.) with a 1 mm path length were used. Holo-SiaA (10 μM) and apo-SiaA (10 μM) were recorded in 10 mM potassium phosphate buffer, pH 7.0 in a spectral window of 190 to 260 nm. The final scan represents an average of ten scans.

2.9. Resonance Raman spectroscopy

Resonance Raman spectra were recorded at ambient temperature using a 0.67-m spectrograph equipped with a 2400 g/mm holographic grating and a LN₂ cooled CCD detector. Spectra were excited with 413.1 nm emission from a Kr⁺ laser or 441.6 nm emission from a HeCd laser. The beam was focused to a line at the sample and scattered light was collected in the 135° backscattering geometry using *f*1 optics and a holographic notch filter to remove Rayleigh scattered light. Samples were contained in 5 mm NMR tubes and spun at ~20 Hz to minimize the risk of laser-induced damage to the samples. Sample integrity after laser irradiation during the rR experiment was verified by comparing UV-visible absorbance spectra with those recorded before laser exposure. Spectra were

calibrated against the Raman shifts of pure toluene and CH₂Br₂ and are reproducible to one wavenumber or less.

2.10. Denaturation studies

The following proteins were monitored with UV-visible spectroscopy at the indicated wavelengths and concentrations upon the addition of GdnHCl: C47A (414 nm, 7 μM), C58A (409 nm, 7 μM), K61A (412 nm, 4 μM), M79A (402 nm, 9 μM), and H229A (403 nm, 7 μM). The refractive index of GdnHCl was used to verify the concentration of the stock GdnHCl solutions (50 mM Tris-Cl, pH 7.0) [69]. GdnHCl was titrated into each protein until the unfolding reached equilibrium which was considered to have been established when the absorbance did not change by more than 0.002 from the previous measurement.

The unfolding curves were analyzed using Equation 1 [69]:

$$y = [(A_F + m_F[D]) + (A_U + m_U[D]) \exp[m([D] - [D]_{1/2})/RT]] / [1 + \exp[m([D] - [D]_{1/2})/RT]]$$

[1]

where y is the absorbance at any point along the fitted denaturation curve, A_F is the absorbance of the folded state, A_U is the absorbance of the unfolded state, m is the slope at the midpoint, and also the dependence of the free energy of unfolding on the denaturant concentration, m_F is the slope of the folded state, m_U is the slope of the unfolded state, $[D]$ is the concentration of GdnHCl, $[D]_{1/2}$ is the concentration of GdnHCl at the midpoint of the unfolding curve, R is the gas constant, and T is the temperature (Kelvin).

In another set of experiments, samples of WT SiaA (5 μM) in 50 mM Tris-Cl, pH 7.0 were incubated for 29 h at room temperature in various concentrations of GdnHCl (5.93 M stock solution). Each sample was monitored by UV-visible spectroscopy before loading onto a washed PD-10 desalting column (GE Healthcare). The column was equilibrated with 25 mL of 50 mM Tris-Cl, pH 7.0. Each SiaA sample (2.5 mL) was allowed to enter the column bed before eluting with 3.5 mL of 50 mM Tris-Cl, pH 7.0. Fractions of 500 μL were collected. The protein eluted in the third fraction. The Soret:280 nm ratios of the desalted protein samples were fit to an unfolding curve. Control experiments were also run on horse heart myoglobin (Sigma-Aldrich).

2.11. Studies of unfolding rates

Solutions (5 μM) in 50 mM Tris-Cl, pH 7.0 of WT, C47A, C58A, and K61A were monitored by optical absorbance at the Soret as a function of time for 24 h at the predetermined $D_{1/2}$ concentrations. The unfolding was fit to the sum of two exponential processes using Equation 2:

$$A_t = A_f \exp^{-k_f t} + A_s \exp^{-k_s t} \quad [2]$$

where A_T is the total absorbance of the solution, A_F is the relative absorbance of the fast phase, k_F is the rate of the unfolding for the fast phase, A_S is the relative absorbance of the slow phase, k_S is the rate of the unfolding for the slow phase, and t is time. Attempts to fit the data to a model with an intermediate were not successful.

2.12. Spectrophotometric pH titrations

The pH titrations were performed by the addition of 1.0 M NaOH aliquots to the protein solutions (5 μ M). A buffer of CAPS (20 mM), CHES (20 mM), and Tris-Cl (20 mM) was utilized throughout the titration. Changes in absorbance in the Soret region were analyzed with Kaleidagraph using Equation 3 [70]:

$$A_{obs} = (A_A \cdot 10^{(pK_a - pH)} + A_B) / [1 + 10^{(pK_a - pH)}] \quad [3]$$

where A_{obs} is the experimental absorbance observed, A_A is the absorbance of the acidic form, and A_B is the absorbance of the basic form.

