Characterization of Heme Uptake Proteins; HtaB, ChtB, and ChtA from Corynebacterium Diphtheriae Approaches to the Reaction of Dechloromonas Aromatica Chlorite Dismutase with Nitrite

Catherine Odhiambo
codhiambo1@student.gsu.edu

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CHARACTERIZATION OF HEME UPTAKE PROTEINS; HtaB, ChtB, AND ChtA FROM
CORYNEBACTERIUM DIPHTHERIAE

APPROACHES TO THE REACTION OF DECHLOROMONAS AROMATICA CHLORITE
DISMUTASE WITH NITRITE

by

CATHERINE A. ODHIAMBO

Under the Direction of Dabney Dixon, PhD

ABSTRACT

Heme is a source of iron for many pathogenic bacteria. Corynebacterium diphtheriae, the
first protein in the system is HtaA, ChtA, and/or ChtC; heme is transferred to HtaB or ChtB. Heme
is then mobilized into the cytosol by the ABC transporter where iron is released by the heme
oxygenase.

Growth and expression temperatures for ChtA were of 37 °C and induction OD$_{600}$ between
0.6 and 0.9. Final IPTG concentration of 0.3 mM. ChtA extinction coefficient is $1.4 \times 10^5$ M$^{-1}$ cm$^{-1}$
at the Soret. ChtA is less stable than HtaA-CR2 to denaturation in guanidinium hydrochloride.

Dechloromonas aromatica chlorite dismutase (DaCld) detoxifies chlorite to chloride and
dioxygen. Chlorite sources often have nitrite. The goal is to identify the structure of this adduct.

Temperatures of growth and expression, 30 and 20 °C, respectively, an induction OD$_{600}$ of
0.30, and a final IPTG concentration of 0.1 mM resulted in the desired holo DaCld.

INDEX WORDS: Corynebacterium diphtheriae, Staphylococcus aureus. Streptococcus
pyogenes, Dechloromonas aromatica, Heme, Heme uptake, Heme transfer, Axial ligand, UV-
visible absorption spectroscopy, Resonance Raman spectroscopy, Unfolding, and Denaturing
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CATHERINE A. ODHIAMBO

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science

in the College of Arts and Sciences

Georgia State University

2020
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CATHERINE A. ODHIAMBO

Committee Chair: Dabney Dixon

Committee: David Wilson
Giovanni Gadda

Electronic Version Approved:

Office of Graduate Services
College of Arts and Sciences
Georgia State University
August 2020
DEDICATION

I dedicate this thesis to my father

Michael Odhiambo Wambani

May your soul rest in peace
ACKNOWLEDGEMENTS

Foremost, I would like to express my sincere gratitude to my advisor, Dr. Dabney Dixon, for her guidance, patience, and support throughout my research. Thank you for believing in me.

Thank you: to my family and friends who directly and indirectly contributed to my Master’s program, to those who have assisted me throughout my research with their expertise and time, especially my colleagues in our research lab, Brandon Ferrell, Rosemary Andrews, Lauren Ekeleme, Daniel Wongo, Anne Jean, and Brian Cole.

I also would like to thank my thesis committee: Dr. Dabney Dixon, Dr. Giovanni Gadda, and Dr. David Wilson for their advice and expertise throughout the completion of my degree.

The work in our lab would not have been possible without our collaborators. Many thanks to Dr. Kenton Rodgers and his research group.
# TABLE OF CONTENTS

ACKNOWLEDGEMENTS ............................................................................................................. V

LIST OF TABLES .......................................................................................................................... XI

LIST OF FIGURES ....................................................................................................................... XII

LIST OF ABBREVIATIONS ......................................................................................................... XXI

1INTRODUCTION .......................................................................................................................... 1

1.1 Binding domains in heme-uptake proteins .............................................................................. 3

  1.1.1 NEAT ............................................................................................................................... 3

  1.1.2 HtaA ............................................................................................................................... 4

1.2 Heme-uptake in *Staphylococcus aureus* ............................................................................ 4

  1.2.1 IsdH .............................................................................................................................. 5

  1.2.2 IsdB .............................................................................................................................. 5

  1.2.3 IsdA .............................................................................................................................. 6

  1.2.4 IsdC .............................................................................................................................. 7

  1.2.5 IsdE .............................................................................................................................. 7

1.3 Heme-uptake in *Streptococcus pyogenes*: Shr .................................................................. 8

1.4 Heme uptake in *C. diphtheriae* ......................................................................................... 8

1.5 Protein conformation via charge distribution envelope ................................................... 10

1.6 The role of heme pocket residues in controlling heme binding ....................................... 10

1.7 Detecting heme transfer via electron spray ionization mass spectroscopy .................. 10
1.8 Heme transfer between specific partners .............................................................. 11

1.9 Heme-iron uptake strategies .................................................................................. 12

1.10 Purpose of Study ................................................................................................. 13

2 CHARACTERIZATION OF HOMOLOGOUS HEME UPTAKE PROTEINS
HTAB, CHTB, AND CHTA FROM CORYNEBACTERIUM DIPHTHERIAE ........ 24

2.1 Introduction ........................................................................................................... 24

2.2 Experimental ......................................................................................................... 26

2.2.1 Reagents and solutions ...................................................................................... 26

2.2.2 Optical spectroscopy .......................................................................................... 26

2.2.3 Circular dichroism ............................................................................................. 27

2.2.4 Constructs .......................................................................................................... 27

2.2.5 Site-directed mutagenesis .................................................................................. 27

2.2.6 Sequence alignments and homology Modeling ...................................................... 28

2.2.7 Expression and purification ................................................................................ 28

2.2.8 Hemin addition .................................................................................................. 29

2.2.9 Hemin transfer from metHb .............................................................................. 30

2.2.10 Time scale of unfolding .................................................................................... 30

2.2.11 Thermal denaturation ....................................................................................... 31

2.2.12 Electrospray ionization (ESI) mass spectrometry .............................................. 32

2.2.13 Resonance Raman (rR) spectroscopy ................................................................. 32
2.3 Results and Discussion ................................................................. 33

2.3.1 Homology modeling ................................................................. 33

2.3.2 UV-visible spectroscopy, heme addition, and pH titrations ................. 34

2.3.3 Hemin transfer between HtaB and ChtB .................................... 36

2.3.4 Resonance Raman spectroscopy of ferric WT HtaB and ChtB ................. 36

2.3.5 Resonance Raman spectroscopy of ferrous HtaB ................................ 38

2.3.6 Resonance Raman spectroscopy of ferrous carbonyl WT HtaB and ChtB .... 39

2.3.7 Heme dissociation in ESI mass spectrometry ................................ 41

2.3.8 Time scale protein unfolding in chemical denaturation ....................... 41

2.3.9 Thermal stability ................................................................. 42

2.4 Relationship to other heme uptake pathways .................................... 43

2.5 Conclusions ............................................................................. 46

2.6 Supplementary information ......................................................... 58

3 CHARACTERIZATION OF C. DIPHTHERIAE CHTA AND PRELIMINARY EXPERIMENTS ................................................................. 77

3.1 General .................................................................................... 77

3.2 Materials and methods ............................................................. 78

3.2.1 Optimal growth and expression condition for ChtA ........................ 78

3.2.2 Titration of ChtA with hemin in DMSO .................................... 80

3.2.3 Reconstitution of ChtA with hemin in DMSO ............................... 80
3.2.4 ChtA hemin in potassium hydroxide sequential reconstitution

3.2.5 Concentration measurement: Bradford assay for ChtA

3.2.6 Extinction coefficient measurement: Pyridine hemochrome assay of ChtA

3.2.7 Investigation of protein secondary structure: Circular dichroism

3.3 Results and discussion

3.3.1 Optimal growth and expression condition for ChtA

3.3.2 Homology modeling

3.3.3 Hemin addition to ChtA

3.3.4 Determination of Soret heme loading and extinction coefficient

3.3.5 Experimental aspects of circular dichroism (CD) spectroscopy

4 APPROACHES TO REACTION OF DECHLOROMONAS AROMATICA
   CHLORITE DISMUTASE WITH NITRITE

4.1 Presence of oxyanion in the environment and analysis

4.1.1 Perchlorate

4.1.2 Chlorate and chlorite

4.1.3 Nitrite

4.1.4 Biochemistry of Dechloromonas aromatica chlorite dismutase (DaCld)

4.1.5 Proposed mechanism reaction of chlorite decomposition

4.2 Materials and methods
4.2.1 *Optimal growth and expression condition for Dechloromonas aromatica clorite dismutase* ................................................................. 111

4.3 Results and discussion ........................................................................................................................................ 113

4.3.1 *Optimal growth and expression condition for Dechloromonas aromatica clorite dismutase* ................................................................. 113

4.4 References ...................................................................................................................................................... 124
### LIST OF TABLES

Table 1.1 C. diphtheriae proteins and mutants binding studies. .......................................................... 15

Table 1.2 C. diphtheriae growth studies. ........................................................................................................ 16

Table 2.2 HtaB site-directed mutagenesis primers. The mutation sites are shown bold. .......... 47

Table 2.1 Comparison of rR core-size marker bands (cm$^{-1}$) of select ferric hemin proteins....... 57

Table 3.1 IPTG concentrations, post-expression OD$_{600}$, and volume of liquid culture............. 88

Table 3.2 Tyrosine-bound heme proteins in heme uptake pathways................................................. 89
Figure 1.1 Medically important Gram-positive Bacteria: Actinobacteria (HtaA domains) and Firmicutes (NEAT domains). ................................................................. 17
Figure 1.2 Clustal Omega alignment of NEAT domains for IsdB, IsdC, IsdH, and non-NEAT domain, IsdA from S. aureus; NEAT domains for Shr and non-NEAT domain, Shp from S. pyogenes. ................................................................. 18
Figure 1.3 A graphical sequence Logo representation of HtaA family (PF04213) generated by profile Hidden Markov Model (pHMM): Y15, H15, and Y148 are equivalent to Y1, H, and Y2, respectively of HtaA-CR2 from C. diphtheriae (1). ......................................... 19
Figure 1.4 The model of the location of the Isd proteins involved in heme import into S. aureus (5). .................................................................................................................. 20
Figure 1.5 The model of the location of proteins involved in heme import into S. pyogenes (12). .................................................................................................................. 21
Figure 1.6 The crystal structure of intact Hb with IsdH\textsubscript{N2N3}. D: The IsdH\textsubscript{N2} domain binding β-Hb (green), and E: the IsdH\textsubscript{N3} positioned near the heme of the α-Hb (blue). PDB 4IJ2 (10). 22
Figure 1.7 Heme binding including the axial Y and its H-bonding partner in IsdA, IsdC, and IsdH (9). .................................................................................................................. 23
Figure 2.1 Schematic of hmu and cht heme uptake proteins of C. diphtheriae. ChtA/C depicts each ChtA or ChtC which exhibit significantly high amino acid identity (11).
Hemoglobin is the representative heme source. CR, conserved domains. ........................ 48
Figure 2.2 I-TASSER homology model (2) of A: ChtB and B: ChtA using the crystal structure of HtaB from C. glutamicum (4) as a template and displayed using UCSF Chimera (13).
Shown are location of, ChtB; S52, Y56 (axial ligand), H121, M220, and Y223: ChtA; S125, Y129 (axial ligand), Y178, F271, H279, and Y272. .......................................................... 49

Figure 2.3 UV-visible spectra of ChtA (orange solid line), HtaA-CR2 (dashed red line), ChtB (blue dashed-dotted line), and HtaB (green dotted line). Spectra were taken in 100 mM Tris-HCl, 150 mM NaCl, pH 8.0 and are normalized to 1.0 at the Soret. ................. 50

Figure 2.4 The UV-visible absorption of as-isolated ferric HtaB (solid line), Y56A (dashed line), and H121A (dotted line). Spectra were taken in 100 mM Tris-HCl, 150 mM NaCl, pH 8.0 and are normalized to 1.0 at the Soret. .......................................................... 51

Figure 2.5 Ferric high frequency rR of HtaB as a function of pH. Glycine (pH 9.6, black), Tris-HCl (pH 8.8, red and 8.0, blue), and phosphate (pH 5.8, green) buffers at 100 mM were used. Resonance Raman scattering was excited with 413.1 nm emission from a Kr+ laser using 10 mW of power. The assignments are made by analogy with other heme proteins and on the basis of depolarization ratios evident from Figure 2.27................................. 52

Figure 2.6 Ferric high frequency rR of ChtB as a function of pH. Tris-HCl (pH 8.8, blue and 8.0, red) and phosphate (pH 5.8, green) buffers at 100 mM were used. The excitation wavelength from 413.1 nm from Kr ion laser with 11.4 mW of power. The assignments were made based on polarization data in Figure 2.28 and by analogy with other heme proteins. The vC=C band shifts under the v10 band as the pH is decreased indicating a change in the interactions between the vinyl groups and the heme pocket. ..................... 53

Figure 2.7 Low-frequency spectra of ferrous HtaB excited with 413.1 nm (10 mW, black, bottom, Kr+) and 441.6 nm (1.6 mW, red, top, HeCd). Both spectra have been normalized to the v7 band which lies outside the shown window. The resonance enhancement pattern with 441.6-nm excitation supports assignment of the 217-cm⁻¹ band to the vFe-His mode,
leading to the conclusion that part of the heme is bound to the protein through a Fe–His bond (97).

Figure 2.8 Backbonding correlation plot, relating $\nu_{\text{Fe-CO}}$ and $\nu_{\text{C-O}}$ frequencies for groups of proteins having the same trans (proximal) ligand. The two forms of HtaB–CO are shown as filled red stars, while the two forms of ChtB–CO are filled green stars. HmuT and its mutants, unfilled blue stars (63, 77); catalase, blue hexagon (112); and SmHasA(WT), SmHasA(H83A), SmHasA(H32A), magenta ○ (93). The data for HRP, ▲ and globins, ■ have been compiled previously (93). The dotted line is the least squares fit for six-coordinate heme carbonyls in which the proximal ligand is neutral imidazole from a His residue (93, 102, 103) (and references therein). The solid blue line represents a compilation of heme proteins in which the ligand trans to CO is coordinated through an O atom that is hydrogen bonded to an Arg (two hydrogen bonds) (77, 112). The solid magenta line is the least squares fit for six-coordinate Fe–CO adducts which are coordinated through an O atom having a single hydrogen bond to His or Tyr (93).

Figure 2.9 Absorbance versus wavelength of a solution of heme-loaded HtaB before and after incubation with heme-loaded ChtB. B: Absorbance versus wavelength of a solution of heme-loaded ChtB before and after incubation with as-isolated HtaB. The protein solutions were prepared in 100 mM Tris-HCl, 150 mM NaCl pH 8.0.

Figure 2.10 Clustal Omega alignment of CR domains for HtaA-CR1, HtaA-CR2, HtaB, ChtB, ChtA, and ChtC from C. diphtheriae; HtaA-CR1, HtaA-CR2, and HtaB from C. glutamicum.

Figure 2.11 UV-visible spectra of as-isolated HtaB (dotted line), titrated with hemin (solid line), and reconstituted on a Strep-tag column with hemin from metHb (dashed line). Spectra
were taken in 100 mM Tris-HCl, 150 mM NaCl, pH 8.0 and are normalized to 1.0 at the Soret. 

Figure 2.12 UV-visible spectra of as-isolated HtaB H121A (dashed line) and titrated with hemin (solid line). Spectra were taken in 100 mM Tris-HCl, 150 mM NaCl, pH 8.0 and are normalized to 1.0 at the Soret. 

Figure 2.13 UV-visible spectra of as-isolated HtaB Y56A (dotted line), titrated with hemin (solid line), and reconstituted on a Strep-tag column with hemin from metHb (dashed line). Spectra were taken in 100 mM Tris-HCl, 150 mM NaCl, pH 8.0 and are normalized to 1.0 at the Soret. 

Figure 2.14 UV-visible spectra of as-isolated ChtB (dashed line) and reconstituted on a Strep-tag column with hemin from metHb (solid line). Spectra were taken in 100 mM Tris-HCl, 150 mM NaCl, pH 8.0 and are normalized to 1.0 at the Soret. 

Figure 2.15 UV-visible spectra of heme-loaded ChtA before and passing through a Sephadex G25 superfine column. Dotted: before treatment. Dashed/dotted line: One pass. Dashed line: Two passes. Solid line: Three passes. Spectra were taken in 100 mM Tris-HCl, 150 mM NaCl, pH 8.0 and are normalized to 1.0 at the Soret. 

Figure 2.16 Low frequency rR spectra of WT ChtB as a function of pH. Tris-HCl (pH 8.8, blue and 8.0, red) and phosphate (pH 5.8, green) buffers were used at 100 mM. Raman scattering was excited with 413.1-nm emission from a Kr laser, 11.4 mW. The band assignments were made based on depolarization ratios (data not shown) and by analogy to other heme proteins. Changes in relative intensities of bands attributable to propionate and vinyl bending suggest that the heme-protein interactions near the heme edge are sensitive to pH. Changes in relative intensities and frequencies of bands near 740 and 760
cm\(^{-1}\) are suggestive of a pH-dependent change in deviation of the porphin core from planarity.

Figure 2.17 Ferrous high frequency rR spectra of pH 8.8 HtaB as excited with 413.1 nm Kr\(^+\) laser with 4.8 mW power. Parallel polarized (black) and perpendicular polarized (red) spectra allow for the identification of totally symmetric and non-totally symmetric core size marker bands. Inset shows the UV-visible spectrum of ferrous HtaB.

Figure 2.18 High-frequency rR spectra of ferrous ChtB at pH 8.8, excited with 413.1-nm emission from a Kr\(^+\) laser with power of 10.2 mW. Parallel polarized (black) and perpendicular polarized (red) spectra allow for the identification of the totally symmetric and non-totally symmetric core size marker bands. Inset shows the UV-visible spectrum of ferrous ChtB.

Figure 2.19 Resonance Raman spectra of the heme carbonyls of HtaB-WT. Raman scattering was excited using 413.1-nm emission from a Kr\(^+\) laser; 5.7 mW. Natural-abundance HtaB–CO (top), HtaB–\(^{13}\)CO (middle) and \(^{12}\)CO–\(^{13}\)CO difference (blue, bottom) spectra are shown in the \(\nu_{Fe-CO}\), \(\delta_{FeCO}\) and \(\nu_{C-O}\) regions. Bands in the low-frequency region were fit using Gaussian peak functions (gray). These bands were used to calculate the red difference spectrum superimposed on the blue points in the bottom left spectrum. The bands sensitive to \(^{13}\)CO substitution are labeled with their respective mode designations. Spectra were recorded at pH 8.8. Inset: UV-visible absorbance spectrum of HtaB.

Figure 2.20 Resonance Raman spectra of the ferrous carbonyls of WT ChtB with conditions and interpretation as described in the legend of Figure 2.19.

Figure 2.21 Electrospray ionization mass spectrometry detection of heme-bound HtaB-WT (solid line), HtaB H121A (dashed line), and HtaB Y56A (dotted line) as a function of collision
energy voltage. The protein solutions were prepared in 20 mM ammonium acetate, pH 6.8.

