Characterization of the Outer Membrane Receptor ShuA from the Heme Uptake System of Shigella dysenteriae

SUBSTRATE SPECIFICITY AND IDENTIFICATION OF THE HEME PROTEIN LIGANDS*

Received for publication, December 4, 2006, and in revised form, March 19, 2007 Published, JBC Papers in Press, March 26, 2007, DOI 10.1074/jbc.M611121200

Kimberly A. Burkhard and Angela Wilks1

From the Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland, Baltimore, Maryland 21201

Shigella dysenteriae, like many bacterial pathogens, has evolved outer membrane receptor-mediated pathways for the uptake and utilization of heme as an iron source. As a first step toward understanding the mechanism of heme uptake we have undertaken a site-directed mutagenesis, spectroscopic, and kinetic analysis of the outer membrane receptor ShuA of S. dysenteriae. Purification of the outer membrane receptor gave a single band of molecular mass 73 kDa on SDS-PAGE. Initial spectroscopic analysis of the protein in either detergent micelles or lipid bicelles revealed residual heme bound to the receptor, with a Soret maximum at 413 nm. Titration of the protein with exogenous heme gave a Soret peak at 437 nm in detergent micelles, and 402 nm in lipid bicelles. However, transfer of heme from hemoglobin yields a Soret maximum at 413 nm identical to that of the isolated protein. Further spectroscopic and kinetic analysis revealed that heme in the oxidized state is the most likely physiological substrate for ShuA. In addition, mutation of the conserved histidines, H86A or H420A, resulted in a loss of the ability of the receptor to efficiently extract heme from hemoglobin. In contrast the double mutant H86A/H420A was unable to extract heme from hemoglobin. These findings taken together confirm that both His-86 and His-420 are essential for substrate recognition, heme coordination, and transfer. Furthermore, the full-length TonB was shown to form a 1:1 complex with either apo-ShuA H86A/H420A or the wild-type ShuA. These observations provide a basis for future studies on the coordination and transport of heme by the TonB-dependent outer membrane receptors.

The ability to acquire iron is essential for the survival and virulence of the large majority of pathogenic bacteria (1). Bacterial pathogens have therefore evolved sophisticated systems to acquire a variety of iron and heme-containing proteins (2, 3). These complex transport systems in Gram-negative bacteria all require a TonB-dependent high affinity outer membrane receptor to facilitate transport across the outer membrane (4). The transport of heme across the outer membrane is driven by coupling the cytoplasmic membrane potential via the TonB-ExbBBD complex to the receptor. Following transport across the outer membrane, a periplasmic-binding protein shuttles the heme to the cytoplasmic ATPase/permease, where the heme is internalized and further utilized (5–7).

A number of Gram-negative pathogens have been shown to utilize a wide spectrum of iron and heme-containing proteins, including Yersinia sp. (8–10), Neisseria sp. (11–13), Vibrio sp. (14), and the opportunistic pathogen Pseudomonas aeruginosa (15). The well characterized Yersinia enterocolitica heme operon (hemRSTUV) encodes the outer membrane receptor HemR and the periplasmic/cytoplasmic components (HemSTUV) of the transport system (8, 9). In addition some pathogenic organisms, including Yersinia sp. (10), Serratia marcescens (16), and P. aeruginosa (15, 17) secrete extracellular soluble hemophores that can acquire heme from a number of heme sources.

In Shigella dysenteriae, the sloughing off of intestinal epithelial cells and the excretion of the Shiga toxin, which causes apoptosis in intestinal cells leading to dysentery and hemolytic uremia, suggests that heme in the form hemoglobin may be a major source of iron (18, 19). The heme operon of S. dysenteriae, Shigella heme uptake (shu) encodes an outer membrane receptor (ShuA), a periplasmic heme-binding protein (ShuT), and the ATPase/permease genes (ShuU and -V) required for heme-uptake across the cytoplasmic membrane. The cytoplasmic heme-binding protein (ShuS) was recently characterized and shown to bind DNA as well as heme (20).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence should be addressed: Dept. of Pharmaceutical Sciences, School of Pharmacy, 20 Penn St., University of Maryland, Baltimore, MD 21201. Tel.: 410-706-2537; Fax: 410-706-5017; E-mail: awilks@rx.umd.edu.

2 The abbreviations used are: heme, iron protoporphyrin IX irrespective of oxidation state; metHb, dimeric hemeoglobin in the ferric (Fe3+) state; oxy-Hb, oxidized tetrameric hemeoglobin in the ferric (Fe3+) state; metMb, myoglobin in the ferric (Fe3+) state; Ni-NTA, nickel-nitrilotriacetate acid; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride.

* This work was supported by National Institutes of Health Grant AI-48551. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.© 2007 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
oxygenase (22). Although a heme oxygenase has not yet been identified in the *S. dysenteriae* genome, it is evident from genetic studies that the cytoplasmic heme-binding proteins are required for efficient heme utilization.

Although there is a great deal of evidence for the direct utilization of hemoproteins by the outer membrane receptors of Gram-negative pathogens, much of it comes from *in vivo* studies (reviewed in Refs. 2 and 3). Specifically, the hemoprotein preference of the ShuA receptor *in vitro* has not previously been determined. We herein report for the first time the purification and characterization of the ShuA receptor ShuA of *S. dysenteriae*. Furthermore we have determined the ligand specificity and oxidation state preference of the *S. dysenteriae* ShuA receptor. We further show that the conserved histidine residues His-86 and His-420 are critical for the initial binding and sequestration of heme by the ShuA receptor. Finally, we provide the first evidence that a full-length TonB protein forms a 1:1 complex with the ShuA receptor.

**MATERIALS AND METHODS**

**Bacterial Strains—** DNA manipulations were carried out in *Escherichia coli* strain DH5α (F, ara D (lac-proAB) rpsL φ80lacZ DM15 hasd R17) and *E. coli* strain BL21(DE3) (B F- dcmm ompT hsdS (rB- mB-)) gal λ (DE3)) was utilized for the production of ShuA. *E. coli* strain M15(pREP4) (F-, nls, str, rif-, his-, lac-, ara, gal, mtl, recA+, uvr+, lon+) was used for TonB production.