2.13. Reduction potential determination

Midpoint potentials were determined by spectroelectrochemical titration of the protein in a homemade cell using a Pt working electrode, a Ag|AgCl reference electrode, and a standard pH meter in its mV mode. The absorbance contributions of all non-heme chromophores had been subtracted from each spectrum for the following experiments. The cell was loaded with ~6 mL of solution at holoprotein concentrations such that absorbance at the Soret maximum was between 0.2 and 0.6. The solution was exhaustively equilibrated under an atmosphere of H₂O-saturated N₂ and the absorbance spectrum of the ferric protein was recorded. The heme was then completely reduced by anaerobic addition of 20 mM aqueous S₂O₄²⁻ and the absorbance spectrum of the ferrous heme was recorded. Electron transfer dye cocktail was added to the protein solution to reach a final concentration of 10 μ M in each dye. The resulting solution was titrated with S₂O₄²⁻ until the UV-visible spectrum of the heme no longer changed and the dyes were completely reduced. Cell potential was monitored using a strip chart recorder to make it easily apparent when equilibrium had been established, at which point the absorbance spectrum was recorded between 350 and 800 nm. The reduced solution was then titrated by anaerobic additions of 40 mM [Fe(CN)₆]³⁻ to oxidize the dyes and heme. Cell potentials and spectra were recorded after each titrant addition and equilibration. Finally, the solution was titrated again with 20 mM S₂O₄²⁻. After the second cathodic titration, the reference electrode was calibrated against the quinhydrone half cell so that cell potentials could be corrected to the SHE reference. Absorbance at the Soret maximum for the ferrous heme was plotted vs. cell potential and fit to the Nernstian relationship in Equation 4 for both the cathodic and anodic titrations. The resulting midpoint potentials were within 10 mV of one another.

$$A_{434} = [A_{\infty} + A_0 \exp([E - E_m]/0.0257 \text{ V})] / [1 + \exp([E - E_m]/0.0257 \text{ V})] \quad [4]$$

3. Results

3.1. Spectroscopic studies

WT SiaA and the C47A, C58A, and K61A mutants were purified as red colored proteins (Figure 3). The Soret band of the WT SiaA was observed at 413 nm, consistent with previous reports [41,45]. UV-visible absorbance spectra of the C47A and K61A mutants were very similar. The Soret maximum of the C58A mutant occurred at 409 nm. All three mutants and WT SiaA showed α - and β -bands near 532 and 567 nm.

In contrast to these three mutants, the purified mutants having axial ligand mutations (M79A and H229A) were pale yellow in color, suggesting low heme loading. Additionally, M79A and H229A had much larger spectral shifts than the other mutants, with Soret bands at 402 and 403 nm, respectively, similar to those reported in the literature [47].

CD spectroscopy was used to probe the overall fold of holo-SiaA versus apo-SiaA (Figure 4). Both proteins contained bands around 190, 210, and 222 nm, indicative of α -helical content. The band at 210 nm was more pronounced for the apoprotein compared to the holoprotein. This indicates that the overall fold of the protein is altered upon heme removal from the protein [47].

3.2. Guanidine-induced denaturation of WT SiaA and mutants

Preliminary studies indicated that unfolding of SiaA was very slow. Therefore, unfolding was followed as a function of time for WT, C47A, C58A, and K61A. Fast and slow unfolding phases were observed for all four proteins. Figure 5 shows the data for WT at the GdnHCl midpoint concentration; plots of the data for the mutants are in the Supplemental Information (Figures S1 – S3). For WT, fitting the data to the sum of two exponential processes gave the rate of the fast process (15%) as $0.32 \pm 0.02 \text{ h}^{-1}$ and the rate of the slower process (85%) as $0.026 \pm 0.001 \text{ h}^{-1}$. The percentages of the faster process ranged from 10 – 30% (Table 2). Faster rate constants for the first phase correlated with smaller abundance of this fast phase and slower rate constants for the second phase. C58A showed the largest difference between the two phases, with half-times of 1.5 h for the fast phase (12%) and 46 h for the slow phase (88%).

