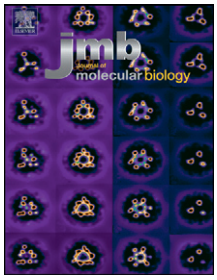

Structural Analysis and Identification of PhuS as a Heme-Degrading Enzyme from *Pseudomonas aeruginosa*

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Abstract

Bacterial pathogens require iron for proliferation and pathogenesis. *Pseudomonas aeruginosa* is a prevalent Gram-negative opportunistic human pathogen that takes advantage of immunocompromised hosts and encodes a number of proteins for uptake and utilization of iron. Here we report the crystal structures of PhuS, previously known as the cytoplasmic heme-trafficking protein from *P. aeruginosa*, in both the apo- and the holo-forms. In comparison to its homologue ChuS from *Escherichia coli* O157:H7, the heme orientation is rotated 180° across the α-γ axis, which may account for some of the unique functional properties of PhuS. In contrast to previous findings, heme binding does not result in an overall conformational change of PhuS. We employed spectroscopic analysis and CO measurement by gas chromatography to analyze heme degradation, demonstrating that PhuS is capable of degrading heme using ascorbic acid or cytochrome P450 reductase-NADPH as an electron donor and produces five times more CO than ChuS. Addition of catalase slows down but does not stop PhuS-catalyzed heme degradation. Through spectroscopic and mass spectrometry analysis, we identified the enzymatic product of heme degradation to be verdoheme. These data taken together suggest that PhuS is a potent heme-degrading enzyme, in addition to its proposed heme-trafficking function.

Introduction

Iron is an essential nutrient that is required for almost all living organisms and helps facilitate numerous cellular processes such as electron transport, peroxide reduction, and nucleotide biosynthesis [1,2]. However, free iron is scarcely available inside the host (~10^{-18} M concentration within humans) for direct utilization by bacterial pathogens, although it is the second most abundant metal on Earth [3]. The competition for iron between the host and invading pathogen has shaped the host's defense strategies, as well as the iron-acquisition mechanisms utilized by the pathogen [4–6]. Efficient iron acquisition is crucial for an invading pathogen's survival and virulence in the host. As a result, many pathogens have developed sophisticated mechanisms to capture the host's heme-containing proteins as a source of iron [7,8]. In particular, pathogenic bacteria can acquire iron from the host by capturing iron from transferrin, lactoferrin, or ferritin via specific outer-membrane receptors, or they can take up iron from free heme or from heme-containing proteins, such as hemoglobin [2,8–10].

Iron requires specialized mechanisms for uptake and storage due to its limited bioavailability, and in the opportunistic Gram-negative pathogen *Pseudomonas aeruginosa*, the uptake and utilization of heme as an iron source are a receptor-mediated process and involve numerous proteins required for internalization and degradation of heme [11,12]. *P. aeruginosa* encodes two heme-uptake systems in the *phu* (*Pseudomonas heme utilization*) and *has* (*h*eme *a*ssimilation *s*ystem) operons, which are under transcriptional regulation by the *f*erric *u*ptake *r*egulator protein Fur [13]. The *phu* operon encodes for the outer-membrane receptor PhuR, the periplasmic transport protein PhuT, and the soluble cytoplasmic membrane ATP-dependent permease complex PhuUVW [14]. As well, the cytoplasmic heme-binding protein PhuS sequesters heme translocated to the

cytoplasm by PhuUVW [14]. In contrast, the *has* operon encodes a TonB-dependent outer-membrane receptor HasR, which accepts heme from a soluble hemophore HasA, which is secreted, and extracts heme from the serum [14]. However, the *has* system lacks a periplasmic uptake system and is assumed to utilize the *phu*-encoded ATP-binding cassette transporter [10,14].

Heme oxygenases have been identified and characterized in Gram-positive and Gram-negative pathogens including *Corynebacterium diphtheriae*, *Neisseriae spp.*, and *P. aeruginosa* [14]. *P. aeruginosa* encodes the iron-regulated HemO, for which the catalytic mechanism and structural fold are strikingly similar to the more well characterized eukaryotic heme oxygenases [15,16]. However, HemO has been shown to be a δ-regioselective heme oxygenase enzyme, compared to the canonical α-regioselectivity seen in eukaryotic heme oxygenases [16]. *P. aeruginosa* also encodes a second heme oxygenase, BphO, which produces α-biliverdin and acts as a chromophore for the bacteriophytochrome kinase, BphP [14]. Interestingly, the *bphOP* operon is not iron regulated and does not appear to be involved in extracellular heme uptake [14].