**Construction of the Expression Vectors pEShuA and pND43TonB—** The *shuA* gene was PCR-amplified from plasmid pSHU9 (23). The first 27 amino acid residues corresponding to the signal peptide were not included in the final *shuA* gene construct (see Fig. 1A). The forward primer (ShuAF) was designed to include an Mscl site preceding the codon for amino acid 28 of ShuA 5'-GGCCCTGTCGGCATTGGCTACT-GAAACCATTGCACC-3'. In designing the reverse primer (ShuAR) 5'-CTGGGCTCTCGAGGGCCATTTGATAACTCAGGAAAT-3' the stop codon in the *shuA* gene was removed to utilize a His6 tag preceding the XhoI site in the expression vector pQE2 utilizing the BseRI and HindIII sites at the 5' and 3' termini, respectively, to generate pND34.

**Site-directed Mutagenesis of ShuA—** Mutagenesis was carried out by PCR utilizing the QuikChange mutagenesis kit (Stratagene, La Jolla, CA). All mutations were verified by DNA sequencing at the Biopolymer Facility, School of Medicine, University of Maryland, Baltimore.

**Expression and Purification of Wild-type, H86A, H420A, and H86A/H420A ShuA—** *E. coli* BL21(DE3) cells freshly transformed with either wild-type or mutant pEShuA constructs were grown on Luria-Bertani (LB)-ampicillin (100 μg/ml) plates. A 300-ml Terrific Broth-carbenicillin (100 μg/ml) subculture from the freshly transformed cells was grown for 12 h at 37 °C. The subculture was then used to inoculate 1 liter Terrific Broth-carbenicillin (100 μg/ml) cultures. The cells were grown for 36 h at 25 °C and harvested by centrifugation for 10 min at 10,000 rpm in a Beckman JA-10 rotor. The harvested cells were lysed in 100 ml of BugBuster® HT (Novagen) containing 4% (w/v) Triton X-100, 4% (w/v) glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 mM benzamidine, and one protease inhibitor mixture tablet (Roche Diagnostic GmbH). The lysed cells were then subjected to one pass through a Thermo Spectronic French pressure cell at 1000 p.s.i., and further incubated at 25 °C for 30 min to ensure micelle formation.

The resulting cell lysate was centrifuged at 20,000 rpm for 1 h in a Beckman JA-20 rotor. The supernatant was retained, and the resulting pellet was resuspended in 50 mM Tris-HCl (pH 7.8) containing 300 mM NaCl, 4% (w/v) Triton X-100, 4% (w/v) glycerol, and 10 mM benzamidine and incubated at 25 °C for 30 min to promote formation of detergent micelles. The resuspended pellet was again centrifuged, and the supernatants from both extractions were pooled and applied to an Ni-NTA-agarose column (3 x 3 cm) equilibrated in 50 mM Tris-HCl (pH 7.8) containing 2 mM imidazole, 300 mM NaCl, and 0.1% (w/v) Triton X-100. The column was then washed with 10 column volumes of the same buffer containing 20 mM imidazole. The protein was eluted in 50 mM Tris-HCl (pH 7.8) containing 400 mM imidazole, 30 mM NaCl, and 0.1% (w/v) Triton X-100. The fractions containing ShuA, as determined by SDS-PAGE, were pooled and applied to a S-300 Sephacryl size-exclusion column (1 x 15 cm) equilibrated with 20 mM Tris-HCl (pH 7.8) containing 0.1% (w/v) Triton X-100 and 100 mM NaCl. The peak fractions were pooled, and the protein was then concentrated over PBE 94 Polybuffer™ Exchanger (Amersham Biosciences) chromatofocusing column (1.5 x 3.5 cm) equilibrated with 0.052 M imidazole-HCl (pH 7.4) containing 0.1% (w/v) Triton X-100. ShuA was eluted from the column with Polybuffer™ 74 (Amersham Biosciences) at a pH of 5.4, which corresponded to the calculated pI. The fractions containing ShuA were pooled and dialyzed against 20 mM Tris-HCl (pH 7.8) containing 0.1% (w/v) Triton X-100. The protein was further purified and concentrated over a DEAE column equilibrated with 20 mM Tris-HCl (pH 7.8) containing 30 mM octyl-β-D-glucopyranoside. The purified protein eluted from the column in 20 mM Tris-HCl (pH 7.8) containing 500 mM NaCl and 30 mM octyl-β-D-glucopyranoside. Protein concentrations were determined by absorbance at 750 nm using the Bio-Rad DC protein assay.

N-terminal sequencing of the purified ShuA was carried out at the Stanford Protein and Nucleic Acid Biotechnology Center, Beckman Center (Stanford, CA).

**Lipid Bicelle Formation—** The purified ShuA protein was exchanged by successive dialysis into 50 mM HEPES (pH 7.0) containing 30 mM octyl-β-D-glucopyranoside. An 80:20 ratio of lipids 1,2-dimyristoyl-sn-glycero-3-phosphocholine to 1,2-dihexanoyl-sn-glycero-3-phosphocholine was prepared in 50 mM HEPES (pH 7.0) containing 30 mM octyl-β-D-glucopyranoside. Purified ShuA was added to achieve a final lipid to protein ratio of 500:1 (w/w). The lipid-protein solution was incubated at 25 °C for 30 min. The mixed bicelles were then dialyzed against
Outer Membrane Heme Receptor ShuA of *S. dysenteriae*

20 mM HEPES (pH 7.0) containing 150 mM NaCl. The dialysis buffer was changed 3 times at 24-h intervals (24, 25). The protein-lipid bicelles were stored for 2 weeks at 4 °C under nitrogen or at −80 °C.

Expression and Purification of TonB—E. coli M15(pREP4) cells were transformed with pND34 and grown on LB-ampicillin (100 μg/ml) and kanamycin (50 μg/ml) plates overnight. A 250-ml inoculum in LB-Amp/Kan was grown at 37 °C to an *A*$_{600}$ of 0.6. The subculture was then used to inoculate 1-liter LB-Amp/Kan cultures. The cells were grown to an *A*$_{600}$ of 0.6, and protein expression was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 1 mM. The cultures were grown for an additional 4 h at 25 °C, and the cells were harvested by centrifugation at 10,000 rpm for 10 min.