In the measurements of $D_{1/2}$, waiting the times necessary to reach equilibrium resulted in anomalies due to changes in protein concentration. Therefore, a single cuvette titration technique was utilized [71–75] in which increasing concentrations of GdnHCl were added to a single cuvette. This technique can give the approximate relative ease of denaturation in a series. Fitting the C58A data to a two-state model gave a midpoint denaturant concentration of $2.4 \pm 0.1 \text{ M}$ GdnHCl. The C47A, K61A and M79A proteins were also investigated with the same technique and gave $D_{1/2}$ values of $2.9 \pm 0.1 \text{ M}$, $2.5 \pm 0.1 \text{ M}$, and $1.5 \pm 0.1 \text{ M}$, respectively (Figure 6). WT SiaA had a $D_{1/2}$ of $3.1 \pm 0.1 \text{ M}$ as previously reported [45].

The H229A mutant spectrum did not show clear α , β -bands during the titration. For this protein, equilibrium was reached in less than 20 min after each GdnHCl addition. This was a substantially shorter amount of time than that required for WT SiaA to reach equilibrium (which in the transition region was well over one hour). This is consistent with weak binding

of the heme to the protein. The Soret absorbance decreased and shifted toward the red as guanidine was added up to 1.5 M GdnHCl. Fitting the data from 0 to 1.5 M GdnHCl gave a $D_{1/2}$ of approximately 1.1 M.

The unfolding of WT SiaA was also investigated by incubating samples with various concentrations of GdnHCl and then passing the solution through a desalting column to remove loosely-bound heme (Figure S4) [73]. Fitting to a two-state model gave a $D_{1/2}$ of 2.6 ± 0.1 M, somewhat lower than the 3.1 ± 0.1 M in the experiment above, consistent with this not being an equilibrium measurement.

It should be noted that unfolding of WT SiaA and mutants was performed on samples that contained both holo and apo forms of the protein. Freshly prepared apo-SiaA largely precipitated by 24 h after isolation. Thus, it was presumably decreasing in concentration over the long time of the unfolding experiments. However, the effects of protein concentration on unfolding are often small and depend on the exact mechanism of the unfolding process [76].

3.3. Spectrophotometric pH titrations

For the three mutants not involving the heme ligands (C47A, C58A, and K61A), titrations with aliquots of 1 M NaOH gave a Soret absorbance that decreased as the pH increased (representative data from C47A are shown in Figure 7). The transitions were isosbestic for $\text{pH} < 11$. The absorbance at 280 nm also increased above $\text{pH} 11$. This is consistent with high pH leading to unfolding of the protein which in turn results in exposure and deprotonation of some of the 14 tyrosines in the sequence (the extinction coefficient of tyrosinate ($2500 \text{ M}^{-1} \text{ cm}^{-1}$ at 295 nm) is higher than that of tyrosine ($1400 \text{ M}^{-1} \text{ cm}^{-1}$ at 275–280 nm) [77]). Data from $\text{pH} 7$ to approximately $\text{pH} 10.9$ were fit using a two state model to give pK_a values of 9.22 ± 0.03 , 9.04 ± 0.03 and 9.45 ± 0.05 for C47A, C58A, and K61A, respectively. The pK_a of WT SiaA is 9.7 ± 0.1 [45].

3.4. Resonance Raman spectra of ferric and ferrous SiaA mutants

The spectra of ferrous and ferric C58A SiaA are shown in Figure S5 in the Supplementary Material. In contrast to WT SiaA [45], this mutant is susceptible to photo-induced reduction during spectral acquisition. Consequently, the spectra were recorded with low laser power (3.7 mW), and exhibit only a modest signal-to-noise ratio. The spectra are characteristic of hexacoordinate, low-spin (6cLS) hemes and reminiscent of those previously reported for WT SiaA. Thus the spectral signatures for ferric and ferrous C58A SiaA are consistent with the heme conformations and axial ligand set being the same as those for the WT protein. However, the shifted Soret maximum (6 nm to the blue of the WT SiaA) as well as the photolability of the ferric state suggest that its heme environment may be distinct from WT SiaA. By contrast, the spectral signatures and behaviors of the ferrous proteins are rather similar.

Because the K61A SiaA was not photolabile, it was possible to record higher quality rR spectra, as shown in Figure 8. Like the WT protein, it also exhibits rR fingerprints characteristic of 6cLS ferric and ferrous heme proteins. In fact, the rR spectra of WT SiaA and K61A SiaA are nearly identical for both the ferric and ferrous forms. Although

homology modeling suggests that K61 is able to interact with one of the heme propionate groups in the WT protein, the loss of that interaction is not apparent in the rR spectra of K61A SiaA. Specifically, comparison of the WT and K61A rR spectra reveal that the propionate bending bands are virtually identical in the ferric and ferrous forms. Thus, loss of the electrostatic interaction between K61 and the heme periphery does not drive significant changes in propionate conformation. This result is consistent with the insensitivity of the reduction potential (§ 3.5) to the mutation of K61.

