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Characterization of the second conserved domain in the heme uptake protein HtaA from *Corynebacterium diphtheriae*

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ABSTRACT

HtaA is a heme-binding protein that is part of the heme uptake system in *Corynebacterium diphtheriae*. HtaA contains two conserved regions (CR1 and CR2). It has been previously reported that both domains can bind heme; the CR2 domain binds hemoglobin more strongly than the CR1 domain. In this study, we report the biophysical characteristics of HtaA-CR2. UV–visible spectroscopy and resonance Raman experiments are consistent with this domain containing a single heme that is bound to the protein through an axial tyrosine ligand. Mutants of conserved tyrosine and histidine residues (Y361, H412, and Y490) have been studied. These mutants are isolated with very little heme (≤5%) in comparison to the wild-type protein (~20%). Reconstitution after removal of the heme with butanone gave an alternative form of the protein. The HtaA-CR2 fold is very stable; it was necessary to perform thermal denaturation experiments in the presence of guanidinium hydrochloride. HtaA-CR2 unfolds extremely slowly; even in 6.8 M GdnHCl at 37 °C, the half-life was 5 h. In contrast, the apo forms of WT HtaA-CR2 and the aforementioned mutants unfolded at much lower concentrations of GdnHCl, indicating the role of heme in stabilizing the structure and implying that heme transfer is effected only to a partner protein in vivo.

1. Introduction

Corynebacterium diphtheriae is an important Gram-positive human pathogen, causing upper respiratory tract infections in humans [1,2]. Although vaccination is widely available in many countries, diphtheria infections still occur in parts of the world with low vaccine coverage [3]. Analysis of the full genome sequence [2] is consistent with a number of iron uptake systems in this organism. The iron and heme transporter systems are predicted to be coordinately expressed with the toxin [4–7]. This, along with the iron requirement for virulence, suggests that iron acquisition is a key component for pathogenicity. Approximately 80% of heme in vivo is found in hemoglobin (Hb) [8], leading to the role of this prosthetic group as a significant source of iron.

Gram-positive pathogens in general use heme as a key source of the iron required for survival and proliferation. Heme uptake pathways have been the subject of recent reviews [8–14]. The majority of Gram-positive bacteria studied to date employ a series of NEAr-iron Transporter (NEAT) domains in many of the proteins involved in heme transport [14–16]. These domains are generally located in cell membrane-anchored proteins and serve to extract heme from Hb and haptoglobin-hemoglobin (Hp-Hb) and transfer it to additional heme-binding proteins, with final import through an ABC transporter. Classical NEAT domains comprise about 125 amino acids with a β-strand secondary structure; the canonical examples have a conserved YXXXY heme-binding motif, in which the N-terminal tyrosine of the sequence serves as an axial ligand to the heme and the C-terminal tyrosine is a hydrogen-bond partner to the first. On the opposite side of the heme ring, a loop starts with a serine which hydrogen bonds to one of the heme propionates. The first system to be studied in detail was the iron-regulated surface-determinant (isd) pathway in *Staphylococcus aureus* [17]. Of the proteins in this pathway, IsdA, IsdB, IsdC and IsdH bind heme in NEAT domains; leading references can be found in recent work [18–22]. The heme uptake system in *Bacillus anthracis* also uses a series

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; WT, wild-type; 5cHS, pentacoordinate, high-spin.

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of NEAT domains for heme uptake [23,24]. In terms of the reliance on the NEAT architecture, uptake pathways studied to date involving Isd proteins in *Streptococcus lugdunensis* [25] and *Listeria monocytogenes* [26,27], Hal [28] and BslK [29] in *B. anthracis*, and IlsA in *Bacillus cereus* [30,31] all use a NEAT-domain strategy [14]. Heme uptake in *Streptococcus pyogenes* has also been studied in some detail. Here, the heme is abstracted from Hb via Shr (with two NEAT domains) [32–34] and then passed to Shp, a bis-methionine heme binding protein whose structure resembles the NEAT fold [35].

C. diphtheriae is distinct from the other Gram-positive bacteria cited above in that the major heme acquisition pathway studied to date does not employ NEAT domains. The *hmu* gene cluster comprises two heme-binding surface-anchored proteins, HtaA and HtaB, along with a heme binding lipoprotein (HmuT) and an ABC-type heme transporter (HmuUV) [7,36–39] (Fig. 1). HtaA is a 61-kDa, membrane-anchored protein containing two conserved regions, CR1 and CR2, with 50% sequence similarity [37]; enzyme-linked immunosorbent assay (ELISA) experiments showed that HtaA binds Hb and is required for heme uptake from Hp-Hb [7]. HtaB, a 36 kDa protein, has one CR domain that binds heme, but not Hb [37]. HtaA can transfer hemin to HtaB in vitro [37,38]. HmuT, a lipoprotein anchored to the cytoplasmic membrane, is thought to receive its hemin from either HtaB or HtaA and transfer it to the transmembrane permease HmuU, followed by translocation across the membrane in a process driven energetically by ATP hydrolysis catalyzed by the ATPase, HmuV [36]. Deletion of the *htaA* gene, the *hmuTUV* genes or the complete *hmu* gene cluster resulted in significantly decreased growth in comparison to the wild-type (WT) strain when the cells were grown in the presence of Hb as the sole iron source. Recent studies have shown that heme uptake from Hp-Hb necessitates the presence of one or two additional proteins, ChtA and ChtC, each of which has a single CR domain [7].