Figure 2.22 UV-visible absorbance as a function of time for ChtA (orange-dashed line); reaction was carried out in the presence of 4.0 M GdnHCl at 25 °C. HtaA-CR2 (red-solid line); reaction was carried out in the presence of 6.8 M GdnHCl at 37 °C. A 100 mM Tris-HCl, 150 mM NaCl, pH 8.0 buffer A was used for both experiments.

Figure 2.23 UV-visible absorbance as a function of time for HtaB (green-dashed line) and ChtB (blue-solid line); reactions were carried out in the presence of 4.0 M GdnHCl at 25 °C in 100 mM Tris-HCl, 150 mM NaCl, pH 8.0.

Figure 2.24 UV-visible absorbance as a function of temperature for ChtA. The unfolding reaction was carried out in the presence of 1.5 M GdnHCl in 100 mM Tris-HCl, 150 mM NaCl, pH 8.

Figure 2.25 Fraction folded as a function of temperature for the of HtaB (green-dashed line) and ChtB (blue-solid line). Experiments were performed in 1 M GdnHCl in 50 mM NaH$_2$PO$_4$, pH 7.0.

Figure 2.26 Fraction of folded protein as a function of temperature for HtaB (solid line), H121A (dashed line), and Y56A (dotted line) mutants. The unfolding reaction was carried out in the presence of 1.0 M GdnHCl in 50 mM NaH$_2$PO$_4$, pH 7.0.

Figure 2.27 High frequency rR spectrum of ferric HtaB in 100 mM Tris pH 8.0 with 413.1-nm excitation from Kr$^+$ laser with 10 mW of power. Parallel (black) and perpendicular (red) polarized Raman scattering are shown to identify the totally symmetric and non-totally symmetric core size marker bands (7).
Figure 2.28 Polarized high-frequency rR spectra of WT ferric ChtB. Conditions as described for Figure 2.27, except the laser power was 11.4 mW. ................................................................. 76

Figure 3.1 ChtA sequence with a (WSHPQFEK) N-terminal Strep-tag. Conserved tyrosines; Y129 (Y1), Y178 (Y2), and Y272 (Y3) are shown in red (8). .................................................. 90

Figure 3.2 B2: Visual comparison of the cell growth with and without 0.3% glucose in LB after 20 h, B2 : Visual comparison of cell growth with and without 0.3% glucose after 20 h. .. 91

Figure 3.3 Expression of ChtA induced (OD$_{600}$, 0.60) by addition of 0.50 mM (lanes 1 and 5), 1.0 mM (lane 3) IPTG at 37 °C for 3.5 h (lanes 1-4) and 20 °C for 20 h (lanes 5 and 6). Lanes 2, 4, and 6 are without IPTG. ................................................................. 92

Figure 3.4 Expression of ChtA induced by addition of 0.50 mM (lanes 1, 2, and 5), 0.30 mM (lanes 3, 4, and 6) IPTG at 37 °C for 4 h. ................................................................. 93

Figure 3.5 I-TASSER homology model (2) of A: HtaA-CR2 and B: HtaB using the crystal structure of HtaB from C. glutamicum (4) as a template and displayed using UCSF Chimera (13). Shown are location of, HtaA-CR2; S125, Y129 (axial ligand), H178, H179, and F271: HtaB; S52, Y56 (axial ligand), H121, F219, M220, and Y223. ........... 94

Figure 3.6 UV-visible spectra for two-cuvette hemin titration of ChtA (22.6 µM) up to a hemin:protein of 0.90. All spectra were taken in buffer A, pH 8.0. ......................... 95

Figure 3.7 The absorbance at 405 nm plotted as a function of the ratio of the concentration of hemin to ChtA................................................................. 96

Figure 3.8 UV-visible spectra of heme-loaded ChtA before and after treatment with Sephadex G25 superfine column. Dashed line: Two passes after hemin reconstitution of ChtA (34.6 µM) up to a hemin:protein of 0.90. Solid line: Three passes after hemin reconstitution of
ChtA (28.1 µM) that was initially heme loaded (Soret:280 nm ratio of 0.29). Spectra were taken in buffer A, pH 8.0 and are normalized to 1.0 at the Soret. ..............................

Figure 3.9 UV-visible spectra of heme-loaded ChtA after treatment with Sephadex G25 column. Dotted line: Pass through G25 medium. Solid line: Pass through G25 superfine. Spectra were taken in buffer A, pH 8.0 and are normalized to 1.0 at the Soret. ..............................

Figure 3.10 UV-visible spectra for two-cuvette hemin titration of 12.1 µM ChtA with 2.54 µM and 4.85 µM hemin. Hemin used to perform titration was in 100 mM KOH/NaH₂PO₄, pH 7.5. Spectra were taken in buffer A, pH 8.0. ..............................

Figure 3.11 UV-visible spectra of ChtA after hemin loading in DMSO (solid line) and 100 mM KOH/NaH₂PO₄, pH 7.5 (dashed line). Spectra were taken in buffer A, pH 8.0 and are normalized to 1.0 at the Soret. ..............................

Figure 3.12 UV-visible spectra of hemin (dotted line). Heme-loaded ChtA before (dashed line) and after (solid line) one pass through Sephadex G25 superfine column. The spectra of hemin and protein were taken in 100 mM KOH/NaH₂PO₄, pH 7.5 and buffer A (pH 8.0), respectively and are normalized to 1.0 at the Soret. ..............................

Figure 3.13 UV-visible spectra of ChtA before (solid line) dialysis taken in buffer A, pH 8.0 and after (dashed line) dialysis taken in 10 mM ammonium acetate, pH 7.0. Spectra are normalized to 1.0 at 280 nm. ..............................

Figure 3.14 Spectra of BSA with increasing protein concentration. Spectra taken in 10 mM KH₂PO₄, pH 7.0. The inset shows the UV-visible absorbance spectrum. ..............................

Figure 3.15 CD spectra of Mb taken in 10 mM potassium phosphate buffer (solid line) and buffer A (dashed line). ..............................

xix
Figure 3.16 CD spectra of HtaA-CR2 (dotted line), ChtB (dashed line) and HtaB (dashed-dotted line) spectra taken in 10 mM potassium buffer, pH 7.0. HtaA-CR2 spectra taken in 50 mM tris buffer, pH 7.0. ChtA (solid line) spectra was taken in buffer A, pH 7.0. 

Figure 4.1 Heme pocket of DaCld including a nitrite ligand bound to heme-iron and H-bonding with Arg 183 (6).

Figure 4.2 Proposed Cld mechanism involves two successive intermediates (3).

Figure 4.3 Dechloromonas aromatica chlorite dismutase sequence.

Figure 4.4 Expression of DaCld in TB broth with 0.3% glucose, induced by addition of 0.10 mM (lanes 3, 6, and 7), 0.30 mM (lanes 2, 5, and 8) IPTG at 37 °C for 4 h.

Figure 4.5 Expression of DaCld in TB induced by addition of 0.10 mM (lanes 4 and 5), 0.30 mM (lanes 1 and 6) IPTG at 37 °C for 4 h.

Figure 4.6 Expression of DaCld in LB with 0.3% glucose, induced by addition of 0.10 mM (lanes 2 and 4), 0.30 mM (lanes 1 and 3) IPTG at 18 °C for 15 h.

Figure 4.7 Expression of DaCld in LB without glucose, induced by addition of 0.10 mM (lanes 2 and 4), 0.30 mM (lanes 1 and 3) IPTG at 18 °C for 15 h.

Figure 4.8 Visual comparison of cells containing daCld gene induced at an OD$_{600}$ of 0.28 ($Y_1$) and 0.75 ($Y_2$).
LIST OF ABBREVIATIONS

Abbreviations: ABC transporter, ATP-binding cassette transporter; CD, circular dichroism; CO, carbon monoxide; CR, conserved region; CT, charge-transfer; CV, column volume; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; ESI, electrospray ionization; FPLC, fast protein liquid chromatography; GdnHCl, guanidinium hydrochloride; Hb, hemoglobin; Hp, haptoglobin; HS, high-spin; HSA, human serum albumin; IPTG, isopropyl β-D-1-thiogalactopyranoside; LS, low-spin; PMSF, phenylmethanesulfonyl fluoride; rR, resonance Raman spectroscopy; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TB, Terrific Broth; LB, Luria-Bertani; WT, wild-type; 5cHS, pentacoordinate, high-spin.
1 INTRODUCTION

In the mammalian host, the availability and distribution of iron are highly controlled (14). Iron is complexed within the porphyrin ring in hemoglobin (Hb) and myoglobin (Mb). Degradation of heme by heme oxygenases release iron (15). After cell lysis, transferrin, albumin, lactoferrin, and hemopexin readily bind to the free heme to prevent oxidative damage (16). Under physiological conditions, approximately $10^{-9}$ M of free iron is available (15, 17). A much higher concentration of iron ($10^{-6}$ M) is required for optimal bacterial growth. It is, therefore, not surprising that pathogens continue to develop mechanisms to take up iron from the host. Due to constant evolving antibiotic resistance, new ways of controlling bacterial infections are urgently needed. In hopes of developing alternative antibiotic therapeutics, it is useful to study heme uptake in heme proteins.

Corynebacterium diphtheriae is a Gram-positive bacterium that causes infection in the upper respiratory tract and skin (18). In acute cases, bacterial growth remains localized at the infection sites, but diphtheria toxin (DT) is released into the blood, causing severe systematic pathology. Early studies indicated that the tox gene in C7 strains was repressed in a high-iron environment (16, 19, 20). When bound to iron, DtxR-Fe binds to the operator of the tox gene and acts as a transcriptional repressor, which inhibits DT synthesis (21-23). While vaccines exist (e.g., DTPcv-3), as of 2018, 24% of the world population was either under- or un-immunized (24). Consequently, diphtheria infections still occur in parts of the world with low vaccine coverage (25, 26).

In the 1950s, diphtheria was controlled both in the Russian Federation and the United States (27). A diphtheria re-emergence of the gravis biotype started in the Russian Federation in the early 90s and lasted approximately two years. Studying this strain of C. diphtheriae, the
laboratory of Dr. Michael Schmitt identified *hmuTUV* genes involved in the utilization of heme that are homologous to the genes involved in the Gram-negative heme uptake systems (20). HmuT is a lipoprotein that anchors to the cytoplasmic membrane and serves as a heme receptor (8, 28). The protein transfers its heme to the ABC transporter (HmuU and HmuV), in which heme is mobilized into the cytosol, where iron is released by heme oxygenase (HmuO). The *hmuTUV* genes are associated with the *htaABC* gene cluster (29). Additional *chtA*, *chtB*, and *chtC* genes have also been identified (8). Schmitt and colleagues suggested that different isolates of *C. diphtheriae* may utilize different heme uptake systems.

A second project consists of heme nitration in *Dechloromonas aromatica* chlorite dismutase (*DaCld*). Clds are heme proteins that efficiently decompose chlorite (ClO\(^{-}\)) into chloride (Cl\(^{-}\)) and oxygen (O\(_2\)) (30). ClO\(^{-}\) contamination is a rising environmental concern; consequently, Clds may come to play an industrial role in catalyzing the degradation of ClO\(^{-}\). Nitrite (NO\(_2\)^{-}) can often be present with ClO\(^{-}\); therefore, it is essential to understand the products from the reaction of NO\(_2\)^{-} with Clds. Our goal is to identify the products of the reaction of nitrite with *DaCld*.

This thesis consists of four chapters. Chapter 1 is the background of the heme uptake in pathogenic bacteria, including systems based on HtaA and NEAT domains. Chapter 2 is the manuscript entitled “Characterization of homologous heme uptake proteins HtaB, ChTB, and ChTA from *Corynebacterium diphtheriae*,” which is to be submitted to a peer-reviewed journal. Chapter 3 details additional ChTA and experiments that do not appear in the manuscript. Chapter 4 describes our studies to date on the nitration of hemin in chlorite dismutase.

Herein, the Gram-positive heme uptake systems investigated are based on two traditional conserved protein domain families: HtaA and NEAT.
The HtaA domain can be compared with the well-studied NEAT domains (31). Figure 1.1 shows the gram-positive bacteria divided into two groups, Firmicutes (low GC) and Actinobacteria (high GC). Clinically relevant Firmicutes include Bacillus, Clostridium, Listeria, Staphylococcus, and Streptococcus, all of which use the NEAT domain and almost none of which have sequences homologous to HtaA (15). Examples of clinically relevant Actinobacteria include Actinomycetes, Corynebacterium, Propionibacterium, Rothium, and Streptomyces. All of these have sequences homologous to HtaA, but almost none have sequences homologous to NEAT. Thus, there seems to be a divide in the strategies used for the main pathway of heme uptake in clinically relevant examples of these two phyla of bacteria, with Firmicutes using NEAT and Actinobacteria using HtaA.

1.1 Binding domains in heme-uptake proteins

1.1.1 NEAT

NEAT domains are conserved units that bind either or both heme and heme proteins (31-33). The domain is composed of ~125 amino acids and tethered to the extracellular side of the bacterial cell membrane. The conserved region of the NEAT domain is mostly \( \beta \)-strands. Most NEAT domains have a YXXXX heme-binding motif, in which one tyrosine serves as the axial ligand, and the second is the hydrogen-bonding partner of the first. On the opposite side of the heme, an SXXXXY/F sequence is commonly found in the 3\(_{10}\)-helix. In this motif, the serine forms a hydrogen bond with the propionate and the tyrosine stacks with heme. Figure 1.2 shows the alignment of conserved residues thought to be involved in the heme uptake process.

As of this writing, there are 87 structures, 93 architectures, 208 species, and 769 sequences known for NEAT domains (InterPro, IPR006635) (15, 31). It is common to have more than one NEAT domain in a given protein. Because the Isd system of Staphylococcus aureus has
been studied in the most detail, this review will concentrate on this system; the Shr system from *Streptococcus pyogenes* is also discussed at the end of this section.

### 1.1.2 HtaA

HtaA domains are conserved units of heme proteins involved in binding the heme, extracting the heme, and transferring it into the cell (28). As of this writing, there are 49 architectures, 365 species, 1038 sequences, and 9 structures known for HtaA domains (InterPro, IPR007331) (4). It is common to have more than one domain in a given protein. Most HtaA domains coordinate heme iron via tyrosine.

Figure 1.3 shows most HtaA domains have two conserved tyrosines, one (Y1) 15 residues from the beginning of the N-terminus, and the second (Y2) about 133 residues from the first (pfam04213). Y1 is the axial ligand and Y2 forms a hydrogen bond with one of the heme propionates (4). There is also a histidine (H) about 53 residues from the first tyrosine, which has also been considered a hydrogen-bond partner to the axial ligand. The role of the highly conserved tryptophan towards the start of the sequence is not yet understood (8, 20, 28, 34-36).

### 1.2 Heme-uptake in *Staphylococcus aureus*

The *S. aureus* Isd system exploits 12 proteins to bind the heme, extract the heme, transfer it into the cell, and oxidize the tetrapyrrrole ring to release the iron (37). Four of the proteins are surface-anchored (Isd-ABCH). Isd-DEF are heme transporters; IsdI and IsdG are heme monooxygenases (Figure 1.4) (5). *S. aureus* can acquire iron from hemoglobin, the hemoglobin-haptoglobin (Hb-Hp) complex, and myoglobin (Mb).

The first step of the *S. aureus* heme acquisition process is initiated via IsdB or IsdH on the bacterial cell surface (38). The intact heme is sequentially transferred to IsdB, followed by
IsdC, and eventually passed to IsdE. A specific order of heme transfer occurs between IsdA, IsdC, and IsdE: IsdA makes the transfer to IsdC, followed by a transfer to IsdE which eventually transfers the heme to the cytosol through an ABC transporter.

Herein, we focus on the proteins in the pathway which bind hemin via NEAT domains (IsdH, IsdB, IsdA, and IsdC) and also discuss IsdE.

1.2.1 IsdH

The first NEAT domain of IsdH in \textit{S. aureus} (IsdH\textsuperscript{N1}) does not bind heme, but rather hemoglobin (38-40). An x-ray structure shows the details of the interface (PDB 3SZK) (41). Pilpa et al. replaced each of the residues in the binding sequence YYHFF with alanine and found that the Hb binding (K\textsubscript{d} of 17 nM) was reduced by 153-, 134-, 72-, 42-, and 14-fold, respectively (38). IsdH\textsuperscript{N1} also binds Hp with a K\textsubscript{d} of 35 nM. Mutation of any of the YYHFF signature residues to alanine significantly reduces or prevents Hp binding.

The second and third NEAT domains are linked together via a ~70 amino acid \(\alpha\)-helical domain (IsdH\textsuperscript{N2N3}) (39). These domains work in concert to bind Hb and take up the heme. A crystal structure of intact Hb with IsdH\textsuperscript{N2N3} shows the IsdH\textsuperscript{N2} domains each binding one of the Hb chains, while the IsdH\textsuperscript{N3} are each positioned near the corresponding heme (PDB 4IJ2; Figure 1.5) (10). The second NEAT domain of IsdH (IsdH\textsuperscript{N2}) binds Hb, Hp (38, 39), and the Hb-Hp complex (38, 42). No heme binding was observed for IsdH\textsuperscript{N2} (39). The third (IsdH\textsuperscript{N3}) domain binds heme (K\textsubscript{a} of \(3.2 \times 10^6 \text{ M}^{-1}\)) but not Hb (39, 43).

1.2.2 IsdB

The first NEAT domain of IsdB in \textit{S. aureus} (IsdB\textsuperscript{N1}) does not bind heme (31, 38). Consistent with this, this domain does not contain the established heme-binding (YXXXY) motif nor an SXXXXY/F sequence in the \(3_{10}\)-helix; instead, these motifs are replaced by FYHYA and
EEKYD, respectively. The domain binds both $\alpha$-Hb and $\beta$-Hb subunits (44). Mutating F164 and Y167 to alanine resulted in a reduced Hb binding by ~1.8-fold compared to the wild type.

The second domain of IsdB (IsdB$^{N2}$) has a heme-binding conserved motif (YDGQY) and a $3_{10}$-helix sequence (SMMDTF) on the opposite side (15, 31, 38). The crystal structure of heme-bound IsdB$^{N2}$ shows that the M362 and Y440 axial ligands coordinate heme iron from opposite directions (45). Y444 forms a hydrogen bond with Y440, in addition to stacking with the heme porphyrin ring, while S361 forms a hydrogen bond with one of the heme propionates. Mutation of these tyrosine and serine residues to alanine, and methionine to leucine, decrease heme binding by ~1.8-fold.

IsdB$^{N2}$ binds both Hb and the Hb-Hp complex (44). For Hb, IsdB$^{N2}$ prefers binding the $\alpha$-Hb subunit, interacting with histidine of the subunit. No interaction with the $\beta$-Hb subunit was observed.

The first and the second (IsdB$^{N1N2}$) domains are linked together via a ~66 residue $\alpha$-helical domain (46). In the crystal structure of intact Hb with four IsdB$^{N1N2}$, the IsdB$^{N1}$ domain is observed to bind solely to the $\beta$-Hb subunits (PDB 2DN1) (44). Double mutation of the FYHYA motif in the first domain (F164D, Y167D) resulted in a 90% reduction in heme transfer compared to wild type.

