Heme Inhibits the DNA Binding Properties of the Cytoplasmic Heme Binding Protein of *Shigella dysenteriae* (ShuS)†

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ABSTRACT: Heme uptake and utilization by pathogenic bacteria are critical for virulence and disease, since heme and heme proteins are a major source of iron within the host. Although the role of outer membrane heme receptors in this process has been extensively characterized at the genetic and biochemical level, the role of the cytoplasmic heme binding proteins is not yet clear. The *Shigella dysenteriae* cytoplasmic heme binding protein, ShuS, has previously been shown to promote utilization of heme as an iron source at low to moderate heme concentrations and to protect against heme toxicity at high heme concentrations. Herein, we provide evidence that ShuS of *S. dysenteriae* sequesters DNA non-sequence-specifically with a binding affinity of 3.6 μM as determined by fluorescence anisotropy studies. The ability to bind DNA was observed to be restricted to the apoprotein only. The molecular mass of the apo-ShuS−DNA complex was estimated to be ~700 kDa by size exclusion chromatography. Atomic force microscopy (AFM) revealed that apo-ShuS forms aggregates in the presence of DNA and provides a scaffolding matrix from which DNA is observed to loop outward. The AFM images of apo-ShuS−DNA complexes were strikingly similar to the AFM images of the stress-induced *Escherichia coli* protein, Dps, when complexed with DNA; however, unlike the Dps protein, ShuS failed to protect DNA against oxidative stress in vitro and in vivo. Since free heme can generate reactive oxygen species which are damaging to cellular DNA, the ability of ShuS to physically sequester DNA may provide a molecular basis for its role in preventing toxicity associated with high heme concentrations.

Although extremely abundant in nature, iron is rarely found free within the host due to an extremely low solubility of Fe(III) hydroxide at neutral pH (*1*). The ability of Fe^{2+} and H_2O_2 to catalyze formation of hydroxyl radicals via the Fenton reaction further necessitates that iron within cells be sequestered to prevent oxidation of cellular lipids and DNA. This is primarily achieved by sequestering iron in high-affinity iron binding proteins such as transferrin, lactoferrin, and ferritin or complexed as heme[1] in heme proteins (*2*).

Iron is an essential nutrient for survival of all organisms, but for bacterial pathogens, it is also essential for virulence (*3*). Therefore, pathogenic bacteria have evolved multiple mechanisms for acquiring iron, including secretion of siderophores and direct utilization of the host's iron- and heme-containing proteins (*4*, *5*). Heme proteins are a particularly rich source of iron for pathogens as 95% of the iron within the host is in the form of heme (*2*). The ability to utilize heme is characteristic of a growing number of pathogens, including *Bordetella pertussis* and *Bordetella bronchiseptica* (*6*), enterohaemorrhagic *Escherichia coli* O157:H7 (*7*), *Haemophilus influenzae* (*8*−*10*), *Neisseria* spp. (*11*, *12*), *Shigella dysenteriae* (*13*), *Vibrio cholerae* (*14*), and *Yersinia* spp. (*15*, *16*), and many opportunistic pathogens such as *Pseudomonas aeruginosa* (*17*).

In *S. dysenteriae*, the heme uptake locus, *shu*, includes eight genes with *shuAS* and *shuTWXYUV* operons being transcribed from two divergent promoters regulated by the ferric uptake regulator (Fur) (*13*, *18*). The *shuA* gene encodes a TonB-dependent outer membrane receptor that transports the intact heme moiety across the membrane (*19*). The *shuT* gene encodes a periplasmic heme binding protein (*20*) and together with the *shuU* and *shuV* genes, which are homologous to previously characterized inner membrane permeases and ATPases (*21*, *22*), constitutes an ABC transport system that shuttles heme through the periplasm and across the cytoplasmic membrane. The role of the cytoplasmic heme binding protein, ShuS, in heme utilization is not well understood (*23*). The function of ORFs *shuWXY*, which are well conserved in heme transport loci of other bacterial pathogens, has yet to be elucidated (*18*).

This study was undertaken to further characterize the role of the cytoplasmic heme binding protein, ShuS, of *S. dysenteriae* in heme uptake and utilization. A *S. dysenteriae shuS* chromosomal knockout was shown to be defective in heme utilization on the basis of its poor growth at low concentrations of heme and an increased toxicity at higher concentrations of heme (*24*). This is consistent with an earlier study in which HemS, the ShuS homologue in *Yersinia enterocolitica*, was shown to be essential in preventing heme toxicity in *E. coli* when the heme receptor *hemR* was

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[1] Abbreviations: heme, iron protoporphyrin IX irrespective of oxidation state; AFM, atomic force microscopy; Dps, starvation- and stress-inducible DNA-binding protein.