3.5. Spectroelectrochemical titrations

In previous work on WT SiaA, we reported an irreversible midpoint potential that lies between 64 and 83 mV vs SHE [45]. Reanalysis of those data revealed more than one Fe(III)|Fe(II) couple, as shown in Figure S6. After oxidation with $[\text{Fe}(\text{CN})_6]^{3-}$, the reductive titration with $\text{S}_2\text{O}_4^{2-}$ showed a single midpoint potential of 68 ± 3 mV vs SHE. Following complete reduction with $\text{S}_2\text{O}_4^{2-}$, the reverse (oxidative) titration with $[\text{Fe}(\text{CN})_6]^{3-}$ revealed two potentials, one at 15 ± 5 mV, accounting for ~60% of the heme, and the other at 72 ± 10 mV, accounting for ~40% (see Figure S6 for further details). The 68 and 72 mV potentials are indistinguishable with these uncertainties. This finding is consistent with heme oxidation triggering a redox-coupled change in structure or conformation that (a) changes the Fe(III)|Fe(II) potential and (b) is kinetically sluggish to reverse, even after reoxidation of the heme.

Spectroelectrochemical titration of K61A revealed a reversible potential of 61 ± 3 mV. Absorbance spectra and the absorbance at the ferrous Soret maximum are shown in Figure 9. The forward and reverse titrations are superimposable and well modeled by the Nernstian expression shown in Equation 4. Consistent with the rR spectra of WT and K61A, these reduction potentials indicate that the replacement of K61 (which homology modeling suggests is near a heme propionate) with the small hydrophobic methyl group of alanine has only a small effect on the relative stabilities of the oxidized and reduced heme states. Even though the change in reduction potential in response to the loss of the positive charge from K61 is only -7 mV, it is in the direction consistent with stabilization of the ferric heme, which is expected upon loss of the cationic side chain from K61.

In contrast, the reduction of the C58A mutant was < 0 V vs. SHE, significantly more negative than that of the WT protein and irreversible (Figure S7). Although the irreversibility of the reduction precluded reliable determination of its potential, its negative potential indicates that replacement of cysteine 58 with alanine changes the heme pocket so as to destabilize the ferrous state and/or stabilize the ferric state relative to the WT protein. Given that the spectroscopic behavior of the ferric protein is distinct from that of WT SiaA, it is concluded that the negative reduction potential is likely attributable to destabilization of the ferric heme in C58A SiaA.

4. Discussion

4.1. Guanidinium-induced unfolding

WT SiaA in the ferric form undergoes guanidinium-induced unfolding with a $D_{1/2}$ of 3.1 M [45]. To probe specific heme-protein interactions and their influence on heme binding, we

looked at the unfolding of five mutants: C47A, C58A, K61A, M79A, and H229A. Because homology modeling predicts C47 to be distant from the heme, the C47A mutation was studied as a control. The $D_{1/2}$ value of C47A was 2.9 M, similar to the WT $D_{1/2}$ at 3.1 M.

The K61A mutant was chosen because homology modeling showed the residue to be at the entrance to the pocket on the side of M79, close to the propionic acid that is less buried in the protein. K61 is in a similar position to K62 in IsdE (Figure S8), for which the crystal structure is known [48]. In the IsdE structure, K62 hydrogen bonds to a water molecule, which in turn hydrogen bonds to both propionates. K62 in IsdE is also involved in a complex network of non-bonded interactions wherein K62 forms a salt bridge with E265, which in turn hydrogen bonds to H229 via water. E265 also hydrogen bonds directly with Y61 (which corresponds to Y63 in SiaA). Y61 hydrogen bonds to one of the heme propionates, as does the adjacent S60 (conserved in the SiaA model). In view of this complex hydrogen bonding network, it was expected that mutation of the K61 in SiaA would change the stability of the holoprotein. Indeed this was observed, with the K61A mutant ($D_{1/2}$ of 2.5 M) being less stable than the WT protein ($D_{1/2}$ of 3.1 M). The reduction in stability may also be due to loss of a salt bridge between the positively charged lysine and the negatively charged heme propionate. Heme propionates often form salt bridges with nearby cationic residues, with the most prevalent amino acid being arginine; lysine and histidine are common as well [5,49–53,78]. We note, however, that the resonance Raman data indicate that mutation of K61 to alanine in this protein does not induce measurable changes in the propionate vibrational signatures, although it does affect the overall stability of the holoprotein.