1.2.3 IsdA

IsdA has a single NEAT domain (IsdA$^{N}$) with a heme-binding conserved motif (YDGQY) and a $3_{10}$-helix sequence (SHMDDY) on the opposite side (15, 33). As illustrated in Figure 1.6, the crystal structure of heme-bound IsdA (PDB 2ITF) (47) shows that the Y166 and H83 axial ligands coordinate heme iron from opposite directions (9). Ser82 forms a hydrogen
bond with H87 and one of the heme propionates. The heme-iron interacts with Y166 and Y170; the latter also stacks with the heme porphyrin ring.

### 1.2.4 IsdC

IsdC has a single NEAT domain (IsdCN) consisting of ~125 amino acids (48). This domain has an established heme-binding (YXXXY) motif and a 3_{10}-helix sequence SXXXXY (31). The 3_{10}-helix is said to undergo a transition from a flexible to rigid conformation as the heme is captured (48). The crystal structure of the heme-bound IsdC (PDB 2O6P) shows that the Y132 and Ile48 axial ligands coordinate heme iron from opposite directions (9). Ser47 forms a hydrogen bond with Ile48 and one of the heme propionates. Y132 is the axial ligand for the heme (49). The domain binds heme with a $K_d$ of 0.34 ± 0.12 μM. The domain receives heme either directly from IsdH and IsdB or through IsdA and is in turn delivered to IsdE (50, 51). Paoli and colleagues have suggested IsdC is the central conduit through which the heme is passed across the membrane (49).

### 1.2.5 IsdE

IsdE has two non-NEAT substrate-binding domains connected via a single α-helix (52, 53). The protein coordinates the heme iron via M78 and H229. IsdE is reported to bind hemin with a $K_d$ too tight to measure (54); double mutations of the axial ligands to alanine decreased the ability of the protein to bind heme ($K_d$ of 9.1 × 10^{-6} M). IsdE does not transfer heme to IsdA, nor does it accept heme from IsdA. IsdC selectively transfers heme to IsdE (55). The difference in heme dissociation constants partly steers the specific sequential transfer of Isd proteins: IsdA > IsdC >>> IsdE.
1.3 Heme-uptake in *Streptococcus pyogenes*: Shr

The Shr protein of the *S. pyogenes* heme acquisition system has two NEAT domains; Shr\(^{N1}\) and Shr\(^{N2}\) (56). The protein binds the heme and transfers it to Shp; the HtsABC transporter relays heme across the cell envelope (57). Figure 1.7 provides a schematic of the heme uptake pathway (12).

The first NEAT domain (Shr\(^{N1}\)) has an SXXXA sequence in the 3\(_{10}\)-helix and a DXXXY heme-binding motif downstream of the signaling peptide (15, 58). These motifs differ from the canonical 3\(_{10}\)-helix of SXXXXY/F and YXXXY sequences in the domain architecture with heme acquisition proteins. The domain takes up heme from Hb (58). A recent X-ray structure shows that the domain binds Hb (59).

The second NEAT (Shr\(^{N2}\)) domain has an SXXXA sequence in the 3\(_{10}\)-helix and a VXXVK heme-binding motif in the N- and C-terminal regions, respectively (60). This NEAT domain binds heme but not Hb. Eichenbaum and colleagues have suggested that the main functionality for Shr\(^{N1}\) is to deliver heme to Shr\(^{N2}\) and that Shr\(^{N2}\) may serve as a heme storage domain, which can transfer heme back to Shr\(^{N1}\) for movement along the pathway when the heme level drops (61).

The second and the third (Shr\(^{N1N2}\)) domains are linked via a central leucine rich-repeat (LRR) region (62). ELISA studies by Ouattara et al. indicate that Shr binds only the holo form of hemoglobin (61).

1.4 Heme uptake in *C. diphtheriae*

The *C. diphtheriae* heme uptake system exploits eight proteins to bind and transfer heme into the cell (Table 1.1). The first protein in the system is HtaA, followed by HtaB and HmuT. The proteins ChtA/ChtC (homologous to HtaA) and ChtB (homologous to HtaB) are also present.
in the bacterium (1, 28, 36). All of these proteins can acquire iron by utilization of hemoglobin (Hb), myoglobin (Mb), human serum albumin (HSA), and hemopexin (Hpx) heme sources. Table 1.2 shows the effect of mutations on the ability of the bacterium to utilize heme iron from some of these sources. However, only HtaA and either ChtA or ChtC can bind and extract heme from hemoglobin-haptoglobin (Hb-Hp) complex (36).

For the HtaA pathway in *C. diphtheriae*, HtaA transfers heme to HtaB (1, 8). It is known that HtaB and ChtB can substitute for one another (8); ChtB and HtaB can also transfer heme to one another (Chapter 2). Uluisik et al. used biophysical studies (1) to support earlier the conclusion from biochemical studies (8, 36) that tyrosine is the axial ligand. Replacing Y1, Y2, and H of the second HtaA domain (HtaA-CR2) to alanine resulted in reduced heme binding in the order Y1A > HA >> Y2A > wild type (1). The conserved domains of HtaB, ChtB, ChtA, and ChtC only share ~25% identity with the HtaA-CR2.

ChtA has a single conserved domain (ChtA-CR) (Allen et al., 2013). In this domain, there are three conserved tyrosines, two as described in the pfam04213 above; the third (Y3) is in the place of the conserved histidine in the HtaA sequence. ChtA-CR binds heme from both Hb and Hb-Hp complex (36). Mutants Y1A and Y2A resulted in two-fold decreases in heme binding. The domain binds Hb with a $K_d$ of 0.24 μM; the full protein binds Hb with a slightly lower $K_d$ of 0.41 μM (8). ChtA-CR also showed the ability to bind the Hb-Hp complex (EC$_{50}$ of 890 nM) ~two-fold more strongly than full-length ChtA (EC$_{50}$ of > 2 μM) (36). Replacing the third tyrosine with a histidine reduced the ability of *C. diphtheriae* to utilize Hb as a sole iron source. Schmitt has taken these data to indicate that there is a distinction in heme-binding sequence selectivity between ChtA and HtaA (36). Strains with *chtA* gene deletions were still capable of utilizing both Hb and Hb-Hp iron sources, similar to wild type. However, a double gene deletion
(chtA and chtC) affected the bacterial growth. Therefore, ChtA and ChtC may play a similar function in the heme uptake system. The growth was fully abolished for the triple gene deletion (chtC, chtA, and htaA) mutants. ChtA and ChtC are of interest because heme in the Hb-Hp complex can be extracted only when both HtaA and ChtA/C are available (36).

1.5 Protein conformation via charge distribution envelope

Stillman and colleagues have argued that the charge distribution envelope in the mass spectrum of a protein can be used to assess conformation (54). The *S. aureus* M78A and H229A apo-IsdE double mutant had a charge distribution identical to that of wild type. Stillman has taken these data to indicate that mutation of the axial ligands did not affect the overall structure of the protein.

1.6 The role of heme pocket residues in controlling heme binding

Collision-induced mass spectrometry has been used to analyze contributions of various residues that contribute to the binding of the heme in the pocket (63). Draganova et al. replaced H136, R237, Y272, and M292 of *C. diphtheriae* HmuT with alanine and found out that heme was lost as the voltage was increased in the order M292 >> Y272A >>> H136A R237A >> wild type. Dixon and colleagues argue that in addition to the canonical tyrosine ligand, other conserved residues contribute to maintaining heme pocket integrity.

1.7 Detecting heme transfer via electron spray ionization mass spectroscopy

Mass spectrometry has been used to study heme transfer between proteins (54, 64). IsdC transfers heme to IsdE, as indicated by the peak presence of apo-IsdC and holo-IsdE after incubation of holo-IsdC with apo-IsdE. The 100% heme transfer between the two proteins is an indication that IsdC and IsdE may be specific natural partners. IsdE does not transfer heme to IsdC, as indicated by the presence of neither holo-IsdE nor apo-IsdE after holo-IsdC was
incubated with apo-IsdE for one h.

1.8 Heme transfer between specific partners

As described above, electron spray ionization has been used to show that heme transfer occurs between specific partners in the *S. aureus* Isd pathway.

Stillman and colleagues have proposed that mass spectrometry data can be used to establish heme transfer between specific partners (54). No heme gain or loss was observed between holo-IsdE of *S. aureus* and apo-myoglobin, as indicated by the absence of peaks of apo-IsdE or holo-myoglobin after holo-IsdE was incubated with apo-myoglobin. In line with these observations, Stillman and colleagues have suggested that productive protein-protein interactions enable heme transfer from one protein to another (55).

In the *C. diphtheriae* system, the heme uptake protein HtaA-CR2 is very stable, with the half-life of 5 h in 6.8 M GdnHCl at 37 °C (1). Dixon and colleagues argue that the significant stability of HtaA-CR2 is presumably due to the requirement of specific protein-protein interaction with its natural heme-accepting partner for transfer of the heme.

Maresso and colleagues have suggested that the transfer of heme from the NEAT protein BslKN to IsdC in the *Bacillus anthracis* is facilitated by protein-protein interactions (65). No heme gain or loss was observed between holo-BslKN and apo-myoglobin, as indicated by no spectral changes for either the holo- or apo-protein after 24 h of incubation. However, incubation of holo-BslKN with apo-IsdC resulted in a Soret similar to the as-isolated holo-BslKN. Maresso has taken these data to indicate that specific interaction between the two natural partners enables the heme transfer between these proteins.

Lei and colleagues have proposed that the molecular mechanism of heme transfer between holo-Shp and apo-HtsA in *S. pyogenes* is facilitated by complex formation between the
two proteins ($K_d$ of $48 \pm 0.7 \, \mu M$)(66). In holo-Shp, mutation of the axial ligand M66A but not M153A increased the binding constant between the two proteins ($K_d$ of $11.4 \pm 0.3 \, \mu M$). In line with these observations, Lei and colleagues suggested that apo-HtsA must be in close proximity to the two axial ligands of holo-Shp (66). An X-ray structure showed that the heme and its binding sites in the Shp were exposed to the solvent (67). This is consistent with the proposition of Ran et al. that the heme pocket of apo-HtsA changes conformation after complex formation, allowing insertion of its ligands on both sides of exposed heme and simultaneously extracting it from holo-Shp (66).

Other studies have also led to the conclusion that heme transfer between natural partners is facilitated via specific protein-protein interactions. These examples include investigations of the pathways in Isd proteins of *S. aureus* from IsdA$^N$ to IsdC$^N$ (68); *B. anthracis* from IsdX1/IsdX2 to IsdC (69); and from Hbp1 to Hbp2 in *L. monocytogenes* (70).

### 1.9 Heme-iron uptake strategies

A second look at the known HtaA- and NEAT-based heme uptake strategies shows that most of the proteins involved in the first step have at least two conserved domains. One possibility is that multiple proteins are required for efficient transfer of heme. In certain instances, it is known that one domain binds the protein that donates heme, and the other extracts the heme. An example is the approach employed by the NEAT domains of *S. aureus* described above. IsdH$^{N2}$ binds both Hb and the Hb-Hp complex (38, 39, 42), but not heme itself, and IsdH$^{N3}$ domain binds heme (71), but not Hb (39). IsdB$^{N2}$ binds both Hb and the Hb-Hp complex whereas, IsdB$^{N1}$ binds neither of these sources but does bind heme (44).

A similar strategy may be occurring in *C. diphtheriae* HtaA, which can bind both Hb and the Hb-Hp complex. HtaB can receive heme from both HtaA and Hb, but cannot itself bind Hb.
The necessity for one domain to bind the heme donor and the second to receive the heme leads to the assumption that they are in close proximity. In some instances, both domains are part of the single protein.

As described in detail above, it is also the case that ChtA and ChtC work in concert with one another to utilize the Hb-Hp complex iron (36). As described in detail above, Schmitt and colleagues have proposed that ChtA and ChtC work in concert with one another to utilize the Hb-Hp complex iron (36). This was consistent with the finding that mutation of two genes, *chtA* and *chtC*, but not only *chtA* or *chtC*, abolished the bacterial growth in the presence of the Hb-Hp complex.

Two domains may also serve to take up and store heme, as has been proposed for Shr from *S. pyogenes* (61). For this system, the kinetics of heme uptake have been interpreted in terms of heme transfer to Shr$^{N1}$, with Shr$^{N2}$ serving as a heme storage domain. The second domain can transfer heme back to the first for movement along the pathway when the exogenous heme level drops.

1.10 Purpose of Study

Due to constant evolving antibiotic resistance, new ways of controlling bacterial infections are urgently needed. In hopes of developing alternative antibiotic therapeutics, it is useful to study heme uptake in heme proteins.

Most of the heme uptake work has been performed Gram-negative bacteria. These studies focus mainly on the strategies used by Gram-positive bacteria for heme uptake. In the first project, characterized the heme-binding proteins ChtA from *C. diphtheriae*. Spectroscopic techniques were used to determine some biochemical and biophysical characteristics of this protein.
Chapter 2 is a draft of the manuscript entitled “Characterization of homologous heme uptake proteins HtaB, ChtB, and ChtA from Corynebacterium. diphtheriae," which is to be submitted to a peer-reviewed journal. Chapter 3 is the additional experiments that are not part of the manuscript.
### Table 1.1 C. diphtheriae proteins and mutants binding studies.

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References taken from, (8, 28, 34, 36).
Table 1.2 *C. diphtheriae* growth studies.

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<td>(8)</td>
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<td>(8)</td>
<td></td>
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<td>Rel 0.76</td>
<td>(8)</td>
<td></td>
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<tr>
<td>ΔhtaB/chtB</td>
<td>Rel 0.58</td>
<td>(8)</td>
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<td>ΔhtaB</td>
<td>Rel 0.83</td>
<td>(8)</td>
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</tr>
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<td>(36)</td>
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Figure 1.1 Medically important Gram-positive Bacteria: Actinobacteria (HtaA domains) and Firmicutes (NEAT domains).
Figure 1.2 Clustal Omega alignment of NEAT domains for IsdB, IsdC, IsdH, and non-NEAT domain, IsdA from S. aureus; NEAT domains for Shr and non-NEAT domain, Shp from S. pyogenes.
Figure 1.3 A graphical sequence Logo representation of HtaA family (PF04213) generated by profile Hidden Markov Model (pHMM): Y15, H15, and Y148 are equivalent to Y1, H, and Y2, respectively of HtaA-CR2 from C. diphtheriae (1).
Figure 1.4 The model of the location of the Isd proteins involved in heme import into S. aureus (5).
Figure 1.5 The model of the location of proteins involved in heme import into S. pyogenes (12).
Figure 0.1 The crystal structure of intact Hb with IsdH^{N2N3}. D: The IsdH^{N2} domain binding β-Hb (green), and E: the IsdH^{N3} positioned near the heme of the α-Hb (blue). PDB 4IJ2 (10).
Figure 0.2 Heme binding including the axial Y and its H-bonding partner in IsdA, IsdC, and IsdH (9)
2 CHARACTERIZATION OF HOMOLOGOUS HEME UPTAKE PROTEINS HTAB, CHTB, AND CHTA FROM CORYNEBACTERIUM DIPHTHERIAE

This chapter is a draft of the manuscript to be submitted to a peer-reviewed journal. The authors are Catherine Odhiambo a, Rizvan C. Uluisik a, Brandon L. Ferrell a, Gudrun S. Lukat-Rodgers b, Courtni E. Allen c, Michael P. Schmitt c, Kenton R. Rodgers b,*, and Dabney W. Dixon a,* . The expression, purification, and UV-visible spectroscopy of the WT and mutants were performed at Georgia State University.

2.1 Introduction

Corynebacterium diphtheriae is a Gram-positive bacterium that causes communicable infections of the upper respiratory tract and skin in humans. Strains that secrete the iron- and DtxR-regulated diphtheria toxins are associated with severe symptoms (35, 72). Early work showed that strain C7 was able to use both heme and hemoglobin as iron sources in a low-iron medium (73). A screen for heme- and hemoglobin-uptake-deficient mutants discovered a gene cluster expressing a heme transport-associated protein HtaA and a heme-binding protein (HmuT), which transfers heme to HmuUV, an ABC transporter (20). Two additional gene products, HtaB and HtaC, were also expressed from this gene cluster, each transcribed from its own DtxR promoter (28, 29). In later work, two more operons transcriptionally regulated by DtxR and iron were discovered, with gene products ChtA-ChtB and ChtC-CirA (74).

HtaA, HtaB, ChtB, ChtA, and ChtC (Figure 2.1) all have heme-binding domains that show significant sequence similarities (Figure 2.10). HtaA has two of these conserved domains, and the other four proteins each have one. There are two highly conserved tyrosines, one about ten residues from the N-terminus (Y1), and the second about 30 residues from the C-terminus of the domain (Y2). There is also a histidine or tyrosine about one-third of the way through the
sequence. For the second conserved domain in HtaA (HtaA-CR2), Y1 is more important for hemin binding Y2, but both are involved in some way (74, 75). Biophysical studies have showed that the heme has a tyrosine ligand (75).

HtaA, ChtB, ChtA, and ChtC all bind Hb with similar binding constants (74). Heme can be transferred from Hb to HtaA and from HtaA to HtaB (28, 34). HtaB can acquire heme from Hb but cannot itself bind Hb (74).

The Hb-Hp complex may also be a major source of heme iron for bacterial pathogens; almost all free Hb is found in the Hb-Hp complex in vivo (76). In for these *C. diphtheriae* proteins, HtaA takes up the heme from Hb-Hp in conjunction with ChtA and ChtC (which seem to have very similar functions) (11).

Recent structural studies on three conserved domains in the related species, *Corynebacterium glutamicum* (CgHtaA-CR1, CgHtaA-CR2 and CgHtaB), have shown that the heme is positioned on edge of the protein cleft below a short \( \alpha \)-helix (4). In these domains Y1 serves as the axial ligand and Y2 forms a hydrogen bond with a heme propionate. A conserved serine near the N-terminus forms a hydrogen bond with the same propionate. The conserved histidine forms a hydrogen bond with Y1 as well as another tyrosine.

Biophysical studies to date on the *C. diphtheriae* HtaA pathway have concentrated on HtaA (28, 34, 75) and HmuT (63, 77, 78). Herein we report the characteristics of an additional three members of this multifaceted heme uptake pathway. We first compare the properties of HtaB and ChtB, which seem to be able to function for one another in vivo (74). ChtA, which appears to be required for heme uptake from Hb-Hp (11), is then discussed.

Resonance Raman (rR) spectroscopy was used to investigate the structural and electronic features of the heme micro-environment in the binding pocket. The stability of the protein
folding was investigated via chemical and thermal denaturation techniques and collision-induced heme dissociation in the gas phase. We compare the *C. diphtheriae* proteins with those of *C. glutamicum*. These comparisons within and between species lead to a picture of related proteins that are similar in many aspects, but nonetheless have noticeable differences, presumably significant in controlling the process of heme transfer. This work adds to the increasing knowledge of heme uptake in Gram-positive pathogenic bacteria across a variety of species as outlined in recent reviews (15, 79-83). Most pathogenic bacteria need iron for survival. A deeper understanding of the role of hemin uptake in providing this iron (84) may point to new approaches to the increasing problem of bacterial resistance to antibiotics (85).