The harvested cells were lysed, and TonB was solubilized as previously described for ShuA (see above). The resulting supernatant was applied to a Q-Sepharose column (1 × 30 cm) previously equilibrated in 50 mM Tris-HCl (pH 7.8) containing 0.1% (w/v) Triton X-100. The column was washed with 10 column volumes of the same buffer containing 50 mM NaCl. The TonB protein was eluted from the column with a gradient from 50 to 500 mM NaCl. The fractions containing TonB, as determined by SDS-PAGE, were pooled and applied to a S300 Superdex size-exclusion column (1 × 15 cm) equilibrated with 20 mM Tris-HCl (pH 7.8) containing 0.1% (w/v) Triton X-100 and 100 mM NaCl. TonB protein concentrations were determined by Bio-Rad DC protein assay.

Secondary Structure as Measured by CD Spectroscopy—CD spectra were recorded for the wild-type and mutant ShuA proteins in octyl-β-D-glucopyranoside micelles and for the wild-type ShuA in 1,2-dimyrstoyl-sn-glycerol-3-phosphocholine:1,2-dihexanoyl-sn-glycerol-3-phosphocholine lipid bicelles. All samples were recorded at 25 °C in 10 mM HEPES (pH 7.0) on a JASCO J-810 spectropolarimeter from 190 to 260 nm with 0.2-nm resolution and 1.0-cm bandwidth. The mean residue ellipticity (deg cm$^2$ dmol$^{-1}$) was calculated using CDPRO software supplied by the manufacturer.

Heme Binding to ShuA—Heme binding was observed by difference (Δ absorbance) spectroscopy between 300 and 700 nm. Heme binding reactions were carried out in 1-ml reaction volumes containing 5 μM ShuA in 20 mM Tris-HCl (pH 7.6), 30 mM octyl-β-D-glucopyranoside, and 5 μM heme. UV-visible absorbance spectra were recorded at 60-s intervals over a 30-min time period. The rate of heme association with ShuA was determined by measuring the Δ*A*$_{437}$, as a function of time and fit to a first-order reaction. Heme stoichiometry was determined by using the pyridine hemochrome method (26). Heme concentration was determined by measuring the absorbance at 418.5, 526, and 555 nm following the addition of dithionite using the extinction coefficients of 170, 17.5, and 34.4 mM$^{-1}$ cm$^{-1}$. The protein concentration was calculated based on the empirically calculated extinction coefficient of 117.7 mM$^{-1}$ cm$^{-1}$ (ExPASy). Protein concentrations, calculated utilizing the empirically calculated extinction coefficient, were in close agreement with protein concentrations measured with the Bio-Rad DC protein assay. Attempts to calculate the extinction coefficient for the native protein by measuring the absorbance of the unfolded protein and the empirically calculated extinction coefficient were unsuccessful due to the tendency of the protein to remain insoluble on unfolding.

Fluorescence quenching of tryptophan residues was also monitored to determine the heme stoichiometry of ShuA. The protein was excited at a wavelength of 295 nm, and the emission spectrum at 338 nm was recorded on a PerkinElmer Life Sciences LS-50 luminescence spectrometer. Fluorescence emission spectra of ShuA (1 μM) in 20 mM Tris-HCl (pH 7.8) 30 mM octyl-β-D-glucopyranoside were measured over a heme concentration range of 0.01 to 30 μM.

Size-exclusion Chromatography of metHb$_{dimer}$ and metHb$_{tetramer}$ as Heme Substrates for ShuA—metHb$_{dimer}$ (α$_1$β$_1$) was produced by incubation of metHb$_{tetramer}$ (α$_1$β$_1$/α$_2$β$_2$) at 37 °C (27). The quaternary structure of human hemoglobin at 25 °C and 37 °C was confirmed by fast-protein liquid chromatography on a Superdex S200 HR 10/30 column equilibrated in 20 mM Tris-HCl (pH 7.8). Molecular mass markers were also run at both temperatures to generate a standard curve in the range of 669–17 kDa. At the higher temperature the methemoglobin substrate exists as the αβ-dimer, whereas at 25 °C the tetramer was the only species observed (data not shown).

Size-exclusion Separation of the Wild-type and Mutant ShuA Proteins from Hemoglobin following Heme Transfer—The wild-type or His mutant proteins (10 μM) were incubated at 25 °C with 1 μM metHb$_{tetramer}$ for 20 min. ShuA was separated from metHb$_{tetramer}$ over a Sephadex S300 column (1 × 15 cm) equilibrated in 20 mM Tris-HCl (pH 7.8) containing 30 mM β-D-glucopyranoside. Fractions containing ShuA as determined by SDS-PAGE were analyzed by UV-visible spectroscopy. The Soret maxima of the ShuA, and hemoglobin proteins were recorded prior too and following incubation and fast-protein liquid chromatography separation. The absorbance values following chromatography were corrected for any dilution factor. The transfer of heme from metHb$_{tetramer}$ to ShuA was determined by the relative change in absorption of the heme in both ShuA and hemoglobin.

Kinetic Measurement of Heme Transfer from metHb$_{tetramer}$, metHb$_{dimer}$, or Myoglobin—The rate of heme transfer from metHb$_{tetramer}$, metHb$_{dimer}$, omy-Hb, or metMb was measured by stopped-flow spectrometric methods on an Applied Photophysics SX18MV instrument. MetHb$_{tetramer}$, metHb$_{dimer}$, or metMb (100 μM) were prepared in 20 mM Tris-HCl (pH 7.8) containing 500 mM NaCl and 30 mM octyl-β-D-glucopyranoside. The reduced deoxy-Hb$_{tetramer}$ was prepared in a Plas Labs anaerobic chamber by the addition of dithionite to the metHb$_{tetramer}$ solution. Excess reductant was removed over a Sephadex G-25 column, and the ferrous complex was then exposed to air to generate the omy-Hb complex. Reactions were initiated with ShuA (10 μM) in 20 mM Tris-HCl (pH 7.8) containing 500 mM NaCl and 30 mM octyl-β-D-glucopyranoside in syringe A and metHb$_{tetramer}$ (0.25 μM), metHb$_{dimer}$ (0.5 μM), omy-Hb (0.25 μM), or metMb (1 μM) in syringe B. Single wavelength absorbance changes at 413 nm versus time were recorded until no further change in the absorbance was noted. The data for metHb was collected at 25 °C (metHb$_{tetramer}$) and 37 °C (metHb$_{dimer}$) and fit using Pro-Kineticist software supplied by Applied Photophysics.
In Vitro Cross-linking of ShuA and Hemoglobin—ShuA in 20 mM Tris-HCl (pH 7.8) containing 30 mM octyl-$\beta$-D-glucopyranoside was incubated with an equimolar ratio of hemoglobin in a final reaction volume of 500 l at a total protein concentration of 1 mg/ml. The cross-linking agent 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was added at a final concentration of 10 mg/ml, and the samples were incubated at 25 °C for 10 min. The reactions were terminated by the addition of an equal volume of SDS-PAGE loading buffer (28).