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expressed from a high-copy plasmid (*25*). The authors concluded that HemS was most likely a heme-degrading factor or heme oxygenase that releases iron for further utilization. More recently, the crystal structure of ChuS, the ShuS homologue in *E. coli*, has been determined, and on the basis of in vitro studies, ChuS was proposed to be a heme oxygenase (*26*). However, we have previously shown that while ShuS is a heme binding protein it is not a heme oxygenase (*23*). Our recent studies have confirmed that the ShuS homologue in *P. aeruginosa*, PhuS, is also not a heme oxygenase; rather, it functions as a protein that traffics heme to the iron-regulated heme oxygenase (*pa*-HO) (*27*). It has therefore been proposed that ShuS of *S. dysenteriae* may similarly function in the transport of heme from the cytoplasmic membrane to heme-containing or heme-degrading proteins, for efficient utilization of heme as an iron source (*24*). Furthermore, as we will outline below, since free heme can generate reactive oxygen species which are damaging to DNA, the ability of ShuS to physically sequester DNA may provide a molecular basis for its role in preventing toxicity associated with high heme concentrations.

Previous studies have indicated that the ShuS protein has the ability to bind DNA non-sequence-specifically (*23*). In this investigation, we have further characterized the DNA binding ability of ShuS as a function of heme occupancy as assessed in vitro with a gel shift assay. The binding affinity of ShuS for the DNA was determined by fluorescence anisotropy, and the morphology of the ShuS−DNA complex was analyzed by atomic force microscopy (AFM) in the presence and absence of heme. As will be discussed below, the AFM images of the apo-ShuS−DNA complex were strikingly similar to the images of the stress-induced *E. coli* protein, Dps, when complexed with DNA. Dps, a 19 kDa protein, can sequester and protect cellular DNA during periods of nutrient starvation and oxidative stress (*28*). It forms extremely stable complexes with DNA in the cytoplasm of starved bacteria, resulting in the formation of Dps−DNA cocrystals, which have been proposed to represent a general mode of DNA protection during starvation (*29*). Therefore, binding of ShuS to DNA in a non-sequence-specific manner resembling that of the Dps protein led us to investigate if ShuS could functionally complement an *E. coli dps* mutant in protecting against oxidative stress in vivo.

MATERIALS AND METHODS

Materials. All chemicals (ACS grade or better), resins, DNA-modifying enzymes, and oligonucleotides were purchased from commercial sources and used as received. Subcloning and bacterial transformation were carried out as previously described (*30*). Plasmid purification was carried out with the plasmid purification kit from Qiagen. Deionized, doubly distilled water was used for all experiments. The 49 bp fluorescein-labeled double-stranded DNA used in the fluorescence anisotropy studies with the sequence 5′ CTTCAAAGATATAACACTAAT**T**CATTTTAAATAAT-AATTAGTTAATGAA3′ was a kind gift of S. Michel (University of Maryland). The fluorescein-labeleled nucleotide is the boldface character. The oligonucleotide was PAGE-purified, deprotected, and desalted. Concentrations were calculated following resuspension in doubly distilled deionized water.

Bacterial Strains, Plasmids, and Growth Conditions. E. coli strain DH 5α [F′, *ara* D(*lac-proAB*) rpsL ø80d*lacZ*DM15 *hsd* R17] was used for all DNA manipulations, and *E. coli* strain BL21(DE3) *pLysS* [F^- *ompT hsdS*$_B$ ($r_B^-m_B^-$) *gal dcm* (DE3)] was used for expression of the ShuS protein. The *shuS* gene was cloned into the pET21a vector for protein expression (*23*). Bacteria were grown on Luria-Bertani (LB) liquid medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl). Ampicillin at 100 μg/mL was included in the LB media (LB-Amp) wherever applicable. The pUC19 plasmid was used for gel shift assays (*31*). *E. coli* strain ZK126 *dps::kan* (*28*) was kindly provided by R. Kolter (Harvard Medical School, Boston, MA) for in vivo oxidative stress assays.

Expression and Purification of the ShuS Protein. The protein was expressed and purified by modification of a previously described procedure (*23*). Briefly, the plasmid was transformed into *E. coli* strain BL21(DE3) *pLysS*, plated on LB-Amp plates, and incubated at 37 °C overnight. The following day, colonies from the plate were scraped using a sterile inoculating rod and used to inoculate a 100 mL LB-Amp culture. The culture was grown at 250 rpm with shaking at 37 °C to an OD_{600} of 0.6. Subsequently, this culture was used to inoculate 1 L of LB-Amp medium to a final OD_{600} of 0.06. The cells were grown to an OD_{600} of 0.6, induced with 1 mM isopropyl 1-thiogalactopyranoside (IPTG), and grown for an additional 3 h at room temperature. The cells were harvested by centrifugation for 10 min at 8000 rpm in a Beckman JA-20 rotor and lysed by sonication in 10 mL of lysis buffer containing 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 0.2 mM PMSF per liter of culture and one protease inhibitor cocktail tablet per 50 mL of lysis buffer (Roche Diagnostics). DNase I was added to the lysis buffer at a final concentration of 0.5 mg/mL. The cell debris was removed by centrifugation at 18 000 rpm for 35 min in a Beckman JA-20 rotor. The soluble fraction was applied to a Sepharose-Q fast flow column (3 cm × 15 cm) equilibrated in 20 mM Tris-HCl (pH 8.0). The column was washed with 10 column volumes of the same buffer containing 50 mM NaCl, and the protein was then eluted with a linear gradient from 50 to 400 mM NaCl. The ShuS protein eluted at a concentration of 150 mM NaCl; the peak fractions, as judged by SDS−PAGE, were pooled and concentrated to 5 mL, and the apoprotein was stored at −80 °C. Heme reconstitution was carried out as previously described (*23*).

