Characterization of the HEME Uptake Pathway Proteins from Streptococcus Pyogenes and Corynebacterium Diphtheriae

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CHARACTERIZATION OF THE HEME UPTAKE PATHWAY PROTEINS FROM
STREPTOCOCCUS PYOGENES AND CORYNEBACTERIUM DIPHTHERIAE

by

NEVAL AKBAS

Under the Direction of Professor Dabney W. Dixon

ABSTRACT

In *Streptococcus pyogenes*, the protein SiaA (HtsA) is part of a heme uptake pathway system and involved in heme transfer from Shp to the ABC transporter. SiaA mutants, in which alanine replaces the axial histidine (H229) and methionine (M79) ligands, as well as a lysine (K61) and cysteine (C58) located near the heme propionates, are reported. Studies on a mutant of a cysteine expected to be at a distance from the propionates (C47A) are also reported. The coordination state and spin state of the selected mutants were determined via Resonance Raman studies. The $pK_a$ values of mutants ranged from 9.0 to 9.4, which were close to the $pK_a$ of the WT SiaA (9.7). The midpoint reduction potential of lysine (K61A) mutant was determined by spectroelectrochemical titration to be 61 ± 3 mV vs. SHE, similar to the WT protein (68 ± 3 mV). The addition of guanidinium hydrochloride resulted in protein denaturation that could show more than one process and occurred over days. The ease of protein unfolding was directly
related to the extent of interaction of the residues with the heme: changes in the axial ligands resulted in far greater changes in heme protein stability than changes in the residues near the heme propionates.

The causative agent of diphtheriae, Corynebacterium diphtheriae, imports heme via an ABC uptake transporter. In this research, two of the five proteins in the heme uptake pathway of C. diphtheriae were studied. These proteins were HmuT, lipoprotein component of the ABC transporter, and HtaA, the heme receptor. UV-visible spectroscopy and fluorescence spectroscopy showed that HmuT protein as isolated bound a porphyrin, rather than heme. Electrospray ionization mass spectrometry (ESI-MS) studies suggested that two tetrapyrroles were bound. To assess stability of this protein towards heme release, thermal denaturation studies were performed. For HtaA, UV-visible and fluorescence spectroscopy also showed the protein as isolated was also bound a porphyrin, rather than heme. Homology studies showed that HtaA protein is quiet distant from homologous heme uptake proteins and could be a member of novel heme binding domain family.

INDEX WORDS: SiaA, HtsA, Streptococcus pyogenes, Heme, Uptake, ABC transporter, Resonance Raman, Guanidinium denaturation, Mass spectrometry, HmuT, HtaA, Corynebacterium diphtheriae, Gram-positive, PPIX, Axial ligand, Thermal denaturation
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NEVAL AKBAS

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August 2012
DEDICATION

I dedicate this doctoral dissertation to my parents Mümin and Nesrin Yönet, my in-laws Kemal and Huma Akbaş, my husband Erol Akbas, our precious son Cemal Baturalp Akbas and to my sisters Neslihan Yönet-Laçin and Nihan Yönet-Tanyeri.
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TABLE OF CONTENTS

ACKNOWLEDGEMENTS .................................................................................. v

LIST OF TABLES ............................................................................................... xiii

LIST OF FIGURES ............................................................................................ xiv

1 BIOPHYSICAL CHARACTERIZATION OF MUTANTS OF SIAA PROTEIN
FROM STREPTOCOCCUS PYOGENES ................................................................. 1

1.1 Introduction. ............................................................................................... 1

1.1.1 Iron and heme in bacteria. ................................................................. 1

1.1.2 Streptococcus pyogenes........................................................................ 2

1.1.3 Introduction to heme acquisition systems in bacteria. ....................... 2

1.2 ABC transporters. .................................................................................... 3

1.2.1 Overview of ABC transporters............................................................ 3

1.2.2 ABC transporters associated heme acquisition systems in Gram-negative bacteria........................................................................... 5

1.2.3 ABC transporters associated heme acquisition systems in Gram-positive bacteria........................................................................... 6

1.3 Related heme acquisition systems with homologous heme binding proteins...
........................................................................................................................................... 12

1.3.1 Shigella dysenteriae. ............................................................................ 12

1.3.2 Pseudomonas aeruginosa. ................................................................. 15
1.3.3  *Staphylococcus aureus*………………………………………………………………………………… 17
1.3.4  *Porphyromonas gingivalis* ……………………………………………………………………………… 22
1.3.5  *Escherichia coli*………………………………………………………………………………………… 24
1.3.6  *Bacillus anthracis*. ……………………………………………………………………………………… 24
1.4  Summary………………………………………………………………………………………………………… 27
1.5  Reference List………………………………………………………………………………………………… 30

2  HEME-BOUND SIAA FROM *STREPTOCOCCUS PYOGENES*: EFFECTS OF MUTATIONS AND OXIDATION STATE ON PROTEIN STABILITY …………… 40

2.1  Introduction……………………………………………………………………………………………………. 40
2.2  Materials and methods…………………………………………………………………………………………… 42
  2.2.1  Homology modeling.……………………………………………………………………………………… 42
  2.2.2  Materials……………………………………………………………………………………………………… 43
  2.2.3  Preparation of plasmids.…………………………………………………………………………………… 43
  2.2.4  Expression and purification of mutants………………………………………………………………… 44
  2.2.5  Mass spectrometry………………………………………………………………………………………… 45
  2.2.6  UV–visible spectroscopy…………………………………………………………………………………… 45
  2.2.7  Chemical denaturation studies via titration…………………………………………………………… 46
  2.2.8  Chemical denaturation studies with multiple protein samples. …………………………………… 47
  2.2.9  Spectrophotometric pH titration……………………………………………………………………… 47
  2.2.10  Resonance Raman spectroscopy……………………………………………………………………… 48
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2.11 Reduction potential determination</td>
<td>48</td>
</tr>
<tr>
<td>2.2.12 Native polyacrylamide gel electrophoresis</td>
<td>49</td>
</tr>
<tr>
<td>2.3 Results</td>
<td>50</td>
</tr>
<tr>
<td>2.3.1 Spectroscopic studies</td>
<td>50</td>
</tr>
<tr>
<td>2.3.2 Guanidinium-induced denaturation</td>
<td>50</td>
</tr>
<tr>
<td>2.3.3 Spectrophotometric pH titration</td>
<td>52</td>
</tr>
<tr>
<td>2.3.4 Resonance Raman spectra of ferric and ferrous SiaA mutants</td>
<td>53</td>
</tr>
<tr>
<td>2.3.5 Spectrophotometric redox titration</td>
<td>54</td>
</tr>
<tr>
<td>2.4 Discussion</td>
<td>55</td>
</tr>
<tr>
<td>2.4.1 Guanidinium-induced unfolding</td>
<td>55</td>
</tr>
<tr>
<td>2.4.2 The time scale of protein unfolding</td>
<td>57</td>
</tr>
<tr>
<td>2.4.3 Spectrophotometric pH titration</td>
<td>59</td>
</tr>
<tr>
<td>2.4.4 The effect of redox state</td>
<td>60</td>
</tr>
<tr>
<td>2.5 Conclusions</td>
<td>62</td>
</tr>
<tr>
<td>2.6 Abbreviations</td>
<td>63</td>
</tr>
<tr>
<td>2.7 Acknowledgments</td>
<td>64</td>
</tr>
<tr>
<td>2.8 Reference List</td>
<td>76</td>
</tr>
<tr>
<td>3 MUTANTS OF SIAA, A PROTEIN IN THE HEME UPTAKE PATHWAY OF S. PYOGENES</td>
<td>83</td>
</tr>
</tbody>
</table>
3.1 Homology modeling using I-TASSER (Protein Structure and Function Predictions) ........................................................................................................ 83

3.2 Purification of mutants of SiaA ........................................................................ 83

3.3 MALDI Mass spectrometry of mutants of SiaA .............................................. 84

3.4 Charge distribution bands (ESI results) of C47A mutant .............................. 85

3.5 Thermal denaturation studies ........................................................................ 86

3.6 Guanidinium-induced denaturation results of C58A at 3 h, 24 h, 29 h, 9 d 5h and titration method ................................................................. 86

3.7 Native polyacrylamide gel electrophoresis ..................................................... 86

3.8 Spectrophotometric pH titration .................................................................... 87

3.9 Reference List .................................................................................................. 103

4 CHARACTERIZATION OF HMUT, A HEME-BINDING PROTEIN
ASSOCIATED WITH A HEME ABC TRANSPORT SYSTEM IN
CORYNEBACTERIUM DIPHTHERIAE .................................................................. 104

4.1 Introduction ...................................................................................................... 104

  4.1.1 Corynebacterium diphtheriae ...................................................................... 104

  4.1.2 Heme acquisition in C. diphtheriae ........................................................... 106

  4.1.3 Heme acquisition in Yersinia pestis ......................................................... 110

  4.1.4 Scope of this study ..................................................................................... 112

4.2 Experimental ................................................................................................... 113

  4.2.1 Materials .................................................................................................... 113
4.2.2 Expression and purification of HmuT .......................................................... 114
4.2.3 Native polyacrylamide gel electrophoresis .............................................. 115
4.2.4 MALDI mass spectrometry ........................................................................ 115
4.2.5 Electrospray ionization mass spectrometry .............................................. 116
4.2.6 UV–visible spectroscopy ........................................................................... 116
4.2.7 Preparation of apoHmuT ........................................................................... 116
4.2.8 Reconstitution of HmuT ............................................................................ 117
4.2.9 Fluorescence spectroscopy ........................................................................ 117
4.2.10 Thermal denaturation ............................................................................. 118
4.2.11 Spectrophotometric pH titration ............................................................... 118
4.2.12 Homology modeling ................................................................................. 118
4.2.13 ClustalW alignment of HmuT protein to homologous proteins with known crystal structure ........................................................................................................ 119

4.3 Results and discussion .................................................................................. 119
4.3.1 Purification of HmuT protein ............................................................... 119
4.3.2 UV–visible spectroscopy ........................................................................... 120
4.3.3 Fluorescence spectroscopy ....................................................................... 122
4.3.4 MALDI mass spectrometry of HmuT ....................................................... 123
4.3.5 Electrospray ionization mass spectrometry and charge distribution bands of HmuT protein ................................................................. 123
4.3.6 Heme binding to HmuT. ............................................................... 125
4.3.7 Thermal denaturation. ................................................................. 126
4.3.8 Spectrophotometric pH titration. .................................................. 127
4.3.9 ClustalW alignment and homology modeling of HmuT. ................. 127
4.4 Reference List .................................................................................. 155

5 CHARACTERIZATION OF HTAA, A NOVEL HEME-BINDING PROTEIN
FROM CORYNEBACTERIUM DIPHTHERIAE ........................................... 160

5.1 Introduction ................................................................. 160

5.2 Experimental ........................................................................... 160

5.2.1 Materials .............................................................................. 160

5.2.2 Expression and purification of HtaA. .......................................... 162

5.2.3 Native polyacrylamide gel electrophoresis ................................... 162

5.2.4 MALDI mass spectrometry. ....................................................... 162

5.2.5 Electrospray ionization mass spectrometry. ............................... 162

5.2.6 UV–visible spectroscopy .............................................................. 163

5.2.7 Preparation of apoHtaA and Reconstitution of HtaA. ................. 163

5.2.8 Fluorescence spectroscopy ........................................................... 163

5.2.9 Spectrophotometric pH titration .................................................. 163

5.2.10 Homology modeling ................................................................. 163

5.3 Results and discussion ................................................................ 164
5.3.1 Purification of HtaA protein ................................................................. 164
5.3.2 UV–visible spectroscopy ................................................................. 164
5.3.3 Fluorescence spectroscopy ............................................................... 165
5.3.4 MALDI mass spectrometry of HtaA ................................................... 165
5.3.5 Electrospray ionization mass spectrometry and charge distribution bands of HtaA protein .................................................................................. 166
5.3.6 Heme binding to HtaA ....................................................................... 167
5.3.7 Spectrophotometric pH titration ........................................................... 168
5.3.8 Homology modeling and ClustalW alignment of HtaA ....................... 168
5.4 Reference List .......................................................................................... 184
LIST OF TABLES

Table 2.1  Forward and reverse primers for the mutants in this work................................. 72
Table 2.2  GdnCl denaturation values and equilibrium time for selected $b$-type heme proteins
including cytochromes and heme trafficking proteins....................................................... 73
Table 2.3  GdnCl denaturation values and equilibrium time for selected ferric $b$-type heme
proteins: Globins, peroxidases and cytochrome P450...................................................... 74
Table 4.1  Spectral bands of PPIX................................................................. 154
LIST OF FIGURES

Figure 1.1  Heme uptake system in *S. pyogenes* .......................................................... 29

Figure 1.2  *S. pyogenes sia* gene cluster. ................................................................. 29

Figure 2.1  Homology modeling of SiaA. Shown are the locations of C47, C58, K61, M79, and H229................................................................. 65

Figure 2.2  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-HCl, pH 7.0................. 65

Figure 2.3  Relative absorbance at the Soret maximum as a function of time for proteins in the presence of GdnCl. C47A is shown at the midpoint (2.95 M GdnCl). C58A is shown in the pre-transition region (1.46 M GdnCl) and at the midpoint concentrations (2.42 M GdnCl). WT is shown at the midpoint (3.1 M GdnCl). The solutions were in 50 mM Tris-HCl, pH 7.0. .......... 66

Figure 2.4  Fraction folded of the C58A mutant as a function of the concentration of GdnCl at 29 h (squares) and 221 h (triangles) after the completion of addition of denaturant. Lines are the nonlinear least square fits to the equations to a two state unfolding model. The inset shows absorbance as a function of the concentration of GdnCl for these two data sets and data taken at 3 h (diamonds). The solutions were in 50 mM Tris-HCl, pH 7.0................................. 66

Figure 2.5  Fraction folded of the WT SiaA and mutants in this study as a function of the concentration of GdnCl. Data from titrations in 50 mM Tris-HCl, pH 7.0 buffer were fit via nonlinear least squares to a two state unfolding model. ............................................. 67

Figure 2.6  Spectrophotometric pH titration of C47A, titrated with 1.0 M NaOH, in a buffer of 20 mM each CAPS, CHES and Tris-HCl. UV-visible spectra of 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 9.2 ± 0.1.................................................. 67
Figure 2.7  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 μM in holoSiaA and 20 mM in Tris-HCl 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. ............................ 68

Figure 2.8  UV-visible absorbance spectra of K61A SiaA during the course of the anodic spectroelectrochemical titration with dithionite. The inset shows normalized 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 3) yielded a midpoint potential of 61 ± 3 mV vs SHE. Titrations were carried out in 50 mM Tris-HCl at pH 8.0, 100 mM NaCl. .................................................... 69

Figure 2.9  Soret-excited rR spectra of ferric (top) and ferrous (bottom) C58A SiaA. The high frequency (in-plane porphyrin stretching) and low frequency regions are shown. Samples were 50 μM in holoSiaA and 10 mM in Tris-HCl at pH 8.0. Ferrous C58A SiaA was generated by anaerobic introduction of a 10-fold molar excess of buffered dithionite to the ferric protein. Complete reduction was verified by UV-visible absorbance spectroscopy. ................................. 70

Figure 2.10  Spectroelectrochemical titration of WT SiaA. Normalized absorbance at the Soret maximum for ferrous WT SiaA (424 nm) as a function of cell potential (vs SHE reference) reveals electrochemical irreversibility of the Fe(III)|Fe(II) couple. The anodic titration curve was best modeled by three Nernstian waves, yielding midpoint heme potentials of 72 and 15 mV vs SHE. A third, unidentified but very negative, potential resulted from the fitting in order to account for absorbance changes at the low end of the titration range. The cathodic titration curve was well modeled by a single Nernstian wave with a midpoint potential of 68 mV. The 68 and
72 mV potentials are taken to indicate a reversible Fe(III)|Fe(II) couple. The 15 mV potential represents a fraction of the protein that can access a ferrous structure/conformation having a lower potential than the ferric protein and which is kinetically slow to revert back to the ferric conformation. Titrations were carried out in 50 mM Tris at pH 8.0, 100 mM NaCl.

Figure 3.1 Homology model of SiaA built with I-TASSER showing cysteine 47, cysteine 58, lysine 61, histidine 229 and methionine 79.

Figure 3.2 The purification of the C47A mutant protein was conducted at 4 °C using a GE Healthcare ÄKTA fast protein liquid chromatography instrument (FPLC, Amersham BioSciences), and all buffer solutions were pH 8.0. The sample was loaded onto a GE Healthcare HisTrap™ HP column (5 mL, Amersham BioSciences) equilibrated with binding buffer (50 mM potassium phosphate, 250 mM NaCl, and 10% v/v glycerol). Unbound material was washed out with 5 column volumes (CV) of binding buffer. C47A was eluted with buffer containing 50 mM potassium phosphate, 250 mM NaCl, 10% v/v glycerol, and 0.5 M imidazole applied via a linear gradient.

Figure 3.3 The mass of the C47A mutant was evaluated using an ABI Voyager DE-Pro (Applied Biosystems) MALDI reflectron time-of-flight spectrometer in positive ion mode. The sinipinic acid matrix was prepared in 30:70 acetonitrile:water and 0.1% trifluoroacetic acid. The sample solution was mixed with the matrix solution in a 1:1 ratio and the resulting solution spotted onto the MALDI target plate. The mass spectrum of C47A showed a species at 35376.1 Da, consistent with the expected 35455 Da.

Figure 3.4 SDS PAGE of C47A fractions. A 5 to 15% acrylamide gel was used. Lane 1: Marker, lane 2: FPLC fraction #51 of C47A mutant, lane 3: Fraction 40, lane 4: Fraction 33, lane 5: Fraction 22, lane 6: Fraction 15, lane 7: Fraction 8, lane 8: Fraction 4. Samples were run 5 min
under 80 V current and 1h under the 120 V current. A Precision Plus Protein Standard was used to determine the molecular weight.

Figure 3.5 Charge distribution bands (ESI results) of C47A. Electrospray ionization mass spectrometry (ESI) was performed using a Micromass Q-TOF Micro mass spectrometer in positive mode. Samples were prepared in a solution containing 50:50 acetonitrile:water and 0.1% formic acid. The capillary voltage was 3.0 kV and the flow rate was 5 μL/min. Deconvolution of the charge state distribution was performed with the MaxEnt program included with the MassLynx™ software. The peaks are rounded to the nearest Dalton. The apoC47A was seen at 35456.5 Da, consistent with the expected 35455 Da. The holoC47A was seen at 36067.5 Da, consistent with the expected 36071 Da. A second set of peaks was seen for the apoC47A at 35554.5 and the holoC47A at 36165. These are each 98 units higher than the peaks reported above.

Figure 3.6 The purification of the C58A mutant protein was conducted at 4 °C using a GE Healthcare ÄKTA fast protein liquid chromatography instrument (FPLC, Amersham BioSciences), and all buffer solutions were pH 8.0. The sample was loaded onto a GE Healthcare HisTrap™ HP column (5 mL, Amersham BioSciences) equilibrated with binding buffer (50 mM potassium phosphate, 250 mM NaCl, and 10% v/v glycerol). Unbound material was washed out with 5 column volumes (CV) of binding buffer. C58A was eluted with buffer containing 50 mM potassium phosphate, 250 mM NaCl, 10% v/v glycerol, and 0.5 M imidazole applied via a linear gradient.

Figure 3.7 The masses of the C58A was evaluated using an ABI Voyager DE-Pro (Applied Biosystems) MALDI reflectron time-of-flight spectrometer in positive ion mode. The sinipinic acid matrix was prepared in 30:70 acetonitrile:water and 0.1% trifluoroacetic acid. The sample
solution was mixed with the matrix solution in a 1:1 ratio and the resulting solution spotted onto the MALDI target plate. The mass spectrum of C58A showed a species at 35364.7 Da, consistent with the expected 35455 Da.

Figure 3.8  Thermal denaturation of C47A. The temperature was increased 65°C to 95°C using 0.5°C per minute increments. The unfolding was followed at 413 nm. The protein was in 50 mM Tris-HCl, pH 7.0 buffer.

Figure 3.9  Guanidinium-induced denaturation of C58A using the multiple cuvette method at 3 h, 24 h, 29 h, and 9 d 5h time points and with titration method. Data from denaturations in 50 mM Tris-HCl, pH 7.0 buffer were fit via nonlinear least squares to a two state unfolding model. The data at 3 h did not fit to a two state model.

Figure 3.10  Purified protein subjected to 5-12% native polyacrylamide gel electrophoresis. Lane 1: marker, lane 2: WT SiaA, lane 3: C47A mutant, lane 4: C58A mutant, lane 5: K61A mutant, lane 6: marker, lane 7: bovine serum albumin. A NativePAGE Sample Prep Kit from Invitrogen was used with a 5 to 12% acrylamide gel. Samples were run for 15 min with 80 V current and 1.5 h with 150 V current. A NativeMark Unstained Protein Standard was used.

Figure 3.11  Spectrophotometric pH titration of C47A, titrated with 1.0 M NaOH, in a buffer of 20 mM each CAPS, CHES and Tris-HCl. UV-visible spectra (isosbestic) are shown from pH 7.0 to 10.7.

Figure 3.12  Spectrophotometric pH titration of C58A, titrated with 1.0 M NaOH, in a buffer of 20 mM each CAPS, CHES and Tris-HCl. UV-visible spectra (isosbestic) are shown from pH 7.0 to 11.0.
Figure 3.13  Spectrophotometric pH titration of C58A, titrated with 1.0 M NaOH, in a buffer of 20 mM each CAPS, CHES and Tris-HCl. The nonlinear least squares fit of the data at 409 nm gave a single pKₐ; the value was 9.0 ± 0.1.

Figure 3.14  Spectrophotometric pH titration of K61A, titrated with 1.0 M NaOH, in a buffer of 20 mM each CAPS, CHES and Tris-HCl. UV-visible spectra (isosbestic) are shown from pH 7.1 to 10.8.

Figure 3.15  Spectrophotometric pH titration of K61A, titrated with 1.0 M NaOH, in a buffer of 20 mM each CAPS, CHES and Tris-HCl. The nonlinear least squares fit of the data at 408 nm gave a single pKₐ; the value was 9.4 ± 0.1.

Figure 4.1  Genes (from hmu locus) associated heme uptake in C. diphtheriae reproduced from (Allen & Schmitt, 2009).

Figure 4.2  Location of the heme uptake proteins in C. diphtheriae reproduced from (Allen & Schmitt, 2009).

Figure 4.3  FPLC of HmuT on a GE Healthcare ÄKTA instrument at 4 °C. The sample was loaded onto a Strep-Tactin Superflow column equilibrated with buffer A (100 mM Tris-HCl, 150 mM NaCl, pH 8.0). Unbound material was washed out with 5 CV of buffer A. HmuT was eluted with 10 CV of buffer B containing 100 mM Tris-HCl, 150 mM NaCl, and 2.5 mM desthiobiotin, pH 8.0 applied via a linear gradient. The first peak (fractions 22-32, red letters on axis), which is essentially at the end of the flow-through, is designated as Peak 1 and the second peak (fractions 33-38) is designated as Peak 2.

Figure 4.4  SDS PAGE of HmuT fractions using a 5 to 15% acrylamide gel. Lane L is the Precision Plus Protein Standard. The rest of the lanes are the FPLC fractions as indicated by the numbers on the top of the gel. Fraction 23 is associated with Peak 1. Fractions 33, 35, and 37
are associated with Peak 2. Lane 15 is flow-through. Samples were run 5 min under 80 V current and 1 h under the 120 V current. ................................................................. 133

Figure 4.5 FPLC-purified HmuT protein subjected to 5-12% native polyacrylamide gel electrophoresis. Lane 1: Marker (NativePAGE Sample Prep Kit from Invitrogen), lane 2: bovine serum albumin (BSA), lane 5: HmuT Peak 2 from FPLC purification, lane 6: HmuT protein Peak 1 from FPLC purification. The gel was run as described in the Experimental. It can be seen that both Peak 1 and Peak 2 are monomeric HmuT. Peak 2 is purer than Peak 1. Lanes 2 and 3 are HtaA ................................................................. 134

Figure 4.6 FPLC of a second batch of HmuT protein. Conditions were as in the previous figure, except for a much lower protein loading. The sample was loaded onto a Strep-Tactin Superflow column equilibrated with buffer A (100 mM Tris-HCl, 150 mM NaCl, pH 8.0). Unbound material was washed out with 5 CV of buffer A. HmuT was eluted with 10 CV of buffer B containing 100 mM Tris-HCl, 150 mM NaCl, 2.5 mM desthiobiotin, pH 8.0 applied via a linear gradient. ................................................................. 135

Figure 4.7 UV-visible spectrum of Peak 2 of the HmuT as isolated (150 mM NaCl and 20 mM Tris-HCl, pH 7.5). Wavelengths of the peaks are indicated. The Soret was at 406 nm. .......... 136

Figure 4.8 Fluorescence spectrum (in 2-butanone) of the tetrapyroles in HmuT after removal by incubation with cold 2-butanone. The excitation was at 402 nm. The peak is seen at 635 nm. ................................................................. 137

Figure 4.9 Positive ion mode MALDI spectrum of HmuT. Top panel: The mass spectrum of HmuT shows a species at 36166.2 Da, consistent with the expected molecular weight of 36195 Da. Bottom panel: The lower molecular weight area of the spectrum shows peaks for both heme and PPIX. ................................................................. 138
Figure 4.10  Electrospray mass spectrum of apoHmuT. The sample was prepared in a solution containing 50:50 acetonitrile:water and 0.1% formic acid (denaturing conditions). Top panel: Charge distribution bands. Bottom panel: The apoHmuT is seen at 36197.5 Da, consistent with the expected 36195 Da.

Figure 4.11  Electrospray mass spectrum of holoHmuT. The sample was prepared in water. Top panel: The duplication of peaks in the charge envelope is consistent with the protein binding one or two tetrapyrroles. Bottom panel: The holoHmuT is seen at 36783.5 Da, consistent with the holoprotein (expected 36811 Da) or PPIX-bound protein (expected 36757 Da). The peak at 37443.0 Da is appropriate for two tetrapyrroles bound (two hemin molecules, two PPIX molecules, or one hemin and one PPIX). The very broad peaks presumably reflect the mixture of hemin and PPIX bound to this protein.

Figure 4.12  Electrospray mass spectrum of holoHmuT. The sample was prepared in water with 20% aqueous MeOH added in a 1:1 ratio to give a final solution of 10% MeOH. Top panel: Charge distribution bands of HmuT. The duplication of peaks in the charge envelope is consistent with the protein binding one or two tetrapyrroles. Bottom panel: The peak at 36731.5 consistent with the holoprotein (expected 36811 Da) or PPIX-bound protein (expected 36757 Da). The peak to the right presumably represents the protein with bound tetrapyrole dimer.

Figure 4.13  Electrospray mass spectrum of holoHmuT. The sample was prepared in water with 40% aqueous MeOH added in a 1:1 ratio to give a final solution of 20% MeOH. Under these conditions, the peak for binding tetrapyrole dimer is becoming less intense.

