Expression and Characterization of a Heme Oxygenase (Hmu O) from Corynebacterium diphtheriae

IRON ACQUISITION REQUIRES OXIDATIVE CLEAVAGE OF THE HEME MACROCYCLE*

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A full-length heme oxygenase gene from the pathogenic bacterium Corynebacterium diphtheriae has been subcloned and expressed in Escherichia coli. The enzyme is expressed at high levels as a soluble catalytically active protein that results in the accumulation of biliverdin within the E. coli cells. The purified heme oxygenase forms a 1:1 complex with heme (Kd = 2.5 ± 1 μM) and has hemeprotein spectra similar to those previously reported for the purified eukaryotic heme oxygenase. In the presence of an E. coli NADPH-dependent reductase isolated during the purification of Hmu O, the heme-Hmu O complex is catalytically turned over to yield biliverdin IXα and carbon monoxide.

A number of redox partners were investigated for their ability to reconstitute Hmu O activity in vitro. Of these the most efficient appeared to be the recombinate NADH-dependent putidaredoxin/putidaredoxin reductase from Pseudomonas putida. As with the E. coli NADPH-dependent reductase the final products of the reaction were biliverdin IXα and carbon monoxide. This is the first bacterial heme oxygenase to be described to date. The close relationship between iron acquisition and pathogenesis suggests that the release of iron from heme oxygenase may play a crucial role in the pathogenicity of C. diphtheriae.

Corynebacterium diphtheriae is a Gram-positive aerobic bacterium and the causative agent of diphtheria. The systemic symptoms of diphtheria are due to the excretion of the powerful diphtheria exotoxin which can cause severe tissue damage throughout the body (1, 2). Although the diphtheria toxin itself has been well studied at both the genetic and biochemical level, little is known of the molecular mechanisms involved in pathogenesis and infection (3, 4). The diphtheria toxin, the product of the tox gene, has been shown to be under transcriptional control of the iron-dependent diphtheria toxin repressor protein, DtxR (5–7). DtxR is a global iron-dependent repressor akin to the ferric uptake repressor (Fur) protein of Gram-negative bacteria. The DtxR protein was also found to bind the promoter regions of six genes in C. diphtheriae, including irp1 and irp2, as well regulating the expression of the C. diphtheriae siderophore (7–9). Like the Fur protein the expression of DtxR-regulated genes is derepressed in low iron conditions and repressed in an iron-replete environment.

Iron is an essential nutrient required for the survival of most bacteria, and the ability of pathogenic bacteria to acquire sufficient iron during infection is essential for such pathogens to cause disease (10). The levels of extracellular iron available within the host are limited, with most of the free iron being complexed to high affinity binding proteins such as transferrin (11). Intracellularly the majority of iron is found in the erythrocytes as heme (12). To circumvent the low availability of iron pathogens have developed sophisticated mechanisms to utilize the host’s iron- and heme-containing proteins. A number of Gram-negative pathogens such as Shigella dysenteriae, Vibrio cholerae, and Yersinia enterocolitica have developed iron-dependent outer membrane receptors specific for heme (13–15). Heme is transported through such receptors via a TonB-mediated gated pore mechanism (16, 17). In the case of Y. enterocolitica several proteins involved in the acquisition of heme have been identified at the genetic level (18). Once the heme has been transported across the outer membrane by the heme receptor (Hem R), a periplasmic heme binding protein (Hem T) is thought to transport the heme to the cytoplasmic membrane where a classic permease/ATPase (Hem V/Hem U) is thought to actively transport the heme into the cytoplasm (18). Once localized within the cytoplasm it is proposed that there is a heme utilizing protein (Hem S) whose function is to release the iron from the heme (18). It has previously been proposed that the hemS gene encoded for a heme oxygenase protein as hemS mutants were unable to utilize heme and developed heme toxicity. However, no sequence identity with known heme oxygenases was evident, and no enzymatic activity for Hem S was reported (18). In contrast the hmuO gene from C. diphtheriae, which has the consensus DtxR binding site located upstream of hmuO, is repressed by iron and DtxR, and activated in the presence of heme, shows extensive homology with the human heme oxygenase protein (19, 20). Heme oxygenase is the rate-limiting enzyme in heme degradation and catalyzes the NADPH-cytochrome P450 reductase-dependent cleavage of heme to biliverdin with the release of iron and carbon monoxide (Scheme 1) (21). In the present paper we describe the first purification and characterization of a bacterial heme oxygenase (Hmu O) from C. diphtheriae and show that it oxidatively cleaves the heme macrocycle, releasing the iron for subsequent use by the invading pathogen.

