HmuT in the Heme Uptake Pathway of *Corynebacterium diphtheriae:* stability and function

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Abstract

Strategies to use heme as a source of iron are key to the survival and virulence of many bacteria; inhibition of iron uptake pathways may be a new strategy to prevent bacterial infection. *Corynebacterium diphtheriae* is a Gram-positive, pathogenic bacterium that is the causative agent of diphtheria It utilizes proteins in heme uptake pathways to obtain required iron for survival and virulence. One uptake pathway involves an ABC-type transporter encoded by the hmuTUV genes. We analyze the role of HmuT, the protein that donates heme to the ABC transporter. We hypothesize that certain residues in the heme pocket, in addition to the direct heme binding ligands, control heme binding and release in HmuT. Sequence alignment with other heme-binding proteins and I-TASSER homology modeling revealed the following possible essential residues: H136, Y235, Y272, Y349, R237 and M292. Site-directed mutagenesis was used to create alanine mutants for these residues. Mutants studied include H136A, Y235A, Y272A, and M292A. UV-visible spectroscopy was used to compare spectral signatures of the WT to the mutants. In addition, chemical and thermal unfolding experiments were performed to assess the contribution of each residue to heme binding. We have shown that H136 and Y235 are axial ligands to the heme while M292 appears to buttress the axial tyrosine. R237 is a H-bonding partner to Y235. Y272, and Y349 in the heme pocket as well, also affects heme binding. Understanding heme proteins create a possible new strategy to prevent bacterial infection by inhibiting iron uptake pathways.

Introduction

Many living organisms, including pathogenic bacteria, require iron for survival *(1)*. In human, most of the iron is found in heme *(2)*. The tetrapyrrole ring of heme allows usage of the iron as a cofactor for enzymes in activities such as oxygen transport, cellular respiration and signal transduction. Many bacteria have developed pathways to take up heme and degrade the tetrapyrrole ring to release the iron.

Corynebacterium diphtheriae, a Gram-positive pathogenic bacterium that utilizes heme uptake pathways to obtain required iron from it host *(3)*. It is the causative agent of diphtheria, a prevalent upper respiratory tract disease that has a high mortality rate in areas with low vaccine coverage *(4)*. *C. diphtheriae* requires iron for survival and virulence *(5-9)*.

Prior study of *C. diphtheriae* showed that the *hmu* gene cluster encodes a transmembrane protein, the hmuTUV ABC transporter, and surface proteins HtaA and HtaC *(10)*. Other *Corynebacterium* species share the hmuTUV genes sequences. The deletion of the hmuTUV, HtaA or the entire hmu gene cluster resulted in reduced call growth, indicating that the hmu gene

cluster is essential in the uptake and binding of heme. The results indicate that HmuT is essential in the heme-binding pathway of *C. diphtheriae*.

HmuT is a surface-anchored lipoprotein that binds heme and transfers it through the HmuU transporter into the cell*(11)*. Prior studies have identified the HmuT heme binding residues to be Y235 and H136 *(11, 12)*. In this study, we analyze other key conserved residues R237, Y272, M292, and Y349A. Site-directed mutagenesis and thermal unfolding is used to probe the roles of the selected residues in heme binding of *C. diphtheriae*.

Materials and Methods

Expression and purification of *Cd*HmuT: HmuT was expressed and purified from competent *E. coli* cells BL21 (DE3). The N-terminal leader sequence was deleted and replaced with a Strep tag for affinity binding with the resin during purification. The LB media was prepared using 10 g of tryptone, 10 g of NaCl, and 5 g of yeast extract in 1 L of Nanopure water and autoclaved for 20 min. After the LB media had reached room temperature, kanamycin was added to a final concentration of 50 µg/mL. A partial volume (50 mL) of the broth was added to a 125 mL Erlenmeyer flask. The broth was inoculated with the HmuT strain by the sterile flame loop technique and was shaken at 37 ºC / 220 rpm for about 16 h. The content of the Erlenmeyer was transferred to the 1 L of broth and was shaken at 37 ºC /220 rpm until the OD_{600} (optical density) reached 0.5-0.6, protein expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1.0 mM. The cells were induced for 4 h at 27 ºC. The cells were harvested by centrifugation using the Beckmann Coulter Centrifuge with the JLA 8.100 rotor set at 4 ºC / 8000 rpm for 30 min. The cell pellets were resuspended in lysis solution (100 mM Tris-Cl, 150 mM NaCl at pH 8.0) containing 0.1 mM of phenylmethanesulfonylfluoride (PMSF), 10 mM MgCl2, 0.2 mg/mL lysozyme from chicken white, and trace amounts of DNase I recombinant (Roche Diagnostics GmbH) and RNase I (Roche Diagnostics GmbH). The cells were broken using sonication. The lysate was centrifuged at 4 ºC / 6500 RPM; the pellet was discarded, and supernatant was purified.