Previous studies with the cytoplasmic heme-binding protein PhuS have shown that it is a heme-trafficking protein that interacts with the heme oxygenase HemO, but only when PhuS is bound to heme [11,12]. PhuS binds one heme per monomer and holo-PhuS forms a 1:1 complex with HemO [11]. The heme-induced conformational switch in PhuS was thought to drive the protein–protein interaction and the subsequent free energy necessary to drive heme transfer to HemO [11]. As a result, these studies have implied that PhuS is specifically a heme-trafficking protein with no enzymatic activity.

PhuS has homologues in heme utilization systems of other Gram-negative bacteria, including ChuS in *Escherichia coli* O157:H7, HemS in *Yersinia enterocolitica*, HmuS in *Yersinia pestis*, and ShuS in *Shigella dysenteriae* [17–21]. The crystal structures of ChuS and HemS have been solved for both the apo- and the heme-bound forms [17–19]. Interestingly, *E. coli* O157:H7 does not have a homologue for the heme oxygenase HemO, as is present in other Gram-negative pathogens, including *P. aeruginosa* [17]. Suits *et al.* reported that ChuS is a heme oxygenase and is able to break down heme to generate free iron and CO [17]. The structure of ChuS represents the identification of a previously uncharacterized fold and was unique compared to other characterized heme oxygenases [17]. ChuS was shown to be capable of using ascorbic acid or cytochrome P450 reductase-NADPH as electron sources for heme oxygenation and was proposed to be α-regioselective for heme [17,18].

Subsequently, Schneider *et al.* reported the structure of HemS, showing the same characteristic fold as ChuS [19]. It is currently unknown whether HemS is able to break down heme or if it has any binding partner to transfer its heme to. The heme-bound structure of HemS showed that, upon binding of heme, there is an induced-fit conformational change around the heme-binding pocket, where the N- and C-terminal domains pivot to close onto the ligand [19]. The HemS structure therefore switches between an open, apo-form, and a closed, bound state. Recently, the apo-PhuS structure has been reported [22] and displayed close structural similarity to ChuS, as expected. However, the apo-PhuS structure did not provide insights into the heme binding of PhuS and the previously proposed heme transfer to HemO [22]. In the present study, we report the crystal structures of apo- and holo-PhuS at 2.0 and 1.95 Å resolution, respectively, which show structural similarity to ChuS and HemS but with key differences. We further show through spectroscopic analysis that PhuS is capable of breaking down heme using ascorbic acid or cytochrome P450 reductase-NADPH as an electron source. CO detection through gas chromatography showed that PhuS is five times more active in producing CO than ChuS. We identify through mass spectrometry that verdoheme is the product of PhuS heme breakdown. Taken together, these results led us to propose that PhuS is a potent heme-degrading enzyme in *P. aeruginosa*.

Results and Discussion

Crystal structure of PhuS

To determine the structure of PhuS in both the heme-bound and the unbound forms, we overexpressed and purified full-length PhuS. The apo-PhuS crystals were colorless and cube-shaped and were diffracted to 2.0 Å, whereas the holo-PhuS crystals were bright red and were diffracted to 1.95 Å. The PhuS structure is highly similar to and has the same characteristic fold as ChuS (Fig. 1), revealing rmsd (*r*oot-*m*ean-*s*quare *d*eviation) values of 0.95 and 1.16 Å (260 and 263 C^{α} atoms, respectively) when comparing the apo monomers and 0.99 and 1.12 Å (276 and 267 C^{α} atoms, respectively) when comparing the holo monomers. Both PhuS structures are more complete than ChuS, with electron density missing only for the first 13 residues for both PhuS structures due to disorder. The overall structure is composed of a central core of two pleated β-sheets, each consisting of nine anti-parallel β-strands stacked together. Each β-sheet is flanked at its N-terminus by a pair of parallel α-helices and at the C-terminus by three α-helices in an α-loop-α-loop-α motif, forming the two halves of a structurally repeating protein. The two halves are connected by a long stretch of coil from Arg168 to Asp194. For holo-PhuS, both molecules were found with strong density for