EXPERIMENTAL PROCEDURES

General Methods—Plasmid purification, subcloning, and bacterial transformations were carried out as described previously (22). Deionized, doubly distilled water was used for all experiments. Oligonucleotides were synthesized at the Biomolecular Resource Center of the University of California at San Francisco using an Applied Biosystems 380B DNA synthesizer. All absorption spectra of the heme-Hmu O...
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Complexes were recorded on a Carey Varian 1E UV spectrophotometer. Bacterial Strains—Escherichia coli strain DH5a [F’ ara Dlac proAB] was used for DNA manipulation, and E. coli strain BL21 (DE3) [F’ ompT hsdS (r_{52} m_{52}) gal dcm (DE3)] was used for expression of the heme oxygenase

Construction of the Expression Vector pTH793—Plasmid pTH793 which carries the hmuO gene under transcriptional control of the phage T7 promoter was used for the expression and purification of Hmu O. A 0.65-kb DNA fragment carrying the hmuO gene was generated by PCR from plasmid pCD293 using the 5′ sense oligonucleotide (5′-GGGATCATATGACCACT-GCATGCTAAAAGCTCC-3′) which encodes an NdeI site, and the 3′ antisense oligonucleotide (5′-GACCTAGTCTGCCGACAGCCTG-3′) which encodes a SalI site. The NdeI site contains the ATG start codon for hmuO. The 0.65-kb PCR product was digested with NdeI and SalI and ligated into the expression vector pT7-7 giving rise to pTH793 (23).

Construction of the Expression Vector pEHmuO—The hmuO gene encoding the heme oxygenase was amplified by PCR from the plasmid pTH793 containing a 0.65-kb insert encoding the 23-kDa heme oxygenase protein. The 5′ sense oligonucleotide (5′-CCGGATCATATGACCACT-GCATGCTAAAAGCTCC-3′) encoded an NdeI site at the NH2 terminus, and the antisense oligonucleotide (5′-CCTGCTGCTGACAGGCGTTAC-CCAAATCA-3′) encoded an Xhol site and removed the stop codon (TAA).

The PCR product when subcloned into pET23a encoded for a protein with a six-histidine tag at the C terminus. The reaction contained 50 ng of pTH793, 50 pmol each of the forward and reverse primers, 1 mM dNTPs, and 1 μl (10 units) of Vent DNA polymerase in a final volume of 50 μl of 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM (NH4)2SO4, 2 mM MgSO4, and 0.1% Triton X-100. The annealing and extension cycles were as follows: 94 °C for 10 min (1 cycle), 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min (10 cycles), 94 °C for 1 min, 65 °C for 1 min, 72 °C for 1 min (20 cycles), and 72 °C 10 min (1 cycle). Following gel purification of the amplified product the 0.65-kb digested was digested with NdeI and Xhol and cloned into pET23a.

Expression and Purification of Hmu O—A 3-ml inoculum in LB-Amp media was prepared from plates with fresh colonies of transformed pTH793 E. coli BL21 (DE3) cells. One-liter cultures were inoculated (1 ml) from the overnight cultures and then grown at 37°C to an A600 of 0.3–0.5 and induced with a final concentration of 1 mM isopropyl-1-thio-
β-D-galactopyranoside. The cells were grown for an additional 12 h after which they were harvested by centrifugation (10,000 × g for 20 min).