2.2 Experimental

2.2.1 Reagents and solutions

Buffer A was 100 mM Tris-Cl and 150 mM NaCl at pH 8.0. Buffer B was 100 mM Tris-Cl, 150 mM NaCl, and 2.5 mM *d*-desthiobiotin and 1.0 mM of EDTA, pH 8.0. Buffer C was a mixture of 50% Buffer A and 50% Buffer B. Buffer D was a mixture of 30% Buffer A and 70% Buffer B.

2.2.2 Optical spectroscopy

UV-visible absorbance spectra were recorded either with a single beam (Varian Cary 50 Bio) or a dual beam scanning spectrophotometer (OLIS-14). The measurements were made using quartz cuvette having a 1.0 cm path length. HtaB, ChtB, ChtA and, HtaB Y56A, H121A concentrations were calculated from $\varepsilon_{280}$ from ExPASY: 42,985, 46,535, 25,485, 41,495 and, 42,985 M$^{-1}$ cm$^{-1}$, respectively. Hemin (Sigma-Aldrich) concentrations in dimethyl sulfoxide (DMSO) were calculated using $\varepsilon_{624} = 6.23$ mM$^{-1}$ cm$^{-1}$ (64). MetHb (equine, Sigma-Aldrich) concentrations were calculated using $\varepsilon_{409} = 179$ mM$^{-1}$ cm$^{-1}$ (86). The Sephadex G25 medium and
superfine grades were from GE Healthcare. The pyridine hemochrome assay was used to
determine the extinction coefficients (87). An average of three experiments gave an extinction
coefficient of $1.3 \times 10^5 \text{M}^{-1} \text{cm}^{-1}$ for HtaB-WT, $1.1 \times 10^5 \text{M}^{-1} \text{cm}^{-1}$ for ChtB, and, $1.4 \times 10^5 \text{M}^{-1}$
$\text{cm}^{-1}$ for ChtA.

2.2.3 Circular dichroism

Circular dichroism (CD) spectra were recorded using a Jasco J-810 spectropolarimeter
using quartz Suprasil cuvettes with a 1 mm. Protein samples were recorded in 10 mM KH$_2$PO$_4$
buffer at pH 7.0; concentrations were adjusted to 10 $\mu$M, except that a Jasco J-1500 CD
spectropolarimeter and a quartz cuvette having a 1.0 cm path length was used for ChtA with a
protein concentration of 1.5 $\mu$M in Buffer A. The final spectra represent an average of 20 scans.

2.2.4 Constructs

The ChtB and ChtA constructs have been previously described (74). For HtaB-WT, an N-
terminus Strep-tag labeled construct was synthesized and inserted into pET24a(+) plasmid at the
NdeI and HindIII restriction sites. The construct included the peptide sequence between Ala25
and Asn292 (NCBI sequence reference number WP_004566989.1). A TEV cleavage sequence
was inserted between the Strep-tag and HtaB-WT. A linker sequence of SGGGGG was used to
separate the Strep-tag and the TEV cleavage sequence. Primers are given in Table 2.2.

2.2.5 Site-directed mutagenesis

Two mutants of HtaB were prepared by site-directed mutagenesis using a QIAprep Spin
Miniprep Kit according to the manufacturer’s instructions. The primers were from Sigma
Aldrich for Y56A and H121A substitutions (Table 2.2). Primers (500 ng) were mixed with 100
ng of pET24a(+) template plasmid in the reaction mixture. DpnI restriction endonuclease was
used to remove the methylated template. The plasmids were isolated from the reaction mixture
and transformed into competent *Escherichia coli* strain BL21(DE3) cells. Sequence analysis was used to confirm the mutations.

2.2.6 Sequence alignments and homology Modeling

Sequence alignments were performed using the Clustal Omega software from the European Bioinformatics Institute (EMBL-EBI) database. The *C. diphtheriae* proteins HtaB, ChtB, and ChtA, HtaA-CR1, and HtaA-CR2 were aligned with the conserved regions of HtaA-CR1, HtaA-CR2 and HtaB from *C. glutamicum* (4). Homology models were constructed using the I-TASSER software (88).

2.2.7 Expression and purification

The protocol for HtaB is given in detail. Luria-Bertani (LB) medium was used for culturing the *E. coli* cells. The medium contained 50 μg/ml kanamycin with 0.3% glucose. A small-scale culture (10 ml) was incubated at 37 °C/220 rpm for 16 h, added to 990 ml of media and the resulting solution incubated at 37 °C/220 rpm until the OD$_{600}$ reached 0.8. The cultures were induced by adding 50 μM isopropyl-β-D-thiogalactopyranoside (IPTG) and allowed to express for 16 h. The cells were harvested by centrifuging at 8000 rpm/4 °C for 30 min. The pellets were collected and stored in -20°C freezer overnight.

The cell pellet was re-suspended in buffer A. The cells were lysed in a solution containing buffer A, 10 mM MgCl$_2$, 0.1 mM phenylmethanesulfonyl fluoride (PMSF), 0.2 mg/mL lysozyme, 5 μg/mL DNase I (from bovine pancreas, Roche) and 5 μg/mL RNase A (from bovine pancreas, Roche) and incubated on ice for 30 min. The cells were disrupted with a sonicator, and the cell debris was removed by centrifugation at 6500 rpm/4 °C for 45 min.

A GE Healthcare ÄKTA fast protein liquid chromatography instrument (FPLC) was used for purification at 4 °C. The protein solutions were loaded onto a Strep-Tactin Superflow column
(5 mL, IBA BioTAGnology) which had been equilibrated with Buffer A. The unbound material was washed out with 5 column volumes of Buffer A. The Strep-Tactin bound protein was eluted with 10 column volumes of Buffer B. A linear gradient of buffer A and B was used. The purity of the fractions was tested by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Approximately 0.8 mg/L of protein were isolated. The hemin loading was 30%. The HtaB Y56A, H121A, and ChtB were prepared in the same manner.

ChtA was prepared similarly except that Terrific Broth was used with 0.3 mM of IPTG and no glucose. Expression was for 3 h at 37 °C.

2.2.8 Hemin addition

HtaB (14.2 µM) with 30% hemin loading as isolated, prepared in Buffer A, was used for hemin titration. Aliquots of a solution of 4.63 mM hemin in DMSO were added to the protein sample which was allowed to incubate for 30 min after each addition. UV-visible spectra were recorded after each hemin addition up to a hemin:protein ratio of 0.90. The volume of DMSO in solution did not exceed 0.2%.

The HtaB Y56A and H121A (both approximately 4% hemin loading as isolated) hemin addition experiments were performed identically on the concentrations of 15.2 and 21 µM, respectively.

The hemin loading of as-isolated ChtB (60%) was sufficient for spectroscopic experiments.

ChtA (28.1 µM) initially 20% heme loaded (Soret:280 nm ratio of 0.29) in Buffer A, was used for hemin reconstitution. Addition of one equivalent of hemin in DMSO gave a sample that was passed down a Sephadex G25 superfine column three times.
2.2.9 *Hemin transfer from metHb*

HtaB (23.8 µM, 3.8 ml), 30% hemin loading as isolated, prepared in 100 mM Tris-HCl with 150 mM NaCl at pH 8.0, was used for metHb-hemin reconstitution. The protein solution was loaded onto a Strep-Tactin column (5 mL IBA BioTAGnology). A Hb (59.9 µM, 5 mL) solution in the same buffer was loaded onto the column. The column was washed with Buffer A to remove excess Hb. Spectra of the eluent fractions showed that all free heme-containing species had been washed from the column. The column was held at 4°C for 20 h. The column-bound HtaB-WT was eluted using 10 column volumes of Buffer B. The hemin-bound fractions of HtaB-WT were concentrated using an Amicon Ultra-15 centrifugal filter at 6500 rpm and 4°C.

The HtaB Y56A mutant and ChtB Hb-hemin addition experiments were performed similarly except that Hb (100 and 503 µM) was used on the protein concentrations of 39 and 76 µM, respectively.

2.2.10 *Time scale of unfolding*

A solution of HtaB-WT (14.7 µM) and 4.0 M GdnHCl was prepared in Buffer A. The GdnHCl concentration was determined by the refractive index method (89). The solution was stirred for 10 min at 25 °C. The UV-visible spectrum was monitored over time for 18 h at 25 °C. The absorbance at 409 nm was fit to a single-term exponential function using Kaleidagraph (version 4.01, Synergy Software).

\[ A_t = (A_0-A_\infty)\exp(-k_{ty}t +A_\infty) \]
where $A_t$ is the absorbance at any time during the unfolding reaction, $A_0$ is the initial absorbance, $A_{\infty}$, is the absorbance of the completely unfolded protein, $A_0 - A_{\infty}$, is the total $\Delta A$ for complete unfolding, and $k_U$ is the unfolding rate constant.

The ChtB and ChtA unfolding experiments were run similarly on the concentrations of 15.8 and 18.5 µM, respectively.

### 2.2.11 Thermal denaturation

Thermal unfolding of HtaB (2.76 µM) was carried out with the UV-visible spectrophotometer equipped with temperature control (TC 125, Quantum Northwest). A screw top quartz cuvette with 1 cm path length was used. The protein solution was prepared in 50 mM NaH$_2$PO$_4$, pH 7.0 buffer including 1.0 M GdnHCl. Data at 410 nm were recorded every 1°C after a 1 min incubation at each temperature. Kaleidagraph was used to fit the data using a two-state protein unfolding model equation (90): Eq. (1)

$$
A = \frac{(A_F + m_F T) + (A_U + m_U T) \exp \left[ \frac{\Delta H_m}{R(T_m^{-1} - T^{-1})} \right]}{1 + \exp \left[ \frac{\Delta H_m}{R(T_m^{-1} - T^{-1})} \right]}
$$

where $A$ is the absorbance at any point along the unfolding curve, $A_F$ is the absorbance of the folded state, $A_U$ is the absorbance of the unfolded state, $m_F$ is the slope of the folded state, $m_U$ is the slope of the unfolded state, $T_m$ is the temperature at which the protein is half unfolded, $\Delta H_m$ is the enthalpy of unfolding, $R$ is the gas constant, and $T$ is the temperature (Kelvin).

The HtaB Y56A, H121A and ChtB unfolding experiments were run in a similar fashion on concentrations of 2.44, 8.33, and 5.35 µM, respectively.
The ChTA experiment was run on the concentration of 10 µM. However, the protein was not stable in 50 mM phosphate, so a solution of 1.5 M GdnHCl in Buffer A was used.

2.2.12 Electrospray ionization (ESI) mass spectrometry

ESI spectra were obtained using a Waters Micromass Q-TOF spectrometer in the positive mode. The fully holoprotein samples (HtaB-WT, HtaB H121A, and HtaB Y56A) were prepared by adding of hemin to the as-isolated protein (approximately 30% heme loaded) as previously described. The protein solutions (~10 µM) were dialyzed against 20 mM ammonium acetate, pH 6.8. Spectra were recorded (flow rate 10 µl/min) for each sample at the following collision energy voltages: 5, 10, 15, 20, 25, 30, and 40 V. All other parameters were held constant (capillary voltage 3000 V, cone voltage 18 V, extraction voltage 1.5 V, desolvation gas temperature 100 °C and source temperature 80 °C). Deconvolution of the charged state was performed using the MaxEnt program with the MassLynx™ software. Peaks were rounded to the nearest Dalton; peak heights were used to calculate holoprotein percentages at each collision energy voltage.

2.2.13 Resonance Raman (rR) spectroscopy

Resonance Raman (rR) spectra were recorded from ferric samples using the 413.1-nm emission line from a Kr⁺ laser and from ferrous samples with 441.6-nm excitation from a HeCd laser. The Raman shifts for toluene, DMSO, and methylene bromide were used as external standards for spectral calibration. Spectra were recorded at ambient temperature using the 135° backscattering geometry with the laser beam focused to a line on a spinning 5 mm NMR tube. UV–visible spectra were recorded before and after rR experiments to verify that the samples were not altered by their exposure to the laser beam. The buffers used were 100 mM glycine (9.6), Tris-HCl (pH
8.8 or 8.0), and sodium phosphate buffer (pH 5.8). Ferrous samples were prepared under anaerobic conditions by adding buffered dithionite in a 300-fold molar excess over heme. The Fe(II)-CO samples were prepared with a 300-fold molar excess of dithionite under an atmosphere of natural abundance CO or $^{13}$CO at pH 8.8. The final protein concentrations were 30 µM for the HtaB-WT.

2.3 Results and Discussion

2.3.1 Homology modeling

Homology models were built based on the recent structures from *C. glutamicum* (4). Figure 2.2 shows the models for ChtA and ChtB. In ChtA, the model shows the important residues around the heme including the axial tyrosine Y129, the two residues that forms a hydrogen bond with one of the propionates, Y272 and S125, and a phenylalanine that stacks against the heme F271. When heme is bound, Y129 is presumably forms a hydrogen bond with H279 which is in turn hydrogen bonded to Y178. In this model, these three residues are extended away from the heme pocket. Disruption of the Y129-H279-Y178 triad may be an important triggering factor in heme transfer.

ChtB has a less well-defined β-sheet structure on one side of the heme. Y56 is the axial ligand hydrogen bonded to H121. Y223 and S52 are hydrogen bonded to the heme propionate.

All of the structures in the HtaA family show significant overall similarities, with a heme lying between two α-helices, and an axial tyrosine hydrogen bonded to a histidine. A serine four residues away from the axial tyrosine forms a hydrogen bond with one of the heme propionates. Interestingly, this motif is also found in NEAT, another major heme uptake domain (Andrade et al., 2002; Grigg et al., 2011; Honsa et al., 2014). Presumably as the protein docks with its
transfer partner, changes in these hydrogen bonding networks allow the heme to be released from the pocket.

2.3.2 **UV-visible spectroscopy, heme addition, and pH titrations**

Despite the presumed similarities between HtaA (homologous to ChtA/ChtC) and HtaB (homologous to ChtB), these proteins vary greatly in how heme loaded they are as isolated. The goal of this work was to determine whether the reconstituted protein was similar to those as isolated.

HtaB-WT was usually only about 30% heme loaded as isolated. The UV-visible spectrum shows a Soret band at 408 nm, $\beta$ and $\alpha$ bands at 504 and 542 nm, respectively, and a charge-transfer band at 625 nm (Figure 2.3). The charge-transfer band is consistent with a high spin heme-iron interaction with an oxygen species, often seen in tyrosine-coordinated heme proteins (82, 91).

To increase heme loading, hemin in DMSO was titrated into the solution to a total of 1 equivalent of hemin. The final $A_{\text{Soret}}/A_{280}$ ratio was approximately 1.8. The spectra of the as-isolated and hemin-titrated HtaB-WT (Figure 2.11) were similar, suggesting that the added hemin was in the same environment as that found in the as-isolated protein. The heme loading of HtaB-WT was also increased by transferring the hemin from metHb. This spectrum also was very similar to the previous two (Figure 2.11). The strategy of transferring hemin from Hb to tagged protein may prove to be a useful technique for heme transfer under mild conditions.

The HtaB as-isolated breadth was slightly wider, indicating the heterogeneity in the as-isolated heme pocket.

Two HtaB mutants were studied. Y56A and H121A were isolated with less than 5% hemin loading, far less than WT (30%). For H121A, the spectrum of the as-isolated protein
(Figure 2.4) was very similar to that of wild type (Figure 2.3). These data argue that H121 is not an axial ligand of the heme in this protein. Hemin in DMSO was added up to a total of 1 eq hemin. The resulting spectrum was similar as that of the as-isolated H121A (Figure 2.12).

For Y56A, in contrast, the as-isolated protein (only 5% heme loading) had a very different spectrum (Figure 2.13) from that of the WT. The 542 and 625 nm peaks disappeared, while new bands appeared at 547 and 587 nm. The lack a charge transfer band at approximately 625 nm is consistent with a heme that does not have a tyrosine axial ligand.

HtaB Y56A was reconstituted both with hemin and by transferring hemin from metHb. The spectra of the resulting species were similar to one another and WT HtaB (Figure 2.13). As the presumed axial Y56 is no longer present, these spectra may indicate a conformational change to provide another oxygen-coordinated axial ligand, presumably another tyrosine or water.

In contrast to HtaB, ChtB was 60% heme-loaded as isolated; the spectrum was very similar to that of HtaB-WT (Figure 2.2). Addition of hemin via transfer from hemoglobin gave holo protein with the same spectrum as the as-isolated species (Figure 2.14).

ChtA had less than 5% bound hemin as isolated. When 0.9 eq of hemin in DMSO was added to a protein sample that was already 30% heme loaded, a broad Soret band was observed (Figure 2.15). Passing this sample through a Sephadex G25 superfine column resulted in a narrower Soret, but the band was still broader than those of the other conserved domains in this family (Figure 2.15). To determine if the breadth of the ChtA peak was due to remaining surface-bound hemin, the sample was passed through the Sephadex G25 superfine column twice more. Minimal changes were seen (Figure 2.15), leading to the conclusion that the Soret of ChtA is intrinsically broad. Comparison of the reconstituted proteins to HtaA-CR2 (75), were very similar (Figure 2.2).
pH titrations of ChtA, HtaB-WT and ChtB showed no significant spectral changes over the pH range 6.9-11.2 (data not shown). These findings are in line with the previous observation that HtaA-CR2 shows no pH-induced changes over this range (75).

2.3.3 **Hemin transfer between HtaB and ChtB**

Growth studies show that either HtaB or ChtB can serve in terms of the growth of the cell with heme as an iron source (28, 74). It was of interest to determine whether HtaB and ChtB could transfer hemin between them. Previous studies showed that holo HtaA can transfer a heme to HtaB (34). This has been the only transfer shown thus far in the **hmu-cht** heme uptake pathway. HtaB and ChtB both have significant sequence similarities, suggesting that the proteins may have similar functions as intermediate transport proteins.

To assess heme transfer between HtaB and ChtB, heme-loaded strep-tagged ChtB was bound to a StrepTactin column and incubated with as-isolated His-tagged HtaB. The proteins were separated by washing the column with buffer A to elute all His-tagged HtaB followed by the elution of strep-tagged ChtB with a linear gradient of buffer B. The fractions were collected, concentrated, and assessed for purity by SDS-PAGE; only one protein per fraction data not shown. The UV-visible spectra of as-isolated ChtB before and after the transfer are shown overlaid in Figure 2.9. The results show that HtaB and ChtB readily transfer heme to one another (Figure 2.9). This indicates that there may be an extent of crossover of heme transfer between the **hmu** and **cht** heme uptake pathways *in vitro*.