Figure 4.14  Three samples of a solution of 5 μM hemin in 20 mM Tris-HCl and 150 mM NaCl, pH 7.5 titrated to which were added increasing concentrations of apoHmuT (from Peak 1). The spectrum was taken after 5 min of incubation.
Figure 4.15  Three samples of a solution of 5 μM hemin in 20 mM Tris-HCl and 150 mM NaCl, pH 7.5 titrated to which were added increasing concentrations of apoHmuT (from Peak 1). The spectrum was taken after 3 d of incubation. .......................................................... 144

Figure 4.16  Comparison of the spectra of the sample with 5 μM hemin and 10 μM apoHmuT (Peak 1) after 5 min and 3 d of incubation at 4 °C (20 mM Tris-HCl and 150 mM NaCl, pH 7.5). .................................................................................................................. 145

Figure 4.17  A solution of 0.5 μM hemin and 0.5 μM apoHmuT (Peak 2) incubated in 20 mM Tris-HCl and 150 mM NaCl, pH 7.5 for 20 h at 20 °C. Spectra were taken every hour. The Soret is at 406 nm, indicating that the heme is binding as the monomer. There are minimal changes over time, indicating that Peak 2 reconstitutes easily and that once reconstituted, the protein undergoes no further seen in the UV-visible spectra...................................................... 146

Figure 4.18  A solution of 5 μM hemin and 5 μM apoHmuT (Peak 2) incubated in 20 mM Tris-HCl and 150 mM NaCl, pH 7.5 for 20 h at 20 °C. Spectra were taken every hour. The Soret is at 406 nm, indicating that the heme is binding as the monomer. The Soret increases over time, indicating that reconstitution is slower in this experiment, in which both hemin and apoHmuT concentrations are 10x the previous experiment. The band at approximately 380 nm increases slightly over time, which is inconsistent with an initial bound hemin dimer converting to a bound hemin monomer over time................................................................. 147

Figure 4.19  Thermal denaturation of holoHmuT (reconstituted Peak 2). The temperature was increased from 20 °C to 82 °C in 2 °C increments. After each temperature change, the solution was allowed to stand until the temperature was stable (about a minute) before the spectrum was recorded. The protein was in 20 mM Tris-HCl, 150 mM NaCl, pH 7.5 buffer. The spectrum changes smoothly from 20 to 68 degrees. The last spectrum in this series has an absorbance of
approximately 0.63 at the Soret. After this point (which is very close to the midpoint in the fitting), the spectrum changes considerably.  ................................................................. 148

Figure 4.20  Absorbance at 406 nm vs. temperature for heme-reconstituted Peak 2. The full spectra are given in the previous figure. The data were fit to the thermal unfolding equation given in the Experimental.  ........................................................................ 149

Figure 4.21  Absorbance at 394 nm vs. temperature for heme-reconstituted Peak 2. The full spectra are given in the previous figure. At even lower wavelengths (approximately 360 nm) the spectrum first decreases and then increases, consistent with the release of free heme. ............... 150

Figure 4.22  Spectrophotometric pH titration of HmuT (reconstituted Peak 2). The protein, in a buffer that was 20 mM each CAPS, CHES, MOPS and Tris-HCl was titrated with 1.0 M NaOH.
Top panel: spectra as a function of pH. Bottom panel: The absorbance at 417 nm vs. pH. The protein shows essentially no sensitivity to pH, at least up to pH 10.5................................. 151

Figure 4.23  ClustalW alignment of HmuT to homologous proteins with known crystal structures. The axial tyrosine ligands of PhuT (Y71) and ShuT (Y67) are indicated in purple.
The axial ligands of Yp-HmuT (tyrosine 70 and histidine 167) are in blue. The axial ligands of IsdE (methionine 78 and histidine 229) are in orange. The most likely axial ligands of HmuT (methionine 292, histidine 136 and tyrosine 235) are indicated in red......................... 152

Figure 4.24  Homology model of HmuT built with I-TASSER showing histidine 136 (blue), methionine 292 (orange) and tyrosine 235 (magenta). ................................................................. 153

Figure 5.1  Homology model of HtaA built with I-TASSER showing tyrosine 19 (magenta), histidine 70 (blue) and tyrosine 148 (yellow)................................................................. 170

Figure 5.2  FPLC of HtaA on a GE Healthcare ÄKTA instrument at 4 °C. The sample was loaded onto a Strep-Tactin Superflow column equilibrated with buffer A (100 mM Tris-HCl, 150
mM NaCl, pH 8.0). Unbound material was washed out with 5 CV of buffer A. HtaA was eluted with 10 CV of buffer B containing 100 mM Tris-HCl, 150 mM NaCl, and 2.5 mM desthiobiotin, pH 8.0 applied via a linear gradient. The first peak (fractions 22-30, red letters on axis), which is essentially at the end of the flow-through, is designated as Peak 1 and the second peak (fractions 31-37) is designated as Peak 2.

Figure 5.3 SDS PAGE of HtaA fractions using a 5 to 15% acrylamide gel. Lane L is the Precision Plus Protein Standard. The rest of the lanes are the FPLC fractions as indicated by the numbers on the top of the gel. Fraction 22 is associated with Peak 1. Fractions 31, 35, and 37 are associated with Peak 2. Lane 13 is flow-through. Samples were run 5 min under 80 V current and 1 h under the 120 V current.

Figure 5.4 FPLC-purified HtaA protein subjected to 5-12% native polyacrylamide gel electrophoresis. Lane 1: Marker (NativePAGE Sample Prep Kit from Invitrogen); lane 2: bovine serum albumin (BSA), lane 3: HtaA Peak 2 from FPLC purification, lane 4: HtaA protein Peak 1 from FPLC purification. The gel was run as described in the Experimental. It can be seen that both Peak 1 and Peak 2 are multimeric HtaA. Lanes 5 and 6 are HmuT.

Figure 5.5 FPLC of a second batch of HtaA protein. Conditions were as in the previous figure, except for a much lower protein loading. The sample was loaded onto a Strep-Tactin Superflow column equilibrated with buffer A (100 mM Tris-HCl, 150 mM NaCl, pH 8.0). Unbound material was washed out with 5 CV of buffer A. HmuT was eluted with 10 CV of buffer B containing 100 mM Tris-HCl, 150 mM NaCl, 2.5 mM desthiobiotin, pH 8.0 applied via a linear gradient.

Figure 5.6 UV-visible spectrum of Peak 2 of the HtaA as isolated (150 mM NaCl and 20 mM Tris-HCl, pH 7.5). Wavelengths of the peaks are indicated. The Soret was at 408 nm.
Figure 5.7  Fluorescence spectrum (in 2-butanone) of the tetrapyrroles in HtaA after removal by incubation with cold 2-butanone. The excitation was at 402 nm. The peak is seen at 635 nm. 176

Figure 5.8  Positive ion mode MALDI spectrum of HtaA (Peak 2). Top panel: The mass spectrum of HmuT shows a species at 27378.6 Da, consistent with the expected molecular weight of 27318.9 Da. ........................................................... 177

Figure 5.9  Electrospray mass spectrum of apoHtaA. The sample was prepared in a solution containing 50:50 acetonitrile:water and 0.1% formic acid (denaturing conditions). Top panel: Charge distribution bands. Bottom panel: The apo-HtaA is seen at 27324.0 Da, consistent with the expected 27318.9 Da. ........................................................... 178

Figure 5.10  Electrospray mass spectrum of holoHtaA. The sample was prepared in water (diluted 1:1 with water). Top panel: The duplication of peaks in the charge envelope is seen. Middle panel: Deconvolution of the spectrum gave two very broad HtaA peaks. Bottom panel: The lower molecular weight area of the spectrum shows peaks for both heme and PPIX. ......... 179

Figure 5.11  A solution of 0.5 μM hemin and 0.5 μM apoHtaA (Peak 2) incubated in 20 mM Tris-HCl and 150 mM NaCl, pH 7.5 for 20 h at 20 °C. Spectra were taken every hour (24 h). The Soret is at 403 nm, indicating that the heme is binding largely as the monomer. However, the band to the blue may indicate binding of some dimer. There are no significant changes in the positions or the ratios of the bands over time, indicating that the heme, once reconstituted, is in a stable form. It is not yet clear why the spectrum decreases over time – this may be due to protein precipitation (HtaA is multimeric in solution as shown by NATIVE PAGE). ...................... 180

Figure 5.12  A solution of 5 μM hemin and 5 μM apoHtaA (Peak 2) incubated in 20 mM Tris-HCl and 150 mM NaCl, pH 7.5 for 20 h at 20 °C. Spectra were taken every hour (over 18 h). The Soret is at 405 nm. ........................................................................................................ 181
Figure 5.13  Spectrophotometric pH titration of HtaA (reconstituted Peak 2). The protein, in a buffer that was 20 mM each CAPS, CHES, MOPS and Tris-HCl was titrated with 1.0 M NaOH. Top panel: spectra as a function of pH. Bottom panel: The absorbance at 404 nm vs. pH. The protein shows essentially no sensitivity to pH, at least up to pH 10.5.

Figure 5.14  ClustalW alignment of HtaACR2 domains of *C. diphtheria*, *C. aurimucosum*, *C. jeikeium* and *C. ulcerans*). The conserved Tyr19, His70 and Tyr148 residues are shown in red and green.
1 BIOPHYSICAL CHARACTERIZATION OF MUTANTS OF SIAA PROTEIN
FROM STREPTOCOCCUS PYOGENES

1.1 Introduction.

1.1.1 Iron and heme in bacteria.

Iron is a crucial nutrient for almost all living organisms including bacteria (Wandersman & Delepelaire, 2004; Wilks & Burkhard, 2007; Krewulak & Vogel, 2008; Tong & Guo, 2009; Braun & Hantke, 2011; Nobles & Maresso, 2011). The most common oxidation states of iron under the physiological conditions are (+3) [Fe(III), ($d^5$), ferric] and (+2) [Fe(II), ($d^6$), ferrous]. The broad redox potential range of iron makes it capable of playing important role in various cellular and biological processes, including electron transport, reduction of peroxide, transportation of oxygen, enzyme catalysis, electron transfer, photosynthesis and respiration. Although ferric iron is the most abundant form of iron in the earth’s crust, it has very low solubility and is therefore biologically unavailable (Chipperfield & Ratledge, 2000; Nobles & Maresso, 2011). In addition, the presence of excess iron in cells causes toxicity through hydroxyl radicals that are produced via the Fenton reaction. These reactive radicals target nucleic acids, lipids and proteins (Krewulak & Vogel, 2008; Braun & Hantke, 2011; Nobles & Maresso, 2011). In order to overcome these problems, iron is primarily-found in the protoporphyrin IX macromolecule known as heme (Wilks & Burkhard, 2007; Tong & Guo, 2009; Nobles & Maresso, 2011). It is generally bound to proteins, e.g., transferrin, lactoferrin, ferritin, hemoglobin, haptoglobin, and hemopexin (Tong & Guo, 2009; Krewulak & Vogel, 2008; Braun & Hantke, 2011; Nobles & Maresso, 2011). Heme is the cofactor in hemoglobin (Otto et al., 1992; Wilks & Burkhard, 2007), which is the major source of iron for bacteria infecting humans. In order to fulfill their nutritional requirements, bacteria adopt sophisticated ways to transport heme, into the cytoplasm.
1.1.2 *Streptococcus pyogenes.*

*Streptococcus pyogenes* (group A streptococcus) is a Gram-positive bacterial pathogen causing a wide range of diseases (Cunningham, 2000). Through development of complex virulence mechanisms, it can avoid host defense systems and cause a number of infections. This emerging pathogen can be responsible for diseases as treatable as strep throat to severe deadly infections such as necrotizing fasciitis (“flesh-eating disease”) (Cunningham, 2000). Other clinical manifestations of *Streptococcus* include impetigo, cellulitis, toxic shock syndrome, scarlet fever, septicemia, pneumonia, and meningitis.

*Streptococcus pyogenes* shows increasing resistance to macrolide antibiotics (Bessen, 2009). It does not have the biosynthetic pathway to produce heme; however, it requires heme to be infectious. Therefore, its heme acquisition machinery may be a promising drug therapy target (Skaar et al., 2004; Furci et al., 2007).

1.1.3 *Introduction to heme acquisition systems in bacteria.*

Bacteria can fulfill their need for heme essentially in two different ways (Cavallaro et al., 2008; Wilks & Burkhard, 2007). They can create strategies to take up heme from the host, or produce heme biosynthetically. Some of the bacteria can use both approaches. Cavallaro and co-workers used bioinformatic techniques to characterize heme sources in 474 bacteria (Cavallaro et al., 2008). It was reported that 168 species can use only biosynthesis, 20 species used only heme uptake pathways and 218 species use both methods in order to obtain heme.

In humans, the majority of iron is sequestered in heme and majority of heme found in hemoglobin. Pathogenic bacteria have developed strategies to have access to this major source of iron (Wilks & Burkhard, 2007; Wandersman & Delepelaire, 2004; Nobles & Maresso, 2011).
Proteins in the cell envelope of the bacterial pathogens are involved in heme uptake. The review below outlines a number of these sets proteins.

1.2 ABC transporters.

1.2.1 Overview of ABC transporters.

Heme can be imported into cytoplasm through ABC transporters; the name is derived from ATP-binding cassette transporters (Jones & George, 2004; Davidson & Chen, 2004; Hollenstein et al., 2007; Oldham et al., 2008; Davidson et al., 2008; Stieger & Higgins, 2007; Kos & Ford, 2009; Licht & Schneider, 2011). ABC transporters are integral membrane proteins that convey compounds either into or out of the cell. The energy required for transportation is obtained from ATP hydrolysis. ABC transporters are present in organisms from bacteria to humans. This protein family is divided into 26 prokaryotic and 10 eukaryotic subfamilies; they play roles in different processes such as drug and antibiotic resistance, cholesterol transport and homeostasis (Jones & George, 2004; Davidson et al., 2008; Kos & Ford, 2009; Licht & Schneider, 2011).

Based on their function, ABC transporters can be divided into three subclasses which are importers, exporters, and non-transport proteins (Jones & George, 2004; Davidson et al., 2008; Kos & Ford, 2009; Licht & Schneider, 2011). ABC importers shuttle essential substances into the cell. Importers need binding proteins that bind the substances and transfer them to the surface of the transporters. Exporters transfer unwanted compounds out of the cell. They directly convey substances from inner side of the membrane. Non-transport proteins are not associated with transportation. Instead, they are involved in various cellular processes, e.g., DNA repair, flow of ions and translation of proteins. Prokaryotes contain all three subclasses (ABC importers, ABC
exporters and ABC non-transport proteins). Eukaryotes lack ABC importers but have ABC exporters and ABC non-transport proteins.

Structurally, ABC transporters are formed from two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs) (Jones & George, 2004; Davidson et al., 2008; Kos & Ford, 2009; Licht & Schneider, 2011). The transmembrane domains build the translocation pathway for the compounds. These domains have hydrophobic segments permeating through the lipid bilayers. Sequence similarity among the transmembrane domains is low. In ABC exporters, there are total of ten to twelve transmembrane helices with five to six α-helices in each transmembrane domain (Kos & Ford, 2009). Their sequences and lengths are different. In ABC importers, the number of transmembrane helices varies from ten to twenty, with five to ten in each domain (Hollenstein et al., 2007; Davidson & Maloney, 2007). Transmembrane domains in ABC importers have a conserved sequence, EAA motif or L-loop, which is absent in ABC exporters (Kos & Ford, 2009; Licht & Schneider, 2011). The interaction between the NBD domain and the TMD domain is provided by the L-loop in importers.

The nucleotide-binding domain is the peripheral part of the ABC transporter and appears as an L-shape (Hollenstein et al., 2007; Kos & Ford, 2009). The NBD domains consist of two subdomains, a large RecA-like subdomain and a small helical subdomain. The key role of the nucleotide-binding domain is to bind and hydrolyze ATP. High sequence similarities have been seen among the NBD domains, with several conserved sequence motifs. Mutations in these motifs results in the decrease or elimination of transportation or ATP hydrolysis (Hollenstein et al., 2007; Kos & Ford, 2009; Licht & Schneider, 2011). The first conserved motif is the Walker-A motif, also called the P-loop (phosphate-binding loop). It follows β-strand 3. The function of the Walker-A motif is to bind the nucleotide by forming binding loop around β and γ phosphates of
the ATP (Davidson & Maloney, 2007). The Q loop follows β-strand 6 and connects the RecA-like subdomain to the helical subdomain. The LSGGQ motif is also known as the C-loop, linker peptide or ABC signature motif (Hollenstein et al., 2007; Davidson & Maloney, 2007; Kos & Ford, 2009; Licht & Schneider, 2011). This conserved motif is used as the signature to identify ABC transporters. The LSGGQ motif is located between the Walker A and Walker B motifs. This motif contacts the nucleotide in the ATP-bound state. β-Strand 7 is formed by the Walker B motif. It has several hydrophobic residues and a terminal aspartate residue coordinates a Mg$^{+2}$ ion at the ATPase active site. The C-terminal side of the Walker B motif has the D-loop which forms a contact interface between the two nucleotide-binding domains. B-Strand 8 is followed by the H motif, also known as the switch motif. The role of the H motif is to form a hydrogen bond with the γ-phosphate of ATP through a conserved histidine residue.

1.2.2 ABC transporters associated heme acquisition systems in Gram-negative bacteria.

Much of our current understanding of heme uptake in bacteria is based on studies done on Gram-negative bacteria (Cescau et al., 2007; Wilks & Burkhard, 2007; Tong & Guo, 2009; Nobles & Maresso, 2011; Braun & Hantke, 2011). In the Gram-negative bacterial system, the single outer membrane receptor (located on the bacterial surface) binds heme or heme proteins (hemopexin, hemoglobin, and hemoglobin-haptoglobin). In some of the Gram-negative species, hemophores, which scavenge heme from the extracellular environment, bring heme to the cell surface receptor proteins (Wandersman & Delepelaire, 2004; Cescau et al., 2007). The TonB/ExbB/ExbD complex acts as a membrane receptor to carry heme to the periplasmic binding protein, which transfers heme between the outer and inner membranes. Heme is then shuttled into the cell through ABC transporter, where heme is first bound to the lipoprotein component of
the ABC transporter, next passed to a permease to carry it across the membrane, and finally taken up inside the cytoplasm. The ATPase of the ABC transporter provides energy for this process. Heme oxygenase binds heme and degrades it into biliverdin, carbon monoxide and iron.

1.2.3 ABC transporters associated heme acquisition systems in Gram-positive bacteria.

Unlike the Gram-negative bacteria, Gram-positive bacteria do not have an outer membrane. However, they have a thick peptidoglycan layer which can be as thick as 50–100 nm in diameter (Giesbrecht et al., 1998; Wilks & Burkhard, 2007; Tong & Guo, 2009; Nobles & Maresso, 2011; Braun & Hantke, 2011; Schneewind & Missiakas, 2012). Thus, the acquisition and transportation of heme in this bacterial system through the thick layer is important. Frontier work to understand heme uptake mechanism in Gram-positive bacteria was done on the Corynebacterium diphtheriae. Additional researches on streptococcus, staphylococcus, bacillus and listeria revealed that similar heme transfer system is used by Gram-positive bacteria which contain ABC transporters to shuttle heme across the cytoplasmic membrane. Differences from the Gram-negative bacteria arise from the number and structure of the several membrane anchored heme transfer proteins which provide stepwise transportation of heme across the thick cell wall. The focus of this dissertation is heme uptake. Here, we give an introduction to this system, followed by descriptions of heme uptake pathways in other bacteria.

In S. pyogenes, heme is imported through an ATP binding cassette pathway (an ABC transporter) (Bates et al., 2003) (Figure 1.1). It is termed as the streptococcal iron acquisition or Sia system. In this ABC transporter SiaA (spy1795) is the lipoprotein, SiaB (spy1794) is the membrane permease and SiaC (spy1793) is the ATPase. SiaA system is also known as HtsA (heme transport S. pyogenes) (Lei et al., 2003). SiaA acquires heme and transfers heme to the
SiaB which carries it across lipid bilayers. In this process used energy is provided from ATP hydrolysis by SiaC which is located in the inner side of the membrane. Sia ABC transport system is the part of a ten gene cluster (Figure 1.2) from the thick cell wall of *S. pyogenes*. Two upstream genes, Shr and Shp (streptococcal heme-associated protein (Lei et al., 2002)), are involved in bringing heme to the Sia ABC transporter in a stepwise fashion. Downstream of the ABC transport genes, the cluster contains five additional genes with as yet unknown function.

Shr is the first gene in the ten gene operon of Sia heme up take system (Bates et al., 2003). It is a large hydrophilic protein with molecular weight of 145 kDa comprised of 1,275 amino acid residues. Shr is a hemoprotein binding receptor, so named for streptococcal hemoprotein receptor. Hemoglobin, myoglobin, heme-albumin and haptoglobin complex directly bind Shr; in contrast, apo-haptoglobin does not bind.

Shr has functional domains at the N-terminal end and shows no significant homology to the other heme receptor proteins (Bates et al., 2003; Ouattara et al., 2010). It has a leader peptide (LP), two domains with unknown function (DUF), two NEAT (NEAr Transporter) domains which are separated by EF-hand motif (EF) and a leucine-rich repeat region (LRR) and a transmembrane domain (TM) at the C-terminal end (Andrade et al., 2002; Bates et al., 2003; Ouattara et al., 2010).

Shr does not have conserved motifs similar to Isd system of *S. aureus* e.g, the LPXTG or the QVPTG motifs which covalently attach proteins using sortases to the cell wall at the C-terminal end (Fisher et al., 2008). Instead, a hydrophobic segment and a positively charged tail are present in the C-terminus of the Shr and anchor protein to the cytoplasmic membrane. Shr is exposed to the extracellular environment suggesting that it plays role as an adhesin due to binding of the extracellular matrix (ECM) proteins fibronectin and laminin. In a recent study,
Ouattara et al. showed that both NEAT domains of the Shr bind heme using the methemoglobin as a main heme source (Ouattara et al., 2010). Hemin binding studies to the Shr showed that this protein auto reduces the heme iron. Upon initial addition of heme to the Shr under aerobic conditions, a Soret band was initially seen at 414 nm (the ferric form of heme). Additional hemin aliquots resulted in appearance of the peaks at 427, and at 540 and 564 nm indicating the reduction of the iron. These results were consistent with previous reports in which mixture of ferric and ferrous iron heme bound isolated Shr (Zhu et al., 2008a).

Shr is the heme source for the Shp protein in the Sia heme acquisition system (Zhu et al., 2008a). Heme transfer studies showed that holoShr transferred its heme to apoShp more efficiently than as it lost to apoSiaA. These results suggested that the heme acquisition in the Sia system is a stepwise process which includes transfer of heme from Shr to Shp. In addition Zhu et al. showed that methemoglobin is the direct heme source for Shr but not the immediate heme source for Shp (Zhu et al., 2008a).

Shp is the second gene in the ten gene operon of heme up take system of Sia (Lei et al., 2002; Bates et al., 2003). Shp binds one heme for per Shp. Four peaks (at 275, 370, 417, 530, and 560 nm) appeared at the UV-visible spectrum of Shp (as purified) which consistent with heme-binding proteins. Shp has a secretion signal at the N-terminal end and a transmembrane domain and a charged tail at the C-terminal end. Shp does not have LPXTG motif.

An X-ray crystal structure of Shp (2.1 Å) revealed (Aranda et al., 2007). Shp (heme binding domain) has eight β-strands and one α-helix. It forms a β-sandwich fold in which eight β-strands are divided into three and four-stranded antiparallel β-sheets. Shp has a bis-methionine axial ligation where Met66 is located on the α-helix and Met153 is located between the between β-strands B7 and B8. Two exogenous hemins are present in the Shp, in the crystal structure.
These hemins form a dimer at the interface of two Shp molecules. Full Shp and as well as the heme binding domain of Shp are each monomeric in solution as shown via gel filtration and analytical ultracentrifugation studies.

Shp transfers heme to SiaA protein which is the lipoprotein from ABC transporter component of the Sia system (Liu & Lei, 2005; Nygaard et al., 2006; Ran et al., 2007). Liu and Lei showed that apoShp takes up heme from hemoglobin (Liu & Lei, 2005). HoloShp efficiently transfers heme to apoSiaA, via complex formation (Liu & Lei, 2005; Nygaard et al., 2006). Ferric holoShp binds apoSiaA with a $K_d$ of $48 \pm 7 \mu$M and ferrous holoShp binds apoSiaA with a $K_d$ of $120 \pm 18 \mu$M (Nygaard et al., 2006). The heme transfer from the oxidized Shp to apoSiaA is slower than that of the reduced Shp. The rate constants for heme transfer are $28 \pm 6 \text{s}^{-1}$ and $43 \pm 3 \text{s}^{-1}$ from oxidized Shp to apoSiaA and reduced Shp to apoSiaA, respectively.

Ran et al. showed that heme binding domain of Shp transfers heme to apoSiaA in the same mechanism as seen for full Shp (Ran et al., 2007). The ferric heme binding domain of holoShp binds apoSiaA with a $K_d$ of $90 \pm 5 \mu$M and ferrous heme binding domain of holoShp binds apoSiaA with a $K_d$ of $107 \pm 12 \mu$M. The rate constants for heme transfer were $2.9 \pm 0.9 \text{s}^{-1}$ and $4.4 \pm 0.4 \text{s}^{-1}$ from oxidized heme binding domain of Shp to apoSiaA and reduced heme binding domain of Shp to apoSiaA, respectively. Ran and coworkers were also studied the full length Shp with alanine mutants of axial Met66 and Met153. Full length ferric Shp with M66A mutant bound apoSiaA with $K_d$ of $11.4 \pm 0.3 \mu$M and full length ferric Shp with M153A mutant bound apoSiaA with $K_d$ of $170 \pm 8 \mu$M indicating the critical role of Met153 in heme transfer. It was also reported that association constant of hemin binding to WT Shp, mutants of M66A and M153A were 5,300, 22,000, and 38 µM$^{-1}$, respectively. Combination of kinetic data on full length Shp and heme binding domain of Shp were interpreted as a transfer of heme from Shp to
SiaA where axial ligands of Shp concurrently left their place to groups from SiaA upon complex formation.

SiaA (HtsA) is a small protein with 37 kDa molecular weight and 294 amino acid residues (Bates et al., 2003; Lei et al., 2003). It is the first gene of the ATP binding cassette of Sia system where heme is transferred through the membrane. SiaA (as isolated) had strong red color which was indication of heme binding (Lei et al., 2003). The UV-visible spectrum of SiaA confirmed this with appeared peaks at 275, 370, 417, 530, and 560 nm, appropriate for heme binding proteins. Bates et al. showed that SiaA could bind hemoglobin; in contrast, it could not bind myoglobin or the hemoglobin-haptoglobin complexes (Bates et al., 2003).

Conformational stability studies of SiaA, using GdnCl as a denaturant, showed that midpoints were $3.1 \pm 0.1$ M for the oxidized protein and $5.0 \pm 0.1$ M for the reduced protein (Sook et al., 2008).

Resonance Raman (rR) studies showed that both oxidized and reduced SiaA have a six-coordinate and low-spin heme (Sook et al., 2008). The ferrous SiaA did not bind the CO(g), most likely due to the highly stable six coordinate form of the heme. Spectrophotometric pH titration data gave a pKa value of $9.7 \pm 0.1$ for SiaA which could be due to the deprotonation of the axial histidine.