All of the purification steps were conducted at 4 °C using fast protein liquid chromatography (FPLC, Amersham BioSciences), and all buffer solutions were pH 8.0 unless specified otherwise. About 40 mL of the supernatant sample was loaded onto a Strep-Tactin Superflow column (5 mL, IBA BioTAGnology) equilibrated with buffer A (100 mM Tris-Cl, 150 mM NaCl, pH 8.0). Unbound material was washed out with 5 column volumes (CV) of buffer A. The supernatant was eluted with 10 CV of buffer B containing 100 mM Tris-Cl, 150 mM NaCl, 2.5 mM desthiobiotin (pH 8.0) applied via a linear gradient.

Thermal unfolding of CdHmuT: HmuT and mutants were denatured using a Carey 300 Bio spectrophotometer equipped with temperature control (Quantum Northwest). Supracil 1.5 mL screw-top cuvettes (Spectracell) with 1 cm path lengths were used. Diluted in 50 mM potassium phosphate, pH 7.0, the spectrum of the protein samples were taken at the Soret wavelength(406

nm) in exception of H136A (400 nm) and Y235A (413 nm). The spectrum was taken at every 1 ºC from 25 ºC to 80 ºC with exception of mutant Y235A that was taken every 1 ºC from 25 ºC to 65 ºC with an incubation time of 2 minutes. The data collected from thermal denaturation was fitted in in Kaleidagraph using the equation *(13)*:

$$Y(\mathrm{Abs}) = \frac{A_f + m_f T + (A_u + m_u T)\exp\left[\frac{\Delta H_m}{R}\left(\frac{1}{T_m} - \frac{1}{T}\right)\right]}{1 + \exp\left[\frac{\Delta H_m}{R}\left(\frac{1}{T_m} - \frac{1}{T}\right)\right]}$$

In the equation above, y is the absorbance at any point along the fitted denaturation curve, A_f is the absorbance of the folded state, m_f is the slope of the folded state, A_u is the absorbance of the unfolded state, m_u is the slope of the unfolded state, ΔH_m is the enthalpy of the unfolding, T_m is the temperature at which the protein is half-unfolded, R is the gas constant, and T is the temperature (Kelvin).

Results/Discussion

Sequence alignment and homology modeling: Prior studies have shown that conserved residues H136 and Y235 are the axial ligands of HmuT *(12)*. Using sequence alignment of *C. diphtheriae* HmuT with other HmuT proteins from various Corynebacterium species, our group identified additional conserved residues which could play a role in heme binding: R237, Y272, M292, and Y349 (Fig 1).

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                        R237                          Y272               M292
C_glutamicum            ARGTGGVFFILGDAYGGRDLIEGLGGVDMAAEKGIMDLAPANAEALAELNPDVFVMMSEG  300
C_jeikeium              ARGNGGVFFIMGEGTGAKDLIEGVGAVDVGTENNLSYIEPANAESLARLNPDAFIMMTGG  297
C_diphtheriae           ARGNGGVFFIMGEGTGAKDLIEGVGAKDMGAEYKLSYAEPANAEALAKINPEAIIMMTAG  295
C_ulcerans              ARGNGGVFFIMGEGTGAKDLIEGLSAVDLAAEHKLSYAEPANAEALAKINPEAIIMMSGG  293
C_pseudotuberculosis    ARGNGGVFFIMGDGTGAKDLIEGLSAVDLAAEHKLSYAEPANAEALAKINPEAIIMMSGG  293
                        ***.******:*:.  *.:*****:..  *:.:*  :   *****:**.:**:.::**: *