Samples were separated on a 4–15% Tris-HCl pre-cast gel (Bio-Rad) and transferred to a polyvinylidene difluoride membrane probed with polyclonal rabbit anti-ShuA antibodies at 1:2000 dilution.

Protein-Protein Interactions of the ShuA and TonB Proteins—The His-tagged hol-ShuA, apo-ShuA, or H86A/H420A with TonB were mixed in 2:1 TonB:ShuA molar equivalents. ShuA (100 μg) in 50 μl of Tris-HCl (pH 7.8) containing 30 mM octyl-$\beta$-D-glucopyranoside was added to 100 μl of Ni-NTA resin previously equilibrated and incubated at 4 °C with end-over-end shaking for 20 min. The full-length purified TonB (300 μg) in 20 mM Tris-HCl (pH 7.8) containing 0.1% (w/v) Triton X-100 and 100 mM NaCl was added, and the mixture was incubated for a further 20 min. The resin-bound proteins were then washed ($\times 5$) in 200 μl of 20 mM Tris-HCl (pH 7.8) containing 2 mM imidazole, 300 mM NaCl, and 0.1% (w/v) Triton X-100. The resin-bound proteins were eluted ($\times 2$) in 200 μl of 50 mM Tris-HCl (pH 7.8) containing 400 mM imidazole, 30 mM NaCl, and 0.1% (w/v) Triton X-100. Protein-protein interaction and complex formation were determined by SDS-PAGE analysis of the eluted fractions. The ShuA H86A/H420A mutant was utilized in experiments requiring apo-ShuA due to the inability to obtain the purified wild-type receptor free of heme. Control reactions were carried out without TonB alone to ensure that the protein is efficiently eluted under the wash conditions.

Construction of the ShuA Structural Model—The model was predicted using the XM3 program developed at the Center for Biological Sequence Analysis, BioCenter, The Technical University of Denmark, DK-2800 Lyngby, Denmark (29). A large sequence data base consisting of all of the known structures in the NCBI PDP data base was iteratively searched to find a sequence template from proteins with known structure with an $E$ value of $<0.05$. The method differs from the PDB-BLAST method in that a sequence profile is only made if a template is not readily found in the data base of known structures. Query and template sequences are subsequently aligned using a score based on profile-profile comparisons. Using this
approach the template selected was the ferric-enterobactin receptor (FepA) structure (PDB entry 1UJW), which bears 22% identity to ShuA. The query was then aligned to the template using a local alignment algorithm with a maximum number of gaps set to 20, a first gap penalty of 11, and a gap elongation penalty of 1 (30). The corresponding atoms derived from the alignment were extracted from the template file and used as a starting point for the homology modeling. Missing atoms were added using the segmod program (31) from the GeneMine package (Bioinformatics, UCLA). The structure was then refined using the encad program (32) also from the GeneMine package.

RESULTS

Expression, Purification, and Characterization of the Wild-type, H86A, H420A, and H86A/H420A ShuA Proteins—Analysis of the full-length ShuA protein sequence with the SignalP program (ExPASy) identified a signal peptide cleavage site between amino acid residues 28 and 29 (Fig. 1A). This signal peptide was replaced with the E. coli N-terminal PelB signal peptide upon construction of the expression vector that allowed secretion of the protein to the outer membrane. Following solubilization and purification of the protein a single band of molecular mass 73 kDa was observed on SDS-PAGE analysis (Fig. 1B, inset). N-terminal sequencing of the purified protein confirmed that the PelB signal peptide had been cleaved during cellular processing with the first five amino acids corresponding to Thr-Glu-Thr-Met-Thr.

The UV-visible absorbance spectrum of the purified ShuA in octyl-β-D-glucopyranoside detergent micelles had a Soret maximum at 413 nm, presumably due to residual heme bound to the receptor (Fig. 1B). Reconstitution of the protein by titrating with heme and monitoring the decrease in tryptophan fluorescence at 338 nm as a function of heme concentration gave a stoichiometry of one heme molecule per ShuA monomer (data not shown). However, an accurate Kd could not be determined due to the inability to remove the residual heme from the receptor. The Soret maximum upon reconstitution of the protein with exogenously added heme shifted the Soret from 413 to 437 nm with visible bands at 542 nm and 566 nm (Fig. 2A). It was also noted that upon addition of a 1:1 molar ratio of heme to protein a complete shift of the Soret to 437 nm occurred over a 20-min time period (data not shown). As will be outlined below the substrate specificity and manner in which the heme is delivered to the receptor is critical, and “free” heme is not a physiologically relevant substrate.

To determine if the shift in Soret was the result of structural perturbation on solubilization of the receptor into detergent micelles, the heme-ShuA complex was incorporated into mixed lipid bicelles to better mimic the outer membrane (33). In contrast to the heme-ShuA complex in detergent micelles, the spectrum of the reconstituted heme complex in lipid bicelles gave a broad Soret maximum at 402 nm (Fig. 2B). The CD spectra of ShuA in detergent micelles or lipid bicelles indicates that the cause of the differences in Soret maxima was not due to secondary structure perturbations (Fig. 3A).

Transfer of Heme from metHbetramet, metHbdimet, and Myoglobin to Wild-type, H86A, H420A, and H86/H420A ShuA—The Soret maximum observed for the reconstituted heme complex in lipid bicelles gave a broad Soret maximum at 402 nm (Fig. 2B). The CD spectra of ShuA in detergent micelles or lipid bicelles indicates that the cause of the differences in Soret maxima was not due to secondary structure perturbations (Fig. 3A).