2.3.4 **Resonance Raman spectroscopy of ferric WT HtaB and ChtB**

The rR spectrum of ferric HtaB is pH independent, as evidenced by the unchanging high-frequency spectrum over the pH range from 5.8 to 9.6 (Figure 2.5). The $v_3$, $v_{38}$, and $v_2$ core stretching frequencies of 1483, 1520, and 1561 cm$^{-1}$, respectively, reveal the heme to be in a 6-
coordinate high spin (6cHS) state (7). Another protein in a heme uptake pathway, *Yersinia pseudotuberculosis (Yp)* HasA, has been shown to exist as an equilibrium mixture of 6cHS and 5-coordinate high-spin (5cHS) states (92), the former presumably having with a water molecule as the sixth ligand (Table 2.1). *Yp*HasA also does not show a pH dependence over the pH range from 5 to 10. *Serratia marcescens (Sm)* HasA (93) and *Cd*HmuT are mixtures of 6cHS and 6 coordinate low-spin (6cLS) states (77). Even though the CR domain of HtaB shares approximately 27% sequence identity and 38% similarity with the CR2 domain of HtaA, the hemin binding motifs are distinct because *Cd*HtaA is 5cHS in the ferric form (75). HtaA exhibits a rR spectrum typical of 5cHS hemin that is bound to the protein through a proximal tyrosine ligand. The $v_3$ (1490 cm⁻¹) and $v_2$ (1568 cm⁻¹) rival the intensity of the $v_4$ which is unusual for other hemin proteins but has been observed in bovine liver catalase (94) and *Shigella dysenteriae* ShuT (95), both of which share the proximal Tyr binding motif as seen in Table 2.1.

Like its HtaB homolog, *Cd*ChtB forms a 6cHS hemin complex with spectral characteristics consistent with a proximal Tyr binding motif (Figure 2.6). The $v_3$, $v_{38}$, and $v_2$ core stretching frequencies of 1486, 1522, and 1560 cm⁻¹, respectively, indicate 6cHS hemin (7). Unlike ferric HtaB, peripheral substituent modes in ChtB exhibit slight pH dependences, as seen in the shifts of the in-plane vinyl stretch from 1613 to 1627 cm⁻¹, the in-plane CðπCð stretch from 1560 to 1558 cm⁻¹, the in-plane pyrrole breathing mode from 761 to 754 cm⁻¹, out-of-plane pyrrole fold from 739 to 735 cm⁻¹ and an increase at 666 cm⁻¹, and out-of-plane wags from 329 to 327 cm⁻¹ and from 311 to 308 cm⁻¹ Figure 2.6 and 2.16. None of these vibrations are particularly sensitive to the coordination number or spin state of the heme, as evidenced by the constancy in the $v_4$ and $v_3$ frequencies among these proteins. This indicates that the structural determinants for coordination number and spin state in the hemin binding pockets of HtaB and
ChtB are similar, with the environments that impact conformations of the peripheral substituents being slightly variable.

### 2.3.5 Resonance Raman spectroscopy of ferrous HtaB

Upon reduction with dithionite, HtaB was converted to a mixture of 5cHS and 6cLS ferrous states. This mixture was revealed by a $v_3$ band at 1468 cm$^{-1}$ and a small band at 1494 cm$^{-1}$, respectively (Figure 2.17). The $v_{11}$, $v_2$, and $v_{10}$ core stretching frequencies of 1558 cm$^{-1}$, 1583 cm$^{-1}$ and 1624 cm$^{-1}$ respectively, are consistent with the speciation being dominated by the 5cHS heme state (96); the UV-visible Soret maximum of 428 nm is also consistent with this assignment. This result differs from both $Yp$HasA, which is solely 5cHS ($v_3$, 1470 cm$^{-1}$), and $Cd$HmuT and $Sm$HasA, which do not form ferrous complexes under similar conditions (77, 92, 93).

The rR spectra are also consistent with the UV-visible Soret maximum of 428 nm, suggesting a predominately 5cHS speciation similar to what was reported for $Yp$HasA (92) (Figure 2.17, inset). When excited with the 441.6-nm laser light, the low frequency spectrum of HtaB contains a small band at 217 cm$^{-1}$, which is a typical Fe$^{II}$-His stretching frequency in 5cHS ferrous heme proteins (Figure 2.7). Because of its red-shifted Soret band, blue (441.6 nm) Raman excitation selectively enhances Raman scattering by the modes of 5cHS having a proximal His ligand. Thus, it is possible to detect the presence of axial His ligation in 5cHS ferrous hemes, even if it is not the majority species (97).

It is possible that a His ligand is replacing the axial tyrosine in a fraction of the ferrous form. This type of displacement has been observed for the ferrous form of $S. pyogenes$ NEAT-A, in which the axial Tyr ligand in the ferric form is replaced by His in the ferrous form of the protein (98). The small relative intensity of the 217 cm$^{-1}$ band, in comparison to that of $v_{Fe-His}$
modes from other proteins, is likely due to a small amount of the protein being 5cHS with the remainder having Tyr coordinated in the axial position. These spectra suggest the ferrous HtaB is a mixture of complexes, comprising predominately a 5cHS Tyr complex with small contributions from a 5cHS His-bound and 6cLS Tyr/X-bound hemes.

Ferrous ChtB was solely 5cHS as evidenced by its ν₃ at 1468 cm⁻¹ and Soret maximum of 425 nm (Figure 2.18). The ν₁₁, ν₂, and ν₁₀ core stretching frequencies of 1556 cm⁻¹, 1584 cm⁻¹ and 1623 cm⁻¹, respectively, indicate a 5cHS heme environment, similar to the 5cHS contribution to the ferrous HtaB spectrum. The absence of bands attributable to a 6cLS form indicates that reduction destabilizes its 6cLS heme to an even greater extent than in HtaB.

### 2.3.6 Resonance Raman spectroscopy of ferrous carbonyl WT HtaB and ChtB

Through the sensitivity of Raman-active Fe-CO group frequencies to π-backbonding and donor strength of the trans (proximal) ligand, resonance Raman spectra of heme protein carbonyls are useful probes of both the bonding and non-bonding properties of the heme pocket. The extent of π-backbonding is evidenced by the ν₉Fe-C, νC–O frequencies which, when placed on a plot of ν₉Fe-CO versus νC–O frequencies, yield insight into the electrostatic and steric properties of the distal heme pocket and the nature of the trans axial ligand (99-102). The Fe⁻¹³CO isotopologs of HtaB and ChtB were used to verify the identity of rR bands corresponding to the Fe–C stretching (ν₉Fe-CO), Fe-CO bending (δ₉Fe-CO) and C–O stretching (νC–O) modes, as shown in Figure 2.19 and Figure 2.20.

Under an atmosphere of carbon monoxide, the UV-visible spectra of ferrous HtaB and ChtB exhibit shifts of their Soret band maxima to 417 and 415 nm, respectively, along with sharpening and shifting of the Q bands (insets Figure 2.19 and 2.20). These absorbance changes are consistent with the formation of the HtaB and ChtB carbonyls.
The Soret-excited rR spectrum of HtaB–CO reveals two $\nu_{\text{Fe-CO}}$ bands at 503 and 532 cm$^{-1}$, which shift to 501 and 527 cm$^{-1}$, respectively, upon $^{13}$CO substitution. Despite having two $\nu_{\text{Fe-CO}}$ stretches, the spectrum reveals only one $\nu_{\text{C-O}}$ band and a single $\delta_{\text{Fe-CO}}$ band at 1949 and 562 cm$^{-1}$, respectively, which shift to 1902 and 554 cm$^{-1}$ in the $^{13}$CO isotopolog (Figure 2.19). ChtB–CO also exhibits two $\nu_{\text{Fe-CO}}$ bands [503(−3) and 531(−5) cm$^{-1}$], one $\nu_{\text{Fe-CO}}$ band at 563(−13) cm$^{-1}$ and one $\nu_{\text{C-O}}$ band at 1953(−47) cm$^{-1}$, similar to what was observed for HtaB–CO (Figure 2.20). Placement of these pairs of $\nu_{\text{C-O}}$ and $\nu_{\text{Fe-CO}}$ frequencies on the $\pi$-backboning correlation plot is consistent with two heme carbonyls having different proximal ligands (Figure 2.8). One form falls near the line correlating $\nu_{\text{C-O}}$ and $\nu_{\text{Fe-CO}}$ of carbonyls having neutral imidazole ligands. The other falls on the correlation line neutral proximal ligands coordinated through an O atom. This position is consistent with a proximal Tyr ligand whose coordinated O atom interacts with a single hydrogen bond donor (vide infra).

The CO ligand in the trans ImH form is in a weak hydrogen bond interaction with the distal pocket as evidenced by its low position along the neutral imidazole line (103). This interaction likely involves the native, conserved Tyr56 axial ligand interacting with the exogenous CO ligand in a manner similar to that reported for CdHmuT mutants R237A and M292A (63). The position of the form having an O-bound proximal ligand, which is likely the conserved Tyr56, is indicative of stabilization of the oxygen by a single neutral hydrogen bonding partner. The position is close to both YpHasA (92) and SmHasA (104) which have been shown to have a trans neutral histidine that is hydrogen bonded to the axial Tyr (92, 105). The trans ligand environments of HtaB and ChtB differ from that of CdHmuT, which has been shown to have a pair of hydrogen bonds from the guanidinium side chain of a nearby Arg residue (63, 77). The positioning of the Arg hydrogen bonding partner of the Tyr ligand in crystal
structure of CgHmuT has been shown (78). A possible neutral hydrogen-bonding partner could be Tyr53 which could stabilize the Tyr56 heme ligand in HtaB, similar to what is seen with IsdB (45).

2.3.7 Heme dissociation in ESI mass spectrometry

The stability of as-isolated HtaB-WT and its mutants was also evaluated by monitoring the mass spectra as a function of collision energy (5 – 40 V). Each as-isolated protein was initially titrated with hemin (~ 0.9 of hemin:apoprotein ratio) to obtain holoprotein concentrations suitable for the mass spectra experiments. Figure 2.21 shows that the percentage of holoprotein decreased approximately from 94% to 30% for WT and the H121A mutant. Y56A, which started with only 67% heme loading, showed a similar loss of hemin with increase in collision energy. In the gas phase, all three-show comparable heme loss with increasing collision energy, whereas in the solution phase, the proteins are in the stability order WT > H121A > Y56A.

This has been observed previously in studies on myoglobin and cytochrome b5 (106) as well as the HmuT heme transfer protein (63) for which gas phase and solution phase data do not necessarily show a direct correlation.

2.3.8 Time scale protein unfolding in chemical denaturation

Denaturant-induced unfolding of heme proteins is used to understand the factors that control heme binding at the active site (89). In previous work, we showed that HtaA-CR2, a domain with significant sequence similarity to HtaB, was very stable in the presence of GdnHCl; e.g., a half-life of 330 min was observed even in the presence of 6.8 M GdnHCl at 37 °C (75). ChtA was less stable than HtaA-CR2; the protein has a half-life of 352 min in the presence of 4.0 M GdnHCl at 25 °C (Figure 2.22).
HtaB and ChtB were significantly less stable toward unfolding. HtaB showed a single first-order process with a half-life of 39 min in the presence of 4.0 M GdnHCl at 25 °C. The unfolding behavior of ChtB was very similar to that of HtaB under the same conditions Figure 2.23.

Although these four domains of proteins in the *C. diphtheriae* heme uptake pathway are structurally homologous both in alignment and in the similarity of their spectra, they are quite different in kinetics of unfolding. HtaB and ChtB have half-lives of approximately 40 min in 4.0 M GdnHCl at 25 °C; ChtA has a half-life of approximately 350 minutes under these conditions, and HtaA-CR2 has a half-life of 330 min in 6.8 M GdnHCl at 37 °C. The varying difficulties of hemin release may be important for directing hemin transfer to specific partner proteins in the pathway.

### 2.3.9 Thermal stability

The thermal stability of these domains was in line with the order observed in the kinetics experiments. HtaA-CR2 had a $T_m$ value of 77 °C in 1.5 M GdnHCl (75). ChtA had $T_m$ value of 59 °C at the same concentration of GdnHCl (Figure 2.24). ChtB and HtaB had $T_m$ values of 61 °C in 1.0 M GdnHCl (Figure 2.25).

To probe the contribution of the axial ligands to the stability of HtaB, thermal unfolding of as-isolated HtaB, and the H121A and Y56A mutants, was performed. In each case, the Soret absorbance gradually decreased as the temperature increased. The apparent $T_m$ values were 61, 46, and 27 °C (1 M GdnHCl) for WT HtaB, and the H121A and Y56A mutants, respectively Figure 2.26. Approximately 90% of the Soret absorbances were recovered for the WT and H121A mutants when the protein solutions were cooled to the initial temperature. In contrast, no recovery was observed for the Y56A mutant. These studies indicate that both H121 and Y56 are
playing a role in heme binding. Y56 has a highly significant role, with a Tm more than 30 °C lower than WT, and no recovery after cooling to room temperature. The importance of these two residues is in line with the rR back-bonding correlation results showing that HtaB is likely a Tyr/His heme-coordinated protein.

The very similar data for HtaB and ChtB in terms of thermal stability, as well as the kinetics of GdnHCl-induced unfolding, are consistent with similar structures and heme binding sites; it is thus reasonable that they play a similar role in the heme uptake. Indeed, Allen and Schmitt showed that a single deletion of either htaB or chtB genes does not affect cell growth in C. diphtheriae, but a double mutation of these genes, significantly affects the growth (28, 74).

The significant differences in ease of unfolding of these four proteins presumably arise from local hydrophobic, electrostatic and hydrogen-bonding interactions in the heme pocket. Schmitt and co-workers have shown that HtaA can bind hemoglobin while HtaB does not. HtaA can transfer heme to HtaB (34). Thus, the presumed pathway for heme uptake involves heme transfer from HtaA to HtaB/ChtB. It is interesting to note that HtaA-CR2 binds hemin more tightly (60 nM, Strep-tag) than HtaB (4.9 µM, no tag) in solution (11, 28). Thus, the role of protein/protein interactions is presumably important in determining the pathway of heme transfer (75, 77).

2.4 Relationship to other heme uptake pathways

The heme uptake pathway studied herein, based on variations of the HtaACR domain, can be compared with the well-studied heme uptake strategy that involves NEAT domains (31). Gram-positive bacteria are largely divided into Firmicutes (low G+C species) and Actinobacteria (high G+C species). Examples of clinically relevant Actinobacteria include Actinomycetes, Corynebacterium, Propionibacterium, Rothium, and Streptomyces. Bioinformatics analysis
shows that all of these species have sequences homologous to HtaACR. Almost none have sequences corresponding to the NEAT domain. Examples of clinically relevant Firmicutes include *Bacillus*, *Clostridium*, *Listerium*, *Staphylococcus*, and *Streptococcus*, all of which use the NEAT domain, as has been studied experimentally for members of each of these classes (15). However, almost none of these Firmicutes have sequences homologous to HtaACR. Thus, there seems to be a divide in the strategies used for the main pathway of heme uptake in clinically relevant examples of these two phyla of bacteria, with Firmicutes using HtaA domains and Actinomycetes using NEAT domains. It should be pointed out that the few sequences of NEAT domains in Firmicutes or HtaACR in Actinobacteria found via a BLAST search may not produce viable protein, as detailed for some HtaACR homologues in Corynebacterium (74).

Comparison of the known HtaA- and NEAT-based heme uptake strategies shows that many of the proteins involved in the first step have two copies of the conserved domain. This design may serve in more than one context. In some instances, one of the domains serves to bind the protein donor, while the second receives the heme. This has perhaps been studied in the most detail for the IsdH system of *Staphylococcus aureus*. The second NEAT domain of IsdH (IsdH\textsuperscript{N2}) (15, 38, 107) binds both Hb and the Hb-Hp complex (38, 42), but not heme itself (107). The third (IsdH\textsuperscript{N3}) domain binds heme (K\textsubscript{d}, 0.32 µM) (43) but not Hb (107). A crystal structure of intact Hb with four IsdH\textsuperscript{N2N3} shows the IsdH\textsuperscript{N2} domains each binding one of the Hb chains, while the IsdH\textsuperscript{N3} are each positioned near the corresponding heme (10). Thus, extraction of heme from Hb via IsdH seems to require two NEAT domains, one to bind the Hb and the second to receive the heme. A similar strategy may be occurring in *C. diphtheriae*; HtaB, which can receive heme from HtaA, cannot itself bind Hb.
Two domains may also serve to take up and store heme, as has been proposed for Shr from *S. pyogenes* (108). For this system the kinetics of heme uptake have been interpreted in terms of heme transfer to Shr$^N_1$, with Shr$^N_2$ serving as a heme storage domain, which can transfer heme back to Shr$^N_1$ for movement along the pathway when the exogenous heme level drops.

In *C. diphtheriae*, HtaA can use heme from both hemoglobin and myoglobin (1, 11). However, heme in the Hb-Hp complex can be extracted only when both HtaA and either ChtA or ChtC are available (11). This is a variation of the ‘two domains in a single protein’ motif and indicates that HtaA and ChtA/C are probably located near one another in three-dimensional space.

In a few instances, the pathway of heme transfer has been described in detail. In NEAT-based systems, kinetic analysis of transfer from IsdA to IsdC to IsdE in *S. aureus* has been interpreted in terms of a “cog-wheel” mechanism, in which heme must be moved to IsdE before IsdC can be reloaded (55); related work on this system includes studies from (52, 64, 68). In *B. anthracis*, heme is transferred from IsdX1 to IsdC and IsdX2 (69) and BslKN to IsdC (65); in *L. monocytogenes* heme is transferred from Hbp1 to Hbp2 (109). In Group A Streptococcus Shr transfers heme to Shp, with the possibility of an intermediate with axial ligands from both proteins (110, 111).

For the HtaA pathway in *C. diphtheriae*, HtaA-CR2 transfers heme to HtaB. It has also been shown that HtaB and ChtB can substitute for one another (8) and ChtB can transfer heme to HtaB (this work). In addition, ChtA and ChtC can substitute for one another (11, 74). The HtaA heme uptake “pathway” is therefore perhaps better envisioned as a series of alternative crossing paths for heme entry, with the usage of the various options being determined by not only the
heme source and specific protein-protein interactions, but also the numbers of various protein species in the membrane as well as their approximation to one another in space.

2.5 Conclusions

Herein, we have investigated HtaB and ChtB from the HtaA heme uptake pathway in *C. diphtheriae*. For both of these domains, sequence alignment, UV-visible spectroscopy of WT and mutants, and the Raman spectra of the WT protein indicate that tyrosine is the heme axial ligand. A conserved histidine is also in the heme pocket, as indicated by the observation of two species for the Fe(II)CO species, one with a *trans* axial histidine and the second with *trans* oxygen-bound ligand. HtaACR domains are significantly stable to unfolding in solution (induced by either denaturants or temperature), consistent with the hypothesis that protein-protein interactions are needed to transfer the heme. The gas phase and solution stabilities are not necessarily related, as observed in other heme protein stability studies.