C_glutamicum            LVSTGGIDGLMERPGIAQTTAGQNQRVLALPDGQSLAFGAQTGELLLRASRELYVQGGE   359
C_jeikeium              LESTGGIEGLLKRPGIAQTTAGQKRRVITIPDGQSLAFGPMTGQTLLRTAKALYDPHG-   355
C_diphtheriae           LESTGGIDGLLARPGVAQTIAGKNRRVITIPDGQSLAFGPMTGQTLLRTAQALYDPQV-   353
C_ulcerans              LESTGGIEGLLSRPGVAQTTAGKNKRVITIPDGQSLAFGPLTGQTLLRTAQALYAPQT-   351
C_pseudotuberculosis    LESTGGIDGLLSRPGVAQTTAGKNKRVITIPDGQSLAFGPLTGQTLLRTAQALYAPQT-   351
                        * *****:**: ***:*** **:::**:::*********  **: ***::: **
                                                                             Y349
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Figure 1. Sequence alignment of the CdHmuT amino acid sequence with various HmuT from Corynebacterium species. Conserved residues R237, Y272, M292, and Y349 were subjected to site-directed mutagenesis

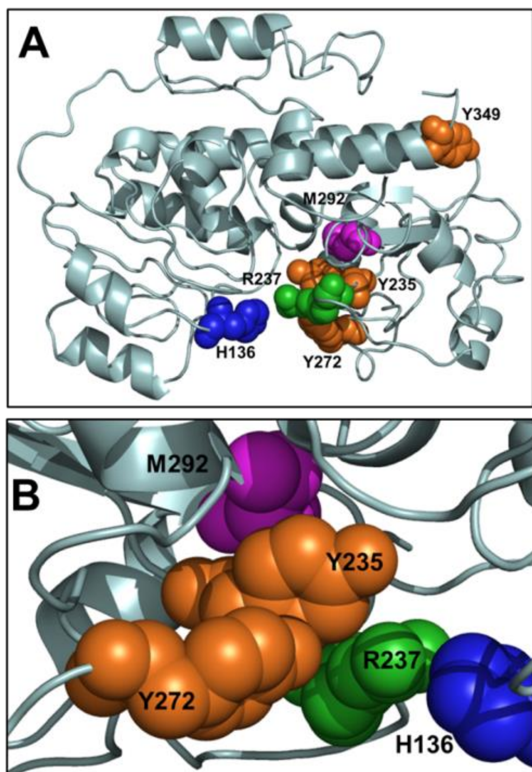

Figure 2. Homology model of HmuT and heme binding pocket. A) I-TASSER homology model of HmuT displayed using PyMOL *(14)*. Shown are the locations of H136 (axial ligand), Y235 (axial ligand), R237, Y272, M292, and Y349. B) Close-up 180° rotation of residues in HmuT heme binding pocket.

Sequence alignment of CdHmuT with other HmuT shows that conserved residues at R237, M292, and Y349 are conserved in all residues. Y272 show consistency in all Corynebacterium species except in *C. glutamicum*. Other conserved residues shown in Figure 1 were not selected because they are known not to bind to the heme iron. Figure 2 is a homology model of HmuT derived from I-TASSER. The model illustrates the different conserved residues and their potential location. All selected residues can be found in the heme pocket with the exception of Y349 (Fig. 2). Additionally, Figure 2b shows a gap between the two axial ligands (H136 and Y235), which is a possible site for the heme in HmuT. Resonance Raman experiments have suggested that R237 is hydrogen bonded to the axial ligand Y235 *(12)*.

Thermal denaturation: To analyze the stability of HmuT in the absence of key residues, thermal unfolding studies were performed on WT HmuT, H136A, Y235A, R237A, Y272A, M292A, Y349A, and Y349F (Figure 3). The stability of the HmuT and mutants was analyzed using T_m, the temperature at which the protein is half folded and half unfolded. Thermal unfolding of HmuT and mutants show a standard two-state process. The WT unfolded with a melting temperature (T_m) of 66.8 ± 0.1 ˚C. Table 1 provides the T_m values for each protein.

HmuT Y235A, R237A, and Y349A show a lower T_m compared to the WT and other mutants in T_m with values at 50 ± 1.0 ˚C, 54.0 ± 0.1 ˚C, and 58.8 ± 0.1 ˚C, respectively.