Transfer of Heme from metHbetramet, metHbdimet, and Myoglobin to Wild-type, H86A, H420A, and H86/H420A ShuA—The Soret maximum observed for the reconstituted heme-ShuA complex in either detergent or lipid was significantly different than that of the residual
heme bound on purification of ShuA. This led us to hypothesize that free heme is not the physiological substrate for the ShuA receptor. Therefore, we tested hemoglobin (metHb<sub>tetramer</sub>), hemoglobin dimer (metHb<sub>dimer</sub>), and myoglobin as potential substrates for heme transfer to ShuA. Interestingly, the first notable observation on incubation of ShuA with metHb<sub>tetramer</sub> was a band on Western blot analysis corresponding to a molecular weight of hemoglobin plus ShuA (Fig. 3A). This suggested that metHb is the physiological substrate for the ShuA outer membrane receptor.

Direct evidence of a protein-protein interaction between ShuA and metHb<sub>tetramer</sub> was confirmed upon incubation of ShuA and hemoglobin in the presence of the cross-linker EDC. Following reaction with EDC a band corresponding to the molecular weight of hemoglobin plus ShuA was observed on Western blot analysis with anti-ShuA polyclonal antibodies (Fig. 4). Similarly, the double mutant H86A/H420A ShuA also gave a band on Western blot analysis corresponding to a ShuA-Hb complex. However, the weaker band observed for the H86A/H420A ShuA mutant was most likely due to the inability to extract heme from Hb resulting in a destabilization of the ShuA-Hb complex (Fig. 4). These data would suggest that heme is also critical in formation of the ShuA-Hb complex. In contrast, reaction of ShuA with bovine serum albumin under identical conditions did not yield a band indicative of a protein-protein interaction (Fig. 1B).

Significant sequence identity (65–25%) exists within the outer membrane heme receptors of Gram-negative organisms (Fig. 1A). Sequence alignment of ShuA with a number of outer membrane heme receptors revealed two absolutely conserved histidine residues, corresponding to His-86 and His-420, in the mature ShuA protein. It has previously been shown that the corresponding histidine residues in the homologous HemR receptor of Yersinia enterocolitica and HasR of Serratia marcescens are required for heme uptake in vivo (34, 35). Site-directed mutagenesis of His-86 and His-420 either singly or in combination was carried out to address the specific role of these conserved residues in heme binding and transport.

Initial analysis of the purified H86A, H420A, and H86A/H420A ShuA proteins by CD spectroscopy indicated that all three of the mutants retained a similar secondary structure to the wild-type ShuA in lipid bicelles. The spectrum of the H86A/H420A ShuA protein is shown in Fig. 3B. Although the heme content and spectra of the H86A and H420A mutants upon purification were similar to that of the wild-type ShuA, they were unable to efficiently extract heme from metHb<sub>tetramer</sub>. Incubation of ShuA with metHb<sub>tetramer</sub> followed by separation of the proteins on size-exclusion chromatography, showed that heme was efficiently transferred from metHb<sub>tetramer</sub> to the wild-type ShuA (Fig. 5). However, incubation of either H86A or H420A ShuA with metHb<sub>tetramer</sub> resulted in no heme transfer from metHb<sub>tetramer</sub> to the receptor (Fig. 5). The H86A/H420A double mutant, which had no detectable heme bound to the protein on purification, as expected was unable to extract heme from metHb<sub>tetramer</sub> (data not shown). Calculation of the heme content by the pyridine hemochrome method indicated a heme to protein molar ratio of 1:1 in agreement with the previous fluorescence titration studies. In contrast the H86A and H420A mutants revealed a 0.25:1 molar ratio in agreement with the kinetic studies, which indicate that both residues are required for extraction and ligation of the heme.

**Kinetics of Heme Transfer from Hemoglobin and Myoglobin to the Wild-type, H86A, H420A, and H86A/H420A ShuA Mutants**—The absorbance time course for the transfer from metHb<sub>tetramer</sub> or metHb<sub>dimer</sub> to wild-type ShuA indicated that the transfer was rapid and unidirectional (Fig. 6, A and B). All heme-protein substrates analyzed had significantly increased rates of heme transfer on the order of ~10<sup>4</sup> times faster compared with the rate of free heme association with the receptor (Table 1). The transfer from metHb<sub>tetramer</sub> to ShuA was again on the order of ~10<sup>4</sup> faster than from either oxy-Hb or metMb. The slow rate of transfer from oxy-Hb most likely reflects the rate of oxidation to the metHb<sub>tetramer</sub> prior to transfer. These data suggest that hemoglobin in the tetramer or dimer is a viable physiological substrate, and the preferred oxidation state of the heme-iron is ferric (Fe<sup>3+</sup>), which is consistent with previous reports.

Interestingly, heme transfer did not occur from metHb<sub>tetramer</sub> to either the single or double His-mutant proteins. However, transfer from metHb<sub>dimer</sub> to H86A and H420A showed bipha-
sic kinetics (Fig. 6, C and D). The initial time course we attribute to heme transfer to ShuA, and the second phase represents the back transfer from the ShuA mutants to metHb\textsubscript{dimer}. The apparent rate constants for the forward reaction from metHb\textsubscript{dimer} to either H86A or H420A ShuA are similar to the wild-type ShuA (Table 1). However, loss of heme from the receptor occurs at a significant rate. The inability of the His mutants to extract heme from metHb\textsubscript{tetramer}, together with the observed rates for heme transfer from metHb\textsubscript{dimer}, merely reflects a distribution of heme based on the comparative heme affinities of the ShuA mutants with metHb\textsubscript{dimer}. The heme equilibrium between ShuA H86A or H420A and metHb\textsubscript{dimer} is most likely a result of the decreased heme affinities of metHb\textsubscript{dimer}, α- and β-subunit versus the values for the metHb\textsubscript{tetramer}. The rate of heme dissociation from the hemoglobin α-subunit in metHb\textsubscript{dimer} versus metHb\textsubscript{tetramer} has been reported to increase from $1.08 \times 10^3$ to $2.16 \times 10^3$ s$^{-1}$, and for the β-subunit from $5.4 \times 10^2$ to $5.4 \times 10^4$ s$^{-1}$ (27). Although, the direct interaction of ShuA with Hb most likely involves a conformational change that promotes heme transfer, the increased dissociation constants for the Hb\textsubscript{dimer} versus Hb\textsubscript{tetramer} with the lower binding affinities of the single His-ShuA mutant would allow for back transfer of the heme.