Bioinformatics analyses of currently available species indicate that many species of *Actinobacteria*, in addition to *Corynebacteria*, use HtaA domains. The conserved (CR) domains of HtaA-type proteins are increasingly understood as a fundamental heme transfer motif. The apparently interchangeability of HtaB and ChtB in transferring heme from HtaA/ChtA/ChtC to HmuT may indicate that heme uptake does not follow a simple linear pathway, but rather proceeds along a network of pathways, taking advantage of multiple protein-protein interactions, as well as protein organization in space, to maximize internalization of this important iron source.
Table 2.1 HtaB site-directed mutagenesis primers. The mutation sites are shown bold.

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Figure 2.1 Schematic of hmu and cht heme uptake proteins of *C. diphtheriae*. ChtA/C depicts each ChtA or ChtC which exhibit significantly high amino acid identity (11).
Figure 2.2 I-TASSER homology model (2) of A: ChtB and B: ChtA using the crystal structure of HtaB from C. glutamicum (4) as a template and displayed using UCSF Chimera (13). Shown are location of ChtB; S52, Y56 (axial ligand), H121, M220, and Y223: ChtA; S125, Y129 (axial ligand), Y178, F271, H279, and Y272.
Figure 2.3 UV-visible spectra of ChtA (orange solid line), HtaA-CR2 (dashed red line), ChtB (blue dashed-dotted line), and HtaB (green dotted line). Spectra were taken in 100 mM Tris-HCl, 150 mM NaCl, pH 8.0 and are normalized to 1.0 at the Soret.
Figure 2.4 The UV-visible absorption of as-isolated ferric HtaB (solid line), Y56A (dashed line), and H121A (dotted line). Spectra were taken in 100 mM Tris-HCl, 150 mM NaCl, pH 8.0 and are normalized to 1.0 at the Soret.
Figure 2.5 Ferric high frequency rR of HtaB as a function of pH. Glycine (pH 9.6, black), Tris-HCl (pH 8.8, red and 8.0, blue), and phosphate (pH 5.8, green) buffers at 100 mM were used. Resonance Raman scattering was excited with 413.1 nm emission from a Kr$^+$ laser using 10 mW of power. The assignments are made by analogy with other heme proteins and on the basis of depolarization ratios evident from Figure 2.27.
Figure 2.6 Ferric high frequency $rR$ of ChtB as a function of pH. Tris-HCl (pH 8.8, blue and 8.0, red) and phosphate (pH 5.8, green) buffers at 100 mM were used. The excitation wavelength from 413.1 nm from Kr ion laser with 11.4 mW of power. The assignments were made based on polarization data in Figure 2.28 and by analogy with other heme proteins. The $\nu_{\text{C} = \text{C}}$ band shifts under the $\nu_{\text{10}}$ band as the pH is decreased indicating a change in the interactions between the vinyl groups and the heme pocket.
Figure 2.7 Low-frequency spectra of ferrous HtaB excited with 413.1 nm (10 mW, black, bottom, Kr+) and 441.6 nm (1.6 mW, red, top, HeCd). Both spectra have been normalized to the ν7 band which lies outside the shown window. The resonance enhancement pattern with 441.6-nm excitation supports assignment of the 217-cm\(^{-1}\) band to the ν\(_{\text{Fe−His}}\) mode, leading to the conclusion that part of the heme is bound to the protein through a Fe−His bond (97).
Figure 2.8 Backbonding correlation plot, relating $v_{\text{Fe-CO}}$ and $v_{\text{C-O}}$ frequencies for groups of proteins having the same trans (proximal) ligand. The two forms of HtaB-CO are shown as filled red stars, while the two forms of ChtB-CO are filled green stars. HmuT and its mutants, unfilled blue stars (63, 77); catalase, blue hexagon (112); and SmHasA(WT), SmHasA(H83A), SmHasA(H32A), magenta ○ (93). The data for HRP, ▲ and globins, ■ have been compiled previously (93). The dotted line is the least squares fit for six-coordinate heme carbonyls in which the proximal ligand is neutral imidazole from a His residue (93, 102, 103) (and references therein). The solid blue line represents a compilation of heme proteins in which the ligand trans to CO is coordinated through an O atom that is hydrogen bonded to an Arg (two hydrogen bonds) (77, 112). The solid magenta line is the least squares fit for six-coordinate Fe-CO adducts which are coordinated through an O atom having a single hydrogen bond to His or Tyr (93).
Figure 2.9 Absorbance versus wavelength of a solution of heme-loaded HtaB before and after incubation with heme-loaded ChtB. B: Absorbance versus wavelength of a solution of heme-loaded ChtB before and after incubation with as-isolated HtaB. The protein solutions were prepared in 100 mM Tris-HCl, 150 mM NaCl pH 8.0.
Table 2.2 Comparison of rR core-size marker bands (cm⁻¹) of select ferric hemin proteins.

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2.6 Supplementary information

![Supplementary information from C. glutamicum](image)

Figure 2.10 Clustal Omega alignment of CR domains for HtaA-CR1, HtaA-CR2, HtaB, ChtB, ChtA, and ChtC from C. diphtheriae; HtaA-CR1, HtaA-CR2, and HtaB from C. glutamicum (4).
Figure 2.11 UV-visible spectra of as-isolated HtaB (dotted line), titrated with hemin (solid line), and reconstituted on a Strep-tag column with hemin from metHb (dashed line). Spectra were taken in 100 mM Tris-HCl, 150 mM NaCl, pH 8.0 and are normalized to 1.0 at the Soret.
Figure 2.12 UV-visible spectra of as-isolated HtaB H121A (dashed line) and titrated with hemin (solid line). Spectra were taken in 100 mM Tris-HCl, 150 mM NaCl, pH 8.0 and are normalized to 1.0 at the Soret.
Figure 2.13 UV-visible spectra of as-isolated HtaB Y56A (dotted line), titrated with hemin (solid line), and reconstituted on a Strep-tag column with hemin from metHb (dashed line). Spectra were taken in 100 mM Tris-HCl, 150 mM NaCl, pH 8.0 and are normalized to 1.0 at the Soret.
Figure 2.14 UV-visible spectra of as-isolated ChtB (dashed line) and reconstituted on a Strep-tag column with hemin from metHb (solid line). Spectra were taken in 100 mM Tris-HCl, 150 mM NaCl, pH 8.0 and are normalized to 1.0 at the Soret.
Figure 2.15 UV-visible spectra of heme-loaded ChtA before and passing through a Sephadex G25 superfine column. Dotted: before treatment. Dashed/dotted line: One pass. Dashed line: Two passes. Solid line: Three passes. Spectra were taken in 100 mM Tris-HCl, 150 mM NaCl, pH 8.0 and are normalized to 1.0 at the Soret.
Figure 2.16 Low frequency rR spectra of WT ChTB as a function of pH. Tris-HCl (pH 8.8, blue and 8.0, red) and phosphate (pH 5.8, green) buffers were used at 100 mM. Raman scattering was excited with 413.1-nm emission from a Kr\(^+\) laser, 11.4 mW. The band assignments were made based on depolarization ratios (data not shown) and by analogy to other heme proteins. Changes in relative intensities of bands attributable to propionate and vinyl bending suggest that the heme-protein interactions near the heme edge are sensitive to pH. Changes in relative intensities and frequencies of bands near 740 and 760 cm\(^{-1}\) are suggestive of a pH-dependent change in deviation of the porphin core from planarity.
Figure 2.17 Ferrous high frequency rR spectra of pH 8.8 HtaB as excited with 413.1 nm Kr⁺ laser with 4.8 mW power. Parallel polarized (black) and perpendicular polarized (red) spectra allow for the identification of totally symmetric and non-totally symmetric core size marker bands. Inset shows the UV-visible spectrum of ferrous HtaB.
Figure 2.18 High-frequency rR spectra of ferrous ChtB at pH 8.8, excited with 413.1-nm emission from a Kr⁺ laser with power of 10.2 mW. Parallel polarized (black) and perpendicular polarized (red) spectra allow for the identification of the totally symmetric and non-totally symmetric core size marker bands. Inset shows the UV-visible spectrum of ferrous ChtB.
Figure 2.19 Resonance Raman spectra of the heme carbonyls of HtaB-WT. Raman scattering was excited using 413.1-nm emission from a Kr⁺ laser; 5.7 mW. Natural-abundance HtaB−CO (top), HtaB−¹³CO (middle) and ¹²CO−¹³CO difference (blue, bottom) spectra are shown in the $\nu_{\text{Fe-CO}}$, $\delta_{\text{FeCO}}$ and $\nu_{\text{C-O}}$ regions. Bands in the low-frequency region were fit using Gaussian peak functions (gray). These bands were used to calculate the red difference spectrum superimposed on the blue points in the bottom left spectrum. The bands sensitive to $^{13}$CO substitution are labeled with their respective mode designations. Spectra were recorded at pH 8.8. Inset: UV-visible absorbance spectrum of HtaB.
Figure 2.20 Resonance Raman spectra of the ferrous carbonyls of WT ChtB with conditions and interpretation as described in the legend of Figure 2.19.
Figure 2.21 Electrospray ionization mass spectrometry detection of heme-bound HtaB-WT (solid line), HtaB H121A (dashed line), and HtaB Y56A (dotted line) as a function of collision energy voltage. The protein solutions were prepared in 20 mM ammonium acetate, pH 6.8.
Figure 2.22 UV-visible absorbance as a function of time for ChtA (orange-dashed line); reaction was carried out in the presence of 4.0 M GdnHCl at 25 °C. HtaA-CR2 (red-solid line); reaction was carried out in the presence of 6.8 M GdnHCl at 37 °C. A 100 mM Tris-HCl, 150 mM NaCl, pH 8.0 buffer A was used for both experiments.
Figure 2.23 UV-visible absorbance as a function of time for HtaB (green-dashed line) and ChtB (blue-solid line); reactions were carried out in the presence of 4.0 M GdnHCl at 25 °C in 100 mM Tris-HCl, 150 mM NaCl, pH 8.0.
Figure 2.24 UV-visible absorbance as a function of temperature for ChtA. The unfolding reaction was carried out in the presence of 1.5 M GdnHCl in 100 mM Tris-HCl, 150 mM NaCl, pH 8.
Figure 2.25 Fraction folded as a function of temperature for the of HtaB (green-dashed line) and ChtB (blue-solid line). Experiments were performed in 1 M GdnHCl in 50 mM NaH$_2$PO$_4$, pH 7.0.
Figure 2.26 Fraction of folded protein as a function of temperature for HtaB (solid line), H121A (dashed line), and Y56A (dotted line) mutants. The unfolding reaction was carried out in the presence of 1.0 M GdnHCl in 50 mM NaH$_2$PO$_4$, pH 7.0.
Figure 2.27 High frequency rR spectrum of ferric HtαB in 100 mM Tris pH 8.0 with 413.1-nm excitation from Kr\(^+\) laser with 10 mW of power. Parallel (black) and perpendicular (red) polarized Raman scattering are shown to identify the totally symmetric and non-totally symmetric core size marker bands (7).
Figure 2.28 Polarized high-frequency rR spectra of WT ferric ChtB. Conditions as described for Figure 2.27, except the laser power was 11.4 mW.
3 CHARACTERIZATION OF C. DIPHTHERIAE CHTA AND PRELIMINARY EXPERIMENTS

This chapter is additional work on C. diphtheriae ChTA, not reported in Chapter 2. Expression, purification, UV-visible absorption spectroscopy, and thermal unfolding were performed at Georgia State University.

3.1 General

UV-visible spectra were taken on a CaryBio UV-Visible spectrophotometer. Disposable polystyrene cuvettes were used for Bradford assay studies and optical density measurements. Water from a Barnstead water purifier had 18.2 mΩ resistance. Laemmli buffer was commercially available. Protein samples containing SDS sample buffer were vortexed using a VWR Mini Centrifuge C-1200. Mini-PROTEAN TGX Precast Gels from Bio-Rad were used to run sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE). Lysogeny Broth (LB) was prepared by using of 1 g of tryptone, 1 g of NaCl, and 0.5 g of yeast extract in 100 mL of water and autoclaved for 45 min. After the LB media had reached room temperature, kanamycin was added to a final concentration of 50 μg/mL. Terrific Broth (TB) was prepared using 2.4 g of yeast extract, 1.2 g of tryptone, 0.23 g of K$_2$HPO$_4$, 1.64 g of KH$_2$PO$_4$, and 0.4 mL of glycerol in 100 mL of water and autoclaved for 45 min. After the TB media had reached room temperature, kanamycin was added to a final concentration of 50 μg/mL. UV-visible spectra were taken on a CaryBio UV-visible spectrophotometer. Buffer A was 100 mM Tris-Cl and 150 mM NaCl at pH 8.0. Buffer B was 100 mM Tris-Cl, 150 mM NaCl, and 2.5 mM $d$-desthiobiotin and 1.0 mM of EDTA at pH 8.0. Buffer C was a mixture of 70% Buffer A and 30% Buffer B. ChTA concentrations were calculated from $\varepsilon_{280}$ from ExPASY (25,485 M$^{-1}$ cm$^{-1}$). Hemin (Sigma-
Aldrich) concentrations in dimethyl sulfoxide (DMSO) were calculated using $\varepsilon_{624} = 6.23 \text{ mM}^{-1} \text{ cm}^{-1}$ (64). The Sephadex G25 medium and superfine grades were from GE Healthcare.

3.2 Materials and methods

3.2.1 Optimal growth and expression condition for ChtA

3.2.1.1 Comparison of TB and LB

An Erlenmeyer flask containing the *E. coli* strain BL21(DE3) carrying the *chtA* gene on the [pET24a(+)] expression vector (a gift from Dr. Michael Schmitt) (8) was grown in 100 mL Terrific Broth (TB) containing kanamycin (50 µg mL$^{-1}$) was prepared with and without 0.3% glucose. A 1 mL sample was preserved at room temperature. The remaining inoculum was shaken at 20 °C/220 rpm for 20 h. A second sample of the inoculum was taken. The same experiment was repeated with LB.

3.2.1.2 Post-induction temperatures and isopropyl-β-D-thiogalactopyranoside concentration

The cells were grown in 100 mL Terrific Broth (TB) containing kanamycin (50 µg mL$^{-1}$) at 37 °C/220 rpm for 20 h. The culture was inoculated (100 µL each) into four separate Falcon tubes each containing 10 mL TB with kanamycin and incubated under the same conditions until an OD$_{600}$ of 0.6 was reached. Two equal volumes (3 mL) from each culture were transferred to eight additional Falcon tubes. Four of these tubes were induced by adding volumes of 15 and 30 µL of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 and 1.0 mM, respectively. The other four tubes were not induced. The inocula were allowed to express in pairs, each with a pre- and post-induction culture for 3.5 h at 37 °C/220 rpm. The optical density of the pre- and post-expression was used to determine the colony counts per volume of the liquid culture:

$$OD_{600} \times x = 1.0$$
where $OD_{600}$ is the optical density of the expressed culture measured at a wavelength of 600 nm and $x$ is the cell volume. Table 3.1 gives an example of these calculations for $OD_{600}$ values of 1.19, 1.21, 1.29, 1.31, 1.46, and 1.44. The calculated volumes were harvested by centrifugation at 7000 rpm/4 °C for 40 min. The pellets were collected and stored in a 20 °C freezer for 20 h. The cell pellets were re-suspended in 75 µL of 1:19 of SDS sample buffer [(2-mercaptoethanol (50 µL): Laemmli (950 µL)] and boiled at 90 °C for 10 min.

3.2.1.3 Optimal Density at $OD_{600}$ and isopropyl-β-D-thiogalactopyranoside concentration

*E. coli* strain BL21(DE3) carrying chtA gene on the [pET24a(+)] expression vector was grown in 200 mL Terrific Broth (TB) containing kanamycin (50 µg mL$^{-1}$) at 37 °C/220 rpm for 20 h. The culture (150 µL) was then inoculated into three separate Falcon tubes each containing 15 mL TB with kanamycin and incubated under the same conditions until the $OD_{600}$ reached 0.57, 0.86, and 1.15. Two equal volumes (3 mL) from each culture were transferred to new six Falcon tubes and induced by adding volumes of 9 and 15 µL of IPTG to a final concentration of 0.3 and 0.5 mM. The induced cultures were allowed to express for additional 4 h at 37 °C/220 rpm. The optical density of the post-expressed culture was used to determine the colony counts per volume of the liquid culture as described above and harvested by centrifugation at 7000 rpm/4 °C for 40 min. The pellets were collected and stored in a 20 °C freezer for 20 h.

3.2.1.4 SDS PAGE

SDS sample buffer was prepared by adding 50 µL of 2-mercaptoethanol into 950 µL of Laemmli buffer. The cell pellets were re-suspended in 75 µL of 1:19 SDS sample buffer, vortexed and boiled at 90 °C for 10 min. The hot protein samples (4 µL), the protein ladder (4 µL) and myoglobin (10 µL) were loaded into the wells using a pipette. The samples were run through the stacking gel at 70 V for 15 min. The voltage was increased to 118 V and ran for an
additional 45 min. The gel was washed with water and stained in Coomassie Brilliant Blue R-250 for 1 h on an orbital shaker. The staining solution was recycled. To remove the stain, the gel was incubated in 20% methanol and 10% acetic acid (destaining solution) for 16 h on an orbital shaker. The destaining solution was recycled by filtering through an activated carbon filter. The gel was washed with water twice and left to dry.

3.2.2 **Titration of ChtA with hemin in DMSO**

ChtA as-isolated (22.6 µM, essentially entirely in the apo form) was used for a two-cuvette hemin titration in buffer A. Aliquots of a solution of 10.1 mM hemin in DMSO were added sequentially to one cuvette containing the protein and a second cuvette, which served as a blank, with buffer A only. The sample was allowed to stand for 30 min after each addition. The volume of DMSO in solution did not exceed 0.2%. UV-visible spectra were recorded after each hemin addition up to a hemin:protein ratio of 0.90. At the end of the titration, the sample was passed through a Sephadex G25 medium column.

3.2.3 **Reconstitution of ChtA with hemin in DMSO**

Hemin in DMSO (1.89 mM, 2.30 µL) was added to a solution of ChtA (1400 µL, 34.6 µM) in buffer A. The hemin:protein ratio was 0.90. The solution was allowed to incubate for 30 min. Spectra were taken with baselines of buffer only and buffer with an equivalent amount of hemin. The sample was passed down a Sephadex G25 superfine column twice. The experiment was repeated with a sample of ChtA (28.1 µM) that was initially more heme loaded (Soret:280 nm ratio of 0.29). Addition of one equivalent of hemin in DMSO gave a sample that was passed down a Sephadex G25 superfine column three times.
3.2.4 *ChtA hemin in potassium hydroxide sequential reconstitution*

ChtA as-isolated (12.2 μM, essentially entirely in the apo form) was used for a two-cuvette heme titration in buffer A. A 115 μM solution of hemin in 100 mM KOH was diluted 1:50 with 100 mM NaH$_2$PO$_4$, pH 7.5. Two 20 μL aliquots were added sequentially to both the cuvette containing the protein and a cuvette with buffer A only. The samples were allowed to stand for 30 min after each addition. UV-visible spectra were recorded after each hemin addition (hemin:protein ratios of 0.21 and 0.42). At the end of the titration, the sample was passed through a Sephadex G25 superfine column.