Results from NMR, MCD, mutagenesis, UV-visible, EPR and resonance Raman studies suggested that axial ligands of SiaA are methionine 79 and histidine 229 (Sook et al., 2008; Ran et al., 2010). Ran et al. mutated all five histidine residues (H24, H229, H251, H289, and H293) from SiaA sequences into alanine (Ran et al., 2010). The mutants H24A, H251A, H289A, and H293A showed very similar UV-visible spectrum to WTSiaA. In contrast, the absorption spectrum of ferric H229A mutant showed a10 nm blue shift (from 412 to 402) in Soret peak com-
pared to WT SiaA. In H229A peaks appeared in the far UV region at the 482 and 600 nm which were at 532 and 560 nm in WT SiaA. The ferrous H229A mutant showed a Soret with reduced intensity and a lack of the main α-band in the 560 nm region. It was concluded that the heme is not hexacoordinated to two axial ligands in H229A. Thus overall results suggested that H229 is one of the axial ligands of the SiaA protein. In SiaA sequence there are six methionine residues (79, 126, 173, 216, 238, and 270) which were mutated into alanine. Only UV-visible spectrum of the M79A mutant was different than the WT SiaA. The reduced M79A showed a Soret peak with reduced intensity and no α- and β- bands appeared in the UV spectrum. These data are consistent with a heme that is not hexacoordinated. The oxidized M79A mutant had UV spectrum with a Soret peak at 402 nm (in place of 412 in WTSiaA) with a larger intensity. In addition, M79A showed peaks at 498, 536 and 630 nm. Overall, the data were consistent for the WT protein with a hexacoordinated heme with histidine and water axial ligands in oxidized M79A.

The relative heme affinity of WTSiaA, H229A, and M79A was WTSiaA > M79A > H229A as shown by heme transfer experiments from holo SiaA to H64Y/V68F apomyoglobin. Sun et al. showed the same pattern of heme affinities of WTSiaA and axial ligand mutants for heme (WTSiaA > M79A > H229A) via acid-induced denaturation studies (Sun et al., 2010).

The secondary structure of the WTSiaA, M79A and H229A changed upon heme binding to proteins as shown via fluorescence and CD experiments (Sun et al., 2010). Fluorescence quenching at 340 nm was observed upon heme binding. ApoSiaA had 91.5% α-helix and 7.5% β-sheet calculated using qualitative CD analysis. The percentage of the α-helix dropped to 56.5% while percentage of the β-sheet (9.8%) remained similar in holoSiaA. Percentages of the α-helix were 85.4% and 96.9% for apoM79A and apoH229A, respectively. In addition, the percentages of the β-sheet were 0% and 3.1% for apoM79A and apoH229A, respectively. Overall, the data
indicated that heme binding to WTSiaA results in some change of conformation in the protein. The secondary structure of the protein did not show major changes with mutations of M79 and H229 into alanine. In contrast, heme bound states of M79A and H229A mutants showed increase in percentage of α-helix and decrease in percentage of β-sheet.

1.3 Related heme acquisition systems with homologous heme binding proteins.

1.3.1 Shigella dysenteriae.

*Shigella dysenteriae* is a Gram-negative intestinal pathogen causing bacillary dysentery, characterized by bloody diarrhea. The *Shigella* heme uptake (shu) locus has eight genes (Mills & Payne, 1995; Mills & Payne, 1997; Wyckoff et al., 1998; Payne & Mey, 2004; Payne et al., 2006; Wyckoff et al., 2009). Four of these, shuTWXY, are co-transcribed. The shuUV genes are downstream and the ShuA and ShuS genes are upstream (Wyckoff et al., 1998). There are promoter regions next to four of these proteins P1; ShuA, P2; ShuS, P3; ShuT and P4; ShuU.

ShuA protein is an outer membrane receptor and weighs 70 kDa (Mills & Payne, 1995; Mills & Payne, 1997). It takes up heme through the TonB-dependent pathway and transports it across the outer membrane. Mutation of ShuA resulted in *S. dysenteriae* that lost the ability to use heme, indicating that ShuA is vital for heme transportation in the shu system (Mills & Payne, 1997). The ShuA spectrum showed a Soret peak of ShuA at 413 nm with β- and α-bands appearing at 535 and 565 nm, respectively (Burkhard & Wilks, 2007). Kinetic studies showed that heme was transferred to ShuA from the metHb tetramer \((\text{Fe}^{3+})\) approximately \(10^4\) faster than from oxy-Hb \((\text{Fe}^{2+})\) or metMb. This faster transfer rate for metHb may suggest that this is the possible in vivo heme substrate for ShuA. Site-directed mutagenesis studies showed that H86A and H420A had a decreased efficiency in capturing heme from hemoglobin. The H86A/H420A mu-
tant was unable to capture heme from hemoglobin. These studies indicate that these conserved histidines play a role in heme transportation across the outer membrane. It has been also shown that a 1:1 complex is formed between full-length TonB and WT ShuA or the apoShuA double mutant (Burkhard & Wilks, 2007).

Cobessi et al. have solved the crystal structure of apoShuA at a resolution of 2.6 Å (Cobessi et al., 2010). ShuA is a member of the TonB-dependent transporter (TBDT) class of proteins showing the 3D structure characteristic of this group. The N-terminal plug domain from Thr1 to Gln-135 is surrounded by a 22-stranded transmembrane β-barrel consisting of Ser-136 to Trp-632 from the C-terminal end of the protein. Long extracellular loops and periplasmic turns connect antiparallel β-strands. Acidic and basic residues from the β-barrel (Glu-446 and Asp-503) and the plug domain (Arg-64 and Arg-104) interact to form “the lock region.” The buried periplasmic N-terminal Met-4TVTAT-Gly-10 (TonB-box) structure is well defined. TonB-dependent transporter specific signatures FRAP and NPNL are formed by Phe-406 to Pro-409 and Asn-434 to Leu-437, respectively. The putative heme coordinating residues His-86 and His-420 are about 10 Å apart. His-86 is located on the upper side of the plug domain and His-420 is on loop L7. A complex hydrogen bonding network is present in the protein. In the heme pocket, Gln-228 has hydrogen bond interactions with Gly-58 and also with His-86. Gln-59 is also hydrogen bonded to Arg-43. Hydrogen bonding is seen between TonB-box and the loop containing Arg-43. Cobessi et al. have proposed that the lock region may play role to open the channel in this network (Cobessi et al., 2010).

The 36 kDa cytoplasmic heme binding protein ShuS from Shigella dysenteriae is also part of the heme transfer pathway (Mills & Payne, 1995). ShuS is located directly downstream of ShuA (Wyckoff et al., 1998). ShuS is only expressed (in conjunction with ShuA) under iron lim-
iting conditions (Mills & Payne, 1995). Chromosomal mutation studies on ShuS show that this protein is required for heme utilization (Wyckoff et al., 2005). ShuS binds cellular DNA non-specifically; Wyckoff et al. have proposed that ShuS may function to protect DNA from oxidative damage (Wyckoff et al., 2005). ShuS is over-expressed under high heme concentrations perhaps indicating that it could play role in preventing heme toxicity (Wyckoff et al., 2005; Burkhard & Wilks, 2007). It is also known to bind DNA, which has also been speculated to be involved in protecting the cell from heme toxicity (Kaur & Wilks, 2007).

ShuS was shown by CD spectroscopy to be composed of approximately equal amounts of α–helix, β-sheet and random coil; no significant secondary structure change was observed upon heme addition. ApoShuS had a maximum at 337 nm in the fluorescence spectra. Upon addition of heme, the peak decreased in intensity and blue-shifted to 330 nm, indicating a change in the tertiary structure. Heme-reconstituted ShuS has a Soret peak at 410 nm (159 mM$^{-1}$ cm$^{-1}$) and β- and α- bands appearing at 550 and 575, respectively (Wilks, 2001). The ferrous heme-ShuS complex was formed by adding dithionite to a CO-saturated sample. This sample was unstable, however; the ferrous complex autoxidized to the ferric heme-ShuS complex in 10 min. Based on absorption spectroscopy data, ShuS binds the heme with a binding constant ($K_d$) of 13 µM.

ShuT is the heme binding protein of the ABC transport system of the ShuTUV from *Shigella dysenteriae* (Eakanunkul et al., 2005). Transportation of heme from ShuT to ShuS and ATP hydrolysis are coupled in this system based on functional analysis (Burkhard & Wilks, 2008). ShuT has five-coordinate high spin heme with an anionic O-bound proximal ligand (Eakanunkul et al., 2005). Spectroscopic studies using resonance Raman (rR), magnetic circular dichroism (MCD), and UV-visible techniques confirm that Tyr-67 (in the sequence GYWKQ) is the only axial ligand of the heme. Tyr-201 (in the sequence HQQYKS) is also in the heme pock-
et and has a significant influence on the stability of holoShuT. The heme transfer rate of WT ShuT to apoMb is $4 \times 10^{-5}$ s$^{-1}$. These rates are 0.59 s$^{-1}$ and 0.0174 s$^{-1}$ for the Y67A and Y201F mutants, respectively, consistent with the proposed roles of the two tyrosines.

Ho et al. have solved the crystal structure of apoShuT and partially heme-bound ShuT with 2.0 Å resolution (Ho et al., 2007). Each ShuT unit cell has four molecules. Crystallization in the presence of hemin gives a structure with three ShuT in the unit cell without heme and the fourth ShuT partially heme loaded. Both the apo and partially heme-loaded forms have a bilobed structure with a connecting α-helix. Each lobe has a five-stranded β-sheet flanked by α-helices. The axial ligand of the heme is Tyr-67. Its orientation is different in the apo and partially heme-loaded forms of ShuT. The axial ligand Tyr-67 shows flexibility in orientation in the apo form of the protein. There is no significant conformational change in the protein due to heme binding except in the heme binding pocket.

1.3.2 Pseudomonas aeruginosa.

*Pseudomonas aeruginosa* is a Gram-negative bacterium and an opportunistic pathogen causing variety of infections including skin infections, bloodstream infections, urinary tract infections and surgical site infections (Ochsner et al., 2000; Driscoll et al., 2007). Antibiotic resistance to this organism is increasing (Mesaros et al., 2007; Driscoll et al., 2007; Bassetti et al., 2008; El Solh & Alhajhusain, 2009; Lister et al., 2009). The phu (pseudomonas heme uptake) locus has seven genes which are under the control of ferric uptake regulator (Fur) protein (Vasil, 2007; Cornelis, 2010). The system is comprised of the phuSTUVW operon and a phuR gene (Ochsner et al., 2000; Tong & Guo, 2009). The phuR gene encodes an outer membrane receptor protein. PhuT is the periplasmic component of the ABC transport system, PhuU is the mem-
brane permease and PhuV is the ATPase; the function of PhuW is not known. PhuS is a cytoplasmic heme binding protein.

The phuR gene encodes an outer membrane receptor protein that weighs 82 kDa (Ochsner et al., 2000). PhuR has 25 aa signal sequence which is characteristic of exported proteins. Also, PhuR has an N-terminal TonB box motif (STVSVQTR) and two distinct C-terminal motifs (LNAGLYNLTDKKY and LNAGLYNLTDKKY) that are typical of outer membrane receptor proteins. It shows homology to heme and hemoglobin outer membrane receptor proteins from other organisms such as ShuA from *S. dysenteriae*, HutA from *V. cholerae*, ChuA from *E. coli O157:H7*.

The Soret band of periplasmic PhuT was at 400 nm, with β- and α-bands at 500 and 534 nm, respectively (Tong & Guo, 2007). A metal-ligand charge transfer band appeared at 624 nm. After addition of dithionite, the Soret band was red shifted to 420 nm, while the β- and α-bands were red shifted to 560 and 589 nm, respectively. The metal-ligand charge transfer band disappeared upon addition of dithionite. EPR results indicated that PhuT has five-coordinate high spin ferric heme. Calculations based on fluorescence data gave $K_d$ values of 1.2 nM and 14 nM for binding of heme and protoporphyrin IX, respectively. Conformational change upon heme binding was observed via CD.

The crystal structure of holoPhuT shows two globular domains connected through a single helix (Ho et al., 2007; Liu et al., 2008a). The heme is coordinated to a single tyrosine residue (Tyr-71) and located in a narrow N- and C-terminal binding domain. The structures of apoPhuT and holoPhuT are similar; only minor changes are seen in heme binding pocket. Arg-228 and Arg-73 have H-bonding interactions with one of the propionate groups of heme; Arg-73 is also hydrogen bounded to the axial ligand Tyr-71.
PhuS is a cytoplasmic heme binding protein of molecular weight 39 kDa (Lansky et al., 2006; Block et al., 2007). PhuS is located on the inner side of the cell and functions to store heme and pass it to heme oxygenase. Heme reconstituted PhuS has Soret band at 410 nm (122 mM\(^{-1}\) cm\(^{-1}\)) at pH 8.0 and \(\beta\)- and \(\alpha\)-bands appearing at 545 and 570 nm, respectively (Lansky et al., 2006). The heme binding constant \((K_d)\) of PhuS was measured to be 0.18 ± 0.01 \(\mu\)M. Spectrophotometric pH titration of ferric PhuS gave two pK\(_a\) values, 7.2 ± 0.1 (pK\(_{a1}\)) and 10.2 ± 0.5 (pK\(_{a2}\)). Later Block et al. showed that PhuS could be present in monomeric, dimeric, or mixed states (Block et al., 2007). Monomeric PhuS has Soret maximum at 411 nm while Q-bands appear at 540 and 580 nm. Dimeric PhuS has Soret maximum at 413.5 nm and its Q-bands appear at 540, 566 and 636 (charge transfer band) nm. Each monomer of PhuS binds one heme. The heme in monomeric PhuS has His-209 as the axial ligand. Mutation of this residue to alanine resulted in a protein with an alternate heme axial ligand, His-212. The double mutant H209A/H212A binds heme as a five-coordinate hydroxide complex; thus, that absence of histidine ligands does not prevent heme from binding. The mechanism of transfer from PhuS to the heme oxygenase in this system has been studied (Bhakta & Wilks, 2006; Kaur et al., 2009; O’Neill et al., 2012).

1.3.3 *Staphylococcus aureus*.

*Staphylococcus aureus* is a Gram-positive bacterium causing a variety of diseases including bacteremia, endocarditis, and toxic shock syndrome (Corey, 2009; Boucher et al., 2010). The observation of methicillin resistance is an increasing health concern (Boucher et al., 2010). *S. aureus* has two uptake systems, the Isd system (iron-regulated surface determinant) and the Hts system (heme transport system) to acquire iron from heme and hemoproteins (Mazmanian et
al., 2003; Skaar et al., 2004; Reniere et al., 2007; Haley & Skaar, 2012). The Isd system has been studied in considerable detail. The Hts system is encoded by three genes (HtsABC) that comprise an ABC transporter (Skaar et al., 2004). HtsA is believed to be the lipoprotein component of the system, with a role similar to IsdE in the Isd pathway (Grigg et al., 2007a). HtsA shows low sequence homology to IsdE and key residues that are associated with heme binding in IsdE are absent in HtsA. Thus, Grigg et al. have suggested that if HtsA is the heme binding lipoprotein of the system, it binds heme with a different set of axial ligands than does IsdE (Grigg et al., 2007a).

The Isd operon has ten genes which are regulated by the iron-dependent regulatory protein Fur (ferric uptake repressor protein) (Mazmanian et al., 2003). The IsdABCH genes encode cell wall anchored proteins, the IsdDEF genes encode transmembrane proteins; the IsdGI genes encode cytoplasmic proteins, and the SrtB gene encodes a sortase (Skaar & Schneewind, 2004).

There are two sortases (srtA and srtB), in the isd heme acquisition system, which are responsible for cell-wall anchoring of IsdA, B, C and H (Mazmanian et al., 1999; Mazmanian et al., 2002; Skaar & Schneewind, 2004; Schneewind & Missiakas, 2012). Sortase A and sortase B immobilize surface proteins to the peptidoglycan layer through covalent and non-covalent linkages. In the isd system, cell-wall anchoring proteins have a C-terminal sorting signal, consisting of an LPXTG or NPQTN motif, a hydrophobic domain and a positively charged tail (Maresso & Schneewind, 2006). Sortases, functioning as membrane anchored transpeptidases, cleave surface proteins at the sorting signal and link them to the peptidoglycan layer. In the isd system of S. aureus, IsdA, IsdB, and IsdH (also known as HarA) are anchored to the cell wall at the LPXTG motif by sortase A while IsdC (buried in the cell wall) is immobilized at the NPQTN motif by
sortase B. Inactivation of srtA and srtB reduced the heme uptake and virulence of this pathogen (Mazmanian et al., 2003).

In the *S. aureus* system, exposed receptors IsdB and IsdH capture heme and transfer it to IsdA (which may also acquire heme from external heme-proteins) (Mazmanian et al., 2003; Skaar & Schneewind, 2004; Grigg et al., 2010). Heme is then transferred to the cell-wall buried protein IsdC (IsdC is unique in the Isd system that can bind also PPIX as isolated (Pluym et al., 2007b)) and further transferred to the transmembrane proteins of the system. Deletion of any of the four surface proteins (IsdB, IsdH, IsdA and IsdC) reduces the ability of *S. aureus* to bind heme (Mazmanian et al., 2003). Each of these surface proteins has a secretion signal, a sortase anchoring motif, and one to three NEAT domains.

The NEAT (near iron-transport) domains have about 120 conserved residues (Grigg et al., 2010; Andrade et al., 2002). The NEAT domain fold consists of eight β-strands and an α-helix. X-ray crystal structures of holo IsdA-N1 (1.9 Å) (Grigg et al., 2007b), holo IsdB-N2 (1.45 Å) (Gaudin et al., 2011), holo IsdC-N1 (1.5 Å) (Sharp et al., 2007) and holo IsdH-N3 (1.9 Å) (Watanabe et al., 2008) have been solved. Apo IsdC-N1 (Villareal et al., 2008) and apo IsdH-N1 (Pilpa et al., 2006) structures were revealed using NMR, in addition to, apo crystal structures of IsdA-N1 (1.6 Å) (Grigg et al., 2007b) and IsdH-N3 (2.2 Å) (Watanabe et al., 2008). The NEAT domain structures align well (Grigg et al., 2010). The three holo NEAT domain crystal structures showed the largest differences in the loop regions and the terminal ends. Grigg et al. have noted that IsdC-N1 has five additional residues right before the heme coordinating residue which may control heme transfer from IsdC to IsdE (Grigg et al., 2007b).

Heme (five coordinate ferric heme) binds to IsdA-N1, IsdB-N2, IsdC-N1, and IsdH-N3 via an axial Tyr (Vermeiren et al., 2006; Grigg et al., 2007b; Sharp et al., 2007; Watanabe et al.,
In these domains, four residues downstream of the axial ligand, there is another Tyr residue that H-bonds to the axial Tyr and has a \( \pi \)-binding interaction with the pyrrole ring of the heme. All of these domains have a conserved Ser residue which forms a hydrogen bond with the heme propionate. Several hydrophobic contacts with the porphyrin ring are present in these structures. For example, there is conserved \( \pi \)-stacking interaction between the pyrrole ring and a neighboring Tyr87 (IsdA-N1) or Phe. Also, a Trp113 (IsdA-N1) is present next to a vinyl group of the porphyrin ring.

The techniques of site directed mutagenesis, NMR, surface plasmon resonance and enzyme-linked immunosorbent assays have been used to analyze hemoprotein-NEAT domain interactions (Grigg et al., 2010). IsdH, with three NEAT domains, has been the focus of considerable study. IsdH-N1 and IsdH-N2 can bind hemoglobin or haptoglobin, but not heme (Pilpa et al., 2009). In contrast, IsdH-N3 can only bind heme. Gel filtration and analytical ultracentrifugation studies show that a 2:1 complex is formed between IsdH-N1 and metHb (Pilpa et al., 2006). The full protein IsdB binds hemoglobin (Torres et al., 2006; Dryla et al., 2007) and the hemoglobin-haptoglobin complex, but not haptoglobin alone (Dryla et al., 2007). Homology studies of IsdB with IsdH indicate that IsdB-N1 shows high similarity to IsdH-N1 and IsdH-N2, binds hemoglobin. IsdB-N2 is highly similar to IsdH-N3, binds heme (Grigg et al., 2010).

Kinetic studies have showed that heme is transferred from metHb to IsdB with a \( k_{\text{obs}} \) of 0.31 \( \text{s}^{-1} \) (Zhu et al., 2008b). Heme may be transferred from IsdB-N2 to IsdH-N3 or vice versa (Muryoi et al., 2008). The heme transfer rate from IsdB to apo IsdA is 114 \( \text{s}^{-1} \) and to apo IsdC is 15 \( \text{s}^{-1} \) (Zhu et al., 2008b). Holo IsdA forms a complex with apoIsdC and transfers heme to IsdC with a rate of 54.3 ± 1.8 \( \text{s}^{-1} \) (Liu et al., 2008b; Villareal et al., 2011). IsdB-N2 or IsdH-N3 direct-
ly transfer heme to IsdE (Muryoi et al., 2008). IsdC also transfers heme to IsdE with a rate of 0.0062 s\(^{-1}\) (Zhu et al., 2008b). Grigg et al. have noted that heme transfer rates of cell-wall anchored NEAT domain-containing proteins are at least 10,000 times higher than the rate of heme dissociation to the solvent, indicating that complex formation activates the transfer of heme between Isd proteins (Grigg et al., 2010; Villareal et al., 2011; Abe et al., 2012).

The ABC transporter component of the *S. aureus* consists of a heme-binding protein (IsdE) and a membrane permease (IsdF). There is no protein in the gene cluster that appears to play the role of the ATPase; it has been proposed that FhuC serves as this component in this and other iron-importing systems in this bacterium (Speziali et al., 2006; Grigg et al., 2010). IsdE is located between the cytoplasmic membrane and the cell wall envelope (Mazmanian et al., 2003). Magnetic circular dichroism data and site directed mutagenic studies on IsdE indicated that methionine and histidine are axial ligands (Pluym et al., 2007a). The crystal structure of the IsdE confirmed that the axial ligands are Met78 and His229 (Grigg et al., 2007a). Approximately 19% of the heme surface area is solvent exposed, and the propionate groups are mainly buried within the protein. Overall, the IsdE structure has two domains. The N-terminal domain is composed of Gly-32 to Arg-138 and has two \(\beta\)-sheets surrounded by \(\alpha\)-helices. The C-terminal domain is composed of Asn-163 to Lys-289 and has five \(\beta\)-sheets surrounded by \(\alpha\)-helices. This bi-lobed structure is connected by an \(\alpha\)-helix from Lys-139 to Lys-162. Several hydrophilic and small hydrophobic residues form a large interface between two lobes. Several hydrophobic residues from the N-terminal domain (Pro-77, Val-96, and Ile-99) and the C-terminal domain (Val-175, Pro-176, Leu-180, Tyr-208, and Ile-270) form the interior binding pocket and have hydrophobic interactions with the porphyrin ring. A complex hydrogen bonding network is present within the binding pocket. His-229 hydrogen bonds to Glu-265 via water. This same Glu also hydrogen
bonds directly with Tyr-61 and Lys-62. Lys-62 hydrogen bonds to a water molecule, which hydrogen bonds to both propionates. One of the propionates hydrogen bonds directly to Val-41 and Ala-42. The second propionate directly hydrogen bonds to Ser-60 and Tyr-61. Thr-40 and Thr-271 also hydrogen bond to this propionate via a single water molecule. Mutation of each axial ligand with alanine decreased heme binding, and a double axial ligand mutant completely eliminated binding (Grigg et al., 2007a). Each IsdE protein bound only one heme ligand as shown by electrospray ionization mass spectroscopy (Pluym et al., 2007a). This study also showed that holoIsdE and apoIsdE have different conformations.

In *S. aureus* Isd system, IsdG and IsdI proteins are heme oxygenases which degrade the heme to biliverdin, carbon monoxide and free iron for further use (Skaar & Schneewind, 2004; Reniere et al., 2007; Reniere & Skaar, 2008).

Although the *S. aureus* system is currently the best characterized of the heme uptake systems, it is worth noting that recent studies indicate that a number of components of the pathway are not required for heme iron utilization in this bacteria (Hurd et al., 2012; Wright & Nair, 2012).

### 1.3.4 *Porphyromonas gingivalis.*

*Porphyromonas gingivalis* is a black-pigmented, Gram-negative bacterium. It is the causative agent of periodontitis (Jenkinson, 2011; Belstrom et al., 2012). The inflammatory disease periodontitis affects the tissues that support teeth (Olczak et al., 2005). *P. gingivalis* has two known heme uptake systems – the *htrABCD/tlr* and *hmu* systems.

The *P. gingivalis* A7436 strain has an *hmu* gene cluster (heme utilization receptor) with genes for HmuY, a heme-binding lipoprotein, HmuR, an outer-membrane receptor, and four oth-
er proteins (Simpson et al., 2000; Olczak et al., 2005; Olczak et al., 2008). Mutants in hmuY and hmuT were slower growing and less effective in binding hemin and hemoglobin than wild-type.

HmuR has been shown to be important in heme and hemoglobin utilization (Liu et al., 2003; Liu et al., 2004; Liu et al., 2006). A series of mutations indicated that H95, H434, and the NPDL motif were important in binding hemin, hemoglobin and the heme-albumin complex (Liu et al., 2006). A three-dimensional homology model indicated that these residues were on the apical or extracellular loops of the barrel structure of this protein. Conserved glutamates have also been proposed to be involved in heme uptake (Olczak, 2006).

More recent characterization in this system has focused on HmuY, a 23-kDa protein (Wojtowicz et al., 2009c; Wojtowicz et al., 2009a; Wojtowicz et al., 2009b). CD and MCD studies indicate that the heme is six-coordinate and low-spin in ferric state and the heme ligands are histidines 134 and 166 (Wojtowicz et al., 2009c). UV-visible spectroscopy showed a Soret peak of HmuY at 411nm (Olczak et al., 2008). Addition of sodium dithionite gave the reduced species with a Soret at 425 nm and β- and α-bands appearing at 559 and 529 nm, respectively (Wojtowicz et al., 2009c). Ferrous HmuY had low spin state. UV-visible spectra showed no 620 nm (indication of high spin) and 695 nm (methionine axial ligand) bands, which were consistent with bis-histidine ligation. The midpoint potential of the HmuY was 136 mV. The X-ray structure of protein confirmed the ligation of two histidines and showed that protein in the crystal is a tetramer with four buried hemes (Wojtowicz et al., 2009a). The secondary structure of HmuY changes little upon heme binding (Wojtowicz et al., 2009a). Chemical denaturation by guanidinium hydrochloride was reversible and unfolding curve of apo- and holoHmuY were similar. The calculated ΔG was 34.1±12.4 kJ/mol for both forms.
In the \textit{P. gingivalis} W50 strain, the \textit{htrABCD/tlr locus} has been identified as a heme uptake system via homology with other Gram-negative ABC transported heme binding proteins (Slakeski et al., 2000). Upstream of \textit{htrABCD} is the \textit{tlr} gene, which encodes a TonB-associated outer membrane receptor.