The harvested cells were lysed by sonication in 50 mM Tris buffer (pH 8.0) containing 1 mM EDTA and 1 mM phenylmethylsulfonil fluoride. The cells were then spun at 27,000 × g for 60 min. The protein fractionated with the soluble fraction. The soluble fraction was then applied to a Sepharose-Q Fast Flow column (1.5 × 30 cm) equilibrated with 20 mM Tris-HCl (pH 7.5) and washed with an additional 5 volumes of 20 mM Tris-HCl (pH 8.0). The protein eluted in the same buffer with a linear gradient of 0–500 mM NaCl. The protein eluted at a concentration of 150 mM NaCl, and the fractions (30 ml) containing the Hmu O protein were pooled, diluted with 50 mM Tris-HCl (pH 7.5), and concentrated on an Amicon filtration unit. The Sepharose Q fraction (5 ml) was run through a Sephacryl S-200HR column (1 × 50 cm) equilibrated with 100 mM Tris (pH 7.5). The fractions containing the Hmu O protein (20 ml) were pooled and concentrated on an Amicon filtration unit. Protein purity was analyzed by SDS-PAGE on a 20% acrylamide gel (24).

1 The abbreviations used are: kb, kilobase pair; α, HMP, hemoglobin-like protein; heme, iron protoporphyrin IX irrespective of oxidation or ligation state; HO-1, human heme oxygenase; HPLC, high pressure liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.

Expression and Purification of His-Hmu O—The His-tagged Hmu O protein (pEHmuO) was expressed and fractionated as described for the wild type protein. The soluble fraction was applied directly to a nickel-nitrilotriacetic acid agarose column (1 × 5 ml) previously equilibrated with 20 mM Tris-HCl (pH 7.9) containing 0.5 M NaCl and 5 mM imidazole. The column was washed with 10 volumes of equilibration buffer, followed by 10 volumes of wash buffer (20 mM Tris-HCl, pH 7.9, containing 0.5 M NaCl and 60 mM imidazole), and the protein eluted in 20 mM Tris-HCl (pH 7.9) containing 0.25 M NaCl and 500 mM imidazole. The purified protein was exchanged by dialysis into 50 mM Tris-HCl (pH 7.5).

Purification of Hmu O with Heme—The heme-Hmu O complex was prepared as described previously for the heme-heme oxygenase complex (25). Hemin was added to the purified Hmu O to give a final 2:1 heme:protein ratio. The sample was applied to a Bio-Gel HTP column (1.5 × 60 cm) pre-equilibrated with 10 mM potassium phosphate buffer (pH 7.4). The column was then washed with the same buffer (5 volumes), and the protein eluted in 150 mM potassium phosphate buffer (pH 7.4). The fractions containing the heme-Hmu O complex were pooled and dialyzed against 50 mM Tris-HCl (pH 7.5).

Absorption Spectroscopy—The ferrous CO complex was formed by the addition of dithionite to a carbon monoxide-saturated solution of the ferric heme-Hmu O complex. The ferrous CO heme-Hmu O complex was passed through Sephadex G-25 to remove the excess reductant and concentrated into the ferrous oxygent complex.

Heme binding studies were carried out by difference absorption spectroscopy in the Soret region. Aliquots of heme (0.1–10 μM) were added to both the sample (8 μM Hmu O) and reference cuvettes at 25°C. Spectra were recorded 5 min after each addition of heme.

Calculation of the Extinction Coefficient for the Heme-Hmu O Complex—The value of the millimolar extinction coefficient at 404 nm (ε404) was determined by the pyridine hemochrome method (26). The absorbance of a purified heme-Hmu O sample at 404 nm was measured. The sample was diluted with alkaline pyridine, and an excess of sodium dithionite was added. The pyridine hemochrome spectrum was recorded, and the concentration of heme was determined from the extinction coefficient at 557 nm, using the value of 34,530 M−1 cm−1.