HtaA-CR1 and HtaA-CR2 are of interest because they take a position in the overall heme uptake strategy similar to that of the NEAT domains, yet have no significant sequence or structural homology to these more well-studied structures. Both HtaA-CR1 and HtaA-CR2 bind hemin; they bind Hb with less affinity than the complete HtaA, with the CR2 domain binding Hb more strongly than CR1 [37]. The domains bind hemin-albumin (HSA), Hp-Hb, and myoglobin, with the binding to HtaA-CR2 being stronger than that to HtaA-CR1 in each case [7].

Sequence alignment of selected *Corynebacterium* CR domains shows that one histidine and two tyrosine residues are fully conserved (Y361, H412 and Y490 in HtaA-CR2) (Fig. S1) [37]. Mutation of these conserved residues in HtaA-CR2 showed that all three are important for heme binding and uptake. For heme binding, the order was WT > Y490A > H412A ≈ Y361A. HtaA-CR2 Y361A was isolated with the lowest heme loading of the three mutants. Y361A also bound less Hb than either H412A or Y490A; the single amino acid substitutions Y361A and H412A in the full-length HtaA reduced Hb binding by almost 90%. In line with the hypothesis that Y361 is an axial ligand, mutation of this residue to alanine completely abolished the ability of the cells to use heme or Hb as iron sources. In addition, the GST-tagged CR2 Y361A mutant did not bind Hp-Hb, in contrast to the WT domain, which had an EC_{50} of 200 nM, nor did it bind heme-HSA, while the WT domain had an EC_{50} of 350 nM [7].

Given the crucial role of HtaA in heme acquisition by this important pathogen and, specifically, the second conserved domain with its presumably novel structural features, we have undertaken a study of the biophysical characteristics of HtaA-CR2. Resonance Raman (rR) spectroscopy was used to probe structural and electronic features of the bound heme along with binding aspects of the heme pocket. The stability of the protein fold was investigated via chemical and thermal denaturation techniques.

2. Experimental

2.1. Site-directed mutagenesis

The HtaA-CR2 was previously constructed in pET24a(+) [36]. Recombinant HtaA-CR2 mutants were made by site-directed mutagenesis by using a QIAprep Spin Miniprep Kit according to the manufacturer's instructions. The primers were from Sigma Aldrich for Y361A, H412A and Y490A substitutions (Table S1). Primers (500 ng) were mixed

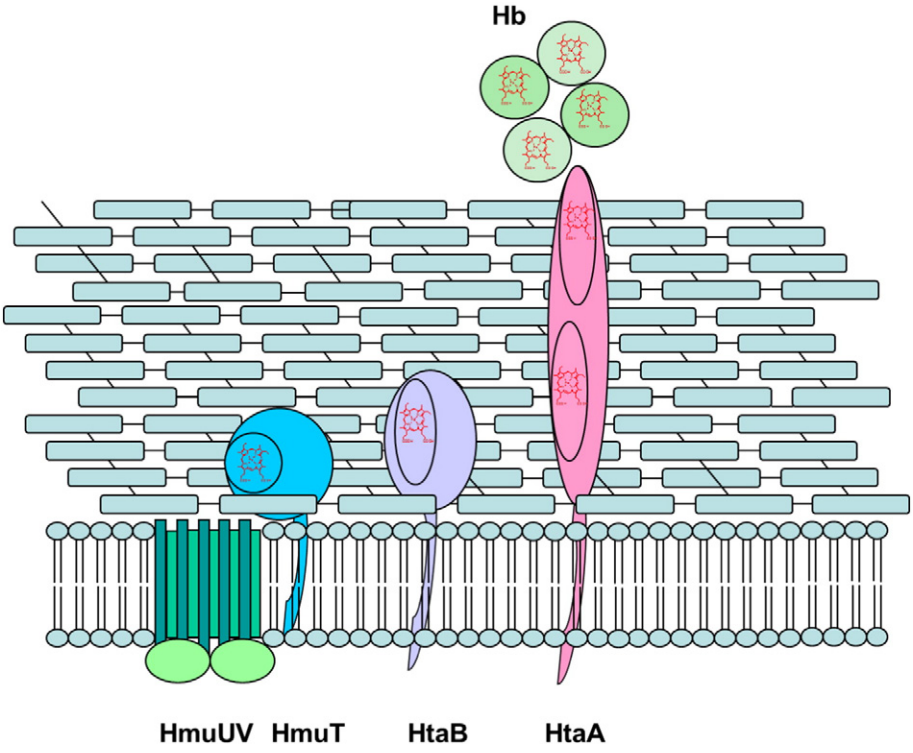

Fig. 1. Schematic of heme uptake proteins in the *hmu* pathway of *C. diphtheriae*.