3.2.5 *Concentration measurement: Bradford assay for ChtA*

A stock solution of bovine serum albumin (BSA) (6.2 μM, $\varepsilon_{280} = 43824$ M$^{-1}$ cm$^{-1}$, Fischer Scientific) was dissolved in H$_2$O and used as a standard for the Bradford assay. The standard solutions were prepared by adding aliquots of 2, 4, 6, and 8 μL of BSA into disposable cuvettes with 798, 796, 794, and 792 μL of buffer A, respectively. Coomassie Blue aliquots (200 μL) of the Bio-Rad were added at 5 min intervals into cuvettes and allowed to sit for 10 min at room temp before the UV-visible spectra were recorded. A similar procedure was used for ChtA (18.2 μM, 4 μL). The absorbance at 595 nm was used with the calibration curve from the Bradford assay to determine the stock concentration of ChtA.

3.2.6 *Extinction coefficient measurement: Pyridine hemochrome assay of ChtA*

The ChtA solution (18.2 μM, 500 μL) used for Bradford Assay was transferred into a capped black quartz cuvette with an equal volume of pyridine solution (4.0 mL pyridine, 6 mL of 400 mM NaOH). A saturated sodium dithionate solution was prepared in water in a capped glass vial; 10 μL of this solution was added to the cuvette containing ChtA and the pyridine hemochrome solution using a glass syringe. The solution mixture was quickly mixed using a
1000-µL glass syringe and UV-visible spectrum was immediately recorded with a baseline of pyridine solution only.

The extinction coefficient for ChtA at Soret was calculated by using Beer’s law from the absorbance value at Soret and molar concentration of heme. The holo-ChtA concentration was determined using this extinction coefficient. The heme loading was estimated by comparing the holo-ChtA with the total (holo-ChtA and apo-ChtA) protein concentration that was determined using Bradford assay described above.

3.2.7 Investigation of protein secondary structure: Circular dichroism

Circular dichroism (CD) spectra of BSA, Mb, and ChtA were recorded using Jasco J-1500 CD spectropolarimeter and a quartz cuvette having a 1.0 cm path length. A 1.5 and 5.0 µM samples of myoglobin and ChtA, respectively, were recorded in each of 10 mM KH₂PO₄ and buffer A at pH 7.0. Except for BSA, four (0.75, 1.5, 3.0, and 5 µM) different concentrations were used in 10 mM KH₂PO₄. The final spectra represent an average of 20 scans.

3.3 Results and discussion

3.3.1 Optimal growth and expression condition for ChtA

The goal of this work was to determine optimal growth and expression conditions for ChtA. There are many factors that can influence protein yields and quality during expression, but this study focused on the comparison of growth medium, carbon sources, temperature, inducer concentrations, and induction conditions. These parameters were tested by involving varying levels of each parameter over a certain range, while holding the other test variables constant.

Our work involved a ChtA construct with an N-terminal Strep-tag® (Figure 3.1)(8). The first step was selecting a medium that would promote the growth of cells. The cell population levels were compared between Lysogeny Broth (LB) and Terrific Broth (TB) with and without
0.3% glucose. There was no growth observed in LB with or without 0.3% glucose after 20 h (Figure 3.2), as determined by the lack absorbance of at 600 nm and no cells settled at the bottom of the cuvette. A higher cell population was observed for TB (Figure 3.2). The OD_{600} for the samples with and without glucose with both approximately 2.8, indicating that glucose is not necessary with TB.

In the second set of experiments, the relative levels of expression were analyzed using the two sets of temperatures (20 and 37 °C) and final IPTG concentrations of 0.5 and 1.0 mM. The temperature seemed to make little difference as seen in the overall intensity of the ChtA bands (Figure 3.3). However, 37 °C conditions seemed to give purer protein and this temperature was used going forward. The two final IPTG concentrations (0.5 and 1.0 mM) gave similar expressions. However, for this study holo-ChtA is preferred. To allow for the possibility of heme biosynthesis to “catch-up” with ChtA synthesis, a final IPTG concentration of 0.5 mM was chosen.

The third set of experiments were run to choose the best induction point in terms of cell growth. The cultures were induced at OD_{600} values of 0.57, 0.86, and 1.15 with final IPTG concentrations of 0.3 mM and 0.5 mM for each. ChtA expression levels for final IPTG concentrations of 0.3 and 0.5 mM were similar (Figure 3.4). Samples induced at OD_{600} values of 0.57 and 0.86 (very similar results) expressed more ChtA than that at 1.15.

The final growth conditions chosen for ChtA were the temperature of 37 °C, an induction OD_{600} between 0.6 and 0.9 and a final IPTG concentration of 0.3 mM.
3.3.2 Homology modeling

Homology models of HtaB, ChtB, ChtA, and HtaA-CR2 from the I-TASSER prediction program were based on the recent structures from *C. glutamicum* (4). The important residues around the heme for the ChtA and ChtB models are described in the manuscript.

The HtaA-CR2 key residues are positioned similarly to those in the ChtA model (figure in Chapter 2 manuscript). In the HtaA-CR2 model, these residues include the axial ligand Y129 and a hydrogen-bond partner to H178 (Figure 3.5). S125 and Y272 both form hydrogen bonds with one of the heme propionates. Phenylalanine residue F271 π-stacks with one of the pyrrole units of the heme. H179 presumably assists in heme transfer.

The HtaB key residues are positioned similarly to those in the ChtB model (Figure in Chapter 2 manuscript). The HtaB model has tyrosine Y56 as the axial ligand; this residue also forms a hydrogen bond with H121 (Figure 3.5). S52 and Y223 both form a hydrogen bond with one of the heme propionates. The canonical π-stacking phenylalanine residue (F271) present in ChtA and HtaA-CR2 is not present in the HtaB model.

It is interesting to note that the two short α-helices located on opposite sides of the heme hold key residues, as is also seen in NEAT domains (31-33). In both HtaA and NEAT SXXXXY motifs, the serine forms a hydrogen bond with one of the heme propionates. The tyrosine, four residues away from serine, π-stacks with one of the pyrrole units of the heme in NEAT domains (45), whereas in the HtaA domains, this ligand is the axial ligand.

3.3.3 Hemin addition to ChtA

Reconstitution. As isolated, ChtA has very little hemin bound (Figure 3.6). Hemin in DMSO was titrated into a ChtA solution to a hemin:protein ratio of 0.90 (Figure 3.6). The increase in the Soret was a linear function of the amount of added hemin (Figure 3.7). Passage of a reconstituted
sample down a Sephadex G25 superfine column gave a narrower Soret, indicating that some of
the red shoulder of this band was probably due to surface-bound hemin (Figure 3.8). Passage of
this sample through the column a second time did not result in a narrower band (data not shown).
A second reconstitution sample, starting with more hemin in the as-isolated ChtA, was passed
through a Sephadex G25 superfine column three times. The Soret was slightly narrower each
time (Figure in Chapter 2 manuscript). A plot of the spectra after one passage in the first
experiment and after three passages in the second experiment shows that they are essentially
identical, indicating that the FWHM of the Soret of holo ChtA is 67.5 nm (Table 3.2). Because
hemin seems to bind to the surface of the protein, it is helpful not to add more than one
equivalent of hemin to the protein in reconstitution efforts. It should be noted that passage
through a Sephadex G25 medium column removed less hemin than did the Sephadex G25
superfine column (Figure 3.9).

ChtA was also reconstituted with hemin in KOH/phosphate solution (Figure 3.10). This gave
broader Soret than the reconstitution with DMSO (Figure 3.11). Figure 3.12 shows the UV-
visible spectra of hemin and the reconstituted sample before and after treatment with Sephadex
G25 superfine column. Even though Sephadex removes unbound hemin, the Soret is still broader
after reconstitution using a KOH solution compared to a DMSO solution of hemin (Figure 3.11).

The spectra of holo-ChtA are quite similar to other tyrosine-bound heme proteins in heme uptake
pathways (Table 3.2). The overlay (Figure in Chapter 2 manuscript) of ChtA with HtaA-CR2,
HtaB, and ChTB from C. diphtheriae shows that all have similar spectra. All of them have a Soret
at about 405 nm and three bands to the red, the β- and α-bands at about 509 and 546 nm,
respectively, and a charge-transfer band at about 626 nm.
3.3.4 Determination of Soret heme loading and extinction coefficient

A ChtA (0.91 μM, 500 μL) solution, determined via Bradford assay, was used for the pyridine hemochrome assay. The reduced bispyridine ferrous heme complex (upon mixing with sodium dithionate), consists of signature β-peak at 525 nm and α-peak at 556 nm (87) as well as a shift in Soret peak to 418 nm (113). Given the extinction coefficient of 34.53 mM$^{-1}$ cm$^{-1}$ at 556 nm (87) the concentration of pyridine hemochrome was 0.60 μM (which is also hemin concentration in the original ChtA solution). Thus, the Bradford assay, in conjunction with the pyridine hemochrome assay showed that heme reconstituted ChtA was 65% heme loaded.

Given that Soret:280 nm ratio was 1.6 for ChtA with 65% heme loading, a Soret:280 nm ratio of 2.5 was calculated for fully heme-loaded ChtA.

The extinction coefficient of 1.4 x 10$^5$ M$^{-1}$ cm$^{-1}$ for ChtA at the Soret was calculated by using Beer’s law with known absorbance (0.833) value at the Soret and corresponding molar (0.60 μM) concentration.

3.3.5 Experimental aspects of circular dichroism (CD) spectroscopy

These studies aimed to determine the optimal conditions required for obtaining reliable ChtA CD data specific to the instrument.

Sample concentration. In the first set of experiments, BSA was used as the model protein to study the concentration effect on the CD data. To take an accurate CD reading, enough photons must reach the detector. Figure 3.13 shows the CD spectra of BSA solutions at varied concentrations. At BSA concentrations above 3 μM, the spectrum becomes distorted; at 5 μM, a Gaussian shape spectrum shifted to lower energy wavelengths was observed. This effect has been observed in another laboratory (114). The absorbance spectrum of 5 μM BSA has a maximum signal at 207 nm, which is twenty-six times more than the signal at 225 nm (Figure
In the 5 µM BSA CD spectrum, not enough photons reach the detector at wavelengths ranging from 194 and 213 nm. At the higher energy wavelengths range from 214 nm to 240 nm, the absorption signal decreased (Figure 3.13, inset), accompanied by a Gaussian shape CD spectrum shifted to lower energy wavelengths (Figure 3.13). Even further to the red, the absorption of the CD signal decreases because BSA does not absorb at those wavelengths.

To measure reliable CD signals specific to BSA enough photons at wavelengths between 205 and 215 nm must reach the detector (115). The two lower (0.75 and 1.5 µM) concentrations gave CD signals (Figure 3.13) very similar to the literature. Hence the upper limit of BSA concentration necessary for a reliable signal for CD instrument used is ~ 2.0 µM.

**Buffer conditions.** In the second set of experiments, Mb was used as a model protein to study the effect of salt on the CD spectra. Figure 3.14 shows the spectra of Mb in buffers A and 10 mM potassium phosphate. The Mb spectrum in potassium phosphate buffer compares very well with the literature (116). A similar phenomenon to that described above was also observed for the Mb in buffer A, indicating that at a higher salt concentration, not enough photons reach the detector; the spectrum shifts from 200 nm to 208 nm compared to that of Mb in potassium phosphate.

ChtA loses its heme under low salt conditions, as shown by the spectra (Figure 3.15) after dialysis from buffer A into 10 mM ammonium acetate. It was necessary to record the spectra under fairly high salt conditions, e.g., buffer A; this resulted in a slight red shift for the spectrum, as was observed for Mb (Figure 3.14).

The overlay CD spectra (Figure 3.16) of ChtA with HtaA-CR2, HtaB, and ChtB from *C. diphtheriae* are similar. All the proteins are largely β-sheets, as indicated by a single local minimum at approximately 215 nm.
Table 3.1 IPTG concentrations, post-expression $OD_{600}$, and volume of liquid culture.

<table>
<thead>
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<th>IPTG mM</th>
<th>Post-expression $OD_{600}$</th>
<th>Culture volume $\mu l$</th>
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<tr>
<td>0.50</td>
<td>1.19</td>
<td>840</td>
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<tr>
<td>0.30</td>
<td>1.21</td>
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<tr>
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<tr>
<td>0.30</td>
<td>1.44</td>
<td>693</td>
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### Table 3.2 Tyrosine-bound heme proteins in heme uptake pathways.

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<th>Protein</th>
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<th>Soret (nm)</th>
<th>Q Bands (nm)</th>
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<td></td>
<td></td>
<td></td>
<td>β</td>
<td>α</td>
</tr>
<tr>
<td><em>S. aureus</em> IsdA-N</td>
<td>Tyr</td>
<td>66.7</td>
<td>403</td>
<td>497</td>
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<td>405</td>
<td>504</td>
<td>538</td>
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<td>402</td>
<td>503</td>
<td>536</td>
</tr>
<tr>
<td><em>S. aureus</em> IsdH-N3</td>
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<tr>
<td><em>C. diphtheriae</em> HtaA-CR2</td>
<td>Tyr</td>
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<td>406</td>
<td>508</td>
<td>546</td>
</tr>
<tr>
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<tr>
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<td>Tyr</td>
<td>50.0</td>
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<td>402</td>
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>ChtA-CR-29.4 kDa

MASWSHPQFEKGAEHVEDAANDSAIVTTDNDQGSAVSEEATADDVVTGDKN
PKGLAETVEIIEDVSDKAITAGEKAVSLKDATERPDAAADAKSTGEALTGW
SSFNNYSGGPEMLDGKQNGTKRFTFQLESVTDYEATEKLEAKFKGGVHYQK
YCDEASHSDCQLDLKIENPRIVIAGGSHVFAKVSSKKYQSSGTYNDGEDDAR
IAQLYTANATFKEEDCKVTWSEIPALLTKDGAEAMFSNFYPVNGLDSTFS

Figure 3.1 ChtA sequence with a (WSHPOFEK) N-terminal Strep-tag. Conserved tyrosines; Y129 (Y1), Y178 (Y2), and Y272 (Y3) are shown in red (8).
Figure 3.2 B2: Visual comparison of the cell growth with and without 0.3% glucose in LB after 20 h, B2: Visual comparison of cell growth with and without 0.3% glucose after 20 h.
Figure 3.3 Expression of ChtA induced (OD\textsubscript{600}, 0.60) by addition of 0.50 mM (lanes 1 and 5), 1.0 mM (lane 3) IPTG at 37 °C for 3.5 h (lanes 1-4) and 20 °C for 20 h (lanes 5 and 6). Lanes 2, 4, and 6 are without IPTG.
Figure 3.4 Expression of ChtA induced by addition of 0.50 mM (lanes 1, 2, and 5), 0.30 mM (lanes 3, 4, and 6) IPTG at 37 °C for 4 h.
Figure 3.5 I-TASSER homology model (2) of A: HtaA-CR2 and B: HtaB using the crystal structure of HtaB from C. glutamicum (4) as a template and displayed using UCSF Chimera (13). Shown are location of, HtaA-CR2; S125, Y129 (axial ligand), H178, H179, and F271: HtaB; S52, Y56 (axial ligand), H121, F219, M220, and Y223.
Figure 3.6 UV-visible spectra for two-cuvette hemin titration of ChtA (22.6 µM) up to a hemin:protein of 0.90. All spectra were taken in buffer A, pH 8.0.
Figure 3.7 The absorbance at 405 nm plotted as a function of the ratio of the concentration of hemin to ChtA.
Figure 3.8 UV-visible spectra of heme-loaded ChtA before and after treatment with Sephadex G25 superfine column. Dashed line: Two passes after hemin reconstitution of ChtA (34.6 µM) up to a hemin:protein of 0.90. Solid line: Three passes after hemin reconstitution of ChtA (28.1 µM) that was initially heme loaded (Soret:280 nm ratio of 0.29). Spectra were taken in buffer A, pH 8.0 and are normalized to 1.0 at the Soret.
Figure 3.9 UV-visible spectra of heme-loaded ChtA after treatment with Sephadex G25 column. Dotted line: Pass through G25 medium. Solid line: Pass through G25 superfine. Spectra were taken in buffer A, pH 8.0 and are normalized to 1.0 at the Soret.
Figure 3.10 UV-visible spectra for two-cuvette hemin titration of 12.1 µM ChtA with 2.54 µM and 4.85 µM hemin. Hemin used to perform titration was in 100 mM KOH/NaH$_2$PO$_4$, pH 7.5. Spectra were taken in buffer A, pH 8.0.
Figure 3.11 UV-visible spectra of ChtA after hemin loading in DMSO (solid line) and 100 mM KOH/NaH₂PO₄, pH 7.5 (dashed line). Spectra were taken in buffer A, pH 8.0 and are normalized to 1.0 at the Soret.
Figure 3.12 UV-visible spectra of hemin (dotted line). Heme-loaded ChtA before (dashed line) and after (solid line) one pass through Sephadex G25 superfine column. The spectra of hemin and protein were taken in 100 mM KOH/NaH₂PO₄, pH 7.5 and buffer A (pH 8.0), respectively and are normalized to 1.0 at the Soret.
Figure 3.13 UV-visible spectra of ChtA before (solid line) dialysis taken in buffer A, pH 8.0 and after (dashed line) dialysis taken in 10 mM ammonium acetate, pH 7.0. Spectra are normalized to 1.0 at 280 nm.
Figure 3.14 Spectra of BSA with increasing protein concentration. Spectra taken in 10 mM KH$_2$PO$_4$, pH 7.0. The inset shows the UV-visible absorbance spectrum.
Figure 3.15 CD spectra of Mb taken in 10 mM potassium phosphate buffer (solid line) and buffer A (dashed line).
Figure 3.16 CD spectra of HtaA-CR2 (dotted line), ChtB (dashed line) and HtaB (dashed-dotted line) spectra taken in 10 mM potassium buffer, pH 7.0. HtaA-CR2 spectra taken in 50 mM tris buffer, pH 7.0. ChtA (solid line) spectra was taken in buffer A, pH 7.0.
**4 APPROACHES TO REACTION OF *DECHLOROMONAS AROMATICA* CHLORITE DISMUTASE WITH NITRITE**

*Dechloromonas aromatica* is a β-proteobacterium that uses the per(chlorate) reduction pathway to catalyze the formation of dioxygen, deriving energy from this process (117). ClO$_4^-$ is reduced to chlorate (ClO$_3^-$), and ClO$_3^-$ to ClO$_2^-$ via perchlorate reductase (PerR). Chloride dismutases (Clds) then detoxify chlorite (ClO$_2^-$) to chloride (Cl$^-$) and dioxygen (O$_2$) (118, 119).

Clds have also been identified in some nitrite-oxidizing bacteria, such as in *Nitrobacter winogradskyi* (120). This microorganism gains energy from oxidation of NO$_2^-$ to NO$_3^-$ via nitrite oxidoreductase (Nxr). The presence of the Cld-like protein in the nitrite-oxidizing enzymes suggests a close relationship between per(chlorate) and nitrite reduction pathways (121).