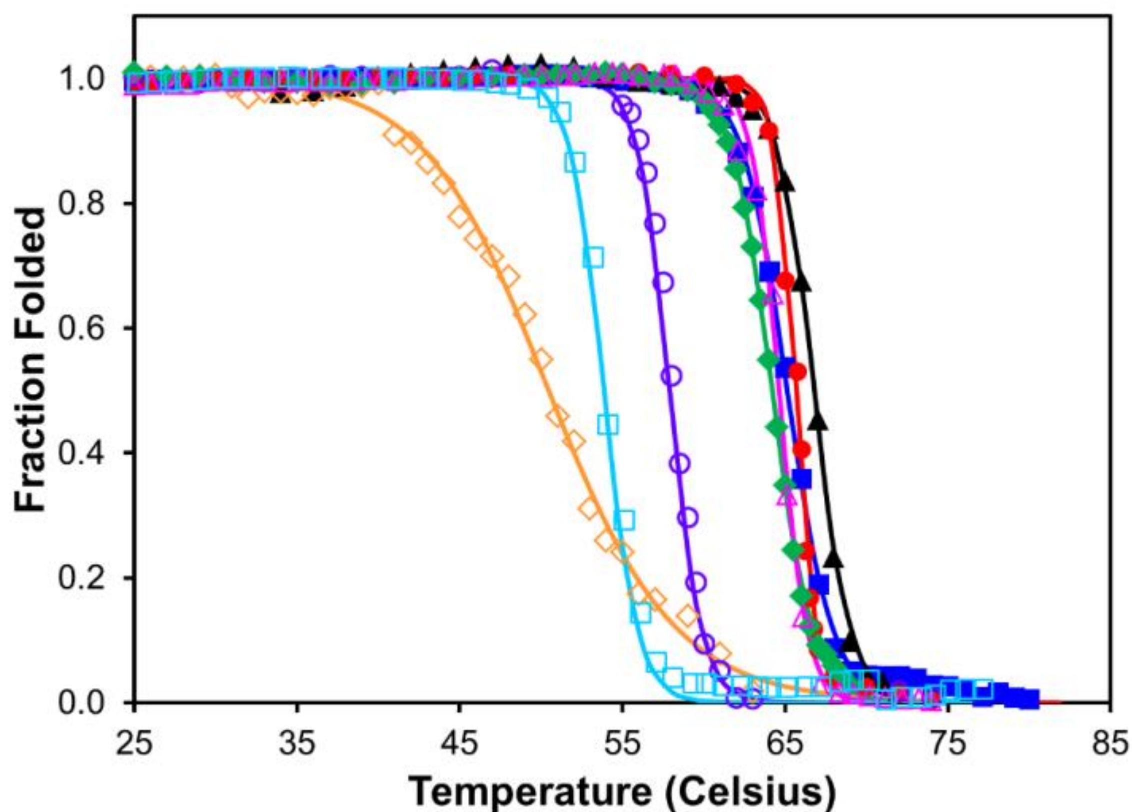

Figure 3. Fraction folded thermal unfolding titrations of WT HmuT (black), H136A (dark blue), Y235A (orange), R237A (cyan), Y272A (red), M292A (pink), Y349A (purple), and Y349F (green). Samples were in 50 mM potassium phosphate, pH 7.0.

Table 1. Thermal unfolding T_m values of WT HmuT and mutants.

Protein	T_m (°C)
WT	66.8 ± 0.1
Y272A	65.6 ± 0.1
H136A	65.1 ± 0.1
M292A	64.6 ± 0.1
Y349F	64.2 ± 0.1
Y349A	58.1 ± 0.1
R237A	54.0 ± 0.1
Y235A	50.5 ± 1.0

The T_m values of H136A, M292A, and Y272A are similar to the T_m of WT indicating that these residues are not crucial for the stability of the protein. Y349 is essential to the stability of

the protein because the replacement of Tyr349 with alanine shows a decrease in the T_m. The protein, however, has a T_m similar to WT when phenylalanine replaces alanine. The aromatic ring provided by tyrosine or phenylalanine may be important in the folding of the protein. Y235 has the lowest T_m, indicating that it is important in the stability of HmuT. This is presumably due to electrostatic interactions between the tyrosinate oxygen and the ferric iron of the heme as well as hydrogen bonding between R237 and Y235. The effect of H-bonding to Y235 is also observed in the T_m of R237A, which is 12° lower than WT.

Conclusions

Thermal denaturation with additional spectroscopy analysis, magnetic circular dichroism and resonance Raman, performed by Draganova et al., support the conclusion that H136 and Y235 are both axial ligands in HmuT; however, only the latter is important in defining the stability of the protein *(12)*. Y349 contributes to the overall stability of the protein but is not involve in heme binding. Y272 stabilize the heme pocket, and M292 support the axial tyrosine in the heme pocket. Finally, R237 is a H-bonding partner to Y235.

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