Reaction of Hmu O with NADH/NADPH Reductases—Several NADPH and NADH reductase systems were reconstituted with the heme-Hmu O complex. A partially purified E. coli NADPH-dependent reductase eluted from the Q-Sepharose FF column, NADH-dependent putidaredoxin reductase (Pseudomonas putida), and the human NADPH-cytochrome P450 reductase. The heme-Hmu O protein (8 μM) had added to it one of the reductase systems in the following molar ratios E. coli reductase (3:1), cytochrome P450 reductase (0:5:1), and putidaredoxin reductase (0.3:1:3:1 putidaredoxin reductase:putidaredoxin:Hmu O) in a final volume of 1 ml of potassium phosphate buffer (pH 7.4). The reaction was initiated by the addition of NADPH or NADH in 10 μM increments to a final concentration of 100 μM. The spectral changes between the wavelengths 300–700 nm were recorded for an additional 30 min. Additional NADPH or NADH (100 μM) was added to each of the assays, and the reactions were incubated for an additional 30 min at 37°C after which the products were extracted for HPLC analysis.

HPLC of the Hmu O Reaction Products—After reaction of the heme-Hmu O complex with NADPH reductase, glacial acetic acid (100 μl) and 5 mM HCl (200 μl) were added to the reaction (1 ml) before extracting into chloroform. The organic layer was washed with distilled water (5 × 1 ml), and the chloroform was evaporated under a stream of argon (27). The residue was analyzed on reverse phase HPLC on a Partisil ODS-3 (5 μ, C18) column eluted with 50:50 (v/v) 20 mM formic acid:acetone at a flow rate of 1 ml/min (28). The eluant was monitored at 380 nm, and the biliverdin standards eluted in the order α (19 min), δ (20 min), β (24 min), and γ (28 min).

Detection of Carbon Monoxide as a Reaction Product—Purified heme-Hmu O (15 μM), partially purified E. coli NADPH-dependent reductase (100 μg), and NADPH (100 μM) in a final volume of 1 ml were placed in both the reference and reaction cuvettes and blanked immediately. The reaction cuvette had added 50 μl of myoglobin (125 μM), and the same volume of buffer was added to the reference cuvette. The spectrum was recorded at 1-min intervals between 300 and 600 nm, and the transition from 404 to 421 nm was monitored.

RESULTS

Expression and Purification of Hmu O—Both the wild type and His-tagged Hmu O were expressed as soluble active proteins. As previously reported for the human and rat heme...
oxygenase proteins, expression of Hmu O in *E. coli* BL21 (DE3) turned the medium green due to the accumulation of biliverdin (28, 29). The bacterial cells have a reductase activity that supports the catalytic turnover of Hmu O, and not yet having identified a reductase activity in *C. diphtheriae*, this fraction was isolated for reconstitution of activity following purification of Hmu O.

Purification of the Hmu O protein by ion-exchange and gel filtration yielded a protein with a single band at 25 kDa on SDS-PAGE (Fig. 1, lane 2). The amount of protein purified from 2 liters of cells, based on a standard protein assay, was 42 mg. The His-Hmu O protein purified on nickel-nitrilotriacetic acid agarose yielded 15 mg/liter and gave a single band on SDS-PAGE (Fig. 1, lane 3).

**Properties of the Heme-Hmu O Complex**—The Soret maximum of the heme-Hmu O complex following the removal of excess heme on a hydroxylapatite column is at 404 nm (Fig. 2). Reduction of the ferric heme-Hmu O complex with dithionite under an atmosphere of CO gives a spectrum typical of a reduced ferrous carbon monoxide complex with a Soret band at 421 nm and α/β bands at 568 and 538 nm, respectively (Fig. 2). Following passage of the ferrous carbon monoxide complex through Sephadex G-25 the Soret band shifts from 421 to 410 nm and the α/β bands to 570 and 540 nm due to formation of the ferrous dioxygen complex (Fig. 2). These values are comparable to those reported for the corresponding complex of the eukaryotic heme oxygenases (28, 29). The histidine-tagged Hmu O (His-Hmu O) gave identical absorption spectra and heme oxygenase activity as the wild type Hmu O protein and therefore only the data for wild type protein are shown. The extinction coefficient (ε404) at 404 nm for Hmu O was calculated from the pyridine hemochrome method to be 121 M⁻¹cm⁻¹.