\subsection*{1.3.5 \textit{Escherichia coli.}}

\textit{Escherichia coli O157:H7} is a Gram-negative bacterial pathogen causing a variety of diseases (Torres & Payne, 1997; Suits et al., 2009). It may infect the intestines to lead diarrhea, hemorrhagic colitis and hemolytic uremic syndrome. When the toxins are transmitted to bloodstream, it may affect other organs causing more serious syndromes such as kidney failure, hemolytic anemia, disorders of central nervous system.

In \textit{Escherichia coli O157:H7} a homologue to SiaA, ChuX, was biophysically analyzed. Resonance Raman spectroscopy of the ferric state indicates that heme is mixture of five-coordinate and high-spin, six-coordinate and low-spin, and six-coordinate and high-spin (Suits et al., 2009). In the ferrous state, the heme is a mixture of five-coordinate and high-spin species (main contributor) with some six-coordinate and low-spin species. The x-ray structure of ChuX shows a dimer with the heme bis-ligated to His-65 and His-98. Absorption spectral analysis shows that ChuX binds heme with 1:1 stoichiometry.

\subsection*{1.3.6 \textit{Bacillus anthracis.}}

\textit{Bacillus anthracis}, the causative agent of anthrax, is a Gram-positive, spore-forming bacterial pathogen (Maresso et al., 2006; Carlson et al., 2009; Honsa & Maresso, 2011). The spores of \textit{B. anthracis} can stay in resting state for many years in environment (Nicholson et al., 2000).
Once host system is infected, the spores are associate with phagocytes and begin to replicate (Dixon et al., 2000; Ruthel et al., 2004). *B. anthracis* may show flu type of symptoms and if it is not treated with an antibiotic at early stages it can cause sepsis, respiratory failure and eventually death (Dixon et al., 1999).

*B. anthracis* requires iron from the host for its rapid replication during infection. The iron acquisition system of *B. anthracis* contains eight genes in an Isd-like operon (Maresso & Schneewind, 2006). The *B. anthracis* has a Fur box which is located on upstream of the genes belong to heme acquisition system (Maresso & Schneewind, 2006; Honsa & Maresso, 2011). The expression of heme acquisition system is regulated by the Fur-like genes. In iron-regulated surface determinants (isd) locus of *B. anthracis* the IsdX1 and IsdX2 genes encode hemophores; the IsdC gene encodes a heme acceptor protein; IsdEFD genes encode ABC transporter proteins; SrtB gene encodes a sortase and IsdG gene encodes a monooxygenase.

The IsdX1 (the smallest gene in the Isd locus) and IsdX2 (the largest gene in the Isd locus) proteins have an N-terminal signal peptide. In contrast they do not have a cell-wall anchoring motif indicating that IsdX1 and IsdX2 are the hemophores secreted by *B. anthracis* into extracellular environment to scavenge heme (Maresso et al., 2008; Fabian et al., 2009; Honsa & Maresso, 2011; Honsa et al., 2011). Both proteins contain NEAT domains (IsdX1 has one NEAT domain and IsdX2 has five NEAT domains) (Gat et al., 2008; Honsa et al., 2011). Purified IsdX1 and IsdX2 bind heme (Maresso et al., 2008). IsdX1 removes heme from hemoglobin and prefers hemoglobin (rather than myoglobin) as heme source. Holo IsdX1 transfers heme to apo IsdC; in contrast, holo IsdC does not transfer heme to apo IsdX1 indicating a heme is transferred directionally from IsdX1 to IsdC (Fabian et al., 2009). IsdX1 also transfers heme to IsdX2. Heme is transferred from holo IsdX1 to apo IsdC and apo IsdX2 with a rate which is about
10,000-fold faster than the loss of hemin into solution. Honsa et al. showed that IsdX2 extracts heme from hemoglobin and transfers it to IsdC (Honsa et al., 2011). In IsdX2 NEAT domains 1, 3, 4 and 5 bind heme; in contrast, NEAT domain 2 does not bind heme. NEAT5 binds heme with the greatest affinity. Even though all NEAT domains could associate with hemoglobin, only NEAT1 and NEAT5 were able to remove heme from hemoglobin. In addition NEAT1, NEAT3 and NEAT4 domains could transfer heme to IsdC.

The Isd locus of \textit{B. anthracis} has IsdC protein (with a single NEAT domain) which functions as a heme receptor (Maresso et al., 2006; Gat et al., 2008). Sortase B covalently attaches IsdC to the cell wall through NPXTG motif. Variations of \textit{B. anthracis} without IsdC or SrtB show deficiency in capturing heme in vitro. IsdC takes up heme from both IsdX1 and IsdX2 proteins indicating its major role in heme acquisition in \textit{B. anthracis}.

Tarlovsky et al. proposed that \textit{B. anthracis} may have alternative mechanisms to import heme through proteins which are not belong to Isd locus (Tarlovsky et al., 2010). \textit{B. anthracis} S-layer (S-layer covers cell surface of bacteria (Fouet, 2009)) protein K (BslK) gene is located at a distant position from the Isd locus; however, it contains one NEAT domain which binds heme. In addition to a single NEAT domain, the BslK protein has three SLH (S-layer homology) domains. Initial studies indicated that BslK is non-covalently associated to cell surface. BslK can transfer heme to the IsdC protein indicating the Isd system is able to receive heme from various sources. A second non-Isd locus protein (encoded by BAS0520) with NEAT domain has been also found (Tarlovsky et al., 2010).
1.4 Summary.

The following chapter is the direct copy of manuscript on mutants of SiaA protein which was submitted for publication: Neval Akbas, Darci R. Block, Brian R. Sook, Yau Fong Chan, You Zhuo, Zehava Eichenbaum, Kenton R. Rodgers, and Dabney W. Dixon.

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

Expression, purification, SDS-PAGE, NATIVE-PAGE, equilibrium time determination experiments for WT SiaA (transition region), C58A (pre-transition and transition region), C47A (transition region) as well as the protein denaturation experiments of C47A (single cuvette), C58A (multiple cuvette) and spectrophotometric pH titration experiments for C47A, C58A and K61A were performed by Neval Akbas (Department of Chemistry, Georgia State University).

The expression, purification, and SDS-PAGE of axial ligands (H229A and M79A) and the protein denaturation experiments (single cuvette) were performed by Brian Sook. Mutation of the C47A and C58A was done by Kyle Chan (Department of Chemistry, Georgia State University) as well as the expression, purification, and SDS-PAGE of C58A and the protein denaturation experiments of C58A (single cuvette). Mutation of the K61A was done by You Zhuo (Department of Chemistry, Georgia State University) as well as the expression, purification, and SDS-PAGE of K61A and the protein denaturation experiments of K61A (single cuvette). Resonance Raman spectroscopy and electrochemistry experiments for C58A & K61A mutants were done by Professor Kenton Rodgers and Darci Block (North Dakota State University).

Mutation of the M79A and H229A was done by Dr. Zehava Eichenbaum and Armrita Nargund (Department of Biology, Georgia State University). Mass spectrometry was performed by Dr. Siming Wang (Department of Chemistry, Georgia State University), analyzed by Neval
Akbas, Brian Sook, Kyle Chan, Joy Zhou and Dr. Dabney Dixon. Homology studies were performed by Neval Akbas, Brian Sook, Kyle Chan, You Zhuo and Dr. Dabney Dixon.

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Figure 1.1  Heme uptake system in *S. pyogenes*.

Figure 1.2  *S. pyogenes* *sia* gene cluster.
1.5 Reference List


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2 HEME-BOUND SIAA FROM *STREPTOCOCCUS PYOGENES*: EFFECTS OF MUTATIONS AND OXIDATION STATE ON PROTEIN STABILITY

2.1 Introduction.

Iron is a key nutrient for many bacteria. Iron(III), however, has very low aqueous solubility (Chipperfield & Ratledge, 2000; Crumbliss & Harrington, 2009). In the human body, a significant majority of the iron occurs as the soluble heme cofactor (Wilks & Burkhard, 2007; Tong & Guo, 2009). Therefore, strategies to use heme as a source of iron are key to the survival and virulence of many bacteria (Wandersman & Delepelaire, 2004; Wilks & Burkhard, 2007; Krewulak & Vogel, 2008; Tong & Guo, 2009; Anzaldi & Skaar, 2010; Mayfield et al., 2011; Braun & Hantke, 2011; Nobles & Maresso, 2011).

Recently a large number of bacteria have been analyzed with regard to their method of obtaining heme: some of the bacteria acquire heme from their environment, some have a biosynthetic pathway to produce the heme, and some use both approaches (Cavallaro et al., 2008). Using bioinformatics techniques, Cavallaro et al. found that approximately 5% of the species use heme uptake only, 30% use biosynthesis only, 50% use both pathways and 15% use neither. Those which use heme uptake only are of particular interest because, absent a biosynthetic pathway, they may be susceptible to 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 take up heme being pathogenic (Cavallaro et al., 2008).

*Streptococcus pyogenes* is one of the comparatively small number 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 (Cunningham, 2008).
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 (Eichenbaum et al., 1996). *S. pyogenes* is increasingly resistant to macrolide antibiotics (Gattringer et al., 2004; Szczypa et al., 2004; Bessen, 2009; Villaseñor-Sierra et al., 2012), potentially posing significant risks for infected populations.

In *S. pyogenes*, one pathway for heme import is through an ATP binding cassette. This has been termed the streptococcal iron acquisition or Sia system (Bates et al., 2003) and is also known as Hts (heme transport system) (Lei et al., 2003a). In this ABC transporter, SiaA is the lipoprotein-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 Sia heme transport system is a part of a conserved ten-gene cluster (Bates et al., 2003). The two genes upstream are Shr (Bates et al., 2003; Zhu et al., 2008) and Shp (Aranda et al., 2007; Lei et al., 2002). Shr receives heme from hemoglobin (Ouattara et al., 2010) and transfers it to Shp (Zhu et al., 2008). Shp, which has two axial methionine ligands (Aranda et al., 2007), transfers heme to SiaA (Liu & Lei, 2005) with rate constants that are similar in the oxidized and reduced forms (Nygaard et al., 2006; Hanks et al., 2006; Ran et al., 2007).

Previous biophysical studies on wild type (WT) SiaA in our group showed that methionine and histidine are axial ligands (Sook et al., 2008); further spectroscopic analyses have confirmed these findings (Ran et al., 2010). Homology modeling (Figure 2.1) indicated that the specific axial ligand residues were likely to be M79 and H229 and site-directed mutagenesis studies have verified this (Ran et al., 2010; Sun et al., 2010). In the WT protein, the heme was six-coordinate and low-spin in both oxidation states of the protein (Sook et al., 2008).
An understanding of the factors controlling the steps of the heme uptake and release that constitute the pathways of heme acquisition will benefit from biophysical characterization of the proteins. The main contributors are the nature of the axial ligand, electrostatic interactions with the heme propionates and the iron center and hydrophobic interactions to control heme uptake and release (Schneider et al., 2007; Fufezan et al., 2008; Zheng & Gunner, 2008; Tong & Guo, 2009; Smith et al., 2010; Li et al., 2011). 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 at some distance from the heme. In addition, we expand on recent studies of M79A and H229A (Ran et al., 2010; Sun et al., 2010). Homology modeling suggests that cysteine 58 is near the heme propionates and lysine 61 is close to the propionate that extends from the heme binding pocket. All of the residues near the heme have significant effects on heme binding in SiaA as shown herein by stability studies using guanidine hydrochloride (GdnCl) as a denaturant. We also discuss the nature of protein unfolding, which is not readily reversible for SiaA. The reduction potential of the mutants of SiaA has 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 were probed by spectrophotometric pH titration and resonance Raman (rR) spectroscopy.

2.2 Materials and methods.

2.2.1 Homology modeling.

The homology model of SiaA was built by using I-TASSER, a secondary structure prediction program (Zhang, 2008; Roy et al., 2010). The program chooses ten similar proteins to create the model. IsdE from Staphylococcus aureus (PSI-Blast results for SiaA show 45% iden-
tities to IsdE with 69% positives) was the closest protein; the root mean square difference (RMSD) between the model and IsdE was 1.352. The model was visualized using Pymol (DeLano, 2009).

2.2.2 Materials.

*E. coli* strain Top10 competent cells, ShuAf, ShuAr primers and Top10/pSiaA-His cells were made as described previously (Sook et al., 2008). 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).

2.2.3 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 (Bates et al., 2003). The forward and reverse primers (underlined letters indicate the mismatches) for each mutant are shown in the Table. 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<sub>BAD</sub> as described previously (Bates et al., 2003). Taq PCR Master Mix Kit was used to amplify SiaA DNA segments, and the sequence of the wild type and mu-
tant 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.

2.2.4 *Expression and purification of mutants.*

The proteins were expressed and purified from the appropriate plasmids as previously described (Sook et al., 2008) 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 cooled on ice between lysing cycles. It was centrifuged for 20 min at 35,000 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 (50 mM potassium phosphate, 250 mM NaCl, and 10% v/v glycerol). Unbound material was washed out with 5 column volumes (CV) of binding buffer. C58A was eluted with buffer containing 50 mM potassium phosphate, 250 mM NaCl, 10% v/v glycerol, and 0.5 M imidazole via a 30 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. 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 column volumes of Buffer A. M79A was eluted with 10 column volumes
of Buffer A containing 1 M NaCl via a linear gradient (0 - 100% NaCl).

2.2.5 Mass spectrometry.

The masses of the mutants were evaluated using matrix-assisted laser desorption ionization
(MALDI) mass spectroscopy using an ABI Voyager DE-Pro (Applied Biosystems) MALDI
reflectron time-of-flight spectrometer in positive ion mode. The sinipinic acid matrix was pre-
pared in 30:70 acetonitrile:water and 0.1% trifluoroacetic acid. The sample solution was mixed
with the matrix solution in a 1:1 ratio and the resulting solution spotted onto the MALDI target
plate.

2.2.6 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, Spo-
kane, WA) was used to set the temperature of the cuvette compartment to 25 °C. Quartz black-
masked Supracil cuvettes (Spectrocell, Inc.) with a 1 cm path length were used. The solution was
stirred throughout the spectrophotometric measurement.
2.2.7 *Chemical denaturation studies via titration.*

Refractive index was used to verify the GdnCl concentration in the stock solutions (50 mM Tris-HCl, pH 7.0) (Pace & Scholtz, 1997). The unfolding curves were analyzed using Equation 1 (Pace & Scholtz, 1997):

\[
y = \frac{(y_F + m_F[D]) + (y_U + m_U \exp\left[\frac{m([D] - [D]_{1/2})}{RT}\right])}{1 + \exp\left[\frac{m([D] - [D]_{1/2})}{RT}\right]}
\]

where \( y \) is the absorbance at any point along the fitted denaturation curve, \( y_F \) is the absorbance of the folded state, \( y_U \) 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 GdnCl, \([D]_{1/2}\) is the concentration of GdnCl at the midpoint of the unfolding curve, \( R \) is the gas constant, and \( T \) is the temperature (Kelvin). Kaleidagraph (version 4.01, Synergy Software) was used to fit the data.

Denaturation experiments were performed with the full constructs (including the His tag). Optical absorbance was monitored at the indicated wavelengths for C47A (414 nm), C58A (409 nm), K61A (412 nm), M79A (402 nm), and H229A (403 nm). GdnCl was titrated into each mutant solution which was allowed to reach equilibrium, considered to have been established when the absorbance did not change by more than 0.002 from the previous measurement. For C58A, the sample stood for 17 h between 2.5 and 2.6 M GdnCl.
2.2.8 Chemical denaturation studies with multiple protein samples.

To follow the approach toward equilibrium, solutions of C58A were prepared in 1.46 (expected pre-transition region) and 2.42 M (expected midpoint) guanidine hydrochloride (GdnCl). Spectra were recorded as a function of time at 409 nm for each of these samples. For the unfolding experiment, a series of samples were prepared at the desired concentrations of GdnCl by mixing appropriate volumes of stock GdnCl solution and Tris-HCl buffer (50 mM Tris-HCl and pH 7.0). Final volumes of these samples were 400 µL. A volume of 55 µL of C58A stock solution was added to each sample. The resultant solutions, with initial absorbance of 0.676, were allowed to equilibrate at room temperature (~22 °C) for 3 h. The cuvette was left in the spectrophotometer throughout the measurement and not rinsed between samples, following the literature protocol (Pace & Scholtz, 1997). In each case, the previous sample was removed using a Pasteur pipette and the next sample was added. The spectrum was then recorded for each solution. The spectra of all samples were also recorded after a total of 29 h and 221 h after mixing. The data were fitted using Equation 1.

2.2.9 Spectrophotometric pH titration.

The titrations were run by adding aliquots of 1.0 M NaOH to the protein in a buffer that was 20 mM each of CAPS, CHES and TrisHCl. A 1 cm cuvette was used with a pH electrode (Thermo Scientific Orion 8220BNWP); the sample was stirred. Changes in absorbance in the Soret region were analyzed with Kaleidagraph using Equation 2 (Ying et al., 2009):

\[
y = \frac{y_A \times 10^{(pK_a-pH)}}{1 + 10^{(pK_a-pH)}} + y_B \quad [2]
\]
where $y$ is the absorbance, $y_A$ is the absorbance of the acidic form, and $y_B$ is the absorbance of the basic form.

### 2.2.10 Resonance Raman spectroscopy.

Resonance Raman spectra were recorded at ambient temperature using a 0.67-m spectrophotograph equipped with a 2400 g/mm holographic grating and a LN$_2$ 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 f1 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 for the rR experiment was checked by comparing UV-visible absorbance spectra with those recorded before laser exposure. Spectra were calibrated against the Raman shifts of pure toluene and CH$_2$Br$_2$ and are reproducible to one wavenumber or less.

### 2.2.11 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 the mV mode. 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 H$_2$O-saturated N$_2$ and the absorbance spectrum of the ferric protein was recorded. The heme was then completely reduced by anaerobic addition of 20 mM aqueous S$_2$O$_4^{2-}$ using a 10 μL gas-tight syringe and the absorbance spectrum of the
ferrous heme was recorded. At this point, enough stock of electron transfer dye cocktail (Sook et al., 2008) was added to the protein solution to make it 10 μM in each dye. The resulting solution was titrated with \( S_2O_4^{2-} \) 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 \( S_2O_4^{2-} \). After the second cathodic titration, the reference electrode was calibrated against the quinhydronate 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 3 for both the cathodic and anodic titrations. The resulting midpoint potentials were within 10 mV of one another.

\[
A_{434} = \frac{A_n + A_0 \exp \left( \frac{E - E_m}{0.0257 \text{ V}} \right)}{1 + \exp \left( \frac{E - E_m}{0.0257 \text{ V}} \right)}
\]

[3]

2.2.12 Native polyacrylamide gel electrophoresis.

A Native PAGE Sample Prep Kit from Invitrogen was used with a 5 to 12% acrylamide gel. Samples were run 15 min under 80 V current and 1.5 h under 50 V current. A NativeMark Unstained Protein Standard was used.
2.3 Results.

2.3.1 Spectroscopic studies.

WT SiaA and the C47A, C58A, and K61A mutants were purified as red colored proteins. Native polyacrylamide gel electrophoresis showed that all of these proteins were monomeric. MALDI showed the expected molecular weight for all mutants. Based on a Soret/280 nm ratio for 18% heme loaded WT SiaA (Lei et al., 2003b), the mutants had approximate holoprotein percentages of: 45% (WT SiaA), 40% (C47A), 25% (C58A), and 30% (K61A). The Soret band of the WT SiaA was observed at 413 nm, in accordance with previous reports (Liu & Lei, 2005; Sook et al., 2008). The C47A and K61A mutants were very similar (Figure 2.2). The C58A mutant showed a 4 nm blue shift, with an absorption peak at 409 nm. All three mutants and WT SiaA showed α- and β-bands at about 532 and 567 nm.

In contrast to these three mutants, the two with mutations in the axial ligands (M79A and H229A) showed only a pale yellow color upon isolation, suggesting low heme loading of the protein. This is expected, as the heme is presumably not as tightly bound to the protein. 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 (Sun et al., 2010). The α- and β-band absorptions were weak for M79A; H229A did not show any clear absorption band in this region.

2.3.2 Guanidinium-induced denaturation.

To determine the best time for data analysis, unfolding of C58A was followed at both 1.46 M GdnCl (pre-transition region) and 2.42 M GdnCl (the approximate midpoint of the transition). Figure 2.3 shows that the protein continued to unfold slowly and smoothly over the entire
time of the experiment (23 h) at both GdnCl concentrations. WT SiaA and C47A were also fol-
lowed at their approximate midpoints (3.1 M and 2.95 M GdnCl, respectively). The data could
be fit to single kinetic processes with rate constants of 0.077 and 0.11 h\(^{-1}\) (halftimes of 9 and 7 h)
respectively. The C58A data showed at least two processes. Two exponentials did not give a
unique fit; a range of values for the absorbance change and rate constant of the slower process
gave good approximations of the data. Best approximations showed that the faster process had a
relatively small absorbance change and a rate constant of about 0.5 h\(^{-1}\). The slower process had a
larger absorbance change and a haltime of 30 – 70 h.

Initial experiments were performed with a series of samples of identical protein concen-
tration but varying concentration of GdnCl. We studied C58A in the most detail, first measuring
the samples at 3 h. However, the data at this time point did not show any significant pre- and
post-transition regions and could not be fit to a standard two state unfolding model (Figure 2.4).
We then remeasured the samples after 29 h. The data in this case did fit a two state unfolding
model, with a transition midpoint of 2.3 ± 0.1 M GdnCl. The samples were still heme-colored
after a week and data at 221 h are also shown for comparison. Again, the data fit a two state un-
folding model, with a \(D_{1/2}\) value of 1.7 ± 0.1 M GdnCl. Thus, there was a significant dependence
of the calculated \(D_{1/2}\) on the time that the solutions were allowed to stand, indicating that the sys-
tem was not at equilibrium when absorbance measurements were made at shorter times in these
experiments.

In that the system was not at equilibrium, we turned to a single cuvette titration technique
(Arnesano et al., 1998; Wittung-Stafshede, 1999; Moczygemba et al., 2000; Andersen et al.,
2002; Roncone et al., 2005), which would use far less heme protein per unfolding experiment. In
this titration method, increasing concentrations of GdnCl were added to a single cuvette. After
each addition, the system was allowed to stand until the change in the absorbance at the Soret was less than 0.002 from the previous measurement. The time between measurements generally varied from 10 min to 2 h, with even longer times needed for guanidine concentrations near the mid-point, as expected (Bowler, 2007). Fitting this C58A data set to a two state model gave a midpoint denaturant concentration of $2.4 \pm 0.1$ M GdnCl, within experimental error of the $D_{1/2}$ at 29 h in the multiple sample experiment. The WT, C47A, K61A and M79A proteins were also investigated with the titration technique (Figure 2.5).

The H229A mutant spectrum did not show clear $\alpha$- or $\beta$-peaks. For this protein equilibrium was reached in less than 20 min after each GdnCl addition. This was a substantially shorter time than that required for WT SiaA to reach equilibrium (which in the transition region was well over one hour), 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 GdnCl. After that point, there was a blue shift in the Soret, with the absorbance first increasing and then decreasing. It is assumed that these latter changes involved various nonspecific interactions of the heme with the unfolding protein. Fitting the data from 0 to 1.5 M GdnCl gave a $D_{1/2}$ of approximately 1.1 M.

### 2.3.3 Spectrophotometric pH titration.

For all three mutants, titration with aliquots of 1 M NaOH gave a Soret absorbance that decreased as the pH increased (representative data from C47A are shown in Figure 2.6). The transitions were isosbestic for pH < 11. The absorbance at 280 nm also increased above 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 M$^{-1}$ cm$^{-1}$ at 295 nm) is higher than that of tyrosine (1400 M$^{-1}$ cm$^{-1}$ at 275 – 280 nm) (Atkins et al., 1993)]. At 380 nm, the spectra first decreased and then, above pH 11, increased. This may indicate loss into solution of hemin, which absorbs in this region. Data from pH 7 to approximately 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 (Sook et al., 2008).

2.3.4 Resonance Raman spectra of ferric and ferrous SiaA mutants.

Soret-excited rR spectra were recorded with 413.1 nm emission from a Kr$^+$ laser. The spectra of ferrous and ferric C58A SiaA are shown in Figure 2.9. In contrast to WT SiaA, this mutant is susceptible to photo-induced reduction during spectral acquisition. Consequently, the spectra were recorded with low laser power (3.7 mW), resulting in a modest signal to noise ratio. Nevertheless, the spectra are both 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 or similar 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 2.7. 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 Lys61 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, any electrostatic interaction between K61 and the heme periphery is concluded to be sufficiently weak that its absence does not drive significant changes in propionate conformation. This result is consistent with the insensitivity of the reduction potential (see Results, Section 3.5) to the mutation of K61.

2.3.5 Spectrophotometric redox titration.

In previous work on WT SiaA (Sook et al., 2008), we reported an irreversible midpoint potential of 74 ± 3 mV. Those data have been reanalyzed and shown to yield a reversible potential of 68 ± 3 mV (Figure 2.10). The reanalysis revealed that after oxidative titration, the cathodic titration showed two midpoint potentials. The fraction of the heme having the same midpoint potential reported by the anodic titration was only on the order of 10%. Based on this, we conclude that upon oxidation of the heme, the protein undergoes a change in structure or conformation that is kinetically sluggish to reverse, even after re-reduction of the heme.

In this work, spectroelectrochemical titration of the K61A mutant revealed a reversible potential of 61 ± 3 mV. Absorbance spectra and the absorbance at the ferrous Soret maximum are shown in Figure 2.8. The forward and reverse titrations are superimposable and are well modeled by the Nernstian expression shown in Equation 3. Consistent with the rR spectra of WT and K61A, these reduction potentials indicate that the replacement of lysine 61, 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 $-7 \text{ mV}$, 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 $< 0 \text{ V vs. SHE}$, significantly more negative than that of the WT protein and irreversible (data not shown). 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.

2.4 Discussion.

2.4.1 Guanidinium-induced unfolding.

WT SiaA in the ferric form undergoes guanidinium-induced unfolding with a $D_{1/2}$ of 3.1 M (Sook et al., 2008). 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, very similar to WT at 3.1 M.

The K61A mutant was chosen because homology modeling showed it 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, for which the crystal structure is known (Grigg et al., 2007). In the IsdE structure, K62 hydrogen bonds to a water molecule, which in turn hy-
drogen bonds to both propionates. K62 in IsdE is also involved in a complex network of nonbonded 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 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 (Guallar & Olsen, 2006; Schneider et al., 2007; Fufezan et al., 2008; Zheng & Gunner, 2008; Tong & Guo, 2009; Smith et al., 2010; Li et al., 2011). We note, however, that the Raman data indicate that mutation of lysine 61 to alanine in this protein does not result in significant changes in propionate conformation.