Homology modeling also suggested that C58A is near the heme propionates on the methionine side of the heme. Although there is no expected electrostatic interaction between C58 and the heme, loss of this residue clearly lowers the thermodynamic cost of unfolding this protein. Cysteine 58 is near the H-bonding network involving S60, described above. It is also near a second hydrogen bonding network involving S40, S271 and a water molecule (T40, T271 and H₂O in IsdE). In IsdE, the water molecule in this network hydrogen bonds to one of the heme propionates [48]. Mutation of the cysteine to alanine in SiaA results in a decrease in the $D_{1/2}$ value to 2.4 M, consistent with disruption of these hydrogen-bonding patterns.

The two axial ligand mutants (M79A and H229A) released heme more easily than the other three mutants. The M79A protein was expected to be significantly less stable than WT, because the presumed contribution of the Fe–S_{Met79} bond to the stability of the protein fold is eliminated in this mutant. Consistent with this reasoning, the midpoint of the transition occurred at 1.5 M GdnHCl, indicating the importance of this iron-ligand bond in stabilizing the holo-SiaA fold. Spectroscopy has indicated that the M79A is hexacoordinate, presumably with a water molecule replacing the methionine [46]. Finally, the H229A mutant had the lowest $D_{1/2}$ of approximately 1.1 M, consistent with the expected importance of the H229-heme interaction (the heme is pentacoordinate in this mutant [46]). This order of unfolding is consistent with previous studies using other techniques; Ran et al. found that the extent of heme transfer from holo-SiaA to an apo-myoglobin mutant was H229A > M79A > WT [46]. The acid-induced unfolding showed the same pattern. H229A and M79A both showed single kinetic processes with the former faster than the latter. These were both faster

than the WT protein (considering the slower step of the two step process was for the WT) [47]. Equilibrium dialysis and inductively coupled plasma mass spectrometry (ICP-MS) experiments indicate that the relative order of heme affinity is WT > M79A > H229A [47]; Ran et al. have concluded that all three of these proteins have binding constants of $> 10^{12} \text{ M}^{-1}$ [46,65].

Heme protein unfolding of *b*-type heme proteins can have intermediates in which the heme is bound to a partially unfolded structure. For example, in an early study, it was postulated that ferric hemoglobin unfolds in a way that allows the heme to be released while the protein is unfolding [79]. In contrast, it was proposed that carboxyhemoglobin unfolds completely while the heme moiety stays bound at the active site until its release [79]. Data for horse heart myoglobin unfolding as a function of GdnHCl concentration were interpreted as an initial unfolding ($D_{1/2} = 1.5 \text{ M}$) followed by loss of heme only at high concentrations of denaturant ($> 5 \text{ M}$) [73]. Sperm whale myoglobin unfolding has been fit to a model involving native, intermediate, and unfolded states as well as their heme-bound counterparts [57,76].

4.2. The time-scale of protein unfolding

The kinetic data for WT SiaA and its mutants were well fit by a two-term exponential function with the majority of the protein (~60 – 90%) unfolding with the slower rate constant. This two-phase process may reflect forms of the protein with two different orientations of the heme in the pocket related by a 180° rotation around the α, δ -*meso* axis [80]. The fast and slow phases had half-lives of 2 – 3 and 70 – 90 h, respectively. Although there are extensive studies of guanidinium denaturation of heme proteins, kinetic studies are more limited. For example, bovine microsomal cytochrome *b*₅ has major and minor heme-bound forms that occur in an 8:1 ratio, as shown by NMR [81,82]. Unfolding of this protein had a fast and slow phase, interpreted as arising from the two forms of the protein with different heme orientations, with a half-time of ~ 30 sec for the first process and a second process that was 3–4 times slower at the $D_{1/2}$ of 3 M GdnHCl [60,83]. Horse heart myoglobin shows a similar pattern, with the first process (20%) having a half-time of seconds and the second process (80%) ~10 times slower at the $D_{1/2}$ of 1.6 M GdnHCl [73]. Circular dichroism studies on the horse heart protein were in line with this ratio of isomers. Sperm whale myoglobin has a heme isomer ratio of 15:1 [84], and thus unfolding would not be expected to show two isomers. Olson and co-workers have looked at the unfolding process in detail and proposed a kinetic scheme with one form of the protein, but with one or more intermediates in the unfolding pathway [57,76,85]. Horseradish peroxidase (HRP) exists almost exclusively as a single isomer [80]. HRP has a $t_{1/2}$ of 520 sec at 6.0 M GdnHCl (pH 7) [86,87]; the apparent $D_{1/2}$ is 5.5 M. Soybean peroxidase unfolds approximately 200–300-fold more slowly than horseradish peroxidase [87]. The rates for bovine microsomal cytochrome *b*₅ and horse heart myoglobin differed approximately by a factor of ten. WT SiaA and C47A are similar. These results are consistent with the two unfolding phases of SiaA being attributable to the two heme orientations. The amplitude ratios of the faster and slower processes for SiaA are in the order of C58 > K61 > WT > C47, with the values of $46 > 34 > 12 > 7.3$, respectively. Mutations near the propionates of the heme edge (C58A and K61A) gave the largest ratios and largest percentages of the slow phase.