The double mutant, H86A/H420A ShuA, was unable to extract heme from either metHb\textsubscript{tetramer} or metHb\textsubscript{dimer}. Furthermore, residual heme in the ShuA H86A/H420A receptor on purification (which was undetectable by UV-visible spectroscopy) was released upon incu-

**TABLE 1**

| ShuA protein | Heme substrate | $k_{H}$ | $k_{H^{-1}}$ |
|--------------|----------------|--------|-------------|
| Wild type    | Heme           | $1.4 \times 10^{-4}$ | ND*        |
|              | Oxymoglobin (tetramer) | $9.46 \times 10^{-3}$ | 0          |
|              | Methemoglobin (tetramer)$^{a}$ | 21 | 0          |
|              | Methemoglobin (dimer)$^{a}$ | 32 | 0          |
| H86A         | Metmyoglobin   | $1.3 \times 10^{-3}$ | 0          |
| H420A        | Methemoglobin (tetramer) | 0 | 0          |
|              | Methemoglobin (dimer) | 20 | 0.3        |
| H86A/H420A   | Methemoglobin (tetramer) | 0 | 1.00       |
|              | Methemoglobin (dimer) | 9.7 | 0.13       |
|              | Methemoglobin (dimer) | 0 | 0.16       |

$^{a}$ ND, not determined.
$^{b}$ Reactions with methemoglobin dimer were performed at 37 °C.
$^{c}$ Reactions with methemoglobin tetramer were performed at 25 °C.
The ability to utilize heme as an iron source has led to the evolution of a number of receptor-mediated pathways by which bacterial pathogens exploit hemeproteins such as hemoglobin, hemopexin, and the hemoglobin-haptoglobin complex (2, 3). Many outer membrane receptors such as HemR of Y. enterocolitica are able to utilize heme from a wide variety of hemoproteins (34). Still other receptors are limited to a particular hemoprotein (34, 36–38). The specificity of these receptors may in part be due to the infectivity profile and physiology of the invading pathogen. Shigella dysenteriae colonizes the intestinal mucosa where heme in the form of hemoglobin is a readily available source of heme from dietary intake, as well as hemo- lytic damage due to the shearing of cells from the intestinal surface layers. Additionally, S. dysenteriae infections can cause hemolytic uremia syndrome characterized by gastrointestinal bleeding (18). This would lead to methHb tetramer being a readily available source of iron to the invading bacteria.

In the present report we show that the S. dysenteriae outer membrane receptor ShuA has high specificity for methHb as the heme substrate. Heme transfer from methHb tetramer is on the order of ~10^4 faster than that from either oxy-Hb or methMb. The association of free heme with ShuA involves release of the hydrophobic heme molecule from the lipid/detergent phase to the receptor and, therefore, may not be directly comparable to transfer of heme from the water-soluble hemoglobin to ShuA. However, the data do highlight that free heme association is ~10^5 slower than transfer from methHb tetramer, and most likely does not reflect a physiological mechanism. In addition the broad Soret absorption observed on the addition of free heme to the ShuA receptor suggests nonspecific binding of heme to the protein. In contrast heme supplied in the form of methHb tetramer or methHb dimer yielded a spectrum and Soret maxima identical to that of the heme complex following purification of the protein. This suggests that the receptor has specificity that requires delivery of the heme from a protein scaffold. The specificity of heme transfer from methHb tetramer to ShuA was confirmed by complex formation in the presence of the cross-linking reagent EDC. Taken together these data suggest that the oxidation state of the iron is critical, and a specific protein-protein interaction induces a conformational change triggering heme transfer to ShuA. Furthermore, the pathophysiology of S. dysenteriae infection (18, 19), and the fact that red cell lysis results in the rapid autoxidation of the oxy-Hb tetramer to the methHb tetramer and eventually methHb dimer (27, 39), is consistent with methHb in the tetrameric or dimeric form being the substrate for ShuA. Previous studies of the Gram-positive Streptococcus pyogenes outer membrane lipoprotein (Shp) have also shown a preference for heme from Hb (40, 41). Furthermore, the holo-Shp transfers heme to the heme specific ABC-transporter HtsA (42).

At the present time there are no available three-dimensional structures of an outer membrane heme receptor from any Gram-negative pathogen. However, the high sequence identity (22–30%) with the well characterized siderophore outer membrane receptors suggests that the overall fold will be similar (43–46). The siderophore outer membrane receptors have a unique and highly conserved β-barrel pore that is closed off from the periplasmic face by an N-terminal plug that sits within the barrel. On the extracellular face of the barrel are a series of extended loops. It is proposed that the loops play an important role in binding the substrate heme to the outer membrane receptor and the loop that is in contact with the inner membrane receptor may be involved in the transfer of heme to the inner membrane receptor. The importance of the loop that is in contact with the inner membrane receptor suggests that it is involved in the transfer of heme to the inner membrane receptor. The loop that is in contact with the inner membrane receptor may be involved in the transfer of heme to the inner membrane receptor. The loop that is in contact with the inner membrane receptor may be involved in the transfer of heme to the inner membrane receptor. The loop that is in contact with the inner membrane receptor may be involved in the transfer of heme to the inner membrane receptor. The loop that is in contact with the inner membrane receptor may be involved in the transfer of heme to the inner membrane receptor.
role in the initial recognition of the ligand (44, 47, 48). A number of in vivo studies in which a series of loop deletions in the cobalamin outer membrane receptor (BtuB) of E. coli (49) or the hemoglobin receptor (HmbR) of Neisseriae meningitidis (48) have shown that specific loops are involved in ligand binding, whereas others are essential for transport. Although there appears to be a common mechanism for ligand acquisition and transport, subtle differences in the recognition and coordination of diverse substrates account for the ligand selectivity and specificity of the receptors (49).