Homology modeling also suggests that C58A is near the heme propionates on the methionine side of the protein. Although there is no expected electrostatic interaction between cysteine 58 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 in this network hydrogen bonds to one of the heme propionates (Grigg et al., 2007). 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 patterns.
The two axial ligand mutants (M79A and H229A) released heme more easily than the other three mutants. The midpoint transition values for M79A and H229A were 1.5 M and 1.1 M, respectively. The M79A mutant was expected to be significantly less stable than WT, because the presumed contribution of the Fe–S
$\text{Met79}$ 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 GdnCl, indicating the importance of this iron-ligand bond in stabilizing the holoSiaA fold. Spectroscopy has indicated that the M79A is hexacoordinate, presumably with a water molecule replacing the methionine (Ran et al., 2010). Finally, the H229A mutant had the lowest D$_{1/2}$ of approximately 1.1 M, consistent with the expected importance of the histidine-heme interaction (the heme is pentacoordinate in this mutant (Ran et al., 2010)). This order of unfolding is consistent with previous studies; Ran et al. found that the extent of heme transfer from holoSiaA to a mutant myoglobin was H229A > M79A > WT (Ran et al., 2010). The acid-induced unfolding showed the same pattern with the H229A mutant losing heme the fastest, followed by the M79A mutant and WT (slow step of a two step process) (Sun et al., 2010). Equilibrium dialysis and inductively coupled plasma mass spectrometry (ICP-MS) experiments indicate that the relative order of binding is WT > M79A > H229A (Sun et al., 2010); Ran et al. have concluded that all three of these proteins have binding constants of $> 10^{12}$ M$^{-1}$ (Ran et al., 2010).

2.4.2 The time scale of protein unfolding.

SiaA and its mutants unfold exceedingly slowly, along a pathway that may involve two or more processes, with an overall half time of many hours. There are limited kinetic studies of heme proteins unfolding in the presence of guanidine hydrochloride. For example, for bovine microsomal cytochrome $b_5$, a bishistidine $b$-type heme protein, the process was biphasic, with a
half time of seconds for the faster process and < 5 min for the slower process at the $D_{1/2}$ of 3 M GdnCl (Manyusa et al., 1999). Horse heart myoglobin shows a similar pattern, with a faster process having a half time of seconds and a slower process with a half time of < 5 min at the $D_{1/2}$ of 1.6 M GdnCl (Moczygemba et al., 2000). Cytochrome $c_{552}$ from *Thermus thermophilus* has a $D_{1/2}$ of approximately 5.5 at pH 7.0; under these conditions, the half-time for unfolding is about a minute (Travaglini-Allocatelli et al., 2003). Cytochrome $c_2$ from *Rhodobacter capsulatus* has a half-time for unfolding of < 10 seconds at the $D_{1/2}$ of approximately 2.0 M GdnCl (Sauder et al., 1996). Cytochrome $f$ from *Chlamydomonas reinhardtii* shows biphasic unfolding, with a fast process (< 5 min) and a much slower process (hours) (Sabahi & Wittung-Stafshede, 2002).

Horseradish and soybean peroxidases unfold slowly. Horseradish peroxidase has a $t_{1/2}$ of a few minutes at 6.0 M GdnCl (pH 7) (Tsaprainis et al., 1998; Kamal & Behere, 2008). This guanidine concentration is considerably above the apparent $D_{1/2}$ (Pappa & Cass, 1993; Moosavi-Movahedi & Nazari, 1995; Tsaprainis et al., 1998). If the usual formalism of linear dependence of the rate of unfolding on the concentration of denaturant applies (Bowler, 2007; Kamal & Behere, 2008) the unfolding of the protein at the $D_{1/2}$ would be expected to be very slow. Soybean peroxidase unfolds approximately 200-fold more slowly than horseradish peroxidase (Kamal & Behere, 2008).

Another approach to understanding the rate of heme protein unfolding in guanidine solutions is to examine the reported times at which spectral measurements are taken after the addition of GdnCl. It is presumed that this time has allowed equilibrium to be reached. In the literature for guanidinium unfolding of $b$-type heme proteins, data have been taken after a wide range of equilibration times, ranging from 5 min (Sabahi & Wittung-Stafshede, 2002) to at least 24 h (Yu et al., 1995). Literature examples for $b$-type heme proteins are given in the Table 2.2 and Table
2.3. All of these reported times are shorter than the times for unfolding observed in the current study. For SiaA and its mutants, we have observed that the unfolding steps are sensitive to mutations in the heme pocket, where bonded and nonbonded interactions must be made and broken in the course of fairly rapid heme transfer reactions. However, overall unfolding rates are very slow. It is possible that SiaA has a facile unfolding coordinate for which the kinetic barrier is relatively low and along which the heme can be released. This might allow the protein to be sufficiently unfolded to release the heme without the risk of it proceeding along a steep, cooperative unfolding pathway that could leave it extensively unfolded and perhaps dysfunctional. Hence, it may be reasonable to consider that biphasic (or multiphasic) kinetics, with comparatively facile but slight unfolding to bind or release heme but resistance to extensive unfolding to preserve the three dimensional form of the protein for further uptake and release, may be a characteristic property of heme trafficking proteins.

2.4.3 Spectrophotometric pH titration.

When titrated with base, all of the mutants gave spectra that were isosbestic from pH 7 to approximately pH 10.9. These data were fit with a two state model to give pK\textsubscript{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\textsubscript{a} of WT SiaA (9.7 ± 0.1). We have proposed (Sook et al., 2008) that this pK\textsubscript{a} is due to deprotonation of the axial histidine, which falls in the range of 8 - 11 for heme proteins (Gadsby & Thomson, 1982; Moore et al., 1985; La Mar et al., 1990; Banci et al., 1992; Saraiva et al., 1992; Bogumil et al., 1995; Arnesano et al., 2000).

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\textsubscript{a} by approximately 0.7 units from WT protein. In IsdE, the corresponding residue is a proline. 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. For SiaA, this rigidity may allow significant change in the pK\textsubscript{a} of the protein upon replacement of C58 with alanine. Even mutation of cysteine 47 has a significant effect on the pK\textsubscript{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 (see Discussion, Section 4.1) (Grigg et al., 2007). The reduction in pK\textsubscript{a} for the K61A mutant may have to do with changes in the hydrogen bonding network arising from the substitution.

2.4.4 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 (Bixler et al., 1992; Telford et al., 1998; Reedy & Gibney, 2004). That cycle is illustrated in Scheme 1 for SiaA. The difference in the free energies of folding of SiaA around the ferric and ferrous hemes (\(\Delta\Delta G_{\text{fd}}^{(\text{III-II})}\)) 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 4. 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 $68 \pm 3$ mV. Taken together with the $-56 \pm 34$ mV reduction potential of free heme (B.R. Gibney, personal communication), the thermodynamic cycle indicates that the folding of SiaA around the ferrous heme is more strongly driven than folding around hemin by only approximately 12.0 kJ·mol$^{-1}$ for the WT; the corresponding value for the K61A mutant is very similar at 11.3 kJ·mol$^{-1}$.

\[
\Delta \Delta G_{\text{fld}}^{(\text{III}\rightarrow \text{II})} = nF \left[ -0.056 (\pm 0.034) \text{ V} - 0.068 (\pm 0.003) \text{ V} \right] = -12.0 \pm 3.3 \text{ kJ·mol}^{-1}
\]

\[
\Delta \Delta G_{\text{fld}}^{(\text{III}\rightarrow \text{II})} = nF \left[ -0.056 (\pm 0.034) \text{ V} - 0.061 (\pm 0.003) \text{ V} \right] = -11.3 \pm 3.3 \text{ kJ·mol}^{-1}
\]

The heme transfer pathway \textit{in vivo} is from Shp to SiaA (Bates et al., 2003; Lei et al., 2003a; Liu & Lei, 2005). 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 (Nygaard et al., 2006). They fit the kinetics to a model involving an equilibri-
um for binding of the two proteins, followed by intramolecular transfer of the iron porphyrin. The dissociation constants for ferrous and ferric Shp with apoSiaA were 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 apoSiaA. The heme transfer rate constants within the Shp-SiaA complex were calculated to be 28 ± 6 s⁻¹ and 43 ± 3 s⁻¹, respectively. This model indicates that the two oxidation states of the heme were transferred within the complex with very similar rate constants, consistent with our electrochemical data indicating that both oxidation states of the heme are released with comparable ease.

2.5 Conclusions.

SiaA is part of a pathway that allows S. pyogenes to take up heme. Guanidinium-induced denaturation showed that, as expected, the axial ligands (M79 and H229) play significant roles in the stability of the holoSiaA 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 was not a simple reversible process. Rather, unfolding could be at least biphasic and the protein continued to unfold very slowly over 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 cycles of the transfer process. Spectrophotometric pH titration studies gave pKₐ 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, very similar to the potential of 68 ± 3 mV potential of WT SiaA. The midpoint potential is about 115 mV different from that of free heme, indicating that the re-
duced protein is only 12 kJ/mole more difficult to unfold than the oxidized protein. These results, together with previously published kinetic data (Nygaard et al., 2006) reveal that the thermodynamic stabilities of the extracellular heme acquisition protein SiaA are balanced so as to be nearly insensitive to the oxidation state of the heme. This may indicate that this system has the flexibility to acquire heme in both oxidation states.

### 2.6 Abbreviations.

- **CAPS**: 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
- **CCD**: charge-coupled device
- **CHES**: N-cyclohexyl-2-aminoethanesulfonic acid
- **ESI**: electrospray ionization mass spectrometry
- **GAS**: Group A streptococcus
- **GdnCl**: guanidine hydrochloride
- **Hts**: heme transport Streptococcus pyogenes
- **ICP-MS**: inductively coupled plasma mass spectrometry
- **LN₂**: liquid nitrogen
- **MALDI**: matrix-assisted laser desorption ionization
- **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

### 2.7 Acknowledgments.

We thank Brian Basden, Giselle Delgado, Armrita Nargund, and P.N. Nguyen for assistance with the experiments. We thank Dr. Kara Bren for useful discussions. This work was supported by National Institutes of Health Grants AI057877 (ZE) and AI072719–02 (KRR), and the Research Corporation (DWD).
Figure 2.1  Homology modeling of SiaA. Shown are the locations of C47, C58, K61, M79, and H229.

Figure 2.2  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-HCl, pH 7.0.
Figure 2.3 Relative absorbance at the Soret maximum as a function of time for proteins in the presence of GdnCl. C47A is shown at the midpoint (2.95 M GdnCl). C58A is shown in the pre-transition region (1.46 M GdnCl) and at the midpoint concentrations (2.42 M GdnCl). WT is shown at the midpoint (3.1 M GdnCl). The solutions were in 50 mM Tris-HCl, pH 7.0.

Figure 2.4 Fraction folded of the C58A mutant as a function of the concentration of GdnCl at 29 h (squares) and 221 h (triangles) after the completion of addition of denaturant. Lines are the nonlinear least square fits to the equations to a two state unfolding model. The inset shows absorbance as a function of the concentration of GdnCl for these two data sets and data taken at 3 h (diamonds). The solutions were in 50 mM Tris-HCl, pH 7.0.
Figure 2.5  Fraction folded of the WT SiaA and mutants in this study as a function of the concentration of GdnCl. Data from titrations in 50 mM Tris-HCl, pH 7.0 buffer were fit via nonlinear least squares to a two state unfolding model.

Figure 2.6  Spectrophotometric pH titration of C47A, titrated with 1.0 M NaOH, in a buffer of 20 mM each CAPS, CHES and Tris-HCl. UV-visible spectra of 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 pKₐ; the value was 9.2 ± 0.1.
Figure 2.7  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 μM in holoSiaA and 20 mM in Tris-HCl 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 2.8  UV-visible absorbance spectra of K61A SiaA during the course of the anodic spectroelectrochemical titration with dithionite. The inset shows normalized 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 3) yielded a midpoint potential of 61 ± 3 mV vs SHE. Titrations were carried out in 50 mM Tris-HCl at pH 8.0, 100 mM NaCl.
Figure 2.9  Soret-excited rR spectra of ferric (top) and ferrous (bottom) C58A SiaA. The high frequency (in-plane porphyrin stretching) and low frequency regions are shown. Samples were 50 μM in holoSiaA and 10 mM in Tris-HCl at pH 8.0. Ferrous C58A SiaA was generated by anaerobic introduction of a 10-fold molar excess of buffered dithionite to the ferric protein. Complete reduction was verified by UV-visible absorbance spectroscopy.
Figure 2.10 Spectroelectrochemical titration of WT SiaA. Normalized absorbance at the Soret maximum for ferrous WT SiaA (424 nm) as a function of cell potential (vs SHE reference) reveals electrochemical irreversibility of the Fe(III)|Fe(II) couple. The anodic titration curve was best modeled by three Nernstian waves, yielding midpoint heme potentials of 72 and 15 mV vs SHE. A third, unidentified but very negative, potential resulted from the fitting in order to account for absorbance changes at the low end of the titration range. The cathodic titration curve was well modeled by a single Nernstian wave with a midpoint potential of 68 mV. The 68 and 72 mV potentials are taken to indicate a reversible Fe(III)|Fe(II) couple. The 15 mV potential represents a fraction of the protein that can access a ferrous structure/conformation having a lower potential than the ferric protein and which is kinetically slow to revert back to the ferric conformation. Titrations were carried out in 50 mM Tris at pH 8.0, 100 mM NaCl.
Table 2.1  Forward and reverse primers for the mutants in this work.

<table>
<thead>
<tr>
<th></th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tbody>
<tr>
<td>C47Af</td>
<td>5'-CCACTTCGTTGGCTGATATCGCTGGTTTAAATTTA-3'</td>
<td></td>
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<tr>
<td>C47Ar</td>
<td>5'-TAAATTTAAAACGGTACAGCAGTATCAACCACAGCAACCGAAGTGG-3'</td>
<td></td>
</tr>
<tr>
<td>C58Af</td>
<td>5'-TTTAGACCTCGTTGCGATTAGTAAA TTATACCCCTTCC-3'</td>
<td></td>
</tr>
<tr>
<td>C58Ar</td>
<td>5'-GGAAGGGTATATAATTACTATCAGC AACCCCAACGAGGTCTAAA-3'</td>
<td></td>
</tr>
<tr>
<td>H229Af</td>
<td>5'-CTTGATTTTACGAACAGCTGCTGCCATTCCAGACAAGG-3'</td>
<td></td>
</tr>
<tr>
<td>H229Ar</td>
<td>5'-CTTTCTTGGCATGGCAAGCAAGCTGGTTCCGTAAAATCA AG-3'</td>
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<tr>
<td>M79Af</td>
<td>5'-GCGTGTTTTTCTCCGTATATAGAGTTGATTG-3'</td>
<td></td>
</tr>
<tr>
<td>M79Ar</td>
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<td>K61Ar</td>
<td>5'-GCGTTAGGAGGTATATTGCACACTATCAAAAAACGCCGAGG-3'</td>
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Table 2.2  GdnCl denaturation values and equilibrium time for selected $b$-type heme proteins including cytochromes and heme trafficking proteins.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Source of Protein</th>
<th>Ligands</th>
<th>Equilibrium Time &amp; Temp</th>
<th>pH</th>
<th>Buffer</th>
<th>[GdnCl] Midpoint Oxidized</th>
<th>Oxidized Reduced</th>
<th>$\Delta G(H_2O)$ kcal mole$^{-1}$ Oxidized Reduced</th>
<th>Reference</th>
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<tr>
<td>OM Cytochrome $b_5$</td>
<td>rat microsomal</td>
<td>His-His</td>
<td>20 h 20°C</td>
<td>7.0</td>
<td>50 mM potassium phosphate</td>
<td>3.64 ± 0.1</td>
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<td>(Silchenko et al., 2000)</td>
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<td>OM Cytochrome $b_5$</td>
<td>rat microsomal</td>
<td>His-His</td>
<td>titration</td>
<td>7.0</td>
<td>100 mM phosphate buffer</td>
<td>2.6 ± 0.3</td>
<td>6.9 ± 0.7</td>
<td>(Arnesano et al., 1998)</td>
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</tr>
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<td>MC Cytochrome $b_5$</td>
<td>bovine microsomal</td>
<td>His-His</td>
<td>60 min 25°C</td>
<td>7.0</td>
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<td>(Manyusa et al., 1999)</td>
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<td>Cytochrome $b_{562}$</td>
<td>E. coli</td>
<td>His-Met</td>
<td>at least 1 h 20°C</td>
<td>7.0</td>
<td>50 mM potassium phosphate and 100 mM KCl</td>
<td>2.15 ± 0.2</td>
<td>7.0 (Hay &amp; Wydrzynski, 2005)</td>
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<td>E. coli</td>
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<td>5.0</td>
<td>20 mM acetate</td>
<td>2.1 ± 0.2</td>
<td>7.2 ± 0.1</td>
<td>(Arnesano et al., 2000)</td>
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<tr>
<td>Cytochrome $b_{562}$</td>
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<td>His-Met</td>
<td>titration</td>
<td>7.0</td>
<td></td>
<td>1.8</td>
<td></td>
<td>(Wittung-Stafshede et al., 1999)</td>
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<tr>
<td>SiaA</td>
<td>Streptococcus pyogenes</td>
<td>His-Met</td>
<td>titration 22°C</td>
<td>7.0</td>
<td>50 mM Tris-HCl</td>
<td>3.1 ± 0.1</td>
<td>7.3 ± 0.8</td>
<td>(Sook et al., 2008)</td>
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<tr>
<td>HasA</td>
<td>Serratia marcescens</td>
<td>His-Tyr</td>
<td>overnight room temp</td>
<td>7.0</td>
<td>20 mM sodium Phosphate</td>
<td>2.7 ±0.2</td>
<td>11.5 ±4.7</td>
<td>(Wolff et al., 2003)</td>
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Table 2.3  GdnCl denaturation values and equilibrium time for selected ferric $b$-type heme proteins: Globins, peroxidases and cytochrome P450.

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<tr>
<th>Protein</th>
<th>Source of Protein</th>
<th>Ligands</th>
<th>Equilibrium Time &amp; Temp</th>
<th>pH</th>
<th>Buffer</th>
<th>[GdnCl] Midpoint</th>
<th>m kcal mole$^{-1}$ M$^{-1}$</th>
<th>$\Delta$G[H$_2$O] kcal mole$^{-1}$</th>
<th>Reference</th>
</tr>
</thead>
</table>
| MetMb   | sperm whale      | His    | titration 25 °C         | 6.0 | 200 mM phosphate buffer | 1.46 ± 0.01 | 3.45 ± 0.09 | 4.95 ± 0.13 | (Roncone et al., 2004)
<p>|         |                  |        |                         |     |        |                  |                          |                          |           | (Roncone et al., 2005) |
| MetMb   | horse heart      | His    | 2 – 3 h                  | 8.0 | 50 mM Tris-HCl         | 1.37          | 3.47 ± 0.3 | 4.86 ± 0.5  | (Choi &amp; Terazima, 2002) |
| MetMb   | horse            | His    | titration 20 °C          | 7.0 | 5 mM phosphate, 100 mM NaCl | 1.5          | 3.37 ± 0.45 | 5.45 ± 0.67 | (Moczygemba et al., 2000) |
| MetMb   | sperm whale      | His    | 1 h                      | 8.0 | 50 mM Tris-HCl and 50 mM NaCl | 2            |              |              | (Hargrove &amp; Olson, 1996) |
| MetMb   | opossum          | His    | 15 min 25 °C             | 8.0 | 10 mM sodium phosphate, 250 mM KCl | 1.61         | 4.65 ± 0.21 | 12.5 ± 0.5 [1] | (Kelly et al., 1988) |
| MetMb   | rabbit           | His    | 15 min 25 °C             | 8.0 | 10 mM sodium phosphate, 250 mM KCl | 2.21         | 4.56 ± 0.13 | 16.6 ± 0.5 [1] | (Kelly et al., 1988) |
| MetMb   | raccoon          | His    | 15 min 25 °C             | 8.0 | 10 mM sodium phosphate, 250 mM KCl | 1.69         | 5.29 ± 0.16 | 14.8 ± 0.4 [1] | (Kelly et al., 1988) |
| MetMb   | rat              | His    | 15 min 25 °C             | 8.0 | 10 mM sodium phosphate, 250 mM KCl | 1.84         | 45.13 ± 0.09 | 15.7 ± 0.3 [1] | (Kelly et al., 1988) |
| MetMb   | Cyprinus carpio  | His    | 15 min 25 °C             | 8.0 | 50 mM phosphate, 100 mM KCl | 8.93 ± 0.55  |              |              | (Kelly et al., 1988) |
| MetMb   | sperm whale      | His    | ~ 16 h                   | 7.0 | 10 mM sodium phosphate | 2.36          |              | 14.3        | (Puett, 1973) |
| MetMb   | horse            | His    | ~ 16 h                   | 7.0 | 10 mM sodium phosphate | 1.63          |              | 11.8        | (Puett, 1973) |</p>
<table>
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<tr>
<th>Enzyme</th>
<th>Source</th>
<th>Residue</th>
<th>Time</th>
<th>pH</th>
<th>Buffer</th>
<th>ΔG (kcal/mol)</th>
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<td>Tyr</td>
<td>6 h</td>
<td>4 °C</td>
<td>7.0</td>
<td>0.025 M sodium phosphate</td>
<td>[2]</td>
<td>(Prakash et al., 2002)</td>
</tr>
<tr>
<td>Lactoperoxidase</td>
<td>bovine milk</td>
<td>His</td>
<td>18 h</td>
<td>6.8</td>
<td>0.025 M sodium phosphate</td>
<td>2.1</td>
<td>(Boscolo et al., 2009)</td>
<td></td>
</tr>
<tr>
<td>CooA [3]</td>
<td><em>Rhodospirillum rubrum</em></td>
<td>Cys-Pro Fe(III) His-Pro Fe(II)</td>
<td>2 h</td>
<td>room temp</td>
<td>7.4</td>
<td>25 mM MOPS, 100 mM NaCl</td>
<td>6.3 ± 0.8</td>
<td>18 ± 0.1</td>
</tr>
<tr>
<td>Myeloperoxidase</td>
<td>human</td>
<td>His</td>
<td>18 h</td>
<td>25 °C</td>
<td>7.0</td>
<td>5 mM phosphate</td>
<td>4.3</td>
<td>1.5</td>
</tr>
<tr>
<td>Cytochrome P450</td>
<td>rat liver</td>
<td>Cys</td>
<td>1 h</td>
<td>25 °C</td>
<td>7.4</td>
<td>50 mM potassium phosphate</td>
<td>0.41 ± 0.02</td>
<td>(Kumar et al., 2007)</td>
</tr>
<tr>
<td>Cytochrome P450</td>
<td><em>Bacillus subtilis</em> BioI (CYP107H1)</td>
<td>Cys</td>
<td>15 min</td>
<td>20 °C</td>
<td>7.0</td>
<td>50 mM Tris-HCl, 1 mM EDTA</td>
<td>2.7 ± 0.1 [4]</td>
<td>3.5 ± 0.1 [5]</td>
</tr>
<tr>
<td>Cytochrome P450</td>
<td>rat liver microsomes</td>
<td>Cys</td>
<td>24 h</td>
<td>4 °C</td>
<td>7.5</td>
<td>0.1 M potassium phosphate</td>
<td>1.6</td>
<td>(Yu et al., 1995)</td>
</tr>
</tbody>
</table>

[3] Data are from circular dichroism measurements, two steps observed. Three steps are visible via fluorescence.
2.8 Reference List


3 MUTANTS OF SIAA, A PROTEIN IN THE HEME UPTAKE PATHWAY OF S. PYOGENES

Chapter 1 gave an overview of heme uptake in bacteria. Chapter 2 reproduced from our work, submitted for publication, on a series of SiaA mutants. This chapter gives additional experimental data and literature compilations related to this study.

3.1 Homology modeling using I-TASSER (Protein Structure and Function Predictions).

To obtain a visual picture of WTSiaA, the sequence of this protein from S. pyogenes was threaded into its ten closest homologies using I-TASSER, (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) (Zhang, 2008; Roy et al., 2010). Based on multiple-threading alignments by iterative TASSER assembly simulations, 3D models were built. The chosen model was visualized using Pymol (DeLano, 2009). Figure 3.1 shows the model and the specific amino acids of interest in this work.

3.2 Purification of mutants of SiaA.

Two cycles of French Press were used to lyse the cell pellets from a 1.5 L growth solution. Cells were in 40 ml of buffer solution containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.1% Triton X-100, 10% glycerol and four tablets of protease inhibitor (Roche Complete Mini, EDTA-free); this solution was kept in ice between the two lysis cycles. The lysate was centrifuged at 35,000xg for 20 min at 4 °C and supernatant was syringe-filtered with a 0.45 µm filter (surfactant-free cellulose acetate membrane, Nalgene). Further purification of the protein was conducted at 4 °C using a GE Healthcare ÄKTA fast protein liquid chromatography instrument (FPLC, Amersham BioSciences), and all buffer solutions were pH 8.0 unless specified other-
wise. The sample (all the material from the 1.5 L growth) was loaded onto a GE Healthcare HisTrap™ HP column (5 mL, Amersham BioSciences) equilibrated with binding buffer (50 mM potassium phosphate, 250 mM NaCl, and 10% v/v glycerol). Unbound material was washed out with 5 column volumes (CV) of binding buffer. The SiaA mutant was eluted with buffer containing 50 mM potassium phosphate, 250 mM NaCl, 10% v/v glycerol, and 0.5 M imidazole applied via a linear gradient (Figure 3.2 for C47A and Figure 3.6 for C58A). The proteins were eluted with a 25 CV gradient and 70% target elution buffer concentration. For C47A mutant two major peaks were seen. The purities of the fractions were evaluated using sodium dodecyl sulfate polyacrylamide gel electrophoresis. The first (lane 6 in Figure 3.4) showed a mixture of many species. The second peak (lane 3 in Figure 3.4) was the desired protein. Lane 5 in the gel also showed a significant amount of the desired protein. This protein comes from the first band in the FPLC. Later studies (on a different batch of protein) indicated that the column was being overloaded and some of the desired material was coming out early, at the end of the flow-through.

Fractions containing the individual SiaA mutants were combined, and imidazole and salts were removed by centrifugal filtration (Amicon Ultra-4, 30,000 molecular weight cutoff, Millipore) using a buffer of 50 mM Tris-HCl.

3.3 MALDI Mass spectrometry of mutants of SiaA.

The masses of the mutants were evaluated using matrix-assisted laser desorption ionization (MALDI) mass spectroscopy (Figures 3.3 and 3.7) using an ABI Voyager DE-Pro (Applied Biosystems) MALDI reflectron time-of-flight spectrometer in positive ion mode. The sinipinic acid matrix was prepared in 30:70 acetonitrile:water and 0.1% trifluoroacetic acid. The sample solution was mixed with the matrix solution in a 1:1 ratio and the resulting solution spotted onto
the MALDI target plate. The mass spectrum of C47A and C58A showed a species at 35376.1 Da and at 35364.7 Da, respectively. These results are consistent with the expected molecular weight of C47A and C58A to be 35455 Da.

### 3.4 Charge distribution bands (ESI results) of C47A mutant.

Electrospray ionization mass spectrometry (ESI) was performed using a Micromass Q-TOF Micro mass spectrometer in positive mode (Figure 3.5). Samples were prepared in a solution containing 50:50 acetonitrile:water and 0.1% formic acid. The capillary voltage was 3.0 kV and the flow rate was 5 μL/min. Deconvolution of the charge state distribution was performed with the MaxEnt program included with the MassLynx™ software. The peaks are rounded to the nearest Dalton.