**Calculation of the Heme Binding Constant (K_d)**—The ratio of heme bound to Hmu O was calculated by difference absorption spectroscopy (Fig. 3). Heme titration with Hmu O gave a calculated K_d of 2.5 ± 1 μM, and the Hmu O appeared saturated at a ratio of 1:1 heme to protein. The calculated K_d value is comparable with the 0.84 ± 0.2 μM value reported for the recombinant human heme oxygenase (30).

**Catalytic Turnover of the Heme-Hmu O Complex**—The ferric heme-Hmu O complex is quantitatively turned over to biliverdin in the presence of NADPH by both the *E. coli* reductase and the human NADPH-cytochrome P450 reductase (Fig. 4, A and B). As for the human heme oxygenase, the heme-Hmu O complex proceeded with the formation of the ferrous dioxygen complex with a drop and shift in the Soret band from 405 to 410 nm and the appearance of α/β bands at 570 and 540 nm. Over a period of time (30 min) the Soret maximum decreased further and shifted back toward 400 nm with the disappearance of the α/β bands and the appearance of a broad peak at 680 nm indicative of biliverdin formation. Putidaredoxin/putidaredoxin reductase also turned over the heme-Hmu O complex to biliverdin (Fig. 4C). HPLC analysis of the products formed in the reactions with both the *E. coli* and human NADPH-reductases as well as the NADH-dependent putidaredoxin system, gave a retention time and absorption spectrum identical to that of biliverdin IXα (data not shown). Co-injection of the product from the *E. coli* reductase/Hmu O reaction with biliverdin IXα from the human heme oxygenase reaction gave a single peak verifying that biliverdin IXα is the final product in the Hmu O reaction (data not shown).

Although conversion of heme to biliverdin was observed with the *E. coli* reductase fraction, human NADPH-cytochrome P450 reductase, and putidaredoxin reductase the rates were not quantitatively measured. The spectrophotometric assay for mammalian heme oxygenases involves coupling biliverdin reduction to bilirubin in the presence of biliverdin reductase. However, the ability of mammalian biliverdin reductase to
accept biliverdin from the bacterial heme oxygenase appears to be impaired. The observation that biliverdin bound in the active site of Hmu O is not easily displaced by heme, as is the case with the mammalian heme oxygenases, suggests Hmu O has a higher affinity for biliverdin than its mammalian counterpart, which would explain the lack of reactivity with the mammalian biliverdin reductase. Biliverdin formation is difficult to measure directly due to its low extinction coefficient, overlapping absorbance contributions from heme, and product inhibition due to biliverdin accumulation within the heme oxygenase active site. An assay which monitors iron release by chelation is currently being developed to measure Hmu O activity.

Detection of Carbon Monoxide as a Reaction Product—Detection absorption spectroscopy in the presence of myoglobin was used to determine whether carbon monoxide is generated as a product of oxidative cleavage of the heme. The myoglobin Soret band was monitored at 1-min intervals and shown to shift from 408 nm to 421 nm with the appearance of α/β bands at 568 and 538 nm, respectively (Fig. 5). The complete conversion of the ferric myoglobin to the ferrous carbon monoxide complex indicated that carbon monoxide as well as biliverdin is a product of heme cleavage in C. diphtheriae.

DISCUSSION

In contrast to Gram-negative pathogens such as S. dysenterae (13), V. cholerae (14), and Y. enterocolitica (15) in which proteins involved in the transport and utilization of heme have been described, acquisition of iron from heme or heme-containing proteins has not been well characterized in Gram-positive bacteria. The uptake and utilization of heme in Gram-negative bacteria, like ferric siderophore uptake, involves an iron-regulated, Ton-B-dependent outer membrane receptor and a periplasmic binding protein transport system (18). It has also been proposed that a heme utilizing protein, perhaps a heme oxygenase, would then release iron from the heme once it is transported into the cytoplasm (18).

The mechanisms by which Gram-positive bacteria such as C. diphtheriae acquire heme are not known, but based on the similarities of heme transport with siderophore mechanisms, it is not unreasonable to assume that a similar mechanism would operate in Gram-positive bacteria. A previous report describing C. diphtheriae strains capable of utilizing heme, independent of siderophore uptake, testifies to the existence of such mechanisms (19). Identification of an hmuO gene capable of complementing mutants deficient in heme utilization provided further evidence for a specific mechanism for heme transport and utilization. A search of the protein data base revealed extensive sequence homology between Hmu O and the human heme oxygenase (HO-1) (19).