Sequence alignment of ShuA with other known heme-binding outer membrane receptors revealed two conserved histidine residues, His-86 and His-420 in ShuA, that correspond to residues previously identified by in vivo transport studies to be critical for heme transport (34). A structural similarity model of ShuA was constructed based on the homologous (22% identity) enterobactin receptor FepA of E. coli. Following validation and evaluation of the model the conserved His-86 and His-420 of ShuA are predicted to be on the extracellular face of the N-terminal plug and loop 7 (L7), respectively (Fig. 8). In the current study the H86A, H420A, and H86A/H420A proteins were purified and characterized in terms of their substrate specificity, ligand coordination, and ShuA-TonB complex formation. The initial in vitro characterization of the mutant proteins is consistent with previous in vivo studies, in that none of the mutants were capable of utilizing heme from metHb tetramer. However, consistent with the observation that residual heme is associated with H86A and H420A, both mutants were able to capture heme, albeit transiently from the metHb dimer (Table 1 and Fig. 5). However, at a steady-state equilibrium heme is distributed between the proteins based on their relative heme affinities (Fig. 5). The significant rate of back transfer of heme to Hb dimer indicates that the coordination of histidine residues may interact with the heme propionates, it is likely that such interactions may arise from other critical loops on the extracellular face of the receptor. The coordination between the extracellular face of the N-terminal plug and the loops would then close off the heme-binding pocket from the extracellular environment.

This hypothesis is consistent with the kinetic data in which there is no heme transfer from metHb tetramer to any of the His mutants. Additionally, the observed equilibrium of heme between the H86A or H420A ShuA mutants and metHb dimer suggests that the mono-His ligation does not stabilize the heme within the receptor. Furthermore, the inability of the H86A/H420A ShuA mutant to extract heme from either the intact metHb tetramer or lower affinity metHb dimer confirms that both residues are critical in the capture and stabilization of heme in the extracellular heme binding site of the receptor.

In a recent study of the corresponding histidine mutants (His-73 and His-603) of the outer membrane receptor HasR of Serratia marcescens it was observed that in vivo the single mutants were able to utilize heme from the hemophore (HasA) only at significantly higher heme concentrations (35). In contrast the double mutant was not able to utilize heme even at high heme concentrations. The in vivo studies with HasR are consistent with the present in vitro data, where bacteria expressing the single His mutant proteins, which cannot extract heme efficiently, are able to survive when given significantly higher heme concentrations than is required for bacteria expressing the wild-type protein. However, the double mutant is unable to bind heme, and therefore as would be expected bacteria expressing the double His-mutant receptor cannot survive on heme as the sole source of iron.

In the present study it is significant that in the absence of TonB the ShuA receptor is competent to capture heme from metHb tetramer. This is consistent with a previous report in which the purified HasR receptor of S. marcescens was shown to extract heme from the soluble HasA hemophore in the absence of TonB (35). In contrast to previous in vitro studies in which...
the HasA-HasR complex on formation appeared to be irreversible, the interaction of hemoglobin with the ShuA receptor was clearly a reversible interaction. The reversible nature of the protein-protein interaction was evidenced by the fact that, to obtain the spectra of the heme-ShuA complex following transfer of heme from Hb (Fig. 5), the Hb substrate was separated from ShuA by size-exclusion fast-protein liquid chromatography. As we have previously suggested for the transfer of heme from the heme carrier PhuS to the iron-regulated heme oxygenase (pa-HO) of P. aeruginosa, transfer is most likely driven by the free energy yield upon protein-protein interaction (22). Such interactions presumably involve conformational changes that induce coordination and/or spin state changes that trigger the transfer of heme.

The current model for ligand transport through the outer membrane receptor, once the ligand is coordinated, requires interaction of TonB with the periplasmic surface of the receptor. Coupling of the cytoplasmic membrane potential to the TonB-receptor complex then drives transport of the ligand through the outer membrane (43, 44, 51). The data described herein have extended earlier studies in which truncated soluble fragments of TonB were shown to interact directly with the outer membrane receptor (32, 33). In the present report the expression and purification of a full-length TonB has been shown to interact with the wild-type holo-ShuA and apo-H86A/H420A ShuA mutant as a 1:1 complex. These data confirm that mutation of either or both of the residues required for heme binding at the extracellular face of the receptor does not cause any significant conformational change that compromises the interaction with TonB. Furthermore, it is apparent that ShuA can interact with TonB in either the holo- or apo-form, as has previously been noted for the ferric hydroxamate receptor HasA-HasR complex on formation appeared to be irreversible (10). In contrast to previous reports our data suggest that the full-length TonB forms a 1:1 complex, which is consistent with recent crystal structures of FhuA and BtuB with the C-terminal soluble fragments of TonB (54, 55). The 1:1 complex observed in the crystal structures would appear to preclude a second TonB molecule binding at a lower affinity site. Therefore, the present preliminary data of a 1:1 ShuA-TonB complex would seem to be physiologically relevant, given that release of substrate to the periplasm most likely involves a protein-protein interaction with the periplasmic binding protein (56). We are currently testing this hypothesis by using a variety of biophysical and biochemical approaches.