Perchlorate (ClO$_4^-$), chlorate (ClO$_3^-$), chlorite (ClO$_2^-$), and nitrite (NO$_2^-$) are environmental pollutants (85). Increasing concentration levels of these contaminants have been identified in soil, drinking water, and groundwater. Because chlorate sources often have associated nitrite (122), it is important to study the reaction of Clds with NO$_2^-$ as well as ClO$_2^-$.

The goal of this work is to identify the products of the reaction of NO$_2^-$ with *D. aromatica* chlorite dismutase (*DaCld*). Our experiments to begin this research included growth and expression optimization of *DaCld*.

The sections below give a review of the production of ClO$_2^-$, ClO$_3^-$, ClO$_4^-$, and NO$_2^-$ in the environment, followed by the biochemistry of *DaCld*.

**4.1 Presence of oxyanion in the environment and analysis**

#### 4.1.1 Perchlorate

In the United States, perchlorate (ClO$_4^-$) is manufactured for use in rockets, missiles, and fireworks. ClO$_4^-$ also occurs in the environment, mostly in arid areas by the oxidation of volatile
atmospheric chlorine (123). Much of the knowledge on ClO₄⁻ effects on human health came from clinical studies on the use of potassium perchlorate to treat hyperthyroidism in the 1950-60s (124). In the 1950s, gastrointestinal irritation, skin rash, and lymphadenopathy were reported after treatment of hyperthyroidism with KClO₄ at doses of 400 mg per day. In the 1960s, aplastic anemia and agranulocytosis were reported for patients treated with 400 to 1000 mg ClO₄⁻ per day for up to 33 weeks. These treatments were discontinued in the United States in the mid-1960s.

The toxicological impact has become a growing interest due to the persistent presence of ClO₄⁻ in drinking water (125, 126). ClO₄⁻ competes with the sodium and iodide symporter (NIS); when ingested, the compound is transported to the thyroid and interferes with the iodide uptake. Jackson and colleagues have reported that 89% of U.S adults are either directly or indirectly exposed to ClO₄⁻ from tap water (127). The average concentration of ClO₄⁻ reported was 4 µg L⁻¹ in 160 drinking water supplies located in 26 states and two territories in the United States; 420 µg L⁻¹ was found in a single surface water in Puerto Rico (128). As of May 2020, ClO₄⁻ is an unregulated contaminant in the United States (129).

ClO₄⁻ not taken up with NIS is secreted in urine and milk unchanged (130, 131). A higher concentration has been detected in breast milk (10.5 µg L⁻¹) than in dairy milk (2.0 µg L⁻¹) (132).

### 4.1.2 Chlorate and chlorite

Chlorate (ClO₃⁻) and chlorite (ClO₂⁻) are environmental pollutants (133). These disinfection by-products (DBPs) result from the decomposition of chlorine dioxide (ClO₂), commonly used in the purification of water. Chlorite, the predominant species (63%), is regulated in the United States; concentrations of up to 1.0 mg L⁻¹ in drinking water are allowed
ClO$_3^-$ and ClO$_2^-$ are also by-products of commercial bleach. ClO$_2^-$, ClO$_3^-$, and ClO$_2$ are also used in food processing as decolorizing agents and as additives in paper and products used for food packaging; as a result, ClO$_2^-$ has been detected in some foods such as flour (134).

Early studies showed that exposure to an acute dosage of the DBPs was associated with decreases in brain mass, changes in thyroid functions, and induced hematological responses in animals (134). In one instance, ingestion of 28% per 100 mL volume of sodium chlorite resulted in methemoglobinemia, hemolysis, and acute renal failure (135).

### 4.1.3 Nitrite

Nitrite (NO$_2^-$) can be readily obtained by the reduction of nitrate (NO$_3^-$) (136). These compounds are commonly used as food preservatives and fertilizers. Concentrations up to 49 mg L$^{-1}$ of nitrite detected in farm soil has been attributed to nitrogen fertilizers (122). The occurrence of nitrite in drinking water and groundwater is mainly via nitrate leaching from the soil. In the United States, the maximum contaminant level of NO$_2^-$ allowed in drinking water is 1.0 mg L$^{-1}$ (137). At higher concentrations, the contaminant leads to irreversible conversion of hemoglobin to methemoglobin (138). Potential health effects from long-term exposure of NO$_2^-$ on infants below the age of six months include shortness of breath and blue-baby syndrome (137).

The incorporation of NO$_2^-$ into food chains is commonly through meat and fish products (122). A concentration of up to 99 mg L$^{-1}$ has been detected in farmed fish tissues. In meat products, NO$_2^-$ is used to inhibit bacterial growth and maintain fresh-pink-meat color (139, 140). The NO$_2^-$ concentration required to inhibit microbial growth in meat products is approximately 42000 mg L$^{-1}$ (141). As of 2019, the maximum permissible concentration NO$_2^-$ in finished meat products was 200 mg L$^{-1}$ (142).
In general, \( \text{NO}_3^- \) occurs with \( \text{NO}_2^- \). \( \text{NO}_3^- \) is an important plant nutrient, particularly in leafy and some root vegetables (143). Average concentrations of \( \text{NO}_3^- \) in these sources range from 175 to 1220 mg per serving.

Ingested \( \text{NO}_3^- \) is absorbed in the small intestine; 25% is excreted in the mouth, where oral bacteria reduce it to \( \text{NO}_2^- \) (144). The role of dietary \( \text{NO}_2^- \) and \( \text{NO}_3^- \) are of interest, as they are precursors of \( N \)-nitroso compounds (NOCs), which induce pancreatic tumors.

Under physiological conditions, \( \text{NO}_2^- \) and \( \text{NO}_3^- \) can be recycled in the blood to form nitric oxide (NO) (145). Collectively, the reduction regulates blood flow and tissue responses under hypoxic conditions. Lower concentrations (1.2 nM g\(^{-1}\)) of \( \text{NO}_2^- \) assist in protecting vascular tissues against ischemic damage (146) e.g., during reperfusion injury following pulmonary hypertension and organ transplant. The beneficial effect of \( \text{NO}_2^- \) seems to be mediated by the release of NO under hypoxia. Higher concentrations of NO may cause DNA damage and promote carcinogenesis (147).

### 4.1.4 Biochemistry of Dechloromonas aromatica chlorite dismutase (DaCld)

DaCld is a homo-pentameric protein; each subunit has a heme pocket consisting of 11 residues (Figure 4.1) (6). The heme has residues RLTF on one side and YQWWHEH on the other. The X-ray structure complex with \( \text{NO}_2^- \) (PDB 3Q08) reveals that the substrate interacts directly with the heme iron and the distal R183 residue. E220 forms a hydrogen bond with the axial ligand H170 and one of the heme propionates, whereas the Y227 stacks with the heme porphyrin ring.

The role of Arg183 in DaCld has been probed by replacing it with residues known to be important in the other members of the Cld protein family (3, 6). Blanc et al. found that the
catalytic efficiencies of the mutants were not completely lost but decreased in the order wildtype  
> R183K >> R183Q >>> R183A.

### 4.1.5 Proposed mechanism reaction of chlorite decomposition

Proposed Cld mechanism involves two successive intermediates; one is via heterolytic and the other, via homolytic bond cleavage (Figure 4.2).

**The heterolytic bond cleavage:** The first step involves the formation of the Fe(III)-ClO$_2^-$ complex, followed by heterolytic bond cleavage of ClO$_2^-$, generating the ferryl-porphyrin cation radical, compound I [(Por$^{•+}$)Fe(IV)=O] and a hypochlorite (ClO$^-$) (148, 149). The subsequent nucleophilic attack of ClO$^-$ at the ferryl-oxygen of compound I generates a Fe(III)-OCIO$^-$, followed by elimination of O$_2$ and Cl$^-$. 

**The homolytic bond cleavage:** The first step involves the formation the Fe(III)-ClO$_2^-$ complex, followed by a homolytic bond cleavage of ClO$_2^-$, generating the ferryl-porphyrin, compound II [(Por)Fe(IV)=O] and a hypochloryl radical (ClO$^{•}$) (148, 149). Recombination of the radical allows for the release of O$_2$ and Cl$^-$. 

UV-visible spectroscopy studies reveals that reaction intermediates, compound I and compound II, exist as a mixture (119). As indicated by the isosbestic points at 355 nm and 440 nm, in addition to the Soret shift from 395 nm to 415 nm.

This work is in collaboration with Dr. Kenton Rodgers of North Dakota State University who provided the DaCld plasmid.
4.2 Materials and methods

4.2.1 Optimal growth and expression condition for Dechloromonas aromatica chlorite dismutase

4.2.1.1 Comparison of TB and LB and isopropyl-β-D-thiogalactopyranoside concentration

Two 200 mL flasks of Terrific Broth (TB) containing kanamycin (50 µg mL\(^{-1}\)) were prepared, one with and the other without 0.3% glucose. Two volumes of 5 mL were transferred from these solutions into two separate Falcon tubes. Each Falcon tube was inoculated with a different colony containing the gene daCld in a pET41a(+) expression vector [previously transformed into E. coli strain Rosetta(DE3) pLysS] and incubated at 37 °C/180 rpm for 18 h.

Four additional Falcon tubes were prepared, each with 25 mL TB, two with and two without 0.3% glucose and inoculated with the cell culture (1 mL) from the previously incubated for 18 h samples. One set of two tubes (with and without glucose) was inoculated with one (1 mL) of the 18 h samples and the second set of two tubes with the other. The inoculated samples were left to grow at 37 °C/180 rpm until an OD\(_{600}\) of 0.6 was reached for one set of tubes and 0.9 for the other. Three 5 mL aliquots were transferred from each of these four Falcon tubes to three new falcon tubes to give a total of twelve falcon tubes. Of the sets of three, one was not induced, one was induced by adding a volume of 5 µL of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM and the third was induced by adding a volume of 15 µL of IPTG to a final concentration of 0.3 mM.

The sets of three were allowed to express for 3 h at 37 °C/180 rpm. The optical density of each sample was used to estimate the colony counts per volume of the liquid culture:

\[ OD_{600} \times x = 1.0 \]
where $OD_{600}$ is the optical density of the pre- and post-expression culture measured at a wavelength of 600 nm and $x$ is the volume of liquid culture. The calculated volumes (approximately equal cells) were transferred into twelve separate Eppendorf tubes and harvested by centrifugation at 7500 rpm/4 °C for 45 min. The pellets were collected and stored at 20 °C for 20 h.

The same experiment was repeated for LB with and without 0.3% glucose. However, the induced cultures were allowed to express for additional 18 h at 18°C/180 rpm.

4.2.1.2 Pre- and post-induction temperatures

Terrific Broth (TB) containing kanamycin (50 µg mL$^{-1}$) was prepared with 0.3% glucose. From this solution, 10 mL was transferred into a Falcon tube, and inoculated with one colony containing the gene daCld in a pET41a(+) expression vector [previously transformed into E. coli strain Rosetta(DE3) pLysS] and incubated at 37 °C/180 rpm for 18 h.

Two Erlenmeyer flasks were prepared, each with 494 mL TB with 0.3% glucose and inoculated with the cell culture (2 mL) from the 18 h samples. The inoculated samples were left to grow at 37 °C/180 rpm until an $OD_{600}$ of 0.28 was reached for one flask and 0.75 for the other. From each of these flasks, one 4 mL aliquot was transferred into a Falcon tube. These sets of two tubes were not induced. The remaining samples (490 mL) each, were induced by adding volumes of 490 µL IPTG to a final concentration of 0.1 mM. The inocula were allowed to express in pairs, each with a pre- and post-induction culture for 15 h at 20 °C/180 rpm. To determine the volumes necessary for equal amounts of proteins for SDS PAGE, the optical densities of the pre- and post-expression inocula samples were used to as described in the formula above. The calculated volumes were transferred into separate Eppendorf tubes and harvested by centrifugation at 7500 rpm/4 °C for 45 min. The two large post-induction samples were
harvested separately via centrifugation (JA-18 rotor, 8000g) at 4 °C for 45 min. The pellet from each sample was washed by suspending in 20 mM Tris buffer pH 8.5 (2 mL buffer per gram of cells) and centrifuging at 8500 rpm /4 °C for 45 min. The cell pellet from each was collected and stored in a 20 °C freezer.

4.2.1.3 SDS PAGE

SDS sample buffer was prepared by adding 50 µL of 2-mercaptoethanol into 950 µL of Laemmli buffer. The cell pellets were thawed at room temperature in a water bath and re-suspended in 40 µL of 1:19 SDS sample buffer, vortexed and boiled at 90°C for 20 min. The hot protein samples (5 µL), and a protein ladder (4 µL) were loaded into the wells using a pipette. The samples were run through the stacking gel at 70 V for 15 min. The voltage was increased to 118 V and run for an additional 45 min. The gel was washed with water and stained in Coomassie Brilliant Blue R-250 for 30 min on an orbital shaker. The staining solution was recycled. To remove the stain, the gel was incubated in 20% methanol and 10% acetic acid (destaining solution) for 16 h on an orbital shaker. The destaining solution was recycled by filtering through an activated carbon filter. The gel was washed with water twice and left to dry.

4.3 Results and discussion

4.3.1 Optimal growth and expression condition for Dechloromonas aromatica chlorite dismutase

The goal of this work was to determine optimal growth and expression conditions for a (Dechloromonas aromatica chlorite dismutase) DaCld construct (Figure 4.3). There are many factors that can influence protein yields and quality during expression, but this study focused on the comparison of growth medium, carbon sources, temperature, optimal density at induction,
and inducer concentrations. These parameters were tested by involving varying levels of each parameter over a certain range, while holding the other test variables constant.

The first step involved selecting a medium that would promote the growth of cells. The cell population levels were compared with Terrific Broth (TB) and Lysogeny Broth (LB) after three and 18 h post-induction growth, respectively. A higher cell population was observed in TB than in LB as indicated by larger OD$_{600}$ values for TB compared to those of LB. This indicates that a rich growth medium (TB) is preferred.

The second set of experiments looked at the effect of glucose. Glucose seemed to make no difference in the cell population of cultures grown in TB as indicated by similar OD$_{600}$ values of approximately 1.33 at harvest. For LB, a higher cell population was observed with glucose, as indicated by OD$_{600}$ values at harvest of 1.24 and 0.93 for LB with and without glucose, respectively.

Looking at the levels of expression itself via SDS PAGE, cells grown in TB with glucose expressed more DaCld than those grown in TB without glucose (Figure 4.4 and 4.5). No expression was observed for LB without glucose as indicated by no presence of a protein band at ~28.1 kDa (Figure 4.6). DaCld expression was observed for LB with glucose (Figure 4.7) but significantly less compared to TB with and without glucose (Figure 4.4 and 4.5). Overall, these data indicate that a richer medium enhances the expression of DaCld.

The third set of experiments were run to choose the best induction point. The relative levels of expression were analyzed for two sets of OD$_{600}$ (0.6 and 0.9) both for TB with and without glucose, and LB with and without glucose. There were no differences observed in expression levels between these two sets of OD$_{600}$ values for each of the media sets. Hence, any induction OD$_{600}$ between 0.6 and 0.9 was acceptable.
In the fourth set experiments, the relative levels of expression were analyzed using the two sets of final IPTG concentrations of 0.1 and 0.3 mM. The IPTG concentration seemed to make little difference as seen in the overall intensity of the DaCld bands. However, 0.1 mM of IPTG seemed to give purer protein (Figure 4.4). Additionally, for this study holo-DaCld is necessary. To allow for the possibility of heme biosynthesis to “catch-up” with DaCld synthesis, a lower IPTG concentration is preferred. In the end, a final IPTG concentration of 0.1 mM in TB with 0.3% glucose was used.

In the fifth set of experiments, lower temperatures of growth and expression, 30 and 20 °C, respectively, were implemented in an effort to achieve heme-loaded DaCld (117). Heme loading is indicated by pink and red color. The relative levels of expression were analyzed for two sets of OD<sub>600</sub> (0.28 and 0.75) using these lower temperatures. An induction OD<sub>600</sub> of 0.28 resulted to a more colored cell pallet than that of 0.75 (Figure 4.8).

The final growth and expression conditions chosen for *E. coli* strain Rosetta(DE3), growth and expression temperatures of 30 and 20 °C, respectively, an induction OD<sub>600</sub> between 0.25 and 0.30 and a final IPTG concentration of 0.1 mM.
Figure 4.1 Heme pocket of DaCld including a nitrite ligand bound to heme-iron and H-bonding with Arg 183 (6).
Figure 4.2 Proposed Cld mechanism involves two successive intermediates (3).
MQPMQSMKIERGTILTQPGVFGVFTMFKLRPVDWNKVPVAERKGAAEEVKK
LIEKHDENVLDLYLTRGETNSDFFRINAYDLAKAQTFMREFRSTTGV
KNADVFETLGVTKPLNYISDKSPGLNAGLSATYSGPAPRYVIVIPVK
KNAEWWNMSPEERLKEMEVHHTTPLAYLVNVKRKLHYSTGLDDTDFITYF
ETDDLTAFNMLMLSLAQVKENKFHVWRGSPTTLGTHSPEDVIKALAD-
MQPMQSMKIERGTILTQPGVFGVFTMFKLRPVDWNKVPVAERKGAAEEVKK
LIEKHDENVLDLYLTRGETNSDFFRINAYDLAKAQTFMREFRSTTGV
KNADVFETLGVTKPLNYISDKSPGLNAGLSATYSGPAPRYVIVIPVK
KNAEWWNMSPEERLKEMEVHHTTPLAYLVNVKRKLHYSTGLDDTDFITYF
ETDDLTAFNMLMLSLAQVKENKFHVWRGSPTTLGTHSPEDVIKALAD-

Figure 4.3 DaCld chlorite dismutase sequence.
Figure 4.4 Expression of DaCld in TB broth with 0.3% glucose, induced by addition of 0.10 mM (lanes 3, 6, and 7), 0.30 mM (lanes 2, 5, and 8) IPTG at 37 °C for 4 h.
Figure 4.5 Expression of DaCld in TB induced by addition of 0.10 mM (lanes 4 and 5), 0.30 mM (lanes 1 and 6) IPTG at 37 °C for 4 h.
Figure 4.6 Expression of DaCld in LB with 0.3% glucose, induced by addition of 0.10 mM (lanes 2 and 4), 0.30 mM (lanes 1 and 3) IPTG at 18 °C for 15 h.
Figure 4.7 Expression of DaCld in LB without glucose, induced by addition of 0.10 mM (lanes 2 and 4), 0.30 mM (lanes 1 and 3) IPTG at 18 °C for 15 h.
Figure 4.8 Visual comparison of cells containing daCld gene induced at an $OD_{600}$ of 0.28 ($Y_1$) and 0.75 ($Y_2$).
4.4 References


43. Watanabe M, Tanaka Y, Suenaga A, Kuroda M, Yao M, Watanabe N, et al. Structural basis for multimeric heme complexation through a specific protein-heme interaction - The case