Both the apoprotein (35456.5 Da, consistent with the expected 35455 Da) and the holoprotein (36067.5, consistent with the expected 36071) were seen. However, each of these peaks had another peak 98 units higher in mass. The origin of these peaks is not known.

Both of the apoprotein peaks showed a wide charge distribution band (from +43 to +18,) centered at about +30 (e.g., 1112.1 of the 35554.6 species has a charge of +32). The protein has a total of 48 arginines, lysines and histidines; the highly charged species are due to protonation of most of these. The holoC47A had a much narrower charge distribution band (+20 to +13), dominated by the +19 and +20 charge states (e.g., 2004.6 has a charge of +18 for the 36067.5 species). The large difference in the charge states of the apo- and holo-proteins indicates that these two species are likely to have quite different conformations in the gas phase, with the holo species the more highly folded.
3.5 Thermal denaturation studies.

Thermal denaturation technique used to examine to unfolding nature of the C47A mutant (Figure 3.8). A 1.5 mL quartz Supracil cuvette (Spectracell) with a 1 cm path length was used for the thermal denaturation. The absorbance of protein was recorded at the Soret maximum (413 nm) while increasing the temperature of solution at 0.5 °C/min. The protein was half unfolded at about 83 °C. It was felt that the guanidinium denaturation was more useful to analyze the protein unfolding and no further thermal denaturation work was performed on other mutants.

3.6 Guanidinium-induced denaturation results of C58A at 3 h, 24 h, 29 h, 9 d 5h and titration method.

Data from denaturations in 50 mM Tris-HCl, pH 7.0 buffer were fit via nonlinear least squares. The data, except for the measurement at 3 h, fit well to a two state unfolding model, described in Chapter 2 and Section 2.2.7 (Figure 3.9). The D_{1/2} values were: 2.53 ± 0.17 M GdnCl (24 h), 2.32 ± 0.10 M GdnCl (29 h), 1.67 ± 0.12 M GdnCl (9 d 5 h), 2.41 ± 0.03 M GdnCl (titration). The slopes at the midpoint of the transitions (m-values) increased as the time of measurement increased. The m-value of the denaturation curve from the titration experiment was significantly larger than the rest (seen in the much steeper slope).

3.7 Native polyacrylamide gel electrophoresis.

For native PAGE of WTsiA and it mutants, a NativePAGE Sample Prep Kit from Invitrogen and a 5 to 12% acrylamide gel were used. Samples were run 30 min under 80 V current and 2 h under 150 V current. A NativeMark Unstained Protein Standard (Invitrogen) was used. Figure3.10 native PAGE shows that WTsiA and mutants are found largely as monomers.
3.8 Spectrophotometric pH titration.

C47A, C58A, and K61A were titrated with aliquots of 1 M NaOH in a buffer of 20 mM each CAPS, CHES and Tris-HCl. For each mutant, the Soret decreased and blue shifted slightly as the pH increased (Figures 3.11, 3.12, and 3.14). The transitions were isosbestic between pH 7.0 and at least 10.7 Figures 3.11, 3.12, and 3.14. Data were fit using a two state model to give pKₐ values of 9.22 ± 0.03 for C47A (Figure 2.6-in previous chapter), 9.04 ± 0.03 for C58A (Figure 3.13) and 9.45 ± 0.05 for K61A (Figure 3.15).
Figure 3.1  Homology model of SiaA built with I-TASSER showing cysteine 47, cysteine 58, lysine 61, histidine 229 and methionine 79.
Figure 3.2  The purification of the C47A mutant protein was conducted at 4 °C using a GE Healthcare ÄKTA fast protein liquid chromatography instrument (FPLC, Amersham BioSciences), and all buffer solutions were pH 8.0. The sample was loaded onto a GE Healthcare HisTrap™ HP column (5 mL, Amersham BioSciences) equilibrated with binding buffer (50 mM potassium phosphate, 250 mM NaCl, and 10% v/v glycerol). Unbound material was washed out with 5 column volumes (CV) of binding buffer. C47A was eluted with buffer containing 50 mM potassium phosphate, 250 mM NaCl, 10% v/v glycerol, and 0.5 M imidazole applied via a linear gradient.
Figure 3.3 The mass of the C47A mutant was evaluated using an ABI Voyager DE-Pro (Applied Biosystems) MALDI reflectron time-of-flight spectrometer in positive ion mode. The sinipinic acid matrix was prepared in 30:70 acetonitrile:water and 0.1% trifluoroacetic acid. The sample solution was mixed with the matrix solution in a 1:1 ratio and the resulting solution spotted onto the MALDI target plate. The mass spectrum of C47A showed a species at 35376.1 Da, consistent with the expected 35455 Da.
Figure 3.4  SDS PAGE of C47A fractions. A 5 to 15% acrylamide gel was used. Lane 1: Marker, lane 2: FPLC fraction #51 of C47A mutant, lane 3: Fraction 40, lane 4: Fraction 33, lane 5: Fraction 22, lane 6: Fraction 15, lane 7: Fraction 8, lane 8: Fraction 4. Samples were run 5 min under 80 V current and 1h under the 120 V current. A Precision Plus Protein Standard was used to determine the molecular weight.
Figure 3.5  Charge distribution bands (ESI results) of C47A. Electrospray ionization mass spectrometry (ESI) was performed using a Micromass Q-TOF Micro mass spectrometer in positive mode. Samples were prepared in a solution containing 50:50 acetonitrile:water and 0.1% formic acid. The capillary voltage was 3.0 kV and the flow rate was 5 μL/min. Deconvolution of the charge state distribution was performed with the MaxEnt program included with the MassLynx™ software. The peaks are rounded to the nearest Dalton. The apoC47A was seen at 35456.5 Da, consistent with the expected 35455 Da. The holoC47A was seen at 36067.5 Da, consistent with the expected 36071 Da. A second set of peaks was seen for the apoC47A at 35554.5 and the holoC47A at 36165. These are each 98 units higher than the peaks reported above.
Figure 3.6 The purification of the C58A mutant protein was conducted at 4 °C using a GE Healthcare ÄKTA fast protein liquid chromatography instrument (FPLC, Amersham BioSciences), and all buffer solutions were pH 8.0. The sample was loaded onto a GE Healthcare HisTrap™ HP column (5 mL, Amersham BioSciences) equilibrated with binding buffer (50 mM potassium phosphate, 250 mM NaCl, and 10% v/v glycerol). Unbound material was washed out with 5 column volumes (CV) of binding buffer. C58A was eluted with buffer containing 50 mM potassium phosphate, 250 mM NaCl, 10% v/v glycerol, and 0.5 M imidazole applied via a linear gradient.
Figure 3.7 The masses of the C58A was evaluated using an ABI Voyager DE-Pro (Applied Biosystems) MALDI reflectron time-of-flight spectrometer in positive ion mode. The sinipinic acid matrix was prepared in 30:70 acetonitrile:water and 0.1% trifluoroacetic acid. The sample solution was mixed with the matrix solution in a 1:1 ratio and the resulting solution spotted onto the MALDI target plate. The mass spectrum of C58A showed a species at 35364.7 Da, consistent with the expected 35455 Da.
Figure 3.8  Thermal denaturation of C47A. The temperature was increased 65°C to 95°C using 0.5°C per minute increments. The unfolding was followed at 413 nm. The protein was in 50 mM Tris-HCl, pH 7.0 buffer.
Figure 3.9 Guanidinium-induced denaturation of C58A using the multiple cuvette method at 3 h, 24 h, 29 h, and 9 d 5h time points and with titration method. Data from denaturations in 50 mM Tris-HCl, pH 7.0 buffer were fit via nonlinear least squares to a two state unfolding model. The data at 3 h did not fit to a two state model.
Figure 3.10  Purified protein subjected to 5-12% native polyacrylamide gel electrophoresis. Lane 1: marker, lane 2: WT SiaA, lane 3: C47A mutant, lane 4: C58A mutant, lane 5: K61A mutant, lane 6: marker, lane 7: bovine serum albumin. A NativePAGE Sample Prep Kit from Invitrogen was used with a 5 to 12% acrylamide gel. Samples were run for 15 min with 80 V current and 1.5 h with 150 V current. A NativeMark Unstained Protein Standard was used.
Figure 3.11  Spectrophotometric pH titration of C47A, titrated with 1.0 M NaOH, in a buffer of 20 mM each CAPS, CHES and Tris-HCl. UV-visible spectra (isosbestic) are shown from pH 7.0 to 10.7.
Figure 3.12  Spectrophotometric pH titration of C58A, titrated with 1.0 M NaOH, in a buffer of 20 mM each CAPS, CHES and Tris-HCl. UV-visible spectra (isosbestic) are shown from pH 7.0 to 11.0.
Figure 3.13  Spectrophotometric pH titration of C58A, titrated with 1.0 M NaOH, in a buffer of 20 mM each CAPS, CHES and Tris-HCl. The nonlinear least squares fit of the data at 409 nm gave a single pKₐ; the value was 9.0 ± 0.1.
Figure 3.14  Spectrophotometric pH titration of K61A, titrated with 1.0 M NaOH, in a buffer of 20 mM each CAPS, CHES and Tris-HCl. UV-visible spectra (isosbestic) are shown from pH 7.1 to 10.8.
Figure 3.15  Spectrophotometric pH titration of K61A, titrated with 1.0 M NaOH, in a buffer of 20 mM each CAPS, CHES and Tris-HCl. The nonlinear least squares fit of the data at 408 nm gave a single pKₐ; the value was 9.4 ± 0.1.
3.9 Reference List


4 CHARACTERIZATION OF HMUT, A HEME-BINDING PROTEIN ASSOCIATED WITH A HEME ABC TRANSPORT SYSTEM IN CORYNEBACTERIUM DIPHTHERIAE

4.1 Introduction.

4.1.1 Corynebacterium diphtheriae.

*Corynebacterium diphtheriae* is a gram-positive bacterium which is the causative agent of diphtheria (Schmitt, 1997b; Hadfield et al., 2000; Holmes, 2000; Burgos & Schmitt, 2012). *Corynebacterium* is an aerobic pathogen and forms straight or slightly bent rod-like shapes. It can colonize the upper respiratory tract in humans and secrete the diphtheria toxin (DT) which is encoded by the *tox* gene (Drazek et al., 2000; Schmitt & Drazek, 2001; Kunkle & Schmitt, 2005). The diphtheria toxin repressor protein (DtxR) and iron negatively regulate the *tox* gene. DtxR functions similar to the Fur (ferric uptake repressor) protein. Expression of about fifty genes in *C. diphtheriae* are known to be regulated by DtxR. *C. diphtheriae* can damage tissues throughout the body. Diphtheriae is still common in developing countries due to the low vaccination rate (Mattos-Guaraldi et al., 2003; Wagner et al., 2012).

Similar to a number of other bacterial pathogens, *C. diphtheriae* requires iron to survive and for virulence (Schmitt, 1997b; Drazek et al., 2000; Schmitt & Drazek, 2001). *C. diphtheriae* removes heme from host sources (e.g., transferrin and hemoglobin) using siderophores (low molecular weight molecules) or proteins anchored to the cell wall or membrane. In siderophore-dependent iron uptake pathway, siderophores are secreted by *C. diphtheriae* into the extracellular environment (Drazek et al., 2000; Qian et al., 2002; Kunkle & Schmitt, 2003; Kunkle & Schmitt, 2005). Upon binding of heme, siderophores return to the organism via membrane-associated transport proteins.
In addition to siderophore-specific heme uptake system, *C. diphtheriae* utilizes heme through an ABC-type heme binding protein transporter system. Initial studies revealed three genes associated with heme transportation in *C. diphtheriae* (Drazek et al., 2000). The heme utilization (*hmu*) operon included *hmuT*, *hmuU* and *hmuV* which were proposed to form an ABC transport system. Later studies on the *hmu* locus revealed the existence of an additional three genes, *htaA*, *htaB* and *htaC* (Allen & Schmitt, 2009; Cerdeno-Tarraga et al., 2003). The *htaA* gene is located immediately upstream of the *hmuTUV* locus, and it is followed by promoter region and *htaC* gene (Figure 4.1). Downstream of the *hmuV* gene has a promoter region and the *htaB* gene. The *hmuTUV* and *htaA* genes form a single operon; *htaB* and *htaC* have been shown to transcribe independently. The function of the *htaA*, *htaB* and *htaC* genes has not known yet. However, it has been suggested that they play role in heme uptake due to their link to the *hmuTUV* operon (Allen & Schmitt, 2009). *HmuO* gene is also associated with using iron as energy source in *C. diphtheriae*. *HmuO* encodes a heme oxygenase that degrades heme and releases iron for further use in cellular functions.

Location of the each protein (Figure 4.2) in the cell wall was predicted by the protein localization studies (Allen & Schmitt, 2009). HtaA is thought to be mainly associated with the cell wall while HtaB is mostly associated to the cytoplasmic membrane. Both HtaA and HtaB are exposed to the bacterial surface, indicating that these hemin binding proteins may function as a heme receptor. It was also reported that the HmuT protein is predominantly linked to membrane and may function as a heme trafficking protein.
4.1.2 Heme acquisition in C. diphtheriae.

HtaA, HtaB and HmuT have ability to bind heme. When the hmuTUV or htaA genes were deleted, C. diphtheriae showed a reduced ability to use hemoglobin as an iron source, indicating that HtaA and the ABC-transport system had an important role in up taking heme inside the cell in C. diphtheriae (Allen & Schmitt, 2009). In contrast, deletion of the htaB gene did not show diminished ability of C. diphtheriae to utilize heme from hemoglobin. This result, together with heme binding ability of HtaB, suggests that hemoglobin might not be the heme source for HtaB protein. However, this protein is still involved in heme uptake pathway of C. diphtheriae (Allen & Schmitt, 2009; Nobles & Maresso, 2011). The htaC gene has been suggested to encode a membrane protein; however, it does not show significant sequence similarity to proteins with known functions (Allen & Schmitt, 2009). The heme binding ability of the protein product of the htaC gene has not been determined yet; however, it is transcribed upstream of the HtaA, indicating its possible association with heme uptake of this pathogen.

HtaA is a 61 kDa hemin binding protein (about 150 amino acids) which has a leader peptide at its N-terminal end (Allen & Schmitt, 2009; Allen & Schmitt, 2011). In addition, it has a hydrophobic region at the C-terminal end which is suggested to function in anchoring the protein to the membrane. The protein has two conserved repeats which are highly similar in their amino acid sequence; they have been termed as CR for conserved regions. The CR domains are novel domains that do not share any important sequence similarity with the NEAT domains which are known as heme binding domains from proteins in other Gram-positive bacteria. The function of the CR domains has not been determined yet. HtaB has a single CR domain. Analogously to HtaA, HtaB contains a hydrophobic region at the C-terminal end which is predicted to assist protein to be anchored to the membrane.
Hemin binding studies on CR1 (conserved region one) and CR2 (conserved region two) of the HtaA protein showed that both domains have ability to bind heme and hemoglobin (Allen & Schmitt, 2011). However, CR2 domain showed stronger binding to hemoglobin compared to CR1. HtaB did not have significant binding with hemoglobin.

The CR domains from HtaA and HtaB show high similarity and contain conserved residues which are important in binding heme (Allen & Schmitt, 2011). These two domains have about 31% identity and 50% similarity (Allen & Schmitt, 2011). HtaACR2 and HtaBCR have about 27% identity and 38% similarity. The HtaACR1 domain shares slightly less similarity with HtaBCR. When sequences of CR domains from HatA and HtaB are aligned to sequences from homologous proteins from other Corynebacterium species, there is no significant overall similarity is exhibited. However, Tyr361, Tyr490 and His412 are conserved in all of these sequences. These findings raise the possibility of His/Tyr ligation in C. diphtheriae, as seen in other heme binding proteins from Gram-positive species.

In order to investigate significance of these residues in heme binding, conserved Y361, Y490 and H412 from HtaACR2 was mutated into alanine (Allen & Schmitt, 2011). The other two conserved residues (F486 and W352), also suspected to be important in heme binding, were also mutated into alanine along with the nonconserved H479 residue. UV-visible spectroscopy was used to compare absorbance behavior of each mutant at Soret (406 nm). The Y361A and H12A mutants showed a significant decrease in intensity of Soret compared to WT, suggesting their important role in heme binding of HtaACR2. The Y490A mutant exhibited strong change in Soret; the intensity change for the W352 mutation was more moderate. The H479A and F486A mutants had similar spectra to the WT protein.
Similar mutagenesis studies were performed on two conserved residues from the HtaACR1 domain (Allen & Schmitt, 2011). When compared to WT HtaCR1, the intensity of the Soret significantly decreased upon alanine mutations of Y49 (analogous to Y361 in CR2) and H107 (analogous to H412 in CR2). These findings indicated that the Y49 and H107 residues from HtaACR1 may have similar role for binding heme as do Y361 and H412 in HtaACR2.

Hemoglobin binding properties of the HtaACR1 and HtaACR2 domains have been investigated via mutations (Allen & Schmitt, 2011). Compared to WT HtaACR2, mutations of Y361, H412, Y490, and W352 residues into alanine significantly reduced the ability of the protein to bind hemoglobin. In contrast, the F486 and H479 mutations resulted in no strong effect on hemoglobin binding. On the other hand, mutations of Y49 and H107 showed almost no hemoglobin binding. These findings suggested importance of the designated tyrosine and histidine residues in binding of hemoglobin.

Allen and Schmitt also showed that HtaA transfers hemin to HtaB (Allen & Schmitt, 2011). A proposed heme acquisition mechanism consistent with all of these findings is that HtaA removes heme from hemoglobin and may transfer it to HtaB; in turn, heme is taken up into cytoplasm through the TUV ATP binding cassette.

The first gene product in the ABC transporter from C. diphtheriae is HmuT, a 37 kDa protein with 354 amino acid residues (Drazek et al., 2000; Schmitt & Drazek, 2001). Sequence homology studies on HmuT showed that this protein shares a significant similarity with other heme binding proteins from Gram-negative species. It shows the closest homology to HmuT from Y. pestis with 24.5% identity and 38.7% similarity. In the N-terminus of C. diphtheriae HmuT, the amino acids one to thirty constitute a leader peptide which is significant in lipoproteins. The positively charged amino acids, e.g. arginine and lysine, predominantly present in the
leader peptide. The hydrophobic region and a signal peptide follow the leader sequence at the N-terminal end.

HmuT has been predicted to be associated with cell membrane (Drazek et al., 2000). Localization of HmuT in the cell was investigated by overexpressing HmuT in *E. coli* and *C. diphtheriae*; HmuT was present in the fractions of cell membrane in both species. When expressed in *E. coli* HmuT was capable of binding both heme and hemoglobin. All these findings, together with sequence similarity results, suggested that HmuT is the lipoprotein component of the ABC transporter and functions as a heme receptor in *C. diphtheriae*.

The second gene product in the ABC transporter is HmuU, a 37 kDa protein with 350 amino acid residues (Drazek et al., 2000). HmuU from *C. diphtheriae* showed the highest homology to HmuU from *Y. pestis* with 36% identity and 62% similarity. This protein is predicted to be the permease of the ATP binding cassette and function to transport heme through the membrane upon receiving heme from HmuT.

The last gene product in the ABC transporter is HmuV, a 30 kDa protein with 278 amino acid residues (Drazek et al., 2000). Sequence homology studies revealed that HmuV from *C. diphtheriae* was most similar to HmuV from *Y. pestis* with 40% identity and 56% similarity. HmuV is thought to function as an ATPase in *Y. pestis*. HmuV from both *Y. pestis* and *C. diphtheriae* contains the Walker A motif which is specific to ATP binding proteins (Drazek et al., 2000; Kos & Ford, 2009; Licht & Schneider, 2011). Thus this protein predicted to be the ATPase of the ABC transporter in *C. diphtheriae*.

Once heme is taken inside the organism, HmuO, the heme oxygenase, degrades it and freed iron is used for further cellular functions (Schmitt, 1997a; Schmitt, 1997b; Schmitt, 1999).
The HmuO protein is encoded by \textit{hmuO} gene and it is not a part of the heme uptake gene cluster in \textit{C. diphtheriae}; however, it is regulated by DtxR and iron.

In conjunction with this pathway, it is important to note that deletion mutation studies on \textit{hmu} operon resulted in no effect on growth with heme and hemoglobin as iron sources, which strongly indicated that \textit{C. diphtheriae} has additional heme uptake pathways to fulfill its requirements for iron (Allen & Schmitt, 2009).

4.1.3 \textbf{Heme acquisition in Yersinia pestis.}

\textit{Yersinia pestis} is a Gram-negative pathogenic bacterium and the causative agent of the bubonic and pneumonic plague (Parkhill et al., 2001; Rossi et al., 2001). It has several iron acquisition systems including an iron and manganese uptake system (termed Yfe), and a siderophore (yersiniabactin) dependent pathway (termed Ybt) (Rossi et al., 2001; Pieper et al., 2010).

\textit{Y. pestis} can also take up heme through an ABC transporter dependent pathway, termed \textit{hmu} (heme uptake locus) (Hornung et al., 1996; Thompson et al., 1999). This contains eight open reading frames comprised of \textit{orfXY} and \textit{hmuP’RSTUV}. Homology studies showed that products of five genes, the \textit{hmuRSTUV}, show significant similarity to other heme binding proteins from number of Gram-negative species. It was also shown that they use heme and hemoglobin as iron sources. The upstream of \textit{hmuP’R} and \textit{hmuS} genes each have a single promoter region, called p1 and p2, respectively. It was also shown that p1 has a Fur box which is regulated by iron. HmuR shows high similarity to TonB-dependent outer membrane (OM) receptors including HemR from \textit{P. gingivalis} (88% similarity and 86% identity), ShuA from \textit{S. dysenteriae} (75% similarity and 69% identity) and ChuA from \textit{Campylobacter jejuni} (75% similarity and
69% identity). Thus, the HmuR protein is predicted to be the outer membrane heme receptor in *Y. pestis*.

HmuS is a cytoplasmic protein and predicted to be associated with releasing iron from heme on the basis that it shows strong sequence homology to proteins from several Gram-negative bacteria with similar function. The remaining three genes, *hmuTUV*, encode an ATP binding cassette in which HmuT is a periplasmic binding protein, HmuU is a permease and HmuV is an ATP binding protein. The HmuTUV heme transport system uses hemin, hemin-albumin, and myoglobin as iron sources but does not use hemoglobin, hemoglobin-haptoglobin, or heme-hemopexin to capture iron. In addition mutation studies by deleting *hmu* operon showed no effect on growth in a medium with high hemoglobin concentrations. These findings were consistent with presence of alternative heme uptake systems in *Y. pestis* which may be associated with utilization of hemoglobin (Hornung et al., 1996; Thompson et al., 1999; Rossi et al., 2001).

The HmuT protein from *Y. pestis* has been characterized in considerable details. X-ray crystal structures of apoYp-HmuT (1.5 Å) and holoYp-HmuT (2.5 Å) have been solved (Mattle et al., 2010). Crystal structures of apo and holo forms of the protein were similar with only minor differences. Yp-HmuT has two lobes and they do not share significant sequence homology. Two domains are connected via a backbone helix which is characteristic of periplasmic binding proteins. B-sheets are present at the center of the each domain and numbers of short α-helices surround them.

HoloYp-HmuT was crystallized from a solution of dimethyl sulfoxide (DMSO) dissolved hemin addition in 2:1 molar ratio (Mattle et al., 2010). Two stacked hemes were observed in the binding pocket, positioned in such a way that propionic acids of the first heme were pointing inside the protein while propionic acids of the second heme were pointing outside the protein. The
two heme bound binding pocket was larger than the binding pocket of homologous proteins. Histidine 167 and tyrosine 70 residues were axially ligated to the dimer form. In addition, residues R72, T179, R199, Y200, M165, M255, and L258 were postulated to contribute to keep hemes inside the protein.

Heme binding studies on Yp-HmuT were consistent with the reported structural data (Mattle et al., 2010). Two different experimental approaches using UV-visible spectroscopy were used to investigate heme binding to Yp-HmuT. In the first approach, aliquots of increasing concentrations of heme were added to fixed concentration of Yp-HmuT. This experiment yielded two maximum (approximately at 400 nm and approximately at 375 nm) in the UV-visible spectrum. These were taken to be the monomer- and dimer-bound species, respectively. In the second approach, aliquots of increasing concentrations of Yp-HmuT were added to fixed concentration of heme. The heme solution itself (without Yp-HmuT addition) had a Soret peak at about 404 nm. When Yp-HmuT was titrated into heme solution up to ratio of 2:1 (heme: Yp-HmuT), the Soret blue shifted to 375 nm, indicative of dimeric heme binding. A Yp-HmuT concentrations higher than this, the Soret red shifted back to monomeric binding band at about 400 nm. ITC (isothermal titration calorimetry) experiments interpreted in terms of two heme binding event in two consecutive heme binding events. First step yielded a dissociation constant ($K_D$) of $\sim 0.29$ nM and second step gave a $K_D$ of $\sim 29$ nM. It was proposed that it might be possible that HmuT has ability to bind two hemes for every transport cycle (Mattle et al., 2010).

4.1.4 Scope of this study.

In this chapter we report the biophysical characteristics of wild type HmuT protein from C. diphtheriae via UV-visible spectroscopy, electrospray ionization spectroscopy, MALDI, heme
reconstitution studies, fluorescence spectroscopy and thermal denaturation studies. This protein was chosen to be investigated based on the homology studies. ClustalW alignment (Larkin et al., 2007) of HmuT with four homologous proteins with known crystal structures: PhuT from *P. aeruginosa* (Ho et al., 2007), ShuT from *S. dysenteriae* (Ho et al., 2007), IsdE from *S. aureus* (Grigg et al., 2007) and HmuT from *Y. pestis* (Mattle et al., 2010) indicated that it was possible that HmuT from *C. diphtheriae* would be likely to have a tyrosine as one axial ligand, and a methionine or histidine as the second. The protein was predicted to be unique in that the axial ligands were reversed with respect to the position in the sequence (N-terminal vs. C-terminal) compared to homologous proteins. These findings were also supported by the homology modeling studies via I-TASSER where created model of Cd-HmuT exhibited reverse order of axial ligation as well. The purpose of this study was to investigate distinct termini order of the axial ligand with the aid of possible unique biophysical characteristics of Cd-HmuT.