REFERENCES

1. Braun, V. (2005) Contrib. Microbiol. 12, 210–233
2. Wandersman, C., and Delepelaire, P. (2004) Annu. Rev. Microbiol. 58, 611–647
3. Wandersman, C., and Stojilkovic, I. (2000) Curr. Opin. Microbiol. 3, 215–220
4. Potelle, K. G., and Kadner, R. J. (2003) Mol. Microbiol. 49, 869–882
5. Davidson, A. L. (2002) J. Bacteriol. 184, 1225–1233
6. Koster, W. (2005) Front Biosci. 10, 462–477
7. Higgins, C. F. (2003) Res. Microbiol. 152, 205–210
8. Stojilkovic, I., and Hantke, K. (1992) EMBO J. 11, 4359–4367
9. Stojilkovic, I., and Hantke, K. (1994) Mol. Microbiol. 13, 719–732
10. Rossi, M. S., Fetherston, J. D., Letoffe, S., Carniel, E., Perry, R. D., and Ghigo, J. M. (2001) Infect. Immun. 69, 6707–6717
11. Stojilkovic, I., Hwa, V., de Saint Martin, L., O’Gaora, P., Nassif, X., Heffron, F., and So, M. (1995) Mol. Microbiol. 15, 531–541
12. Stojilkovic, I., Larson, J., Hwa, V., Anic, S., and So, M. (1996) J. Bacteriol. 178, 4670–4678
13. Turner, P. C., Thomas, C. E., Elkins, C., Clary, S., and Sparling, P. F. (1998) Infect. Immun. 66, 5215–5223
14. Henderson, D. P., and Payne, S. M. (1994) Infect. Immun. 62, 5120–5125
15. Ochsner, U. A., Johnson, Z., and Vasil, M. L. (2000) Microbiology 146, 185–198
16. Letoffe, S., Ghigo, J. M., and Wandersman, C. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9876–9880
17. Letoffe, S., Redeker, V., and Wandersman, C. (1998) Mol. Microbiol. 28, 1223–1234
18. Cherla, R. P., Lee, S. Y., and Tesh, V. L. (2003) FEMS Microbiol. Lett. 228, 159–166
19. Sansonetti, P. J. (2001) FEMS Microbiol. Rev. 25, 3–14
20. Wilks, A. (2001) Arch. Biochem. Biophys. 387, 137–142
21. Wyckoff, E. E., Lopreato, G. F., Tipton, K. A., and Payne, S. M. (2005) J. Bacteriol. 187, 5658–5664
22. Lansky, I. B., Lukat-Rodgers, G. S., Block, D., Rodgers, K. R., Ratliff, M., and Wilks, A. (2006) J. Biol. Chem. 281, 13652–13662
23. Mills, M., and Payne, S. M. (1995) J. Bacteriol. 177, 3004–3009
24. Fanucci, G. E., Cadieux, N., Kadner, R. J., and Cafiso, D. S. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 11382–11387
25. Mimms, I. T., Sampighi, G., Nozaki, Y., Tanford, C., and Reynolds, J. A. (1981) Biochemistry 20, 833–840
26. Fujihiro, I. H., and Smith, K. M. (eds) (1975) Porphyins and Metalloporphyins, pp. 804–808, Elsevier, Amsterdam
27. Hargrove, M. S., Whitaker, T., Olson, J. S., Vail, R. J., and Mathews, A. J. (1997) J. Biol. Chem. 272, 17385–17389
28. Lamb, A. L., Torres, A. S., O’Halloran, T. V., and Rosenzweig, A. C. (2000) Biochemistry 39, 14720–14727
29. Lund, O., Nielsen, M., Lundegaard, C., and Worning, P. (2002) CASPS Conference A102
30. Smith, T. F., and Waterman, M. S. (1981) J. Mol. Biol. 147, 195–197
31. Land, M. (1992) J. Mol. Biol. 226, 507–533
32. Levitt, M., Hirschberg, M., Sharon, R., and Dagget, V. (1995) Comput. Phys. Commun. 91, 215–231
33. Fanucci, G. E., Lee, J. Y., and Cafiso, D. S. (2003) J. Am. Chem. Soc. 125, 13932–13933
34. Bracken, C. S., Baer, M. T., Abdur-Rashid, A., Helms, W., and Stojilkovic, I. (1999) J. Bacteriol. 181, 6063–6072
35. Izadi-Pruneyre, N., Huche, F., Lukat-Rodgers, G. S., Lecroisey, A., Gilli, R., Rodgers, K. R., Wandersman, C., and Delepelaire, P. (2006) J. Biol. Chem. 281, 25541–25550
36. Cope, L. D., Yogev, R., Muller-Eberhard, U., and Hansen, E. J. (1995) J. Bacteriol. 177, 2644–2653
37. Jin, H., Ren, Z., Whibey, P. W., Morton, D. J., and Stull, T. L. (1999) Microbiology 145, 905–914
38. Perkins-Balding, D., Ratliff-Griffin, M., and Stojilkovic, I. (2004) Microbiology 150, 141–147; table of contents
39. Bunn, H. F., and Forget, B. G. (1986) Hemoglobin: Molecular, Genetic, and Clinical Aspects, pp. 634–662, W. B. Saunders Co., Philadelphia
Outer Membrane Heme Receptor ShuA of S. dysenteriae

40. Nygaard, T. K., Liu, M., McClure, M. J., and Lei, B. (2006) BMC Microbiol. 6, 82
41. Srikumar, R., Mikael, L. G., Pawelek, P. D., Khamessan, A., Gibbs, B. F., Jacques, M., and Coulton, J. W. (2004) Microbiology 150, 1723–1734
42. Nygaard, T. K., Blouin, G. C., Liu, M., Fukumura, M., Olson, J. S., Fabian, M., Dooley, D. M., and Lei, B. (2006) J. Biol. Chem. 281, 20761–20771
43. Buchanan, S. K., Smith, B. S., Venkatramani, L., Xia, D., Esser, L., Palnitkar, M., Chakraborty, R., van der Helm, D., and Deisenhofer, J. (1999) Nat. Struct. Biol. 6, 56–63
44. Cobessi, D., Celia, H., Folschweiler, N., Schalk, I. J., Abdallah, M. A., and Pattus, F. (2005) J. Mol. Biol. 347, 121–134
45. Ferguson, A. D., and Deisenhofer, J. (2002) Biochim. Biophys. Acta 1565, 318–332
46. Clarke, T. E., Tari, L. W., and Vogel, H. J. (2001) Curr. Top Med. Chem. 1, 7–30
47. Ferguson, A. D., Chakraborty, R., Smith, B. S., Esser, L., van der Helm, D., and Deisenhofer, J. (2002) Science 295, 1715–1719
48. Perkins-Balding, D., Baer, M. T., and Stojilkovic, I. (2003) Microbiology 149, 3423–3435
49. Fuller-Schaefer, C. A., and Kadner, R. J. (2005) J. Bacteriol. 187, 1732–1739
50. Annamalai, R., Jin, B., Cao, Z., Newton, S. M., and Klebba, P. E. (2004) J. Bacteriol. 186, 3578–3589
51. Ferguson, A. D., Hofmann, E., Coulton, J. W., Diederichs, K., and Welte, W. (1998) Science 282, 2215–2220
52. Khursigara, C. M., De Crescenzo, G., Pawelek, P. D., and Coulton, J. W. (2004) J. Biol. Chem. 279, 7405–7412
53. Sauter, A., Howard, S. P., and Braun, V. (2003) J. Bacteriol. 185, 5747–5754
54. Pawelek, P. D., Croteau, N., Ng-Throw-Hing, C., Khursigara, C. M., Moiseeva, N., Allaire, M., and Coulton, J. W. (2006) Science 312, 1399–1402
55. Shultis, D. D., Purdy, M. D., Banchs, C. N., and Wiener, M. C. (2006) Science 312, 1396–1399
56. Carter, D. M., Miousses, I. R., Gagnon, J. N., Martinez, E., Clements, A., Lee, J., Hancock, M. A., Gagnon, H., Pawelek, P. D., and Coulton, J. W. (2006) J. Biol. Chem. 281, 35413–35424