For SiaA and its mutants, we have observed that the unfolding steps are sensitive to mutations in the heme pocket, where bonded and non-bonded interactions must be made and broken in the course of fairly rapid heme transfer reactions. However, overall unfolding rates as probed by GdnHCl denaturation are very slow, with half-lives for the slower process of one to three days at the $D_{1/2}$. This slow intrinsic heme loss may protect the organism from the deleterious effects of free heme. Heme transfer would be accomplished only by direct transfer to a partner protein, along a facile heme release coordinate for which the kinetic barrier is relatively low. This would allow a change of conformation sufficient to release the heme without the risk of the protein proceeding along a steep, cooperative unfolding pathway that could leave it extensively unfolded and perhaps dysfunctional.

4.3. Spectrophotometric pH titrations

When titrated with base, all of the mutants gave spectra that were isosbestic from pH 7 to ~10.9. The data from each mutant were fit with a two state model to give pK_a values for C47A, C58A, and K61A of 9.22 ± 0.03 , 9.04 ± 0.03 , and 9.45 ± 0.05 , respectively. These are all somewhat lower than the pK_a of WT SiaA (9.7 ± 0.1). We have proposed [45] that this pK_a is due to deprotonation of the axial histidine, which falls in the range of 8 – 11 for heme proteins [88–94].

The largest effect is seen for the C58A mutant. As described above, homology modeling indicates that C58 is near the heme propionates; mutation of this residue to alanine reduces the pK_a by approximately 0.7 units from the WT protein. In IsdE, the corresponding residue is a proline [48]. This P58 is near P77, which is adjacent to the axial methionine. P80 is also very near the axial methionine (PMEP). SiaA has homologous prolines in the sequence near the axial methionine (PMNP). These clusters of prolines may result in rigidity of the protein structure near the heme. This rigidity may allow significant change in the pK_a of the protein upon replacement of C58 with alanine. Even mutation of cysteine 47 has a significant effect on the pK_a , indicating that long range effects of slight changes in protein structure are being transmitted to the heme binding site.

K61 in SiaA aligns with K62 in IsdE. In IsdE, K62 is part of a complex network of hydrogen bonds involving the axial histidine, Y61, E265, two water molecules, and both heme propionates [48]. The reduction in pK_a for the K61A mutant may have to do with changes in the hydrogen bonding network arising from the substitution.

4.4. The effect of redox state

For heme protein unfolding that is reversible, a thermodynamic cycle can be constructed from reduction potentials of the free heme and holoprotein, and the free energies of folding of the two oxidation states [95–97]. This cycle is illustrated for SiaA in Scheme 1. The difference in the free energies of folding of SiaA around the ferric and ferrous hemes ($G_{fd}^{(III-II)}$) is given simply as the difference in the free energies of heme reduction in the unfolded protein and in the native holoprotein, as shown in Equation 5. In this work, chemically induced unfolding was not reversible, as shown experimentally. However, the reduction potentials can still be used to estimate the difference in folding free energies of oxidized and reduced SiaA. Reduction potentials of the holoprotein that are similar to that of

free heme give systems in which the energy costs of unfolding of the two oxidation states are similar. For WT SiaA, the reduction potential is 68 ± 3 mV. Taken together with the -60 mV reduction potential of free heme [98], the thermodynamic cycle indicates that the folding of SiaA around the ferrous heme is more strongly driven than folding around hemin, albeit by only ~ 12.4 kJ·mol⁻¹ for the WT; the corresponding driving force for the K61A mutant is similar at 11.7 kJ·mol⁻¹.