### 4.2 Experimental.

#### 4.2.1 Materials.

The HmuT clone was a gift from Dr. Michael Schmitt’s laboratory (Food and Drug Administration, Bethesda, Maryland). The HmuT protein itself is comprised of 353 amino acids, including a leader peptide (sequence shown below).

```
1  mksllracms  vvcacalygc  gvqgydstk  dldeslkap  dvlkprsftg  vsdvrdfddv
61  rpvsesvsp  lpvhldadg  fdvevdvsr  iialdiygt  ttkleglgl  dkivgrtvss
121  tenvldvvpv  vtegghnin  eavlslhpsl  livdhsgpr  daidqirnag  vttvneprtr
181  tidsvaedik  tlgsvvglsl  easilaersv  heisaareai  aaiapsdpmr  vaflargng
```
The N-terminal Strep-tagged HmuT construct was prepared starting from residue 21 (Gly) and extended through to the native stop codon. The nucleic acid and amino acid sequences of the start of the HmuT construct are shown below. The N-terminal leader sequence was deleted and replaced with a Strep-tag. In the construct, the N-terminal region contained an NcoI site (required for cloning and positioning of the ATG start codon) and an eight amino acid Strep Tag sequence. In the sequence below, the NcoI site is italicized, the Strep-tag is in bold and Gly 21 is underlined. The pI of the entire construct is calculated to be 4.71 and the calculated molecular weight is 36326.04.

```
CCATG   GCA   AGC   TGG   AGC   CAC   CCG   CAG   TTC   GAA   AAG
M    A    S    W    S    H    P    Q    F    E    K
G (21)>>
```

4.2.2 Expression and purification of HmuT.

HmuT was expressed and purified from BL21DE3 (pEThmuT) cells. Culture was prepared in a Luria-Bertini (LB) medium containing 50 µg/mL kanamycin. Inoculation was done with an overnight pre-culture and cells were grown at 37°C with shaking at 220 rpm. When the OD<sub>600</sub> of the culture reached 0.5 – 0.6, protein expression was induced by adding IPTG (isopropyl β-D-1-thiogalactopyranoside) to a final concentration of 0.5 mM. The culture was incubated about 3 h at 27°C with shaking at 220 rpm. Cells were harvested by centrifugation for 20 min at 5000 rpm at 4°C. The cell pellet was resuspended in lysis solution (100 mM Tris-HCl, 150 mM NaCl, pH 8.0) Protease inhibitor (Roche Complete Mini, EDTA-free) cocktail was added to the cell suspension to a final concentration to be 1X. The cell pellet was broken twice with a Cell disrupter (Microfluidics). The solution was cooled on ice between lysing cycles. The lysate was
then centrifuged for 20 min at 5000 rpm at 4°C, and the supernatant was syringe-filtered with a 0.45 \( \mu \)m 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 8.0 unless specified otherwise. The sample was loaded onto a Strep-Tactin Superflow column (5 mL, IBA BioTAGnology) equilibrated with buffer A (100 mM Tris-HCl, 150 mM NaCl, pH 8.0). Unbound material was washed out with 5 column volumes (CV) of buffer A. HmuT was eluted with 10 column volumes of buffer B containing 100 mM Tris-HCl, 150 mM NaCl, 2.5 mM desthiobiotin, pH 8.0 applied via a linear gradient. The purities of the fractions were evaluated using sodium dodecyl sulfate polyacrylamide gel electrophoresis.

4.2.3 Native polyacrylamide gel electrophoresis.

The oligomeric state of HmuT was investigated using NativePAGE Sample Prep Kit from Invitrogen and a 5 to 12% acrylamide gel. Protein samples were run 15 min under 80 V current and 2 h under 150 V current. A NativeMark Unstained Protein Standard (Invitrogen) was used.

4.2.4 MALDI mass spectrometry.

The mass of HmuT was evaluated using matrix-assisted laser desorption ionization (MALDI) mass spectroscopy, performed using a positive ion mode in The Mass Spectrometry Facility at Georgia State University. Experimental conditions are described in Chapter 2.
4.2.5 *Electrospray ionization mass spectrometry.*

HmuT was analyzed using electrospray ionization mass spectrometry (ESI) in positive mode; the instrumentation and general experimental conditions are described in Chapter 3. Initially, the protein was studied in a solution containing 50:50 acetonitrile:water and 0.1% formic acid. Spectra were also taken of this solution diluted 1:1 with water, diluted 1:1 with 20% aqueous MeOH and diluted 1:1 with 40% aqueous MeOH.

4.2.6 *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 Supracil cuvettes (Spectrocell, Inc.) with a 1 cm path length were used. The solution was stirred throughout the spectrophotometric measurements. The absorbance of the protein solutions were recorded from 250 to 800 nm. The Cary WinUV program was used to follow the absorption.

4.2.7 *Preparation of apoHmuT.*

ApoHmuT was prepared following the literature procedure using the cold-butanolone method (Mack et al., 2004). The protein after purification by chromatography was dialyzed against water overnight at 4 °C. During heme removal, all solutions were kept in ice throughout the experiment. Ice cold water was added to the HmuT protein solution to give a total volume of 500 µL. An ice cold aqueous HCl solution (1 M) used to drop the pH value of the HmuT protein solution to 2.3 - 2.2. Cold 2-butanolone (500 µL) was added to the HmuT solution. The mixture
was shaken well and allowed to stand on ice for about 30 min to allow the layers to separate. The top (organic) layer (heme + 2-butanone) was removed by transfer pipette. The lower (aqueous) layer was placed into a 13,000 MWCO dialysis bag and dialyzed overnight at 4 °C against the buffer relevant to the experiment to be performed.

4.2.8 **Reconstitution of HmuT**

Fresh hemin solution was prepared for each experiment by dissolving hemin in dimethyl sulfoxide (DMSO) and filtering this solution through Millipore 0.45 µm membrane filter to remove any precipitate. The concentration of hemin was determined spectroscopically using an extinction coefficient of 188 mM$^{-1}$ cm$^{-1}$ at 404 nm (Collier et al., 1979). The desired concentration of hemin was prepared in buffer by diluting the stock DMSO solution into the relevant buffer. Three equivalents of hemin solution were added to one equivalent of HmuT (20 mM Tris-HCl, 150 mM NaCl, pH 7.5). The solution was incubated at 4 °C overnight. The solution was placed in a 13,000 MWCO dialysis bag and dialyzed overnight at 4 °C against the relevant buffer. In one instance, the hemin solution was titrated into the apo protein solution; this is described in more detail in the Results.

4.2.9 **Fluorescence spectroscopy.**

Fluorescence spectra were recorded on a Perkin Elmer LS 55 fluorescence spectrophotometer. A glass cuvette with 1 cm path length was used. The emission of the organic layer was determined using an excitation wavelength of 402 nm.
4.2.10 Thermal denaturation.

Thermal denaturation of heme-reconstituted HmuT was carried out with a UV-visible spectrophotometer (Cary 50 Bio) which was equipped with a temperature control (TC 125, Quantum Northwest). The 1.5 mL quartz Supracil cuvettes (Spectracell) with 1 cm path lengths were used. For the reconstituted HmuT in Tris buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.5), the spectrum (250 – 800 nm) was recorded every 2 °C from 20 °C to 82 °C after the sample was equilibrated at each temperature. The entire experiment took approximately 3 h.

The data were fit to a two-state unfolding model (Swint & Robertson, 1993):

$$Y_{abs} = \frac{(Y_F + m_FT) + (Y_U + m_UT) \exp[\Delta H_m/R(1/T_m - 1/T)]}{1 + \exp[\Delta H_m/R(1/T_m - 1/T)]}$$

where the $\Delta H_m$ is the enthalpy of unfolding, $T_m$ is the temperature at which the protein is half unfolded and the remaining variables are as described above.

4.2.11 Spectrophotometric pH titration.

Initially, aliquots of 1.0 M HCl were added to drop the pH of the HmuT to 6.5 in a buffer that was 20 mM each of CAPS, CHES, MOPS and Tris-HCl. Titrations were run by adding aliquots of 1.0 M NaOH to the protein. A 1 cm glass cuvette was used with a pH electrode (Thermo Scientific Orion 8220BNWP); the sample was stirred.

4.2.12 Homology modeling.

To obtain a visual picture of HmuT, the sequence of this protein from *C. diphtheriae* was threaded into its ten closest homologies using I-TASSER,
Based on multiple-threading alignments by iterative TASSER assembly simulations, 3D models were built. The chosen model was visualized using Pymol (DeLano, 2009).

4.2.13 ClustalW alignment of HmuT protein to homologous proteins with known crystal structure.

To align HmuT with homologous proteins with known crystal structure, the ClustalW alignment tool (http://www.ebi.ac.uk/Tools/msa/clustalw2/) was used (Larkin et al., 2007). The sequence of HmuT from *C. diphtheriae* was aligned with PhuT from *P. aeruginosa* (Ho et al., 2007), *ShuT* from *S. dysenteriae* (Ho et al., 2007), *IsdE* from *S. aureus* (Grigg et al., 2007) and HmuT from *Y. pestis* (Mattle et al., 2010).

4.3 Results and discussion.

4.3.1 Purification of HmuT protein.

The HmuT protein with a Strep-tag was expressed and purified as described in the Experimental. The protein was eluted from the Streptactin column with a 10 CV gradient and 100% target elution buffer concentration; 2 mL fractions were collected during flow-through and 0.5 mL during peak elution. Two colored peaks were seen, a minor one at fractions 22-32 and a major one at fractions 33-38 (Figure 4.3). The purities of the fractions were evaluated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). The two colored eluents appeared the same on SDS PAGE, with a weight of about 37 kDa. Lanes 23, 28 and 32 (Figure 4.4) belong to first lightly colored peak. Lanes 33, 35, 37 and 38 (Figure 4.4) belong to second dark
colored peak. Lane 15 was the flow-through. Native PAGE of Peak 2 showed that the protein was a monomer (Figure 4.5).

Because the two peaks appeared to be identical in SDS PAGE, a later FPLC run was performed with far less protein. In that experiment, a single heme protein peak was observed Figure 4.6 (corresponding to the second and more concentrated peak, in the first FPLC run). This indicated that the column was overloaded in the first FPLC run and some of the material was coming out early, at the end of the flow-through.

For the first FPLC run, separate characterizations were performed on the two peaks and significant differences were observed, as detailed below. For each of the two peaks, salts were removed by centrifugal filtration (Amicon Ultra-4, 10,000 molecular weight cutoff, Millipore) using a buffer of 20 mM Tris-HCl pH 8.0 and 10% glycerol.

4.3.2 UV–visible spectroscopy.

The UV-visible spectrum of HmuT (Peak 2) showed a Soret peak at 406 nm. Unexpectedly, four peaks appeared in the α,β region at 517, 550, 575, and 617 nm Figure 4.7. These four bands indicate that a porphyrin of some type is binding (hemin has two bands in this region; porphyrins have four). It is not clear from the spectrum how much hemin and how much porphyrin is binding.

In the laboratory production of heme proteins, ALA can be added to the medium to increase the production of heme, which otherwise can be a limiting agent. There are many reports of porphyrin production in bacteria cells, primarily in response to addition of ALA to the medium. For example, in early work, Nitzan and Kauffman found that ALA or ALA methyl ester induced the accumulation of uroporphyrin and protoporphyrin within the cells and excretion of
coproporphyrin and protoporphyrin from the cells in *E. coli* (Nitzan & Kauffman, 1999). Dietel et al. have investigated the porphyrins formed by 31 different bacterial strains in the presence of aminolevulinic acid (ALA) (Dietel et al., 2007). Many bacteria produced protoporphyrin IX (PPIX). Some bacteria produced water-soluble porphyrins via oxidation of the corresponding porphyrinogen precursors under anaerobic conditions. The types and amounts of porphyrins varied from one bacterial strain to another; many bacteria could produce more than one fluorescent porphyrin. Fotinos et al. looked at the presence of porphyrins in bacteria treated with ALA and a series of related esters. HPLC revealed the presence of all porphyrins associated with heme biosynthesis (Fotinos et al., 2008). Tatsumi and Wachi found that *E. coli* grown in the presence of ALA accumulated coproporphyrin in the extracellular medium (Tatsumi & Wachi, 2008). The tolC mutants of this bacterium accumulated this porphyrin intracellularly, as shown by fluorescence spectrometry and HPLC. Bruce-Micah et al. have also found porphyrins accumulation in *Mycobacterium phlei* and *Mycobacterium smegmatis* upon ALA treatment (Bruce-Micah et al., 2009). A spontaneous mutant of *Rhodobacter sphaeroides* f. sp. *denitrificans* IL-106 was found to excrete significant amounts of coproporphyrin III, found to be a result of a reduced synthesis of S-adenosylmethionine in this bacterial strain (Sabaty et al., 2010). In engineered systems, ferrochelatase may not always convert all protoporphyrin to heme. For example, Kwon et al. assembled the heme biosynthetic pathway in a three-plasmid system with *Escherichia coli* as a host (Kwon et al., 2003). They noted that overexpressed *Bacillus subtilis* ferrochelatase could not convert PPIX, produced at high levels, into heme and proposed that ferrochelatase may be inactive in vivo due to incompatible enzyme interactions. The three-plasmid system did allow them to produce high levels of various porphyrins.
We know of only one example of a porphyrin-bound (rather than heme-bound) heme protein produced in the absence of addition of ALA; this is IsdC, in the heme uptake pathway in *S. aureus* (Mack et al., 2004). Sudhamsu et al., who have looked at heme incorporation into recombinant proteins, do not report examples of porphyrin binding in the absence of addition of ALA (Sudhamsu et al., 2010).

In our system, the bands positions show significant variation from those observed for PPIX in chloroform, in SDS micelles, and in IsdC (Table 4.1). Either this shift is caused by the protein environment, or another porphyrin, derived from an earlier step in the biosynthesis (e.g., coproporphyrin or uroporphyrin), is binding to the protein. Our experiments using fluorescence spectroscopy confirmed that HmuT is bound to PPIX rather than porphyrins derived from other precursors of the heme biosynthetic pathway. To our knowledge HmuT is the second protein as isolated which binds PPIX in the absence of ALA added to the medium. It may be that the observation of porphyrin is due to the over-expression of HmuT leading to incorporation of PPIX because the ferrochelatase is not able to incorporate iron quickly enough to match the needs of the system for hemin. As a result, HmuT binds both heme and PPIX.

### 4.3.3 Fluorescence spectroscopy.

In this study, the organic layer (e.g., the butanone), was analyzed with fluorescence spectroscopy. UV-visible spectroscopy cannot confirm type of the porphyrin bound because the optical spectra for the porphyrins are fairly similar and presumably significantly affected by interactions with the protein binding site. Fluorescence spectroscopy can differentiate coproporphyrin and uroporphyrin from PPIX (Tatsumi & Wachi, 2008) but cannot readily differentiate coproporphyrin and uroporphyrin from each other (Shindi et al., 2010). The fluores-
cence spectrum of the organic layer derived from HmuT showed a band at 635 (Figure 4.8) nm, which is the signature for the protoporphyrin IX. This was consistent with the mass spectrometry results, which showed PPIX, but not uroporphyrin or coproporphyrin.

4.3.4 MALDI mass spectrometry of HmuT.

The mass of the purified HmuT (Peak 2) was evaluated using matrix-assisted laser desorption ionization (MALDI) mass spectroscopy (Figure 4.9). HmuT showed a peak at 36166.2 Da. This result is consistent with the expected molecular weight of apoHmuT of 36195 Da. The lower mass region of the spectrum confirmed the presence of heme and protoporphyrin IX. The MALDI spectrum of Peak 1 was very similar (36098 Da, not shown).

4.3.5 Electrospray ionization mass spectrometry and charge distribution bands of HmuT protein.

Electrospray ionization mass spectrometry (ESI) was performed on Peak 2 of HmuT. The first sample was prepared in a solution containing 50:50 acetonitrile:water and 0.1% formic acid (denaturing conditions). The deconvoluted mass spectrum showed a peak at 36197.5 Da (Figure 4.10) consistent with the expected molecular weight of apoHmuT (36195 Da). apoHmuT peak showed a wide charge distribution band Figure 4.10 (from +40 to +13) centered at about +25 (e.g., the peak at 1448.9 has a charge of +25).

To determine the charge distribution band of the holoprotein, the sample was prepared again in water (specifically, a sample in water diluted 1:1 with water) Figure 4.11. In the charge distribution spectrum, the peaks appeared as duplicates. These corresponded to protein with one and two tetrapyrrroles bound. For example, the peak at 1938.0 corresponds to protein with a
charge of +19 and one tetrapyrrole. The peak directly to the right of this (1970.4) corresponds to protein with a charge of +19 and two tetrapyrroles bound. For lower charged species, the peak at 3325.8 corresponds to protein with a charge of +11 and one tetrapyrrole. The peak directly to the right of this (3382.2) corresponds to protein with a charge of +11 and two tetrapyrroles bound. Deconvolution of the spectrum gave two very broad holoHmuT peaks, the first in the region of 36784 Da and second in the region of at 37443 Da. Both peaks were very broad, consistent with a mixture of hemin and PPIX binding to the protein (as expected from both the UV-visible spectrum and the mass spectrum in the lower molecular weight region). The mass of the first peak was consistent with the HmuT binding a single tetrapyrrole (between 36757 for PPIX and 36811 for hemin). The mass of the second peak was consistent with binding of two tetrapyrroles (37320 for two PPIX, 37374 for one PPIX/one hemin and 37428 for two hemin).

We also note that the charge distribution envelope showed two very distinct regions, one between +23 and +16, centered at about +20, and a second region of just two charged species, corresponding to charges of +12 and +11. This indicates that the holoprotein is likely to have two different conformations in the gas phase, one more charged than the other. In contrast, the apoprotein showed a single charge envelope.

It is possible that this finding is due to dimerization of HmuT in the gas phase. If the dimer interface protects the protein from protonation, the dimer would show species with a larger m/z in comparison with the monomer. Consistent with this idea, other heme proteins have been found as dimers. For example, the crystal structure of Shp from S. pyogenes revealed that Shp forms dimer (Aranda et al., 2007). The structure showed a heme at the expected binding site as well as two exogenous hemin molecules, which formed a stacked pair at the interface between the two Shp proteins. Another example is the ChaN protein from Campylobacter jejuni (Chan et
Analytical ultracentrifugation studies showed that the protein is predominantly monomeric in its apo form. In contrast, the x-ray structure shows that the holoprotein forms a dimer in which the heme molecules at the two binding sites adopt a stacked cofacial arrangement with one another. IsdH from *S. aureus* also showed partial overlap of hemes at interface between the subunits (Watanabe et al., 2008).

The one and two tetrapyrrole binding aspect of this study was pursued by changing the sample conditions to 20% MeOH (Figure 4.12) and 40% MeOH (Figure 4.13). Increasing the MeOH concentration reduced the relative amount of the species with two tetrapyrroles bound.

### 4.3.6 Heme binding to HmuT

Heme binding studies were initially performed on Peak 1 (the partially purified protein). Samples containing 5 µM hemin in 20 mM Tris-HCl and 150 mM NaCl, pH 7.5 buffer and three concentrations of apo HmuT (3, 6, and 10 µM) were prepared. The UV-visible spectrum of each sample was taken after 5 min at 4 °C. All three solutions showed a peak at approximately 350 nm and approximately 406 nm (Figure 4.14). Following related work in HmuT from *Yersinia pestis* (Mattle et al., 2010), the band at 350 nm is assigned to the protein with a hemin dimer at the active site and the band at 406 is assigned to the protein with a hemin monomer at the active site. After standing at 4 °C for 3 d, the intensity of the peak at 350 nm had decreased and the intensity of Soret band had increased (Figure 4.15). This is consistent with conversion over time of the species with two hemin molecules at the binding site to a species with one hemin molecule at the binding site. Figure 4.16 shows an overlay of the spectra with 5 µM hemin and 10 µM apoHmuT at 5 min and 3 d.
Heme binding studies were then performed on Peak 2 (the protein). In this experiment, 0.5 μM hemin incubated was with 0.5 μM of apoHmuT for 20 h (20 mM Tris-HCl and 150 mM NaCl, pH 7.5, 20 °C) (Figure 4.17). A spectrum was taken every hour; the Soret increased by only about 10% over the time of the experiment and very little change was seen in the 350 nm region. No shift was seen in the position of the Soret band. The second experiment was done with 10-fold higher concentrations of hemin and apoHmuT (5 μM of hemin and 5 μM of apoHmuT) (Figure 4.18). The UV-visible spectrum was recorded every hour for 21 h. Again, the Soret band did not shift. In this case, the intensity increased by 31% over the time of the experiment. We had hoped that incubation with a higher heme concentration might favor binding of the heme dimer to the apoHmuT. However, this was not seen. Rather, we observed that the heme dimer was not seen for the pure protein, but was seen for impure protein. This may indicate that the heme dimer binds to protein that is damaged or complexed in some way. It has been speculated that uptake of a heme dimer might be more effective than uptake of a heme monomer in a biological pathway (Mattle et al., 2010). Our work introduces that idea that different preparations of the protein may bind heme differently, indicating that simple conclusions may not be appropriate.

4.3.7 Thermal denaturation.

Heme loss from holoHmuT (reconstituted Peak 2) due to the protein unfolding was recorded via thermal denaturation (Figure 4.19). Two distinct events were seen. In the first event, smooth spectral changes were seen between 20 °C and 68 °C, with a gradual decrease at 406 nm region. At this point, the Soret had blue shifted about 15 nm. In the second event, a gradual decrease was seen at about 390 nm from 70 °C to 82 °C. Nonlinear least squares fitting of absorb-
ance at 406 nm vs. temperature for the whole data set gave a $T_m$ value of $67.5 \pm 0.1$ °C (Figure 4.20). In the 360 nm region, the spectra decreased in intensity up to 68 °C, then began to increase in intensity; this was consistent with loss of heme. A band at 630 nm showed isosbestic changes up to 68 °C. After this, a slight increase in absorbance was seen at the isosbestic point, indicating that more than two states were involved in the thermal denaturation. A graph of absorbance at 394 nm vs. temperature also indicated the presence of more than one event in thermal denaturation (Figure 4.21).

4.3.8 *Spectrophotometric pH titration.*

Titration (with aliquots of 1 M NaOH) of heme-reconstituted holoHmuT (Peak 2) gave a slight decrease at the Soret (406 nm) with increasing pH (Figure 4.22). The absorbance at 360 nm region increased throughout the experiment. The Soret did not shift upon addition of base between pH 6.5 and 12.3. The protein did not show a significantly pH dependence up to a pH of 10.5. After this point, a slight decrease was seen with increasing pH. The graph of absorbance at 417 nm vs. pH (Figure 4.22) shows that HmuT essentially did not have sensitivity to pH (note the very small change on the y axis).

4.3.9 *ClustalW alignment and homology modeling of HmuT.*

Axial ligands are one of the most important factors in positioning heme at the binding site of a heme protein. The most common axial ligands are His, Met and Tyr (Schneider et al., 2007).

ClustalW alignment (Larkin et al., 2007) of HmuT was performed with four related proteins with known crystal structures: PhuT from *P. aeruginosa* (Ho et al., 2007), ShuT from *S. dysenteriae* (Ho et al., 2007), IsdE from *S. aureus* (Grigg et al., 2007) and HmuT from *Y. pestis*
PhuT \((P. \text{aeruginosa)}\) and ShuT \((S. \text{dysenteriae)}\) have a tyrosine residue as the only axial ligand \((\text{Ho et al., 2007)}\). The axial Tyr in PhuT is hydrogen bonded to an arginine, which also has a hydrogen bonding interaction with the heme propionate.

ShuT, the homologous protein to PhuT, has lysine residue corresponding to arginine in PhuT. However, the crystal structure shows that this is not near the axial Tyr ligand. The tyrosine axial heme ligand \((\text{Tyr67 in ShuT and Tyr71 in PhuT)}\) aligns with Tyr70 of Yp-HmuT \((Y. \text{pestis)}\). Cd-HmuT does not have a tyrosine in this position. However, Cd-HmuT has a histidine two residues toward the C-terminus of the protein that might serve as an axial ligand. This His in Cd-HmuT aligns with the axial Met in IsdE \((S. \text{aureus)}\).

The second axial ligand in Yp-HmuT is His167. In the alignment between Yp-HmuT and Cd-HmuT, His167 of Yp-HmuT is near Tyr235 of Cd-HmuT. In that the axial ligand set of Yp-HmuT is Tyr70 and His167, the axial ligand set of Cd-HmuT may be His136 and Tyr235. Thus, a likely axial ligation set for Cd-HmuT protein is the same as that in Yp-HmuT but in the reverse order in regard to the N/C-termini positions of the residues.

One can also align Cd-HmuT with IsdE, the protein that serves the same function in the heme uptake pathway in \(S. \text{aureus}\). Here, His136 in Cd-HmuT aligns with the axial Met \((\text{Met78)}\) in IsdE. In this alignment Met292 in Cd-HmuT aligns with the axial His \((\text{His229)}\) in IsdE. Thus, a second possibility for an axial ligand set for Cd-HmuT is the same as that in IsdE \((\text{e.g., His and Met)}\) but in the reverse order in regard to the N/C-termini positions of the residues. The distinct termini order for possible axial ligation in Cd-HmuT protein might be indication of unique biophysical characteristic of the protein.

A homology model of HmuT was created by I-TASSER with the Yp-HmuT as one of the templates \((\text{Figure 4.24)}\). The root mean square difference \((\text{RMSD)}\) between the model and Yp-
HmuT was 0.625. The model showed that His136 was located near the heme but on the opposite (N-terminal) side of the heme compared to the axial His residues (C-terminal) of Yp-HmuT and IsdE. Homology modeling of Cd-HmuT also shows both Met292 and Tyr235 to be on the opposite side of the heme from His136 (e.g., on the C-terminal side). Overall, the I-TASSER model would allow either Met292 or Tyr235 to be the second axial ligand for this protein.

There are two b-type heme proteins with known crystal structures and His-Tyr axial ligation sets; they are HasA from *Serratia marcescens* (Arnoux et al., 1999) and HmuT from *Y. pestis* (Mattie et al., 2010). HasA is a hemophore secreted by the Gram-negative bacteria *Serratia marcescens*. Heme is transferred from this protein to the outer membrane receptor HasR. HasA has axial ligands of His32 and Tyr75. The midpoint reduction potential of HasA was reported to be -550 mV (Izadi et al., 1997). The very low midpoint reduction potential of HasA is characteristic of Tyr ligation, due to the negative charge of the tyrosinate ligand that favors the ferric oxidation state. Indeed, HasA and a number of its mutants can only be prepared in the ferrous form under CO, which traps the reduced heme (Lukat-Rodgers et al., 2008). HmuT is also very difficult to reduce; as for HasA, it was necessary to reduce the protein under CO to obtain a stable ferrous form of the protein. These observations are consistent with a His-Tyr axial ligand set.

Yp-HmuT also has the His-Tyr axial ligand set. There is as yet no published electrochemistry on this protein. The crystal structure of Yp-HmuT shows that this protein binds two hemes (as a stacked dimer) in its binding pocket; histidine and tyrosine are the axial ligands. The ESI results on some samples of Cd-HmuT are consistent with two hemes binding to this protein. Thus, the biophysical data for Yp-HmuT are also consistent with a His-Tyr axial ligand set for Cd-HmuT.
Examples of Met-His ligated $b$-type heme proteins with known crystal structures are IsdE from *S. aureus* (Grigg et al., 2007), cellobiose dehydrogenase (CDH) from *Phanerochaete chrysosporium* (Rotsaert et al., 2003), cytochrome $b_{562}$ from *E. coli* (Xavier et al., 1978; Mathews et al., 1979) and EcDOS from *E. coli* (Gonzalez et al., 2002). All these four proteins have C-terminal Met/N-terminal His ligation. Positive reduction potentials have been reported for CDH (Igarashi et al., 1999) and cytochrome $b_{562}$ (Barker et al., 1996). Indeed, all reduction potentials for $b$-type heme proteins with Met and His axial ligands are positive as reported in the Gibney database (Reedy et al., 2008), as is that of the heme uptake protein SiaA in *S. pyogenes* (which also has Met and His axial ligands) (Sook et al., 2008). The electrochemical data argue strongly against a Met-His axial ligation set for this protein.
Figure 4.1 Genes (from *hmu* locus) associated heme uptake in *C. diphtheriae* reproduced from (Allen & Schmitt, 2009).