In this study, we demonstrate that the product of the hmuO gene oxidatively cleaves heme to biliverdin with the release of carbon monoxide and iron. This reaction, like that of the mammalian heme oxygenases, requires an NAD(P)H-dependent reductase system. In the absence of a known NAD(P)H-dependent reductase from C. diphtheriae it was noted that a soluble reducing activity capable of supporting the conversion of heme to biliverdin was expressed in E. coli at the time of hmuO induction. Interestingly, the isolated reductase fraction isolated had a UV/visible spectrum similar to the hemoglobin-like protein (HMP) isolated from E. coli (data not shown). This protein is a flavohemoprotein with a heme Soret at 404 nm and
a shoulder at 540 nm due to flavin absorbance (31). SDS-PAGE of the E. coli reductase showed a major band at 45 kDa, close to the 44 kDa reported for HMP (31). The HMP protein has been reported to have ferric reductase activity in the presence of free flavins, releasing ferrous iron from ferrisiderophore complexes (32). The ferric reductase activity of the HMP protein was described as playing a minor role in vivo, the major activity being attributed to the NAD(P)H flavin oxidoreductase and sulfite reductase (32, 33). In the case of NAD(P)H flavin oxidoreductase, the enzyme catalyzes the reduction of flavins (FMN, FAD, riboflavin) by NADPH into free reduced flavins which then transfer electrons to the iron complex. The specificity of flavin reduction is determined by the affinity of the reductase for free oxidized flavins. However, the subsequent release of free reduced flavins would allow the reductase system to be less specific with respect to reduction of the iron complex, whether the ligand is a ferrisiderophore or a protein (33).

Although the physiological role of the HMP protein has not been determined, it is thought to play a role as an oxygen sensor in monitoring the redox state of the cell (34). It has been proposed that HMP protein may play a role in redox signal transduction by regulating the assembly and disassembly of an iron sulfur [4Fe-4S] cluster in the transcriptional regulator FNR (34, 35). In general, the role of ferric reductases in mobilizing iron has not been well studied although the reaction has been proposed to be inhibited by oxygen, which is a good alternative acceptor of electrons from reduced flavins (33). One could therefore propose HMP would release electrons for flavin reduction under low oxygen tension when conditions are optimal for iron release. The significance of a “HMP-like” protein being involved in Hmu O-dependent heme degradation in vivo is not known, but the observation that the E. coli HMP protein has reductase activity with ferrisiderophores suggests that a similar protein in C. diphtheriae may be responsible for iron release from both heme and ferrisiderophores.

The human membrane-bound cytochrome P450 reductase and putidaredoxin reductase are also capable of turning over the heme-Hmu O complex to biliverdin but with a lower efficiency than that of the E. coli NADPH-dependent reductase. It is interesting to note that both the putidaredoxin and cytochrome P450 reductase enzymes, whose physiological function is to couple electron transfer to cytochrome P450, can also support Hmu O activity. One could speculate that as in mammalian systems where cytochrome P450 reductase supports both cytochrome P450 and heme oxygenase activity, a similar enzyme in C. diphtheriae may function as redox partner to both proteins. Identification of the reductase in C. diphtheriae responsible for the release of iron from heme and perhaps ferrisiderophores is currently underway. Unlike mammalian heme oxygenases, the bacterial enzyme is a soluble protein with a molecular weight of 25,000, much smaller than the 33-kDa membrane-bound HO-1. In previous studies, the human heme oxygenase has been shown to function as a soluble protein on removal of the carboxyl-terminal 23 amino acids that anchor the protein in the membrane (28). It has further been shown that truncation of the protein to 221 amino acids (25 kDa), the approximate size of the Hmu O protein, results in a soluble active enzyme retaining greater than 50% of the wild type activity. In contrast truncation of the protein beyond 221 amino acids to 215 amino acids results in no detectable expression of the protein (32). The evolution-