$$\begin{aligned}
 \Delta G_{\text{fld}}^{\text{red}} + \Delta G_{\text{fld}}^{\text{II}} &= \Delta G_{\text{F}}^{\text{red}} + \Delta G_{\text{fld}}^{\text{III}} \\
 \Delta G_{\text{fld}}^{\text{III}} - \Delta G_{\text{fld}}^{\text{II}} &= \Delta G_{\text{U}}^{\text{red}} - \Delta G_{\text{F}}^{\text{red}} \\
 \Delta \Delta G_{\text{fld}}^{(\text{III}-\text{II})} &= -nF(E_{\text{mU}} - E_{\text{mF}}) \\
 \Delta \Delta G_{\text{fld}}^{(\text{III}-\text{II})} &= -nF[(-0.060 \text{ V}) - E_{\text{mF}}] \\
 \Delta \Delta G_{\text{fld(WT)}}^{(\text{III}-\text{II})} &= nF[(0.060(\pm 0.034) \text{ V}) + 0.068(\pm 0.003) \text{ V}] = 12.4 \pm 3.3 \text{ kJ} \cdot \text{mol}^{-1} \\
 \Delta \Delta G_{\text{fld(K61A)}}^{(\text{III}-\text{II})} &= nF[(0.060(\pm 0.034) \text{ V}) + 0.061(\pm 0.003) \text{ V}] = 11.7 \pm 3.3 \text{ kJ} \cdot \text{mol}^{-1} \quad [5]
 \end{aligned}$$

The heme donor to SiaA *in vivo* is Shp [34,35,41]. It is not yet known in which oxidation state the heme is transferred. Our electrochemical data indicates that the two oxidation states are similar with respect to the free energy of unfolding. Nygaard et al. have looked at the transfer of both heme and hemin from Shp to SiaA [42]. They fit the kinetics to a model involving an equilibrium for complexation of the two proteins, followed by intracomplex transfer of the iron porphyrinate from Shp to SiaA. The reported dissociation constants for ferrous and ferric Shp with apo-SiaA are 120 ± 18 μM and 48 ± 7 μM , respectively. Thus, the two oxidation states of Shp differed only by a factor of 2.5 in their binding affinity for apo-SiaA. The heme transfer rate constants within the Shp:SiaA complex were calculated to be 28 ± 6 s⁻¹ and 43 ± 3 s⁻¹, respectively. Thus, this model indicates that the ferric and ferrous hemes are transferred within their respective complexes with similar rate constants. This kinetic result is consistent with our thermodynamic electrochemical data, indicating that both oxidation states of the heme should be released with comparable thermodynamic and kinetic ease.

5. Conclusions

SiaA is part of a pathway that facilitates heme acquisition by *S. pyogenes*. Guanidinium-induced denaturation showed that, as expected, the axial ligands (M79 and H229) play significant roles in the stability of the holo-SiaA fold. Other residues near the heme, specifically C58 and K61, which are near the propionic acids, are also important in stabilizing the protein fold. Guanidinium-induced denaturation occurred from two forms of the protein, with the slower process having a half-time of one to three days. The very slow unfolding may indicate that heme transfer proteins can unfold sufficiently to release heme, but are resistant to further unfolding that might result in conformations that could not easily bind heme for further heme transfer reaction cycles. Spectrophotometric pH titration studies gave $\text{p}K_{\text{a}}$ values ranging from 9.0 to 9.5 for the mutants studied; these may be due to deprotonation of the axial histidine. Spectroelectrochemical titrations showed that the midpoint reduction potential of the K61A SiaA was 61 ± 3 mV, similar to the 68 ± 3 mV potential of WT SiaA. The midpoint potential differs from that of free heme by 125 mV,

indicating that the reduced protein is only ~12 kJ/mole more difficult to unfold than the oxidized protein. These results, together with kinetic data from the literature, reveal that the thermodynamic stabilities of the surface-bound heme acquisition protein SiaA are balanced so as to be nearly insensitive to the oxidation state of the heme. This result is consistent with the system having the flexibility to acquire heme in both its ferrous and ferric oxidation states.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CCD	charge-coupled device
CHES	<i>N</i> -cyclohexyl-2-aminoethanesulfonic acid
GAS	Group A streptococcus
GdnHCl	guanidine hydrochloride
Hts	heme transport <i>Streptococcus pyogenes</i>
ICP-MS	inductively coupled plasma mass spectrometry
LN₂	liquid nitrogen
NMR	nuclear magnetic resonance
RMSD	root mean square difference
rR	resonance Raman spectroscopy
SHE	standard hydrogen electrode
Sia	streptococcal iron acquisition
Tris	tris(hydroxymethyl)aminomethane
UV–vis	ultraviolet-visible
WT	wild type
6cLS	six-coordinate low-spin

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Figure 1.
Overview of the *S. pyogenes* Sia/Hts heme uptake pathway.