Figure 4.2 Location of the heme uptake proteins in *C. diphtheriae* reproduced from (Allen & Schmitt, 2009).
Figure 4.3  FPLC of HmuT on a GE Healthcare ÄKTA instrument at 4 °C. The sample was loaded onto a Strep-Tactin Superflow column equilibrated with buffer A (100 mM Tris-HCl, 150 mM NaCl, pH 8.0). Unbound material was washed out with 5 CV of buffer A. HmuT was eluted with 10 CV of buffer B containing 100 mM Tris-HCl, 150 mM NaCl, and 2.5 mM desthiobiotin, pH 8.0 applied via a linear gradient. The first peak (fractions 22-32, red letters on axis), which is essentially at the end of the flow-through, is designated as Peak 1 and the second peak (fractions 33-38) is designated as Peak 2.
Figure 4.4  SDS PAGE of HmuT fractions using a 5 to 15% acrylamide gel. Lane L is the Precision Plus Protein Standard. The rest of the lanes are the FPLC fractions as indicated by the numbers on the top of the gel. Fraction 23 is associated with Peak 1. Fractions 33, 35, and 37 are associated with Peak 2. Lane 15 is flow-through. Samples were run 5 min under 80 V current and 1 h under the 120 V current.
Figure 4.5  FPLC-purified HmuT protein subjected to 5-12% native polyacrylamide gel electrophoresis. Lane 1: Marker (NativePAGE Sample Prep Kit from Invitrogen), lane 2: bovine serum albumin (BSA), lane 5: HmuT Peak 2 from FPLC purification, lane 6: HmuT protein Peak 1 from FPLC purification. The gel was run as described in the Experimental. It can be seen that both Peak 1 and Peak 2 are monomeric HmuT. Peak 2 is purer than Peak 1. Lanes 2 and 3 are HtaA.
Figure 4.6  FPLC of a second batch of HmuT protein. Conditions were as in the previous figure, except for a much lower protein loading. The sample was loaded onto a Strep-Tactin Superflow column equilibrated with buffer A (100 mM Tris-HCl, 150 mM NaCl, pH 8.0). Unbound material was washed out with 5 CV of buffer A. HmuT was eluted with 10 CV of buffer B containing 100 mM Tris-HCl, 150 mM NaCl, 2.5 mM desthiobiotin, pH 8.0 applied via a linear gradient.
Figure 4.7  UV-visible spectrum of Peak 2 of the HmuT as isolated (150 mM NaCl and 20 mM Tris-HCl, pH 7.5). Wavelengths of the peaks are indicated. The Soret was at 406 nm.
Figure 4.8  Fluorescence spectrum (in 2-butane) of the tetrapyrroles in HmuT after removal by incubation with cold 2-butane. The excitation was at 402 nm. The peak is seen at 635 nm.
Figure 4.9  Positive ion mode MALDI spectrum of HmuT.  Top panel: The mass spectrum of HmuT shows a species at 36166.2 Da, consistent with the expected molecular weight of 36195 Da. Bottom panel: The lower molecular weight area of the spectrum shows peaks for both heme and PPIX.
Figure 4.10  Electrospray mass spectrum of apoHmuT. The sample was prepared in a solution containing 50:50 acetonitrile:water and 0.1% formic acid (denaturing conditions). Top panel: Charge distribution bands. Bottom panel: The apoHmuT is seen at 36197.5 Da, consistent with the expected 36195 Da.
Figure 4.11  Electrospray mass spectrum of holoHmuT. The sample was prepared in water. Top panel: The duplication of peaks in the charge envelope is consistent with the protein binding one or two tetrapyrroles. Bottom panel: The holoHmuT is seen at 36783.5 Da, consistent with the holoprotein (expected 36811 Da) or PPIX-bound protein (expected 36757 Da). The peak at 37443.0 Da is appropriate for two tetrapyrroles bound (two hemin molecules, two PPIX molecules, or one hemin and one PPIX). The very broad peaks presumably reflect the mixture of hemin and PPIX bound to this protein.
Figure 4.12  Electrospray mass spectrum of holoHmuT.  The sample was prepared in water with 20% aqueous MeOH added in a 1:1 ratio to give a final solution of 10% MeOH.  Top panel: Charge distribution bands of HmuT.  The duplication of peaks in the charge envelope is consistent with the protein binding one or two tetrapyrroles.  Bottom panel: The peak at 36731.5 Da consistent with the holoprotein (expected 36811 Da) or PPIX-bound protein (expected 36757 Da).  The peak to the right presumably represents the protein with bound tetapyrrole dimer.
Figure 4.13  Electrospray mass spectrum of holoHmuT. The sample was prepared in water with 40% aqueous MeOH added in a 1:1 ratio to give a final solution of 20% MeOH. Under these conditions, the peak for binding tetrapyrrole dimer is becoming less intense.
Figure 4.14  Three samples of a solution of 5 μM hemin in 20 mM Tris-HCl and 150 mM NaCl, pH 7.5 titrated to which were added increasing concentrations of apoHmuT (from Peak 1). The spectrum was taken after 5 min of incubation.
Figure 4.15  Three samples of a solution of 5 μM hemin in 20 mM Tris-HCl and 150 mM NaCl, pH 7.5 titrated to which were added increasing concentrations of apoHmuT (from Peak 1). The spectrum was taken after 3 d of incubation.
Figure 4.16  Comparison of the spectra of the sample with 5 \( \mu \)M hemin and 10 \( \mu \)M apoHmuT (Peak 1) after 5 min and 3 d of incubation at 4 °C (20 mM Tris-HCl and 150 mM NaCl, pH 7.5).
Figure 4.17  A solution of 0.5 μM hemin and 0.5 μM apoHmuT (Peak 2) incubated in 20 mM Tris-HCl and 150 mM NaCl, pH 7.5 for 20 h at 20 °C. Spectra were taken every hour. The Soret is at 406 nm, indicating that the heme is binding as the monomer. There are minimal changes over time, indicating that Peak 2 reconstitutes easily and that once reconstituted, the protein undergoes no further seen in the UV-visible spectra.
Figure 4.18 A solution of 5 μM hemin and 5 μM apoHmuT (Peak 2) incubated in 20 mM Tris-HCl and 150 mM NaCl, pH 7.5 for 20 h at 20 °C. Spectra were taken every hour. The Soret is at 406 nm, indicating that the heme is binding as the monomer. The Soret increases over time, indicating that reconstitution is slower in this experiment, in which both hemin and apoHmuT concentrations are 10x the previous experiment. The band at approximately 380 nm increases slightly over time, which is inconsistent with an initial bound hemin dimer converting to a bound hemin monomer over time.
Figure 4.19  Thermal denaturation of holoHmuT (reconstituted Peak 2). The temperature was increased from 20 °C to 82 °C in 2 °C increments. After each temperature change, the solution was allowed to stand until the temperature was stable (about a minute) before the spectrum was recorded. The protein was in 20 mM Tris-HCl, 150 mM NaCl, pH 7.5 buffer. The spectrum changes smoothly from 20 to 68 degrees. The last spectrum in this series has an absorbance of approximately 0.63 at the Soret. After this point (which is very close to the midpoint in the fitting), the spectrum changes considerably.
Figure 4.20  Absorbance at 406 nm vs. temperature for heme-reconstituted Peak 2. The full spectra are given in the previous figure. The data were fit to the thermal unfolding equation given in the Experimental.
Figure 4.21  Absorbance at 394 nm vs. temperature for heme-reconstituted Peak 2.  The full spectra are given in the previous figure.  At even lower wavelengths (approximately 360 nm) the spectrum first decreases and then increases, consistent with the release of free heme.
Figure 4.22  Spectrophotometric pH titration of HmuT (reconstituted Peak 2). The protein, in a buffer that was 20 mM each CAPS, CHES, MOPS and Tris-HCl was titrated with 1.0 M NaOH. Top panel: spectra as a function of pH. Bottom panel: The absorbance at 417 nm vs. pH. The protein shows essentially no sensitivity to pH, at least up to pH 10.5.
Figure 4.23  ClustalW alignment of HmuT to homologous proteins with known crystal structures. The axial tyrosine ligands of PhuT (Y71) and ShuT (Y67) are indicated in purple. The axial ligands of Yp-HmuT (tyrosine 70 and histidine 167) are in blue. The axial ligands of IsdE (methionine 78 and histidine 229) are in orange. The most likely axial ligands of HmuT (methionine 292, histidine 136 and tyrosine 235) are indicated in red.
Figure 4.24  Homology model of HmuT built with I-TASSER showing histidine 136 (blue), methionine 292 (orange) and tyrosine 235 (magenta).
## Table 4.1  Spectral bands of PPIX.

<table>
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<th>Wavelength (nm)</th>
<th>Wavelength (nm)</th>
<th>Wavelength (nm)</th>
<th>Wavelength (nm)</th>
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<td>575</td>
<td>630</td>
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</tr>
<tr>
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<td><strong>IsdC</strong></td>
<td>504</td>
<td>537</td>
<td>576</td>
<td>631</td>
<td>(Mack et al., 2004)</td>
</tr>
<tr>
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<td>CHCl₃ 498</td>
<td>532</td>
<td>569</td>
<td>596</td>
<td>(Smith, 1975)</td>
</tr>
<tr>
<td><strong>Copro TME[^2]</strong></td>
<td>CHCl₃ 498</td>
<td>532</td>
<td>566</td>
<td>594</td>
<td>(Smith, 1975)</td>
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<tr>
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<td>aq SDS 505</td>
<td>541</td>
<td>578</td>
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<td><strong>Copro TME[^2]</strong></td>
<td>aq SDS 499</td>
<td>533</td>
<td>562</td>
<td>615</td>
<td>(Smith, 1975)</td>
</tr>
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</table>

[^1]: DME: dimethyl ester.
[^2]: TME: tetramethyl ester.
4.4 Reference List


5 CHARACTERIZATION OF HTAA, A NOVEL HEME-BINDING PROTEIN FROM 

*CORYNEBACTERIUM DIPHTHERIAE*

5.1 Introduction.

Background information including initial findings on HtaA was given in the introduction of Chapter 4. In this chapter we report the biophysical characteristics of wild type HtaA protein from *C. diphtheriae* using several techniques e.g. UV-visible spectroscopy, electrospray ionization spectroscopy, MALDI, heme reconstitution studies, fluorescence spectroscopy, NATIVE PAGE and spectrophotometric pH titration. This protein was chosen to be investigated based on the homology studies. The created model of HtaA by I-TASSER was mainly unstructured with no close homology to proteins with known structures (Zhang, 2008; Zhang, 2009; Roy et al., 2010). In addition initial studies in the Dr. Schmitt laboratory together with homology studies showed that HtaA has two conserved domains with conserved key residues. They are associated with heme binding and show no homology to previously determined heme binding (NEAT) domains. All these initial findings made HtaA the subject of our research. The purpose of this study was to investigate biophysical characteristics and determine the axial ligation of this novel heme binding protein.

5.2 Experimental.

5.2.1 Materials.

The HtaA clone was a gift from Dr. Michael Schmitt’s laboratory (Food and Drug Administration, Bethesda, Maryland). The HtaA protein contains 591 amino acids, including a leader peptide (sequence shown below). HtaA has two domains; the National Center for Bio-
technology Information (NCBI) defines these as shown below in red and blue. Dr. Schmitt’s laboratory created a construct for the second domain designated as CR2.

The N-terminal Strep-tagged HtaA CR2 construct was prepared starting from residue 309 (Asp) and extended through to 564 (Pro) (considerably extended from the domain as assigned in the NCBI database). The nucleic acid and amino acid sequences of the start of the HtaA CR2 construct are shown below. In the construct, the N-terminal region contained an NcoI site (required for cloning and positioning of the ATG start codon) and an eight amino acid Strep-tag sequence that was followed by two amino acid residues for proper recognition of the Tag. In the sequences below, the NcoI site is italicized, the Strep-tag is in bold and aspartate 309 is underlined. The pI of the entire construct is calculated to be 5.95 and the calculated molecular weight is 27449.90.
5.2.2 Expression and purification of HtaA.

HtaA was expressed and purified using the same methods described for HmuT in Chapter 4. The purities of the fractions were evaluated using sodium dodecyl sulfate polyacrylamide gel electrophoresis.

5.2.3 Native polyacrylamide gel electrophoresis.

The oligomeric state of HtaA was investigated using the same experimental conditions described in Chapter 4.

5.2.4 MALDI mass spectrometry.

Matrix-assisted laser desorption ionization (MALDI) mass spectroscopy performed to evaluate mass of HtaA. The experimental conditions were the same as described in Chapter 4.

5.2.5 Electrospray ionization mass spectrometry.

HtaA was analyzed using electrospray ionization mass spectrometry (ESI) in positive mode; the instrumentation and general experimental conditions were the same as described in Chapter 4. The protein was prepared in water and the original solution diluted 1:1 with water for the ESI. Another spectrum was taken with a solution containing 50:50 acetonitrile:water and 0.1% formic acid.
5.2.6 *UV–visible spectroscopy.*

UV–visible spectra of HtaA were recorded with the same instrumentation and general experimental conditions described on HmuT (Chapter 4).

5.2.7 *Preparation of apoHtaA and Reconstitution of HtaA.*

ApoHtaA and reconstituted holoHtaA were prepared following the same method as for HmuT.

5.2.8 *Fluorescence spectroscopy.*

Fluorescence spectra of HtaA were recorded under the same experimentation described for HmuT. The emission of the organic layer was determined using an excitation wavelength of 402 nm.

5.2.9 *Spectrophotometric pH titration.*

pH sensitivity of HtaA was investigated using the same instrumental and experimental conditions that were for HmuT.

5.2.10 *Homology modeling.*

A homology model of HtaA was built using I-TASSER, (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) (Zhang, 2008; Zhang, 2009; Roy et al., 2010). Based on multiple-threading alignments by iterative I-TASSER assembly simulations, 3D models were built. The chosen model was visualized using Pymol (DeLano, 2009). Figure 5.1 shows the model and the specific amino acids of interest in this work.
5.3 Results and discussion.

5.3.1 Purification of HtaA protein.

The HtaA protein (with a Strep-tag) was expressed and purified as described in the Experimental. The Streptactin column with a 10 CV gradient and 100% target elution buffer concentration was used to elute the protein. Two colored peaks were seen, a minor one at fractions 22-30 and a major one at fractions 31-37 (Figure 5.2). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) was used to evaluate the purities of the fractions. The two colored eluents appeared at the same weight of about 37 kDa region. Lanes 22, 25 and 28 (Figure 5.3) belong to first lightly colored peak. Lanes 31, 35 and 37 (Figure 5.3) belong to second dark colored peak. Lane 13 was the flow-through. Native PAGE of Peak 2 showed that the protein was multimeric (Figure 5.4).

In later experiment, far less protein was purified by FPLC and a single heme protein peak was observed Figure 5.5 (corresponding to the second, and more concentrated, peak in the first FPLC run). It is concluded that the column was overloaded in the first FPLC run and some of the material (as designated Peak 1) was coming out early, at the end of the flow-through.

All biophysical characterization of HtaA was performed on Peak 2. Salts were removed by centrifugal filtration (Amicon Ultra-4, 10,000 molecular weight cutoff, Millipore) using a buffer of 20 mM Tris-HCl pH 8.0 and 10% glycerol.

5.3.2 UV–visible spectroscopy.

The UV-visible spectrum of HtaA (Peak 2) showed a Soret peak at 408 nm. Similar to HmuT, four peaks appeared in the α,β region at 510, 545, 573, and 627 nm Figure 5.6. These
four bands indicated binding of some type of porphyrin (hemin has two bands in this region; porphyrins have four). Later experiments with fluorescence spectroscopy confirmed that HtaA is bound to PPIX, not the other precursors of the heme biosynthetic pathway.

Enhancement of the heme biosynthetic pathway, e.g., by addition of ALA, may cause proteins to bind the immediate precursor of heme in the heme biosynthetic pathway, protoporphyrin IX (PPIX) (Nitzan & Kauffman, 1999). This is because the enzyme ferrochelatase, which catalyzes insertion of iron into PPIX, cannot keep up with PPIX biosynthesis. To our knowledge, the only previous report of protoporphyrin binding to a heme protein as isolated without addition of ALA is that in the production of IsdC from S. aureus (Mack et al., 2004) and our HmuT observations reported above. HtaA is the third example of this kind.

5.3.3 Fluorescence spectroscopy.

Fluorescence spectroscopy was used to analyze the 2-butanone experiment. As described above, fluorescence can distinguish PPIX from copro- and uro-porphyrin (but not the latter two readily from one another) (Tatsumi & Wachi, 2008; Shindi et al., 2010). In our study, the fluorescence spectrum of organic layer for HtaA gave a band at 635 (Figure 5.7) nm which is the signature for the protoporphyrin IX.

5.3.4 MALDI mass spectrometry of HtaA.

The mass of the purified HtaA (Peak 2) was evaluated MALDI mass spectroscopy (Figure 5.8). HtaA showed a peak at 27378.6 Da. This result is consistent with the expected molecular weight of apoHtaA of 27318 Da. The MALDI spectrum of Peak 1 was very similar (27144.5 Da, not shown).
5.3.5 *Electrospray ionization mass spectrometry and charge distribution bands of HtaA protein.*

Electrospray ionization mass spectrometry (ESI) was performed on Peak 2 of HtaA. The first sample was prepared in a solution containing 50:50 acetonitrile:water and 0.1% formic acid (denaturing conditions). The deconvoluted mass spectrum showed a peak at 27324.0 Da (Figure 5.9) consistent with the expected molecular weight of apoHtaA (27318 Da). ApoHtaA peak showed a charge distribution band Figure 5.9 (from +23 to +11) centered at about +17 (e.g., the peak at 1608.2870 has a charge of +17).

In an attempt to determine the charge distribution band of the holoprotein, the sample was prepared again in water (specifically, a sample in water diluted 1:1 with water) Figure 5.10. In the charge distribution spectrum, the peaks appeared as duplicates. The charge distribution band (from +20 to +10) was centered at about +14 (e.g., the peak at 1954.4921 has a charge of +14 associated with the lighter peak). Deconvolution of the spectrum gave two broad HtaA peaks, the first in the region of 27386 Da and second in the region of 27735 Da. The expected molecular weight of apoHtaA is 27318 Da; holoHtaA is expected to be at 27934 and PPIX-HtaA is expected to be at 27880. Thus, neither of the experimental peaks was consistent with apoprotein, or holoprotein, or the PPIX protein. It is possible that the lighter peak in the spectrum is apoHtaA associated with three sodium atoms. The origin of the second peak, 349.0 Da heavier than the first peak, is not yet known. We note that HtaA is very prone to aggregation, appearing as a hexamer in the native PAGE. If it is observed in the gas phase as a hexamer (with the +6 charge), then an aggregate with five apoproteins and one holoprotein could explain the lighter peak while an aggregate with four apoproteins and two PPIX proteins could explain the
heavier peak. It is likely that, if the system is a multimer, a mixture of hemin and PPIX are binding (the lower molecular weight region of the spectrum shows peaks for both heme and PPIX. In other systems, the extent of hemin binding is a function of the aggregation state of the protein (Watanabe et al., 2008).

5.3.6 Heme binding to HtaA.

Heme binding studies were performed on Peak 2 (the protein) of HtaA. In this experiment, 0.5 μM hemin was incubated with 0.5 μM of apoHtaA for 24 h (20 mM Tris-HCl and 150 mM NaCl, pH 7.5, 20 °C) (Figure 5.11). A spectrum was taken every hour; the Soret decreased by about 30% over the time of the experiment with similar change in the 350 nm region. There were no significant changes in the positions or the ratios of the bands over time, indicating that the heme, once reconstituted, is in a stable form. It is not yet clear why the spectrum decreases over time. This might be due to protein precipitation, since HtaA is a domain and multimeric in solution.

A second experiment was done with a 10-fold higher concentration of hemin and apoHtaA (5 μM of hemin and 5 μM of apoHtaA) (Figure 5.12). The UV-visible spectrum was recorded every hour for 18 h. Again, the Soret band did not shift. In this case, the intensity of Soret decreased about 10% between the beginning and the end of the experiment. We note, however, that the Soret, although generally decreasing, increased to a small extent at some time points. The ratio of the Soret to 375 nm intensity remained about constant, indicating that a higher heme concentration did not favor binding of the heme dimer to the apoHtaA (assuming that the bound dimer is seen at about 375 nm).
5.3.7 **Spectrophotometric pH titration.**

Aliquots of 1 M NaOH used for titration of heme-reconstituted holoHtaA (Peak 2). A slight decrease at the Soret was seen with increasing pH (Figure 5.13). The absorbance at 360 nm region increased up to pH of about 9.5 and then decrease up to pH of 12.3. The Soret blue-shifted 7 nm upon addition of base between pH 6.5 and 12.3. The protein did not show significant pH dependence up to a pH of 10.5. After this point, a slight decrease was seen with increasing pH. The graph of absorbance at 404 nm vs. pH (Figure 5.13) shows that HtaA optical spectrum was essentially insensitive to pH (note the very small change on the y axis).

5.3.8 **Homology modeling and ClustalW alignment of HtaA.**

ClustalW alignment (Larkin et al., 2007) of HtaACR2 domains from four Corynebacterium species (C. diphtheria, C. aurimucosum, C. jeikeium and C. ulcerans) was performed (Figure 5.14). This showed high similarity among the HtaACR2 domains of these organisms with conserved Tyr19, His70 and Tyr148 residues. There is no sequence similarity with the NEAT domain.

Allen and Schmitt showed that mutation of His412 and Tyr490 (His70 and Tyr148 in HtaACR2 domain) have an impact on hemin binding; a greater impact was seen with the Tyr361A (Tyr19 in HtaACR2 domain) mutant (Allen & Schmitt, 2011).

A homology model of HtaACR2 domain was created by I-TASSER (Zhang, 2008; Zhang, 2009; Roy et al., 2010) using ten template proteins chosen as probable best models by the program (Figure 5.1). None of the template proteins are heme proteins and they generally have quite different structures from one another. The model is able to predict very little structure; it is mainly random loops with two very shorts α-helices and four very short β-sheets. The locations
of Tyr19, His70 and Tyr148 are shown in the I-TASSER model (Figure 5.1). Although we have indicated a possible placement of the likely axial ligands, we note that the sequence similarity to the template proteins is low. Overall, we conclude that HtaA is significantly different from proteins of known structure.

MCD studies on holoHtaACR2 indicated that Tyr is likely an axial ligand.
Figure 5.1  Homology model of HtaA built with I-TASSER showing tyrosine 19 (magenta), histidine 70 (blue) and tyrosine 148 (yellow).
Figure 5.2 FPLC of HtaA on a GE Healthcare ÄKTA instrument at 4 °C. The sample was loaded onto a Strep-Tactin Superflow column equilibrated with buffer A (100 mM Tris-HCl, 150 mM NaCl, pH 8.0). Unbound material was washed out with 5 CV of buffer A. HtaA was eluted with 10 CV of buffer B containing 100 mM Tris-HCl, 150 mM NaCl, and 2.5 mM desthiobiotin, pH 8.0 applied via a linear gradient. The first peak (fractions 22-30, red letters on axis), which is essentially at the end of the flow-through, is designated as Peak 1 and the second peak (fractions 31-37) is designated as Peak 2.
Figure 5.3  SDS PAGE of HtaA fractions using a 5 to 15% acrylamide gel. Lane L is the Precision Plus Protein Standard. The rest of the lanes are the FPLC fractions as indicated by the numbers on the top of the gel. Fraction 22 is associated with Peak 1. Fractions 31, 35, and 37 are associated with Peak 2. Lane 13 is flow-through. Samples were run 5 min under 80 V current and 1 h under the 120 V current.
Figure 5.4  FPLC-purified HtaA protein subjected to 5-12% native polyacrylamide gel electrophoresis. Lane 1: Marker (NativePAGE Sample Prep Kit from Invitrogen); lane 2: bovine serum albumin (BSA), lane 3: HtaA Peak 2 from FPLC purification, lane 4: HtaA protein Peak 1 from FPLC purification. The gel was run as described in the Experimental. It can be seen that both Peak 1 and Peak 2 are multimeric HtaA. Lanes 5 and 6 are HmuT.
Figure 5.5  FPLC of a second batch of HtaA protein. Conditions were as in the previous figure, except for a much lower protein loading. The sample was loaded onto a Strep-Tactin Superflow column equilibrated with buffer A (100 mM Tris-HCl, 150 mM NaCl, pH 8.0). Unbound material was washed out with 5 CV of buffer A. HmuT was eluted with 10 CV of buffer B containing 100 mM Tris-HCl, 150 mM NaCl, 2.5 mM desthiobiotin, pH 8.0 applied via a linear gradient.
Figure 5.6  UV-visible spectrum of Peak 2 of the HtaA as isolated (150 mM NaCl and 20 mM Tris-HCl, pH 7.5). Wavelengths of the peaks are indicated. The Soret was at 408 nm.
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Figure 5.10  Electrospray mass spectrum of holoHtaA.  The sample was prepared in water (diluted 1:1 with water). Top panel: The duplication of peaks in the charge envelope is seen. Middle panel: Deconvolution of the spectrum gave two very broad HtaA peaks. Bottom panel: The lower molecular weight area of the spectrum shows peaks for both heme and PPIX.
Figure 5.11 A solution of 0.5 μM hemin and 0.5 μM apoHtaA (Peak 2) incubated in 20 mM Tris-HCl and 150 mM NaCl, pH 7.5 for 20 h at 20 °C. Spectra were taken every hour (24 h). The Soret is at 403 nm, indicating that the heme is binding largely as the monomer. However, the band to the blue may indicate binding of some dimer. There are no significant changes in the positions or the ratios of the bands over time, indicating that the heme, once reconstituted, is in a stable form. It is not yet clear why the spectrum decreases over time – this may be due to protein precipitation (HtaA is multimeric in solution as shown by NATIVE PAGE).
Figure 5.12  A solution of 5 μM hemin and 5 μM apoHtaA (Peak 2) incubated in 20 mM Tris-HCl and 150 mM NaCl, pH 7.5 for 20 h at 20 °C. Spectra were taken every hour (over 18 h). The Soret is at 405 nm.
Figure 5.13  Spectrophotometric pH titration of HtaA (reconstituted Peak 2). The protein, in a buffer that was 20 mM each CAPS, CHES, MOPS and Tris-HCl was titrated with 1.0 M NaOH. Top panel: spectra as a function of pH. Bottom panel: The absorbance at 404 nm vs. pH. The protein shows essentially no sensitivity to pH, at least up to pH 10.5.
Figure 5.14  ClustalW alignment of HtaACR2 domains of *C. diphtheria*, *C. aurimucosum*, *C. jejikeium* and *C. ulcerans*). The conserved Tyr19, His70 and Tyr148 residues are shown in red and green.
5.4 Reference List