Figure 2.
Homology model of SiaA. Shown are the locations of C47, C58, K61, M79, and H229.

Figure 3. UV-visible spectra of the Fe(III) forms of C47A, C58A, K61A, M79A, H229A and WT SiaA normalized at the Soret. The solutions were in 50 mM Tris-Cl, pH 7.0.

Figure 4. CD spectra of holo-SiaA (solid line) and apo-SiaA (dashed). The spectra were recorded in 10 mM potassium phosphate, pH 7.0.

Figure 5. Time-scale unfolding of WT SiaA at the $D_{1/2}$ (3.1 M GdnHCl). Data were taken in 50 mM Tris-Cl, pH 7.0. The data were fit using the sum of two exponential processes.

Figure 6.

Fraction of folded WT SiaA and mutants as a function of the concentration of GdnHCl. Data from the titrations were fit via nonlinear least squares to a two state unfolding model. Protein samples were in 50 mM Tris-Cl, pH 7.0.

Figure 7.

Spectrophotometric pH titration of C47A, titrated with 1.0 M NaOH, in a buffer of 20 mM each CAPS, CHES and Tris-Cl. UV-visible spectra are shown from pH 7.0 to 10.7. The inset shows the nonlinear least squares fit of the data at 409 nm to a single pK_a ; the value was 9.22 ± 0.03 .

Figure 8.

Soret-excited rR spectra of ferric (top) and ferrous (bottom) K61A SiaA. The in-plane porphyrin stretching (high frequency) and low frequency regions are shown. Samples were 250 μ M in holo-SiaA and in 20 mM Tris-Cl at pH 8.0. Ferrous K61A SiaA was generated by anaerobic introduction of a 15-fold molar excess of buffered dithionite to the ferric protein. Complete reduction was verified by UV-visible absorbance spectroscopy.

Figure 9.

UV-visible absorbance spectra of K61A SiaA during the course of the spectroelectrochemical titration with dithionite. Spectral contributions from the dyes, ferricyanide, ferrocyanide and dithionite ions have been subtracted from each spectrum to show the clean isosbestic behavior of the system. The inset shows absorbance at the Soret maximum for ferrous K61A SiaA (423 nm) as a function of cell potential (vs SHE reference). The oxidative and reductive titration curves are superimposable and fitting to a single Nernstian wave (Equation 4) yielded a midpoint potential of 61 ± 3 mV vs SHE. Titrations were carried out in 50 mM Tris/Tris-Cl at pH 8.0, 100 mM NaCl.

Scheme 1.

Table 1

Forward and reverse primers for the mutants in this work.

C47Af	5'-CCACTTCGGTTGCTGTGGTTGATATCGCTGACCGTTTAAATTTA-3'
C47Ar	5'-TAAATTTAAACGGTCAGCGATATCAACCACAGCAACCGAAGTGG-3'
C58Af	5'-TTTAGACCTCGTTGGGGTTGCTGATAGTAAA TTATATACCCTTCC-3'
C58Ar	5'-GGAAGGGTATATAATTTACTATCAGCAACCCCAACGAGGTCTAAA-3'
H229Af	5'-CTTGATTTTACGAACAGCTGCTGCCATTCCAGACAAGG-3'
H229Ar	5'-CCTGTCTGGAATGGCAGCAGCTGTTTCGTAAAATCA AG-3'
M79Af	5'-GCGTGTGGGTTTACCCGCCAATCCTGATATAGAGTTGATTG-3'
M79Ar	5'-CAATCA ACTCTATATCAGGATTGGCGGGTAAACCCACACGC-3'
K61Af	5'-CCTCGTTGGGGTTTGTGATAGTGCATTATATACCCTTCCTAAACGC-3'
K61Ar	5'-GCGTTTAGGAAGGGTATATAATGCACTATCACAAACCCCAACGAGG-3'

Table 2

$D_{1/2}$ unfolding rate constants and relative abundances for SiaA and mutants.

Protein	k_{fast} (h^{-1})	Abundance of the Fast Phase (%)	k_{slow} (h^{-1})	Abundance of the Slow Phase (%)	$D_{1/2}$ (M)
WT	0.32 ± 0.02	15	0.026 ± 0.001	85	3.1
C47A	0.24 ± 0.02	30	0.033 ± 0.001	70	2.9
C58A	0.46 ± 0.02	12	0.010 ± 0.001	88	2.4
K61A	0.41 ± 0.04	11	0.012 ± 0.001	89	2.5