Gel Retardation Assay. Reaction mixtures (20 μL) contained 300 ng of pUC19 in 20 mM Tris-HCl (pH 8.0) and the indicated amounts of apo- or holo-ShuS. The reaction mixtures were incubated at room temperature for 1 h, after which they were analyzed by gel electrophoresis on a 1% agarose gel in Tris-acetate buffer (pH 8.0).

Fluorescence Anisotropy Binding Studies. The binding of apo-ShuS to a 49 bp fluorescently labeled oligonucleotide was assessed using fluorescence anisotropy as previously described (*32*). Briefly, the experiment included 500 μL of a 15 nM solution of the oligonucleotide in 20 mM Tris-HCl (pH 8.0) and 0.05 mg/mL bovine serum albumin in a 0.875 mL Spectrosil far-UV quartz cuvette (Starna Cells, Inc.). All measurements were taken on an ISA/Horida group Fluorolog-3 spectrofluorometer configured in the L-format, with excitation and emission wavelengths of 495 and 519 nm, respectively, and band-passes of 2 and 6 nm, respectively.

The anisotropy of the free oligonucleotide was first recorded, following which apo-ShuS was then titrated into the cuvette and the resulting change in anisotropy recorded. Incremental addition of protein was continued until no further change in anisotropy was observed. To fit the data, the anisotropy, *r*, was converted to fraction bound, F_{bound} (the fraction of protein bound to the oligonucleotide at a given DNA concentration), using the following equation:

$$F_{bound} = \frac{r - r_{free}}{(r_{bound} - r)Q + (r - r_{free})}$$

where r_{free} is the anisotropy of the fluorescein-labeled oligonucleotide and r_{bound} is the anisotropy of the oligonucleotide−protein complex at saturation. The quantum yield designated as *Q* is calculated from the changes in fluorescence intensity that occur over the course of the experiment (I_{bound}/I_{free}). F_{bound} was then plotted against the protein concentration using a simple one-site binding model:

$$P + D \rightleftharpoons PD \quad \frac{[P][D]}{[PD]} = K_d$$

$$F_{bound} = \{P_{total} + D_{total} + K_d - [(P_{total} + D_{total} + K_d)^2 - 4P_{total}D_{total}]_{1/2}\}/2D_{total}$$

where *P* is the protein (apo-ShuS) concentration and *D* is the DNA concentration. All concentrations and fluorescence changes were corrected for volume changes, although no changes greater than 10% were noted over the whole experiment.

Atomic Force Microscopy. The apo-ShuS−DNA complexes were prepared by incubating ShuS (600 nM) with pUC19 plasmid DNA (4 nM) at room temperature for 1 h, in 20 mM Tris-HCl (pH 8.0). The samples were deposited onto freshly cleaved mica, incubated for 5 min, rinsed with 200 μL of water, and dried with a weak stream of compressed air. The images were collected by tapping in air in magnetic A/C imaging mode on a PicoPlus AFM instrument (Molecular Imaging) fitted with a 1 μm × 1 μm scanner using magnetically coated type II MacLevers (Molecular Imaging). All operations were carried out at room temperature. Images were collected with a total scan size of 0.5−3.5 μm, at a scan rate of one scan line per second. The AFM imaging software supplied by the manufacturer (Molecular Imaging) was used to export the images as .tif images.

Size Exclusion Chromatography. Apo-ShuS (400 μg) or holo-ShuS (300 μg) was incubated with pUC19 (1 μg) and analyzed in separate runs on a Pharmacia AKTA FPLC system fitted with a Superdex S200 HR 10/30 column previously equilibrated in 20 mM Tris-HCl (pH 8.0). The sample was injected onto the column, and the elution profile was monitored at 280 nm. The apo- and holo-ShuS proteins in the absence of DNA were analyzed in separate experiments as a control. Molecular mass markers (250 μL) were run to obtain a standard curve across the range of 669−23 kDa.

Oxidative Stress Assay. The ability of ShuS to protect DNA against oxidative damage in vitro and in vivo was analyzed by modification of a previously reported procedure (*33*). Briefly, for the in vitro assay, supercoiled pUC19 (300 ng) was incubated with $FeSO_4$ (0.1 mM) and H_2O_2 (10 mM) with or without purified apo-ShuS (25, 100, 200, and 300 μg) in a total volume of 20 μL. After incubation at room temperature for 1 h, reactions were terminated by addition of 5 mM dipyridyl, and the DNA was resolved on a 1% TAE agarose gel. In a parallel set of experiments, the reaction mixtures were extracted with phenol and chloroform to remove the ShuS protein and analyzed by gel electrophoresis. For the in vivo protection assay, ShuS was expressed from an inducible promoter in a Dps deficient strain of *E. coli* [*dps::kan*] and the ability of ShuS to complement Dps was assessed by exposing actively growing cells to varying concentrations of hydrogen peroxide and measuring the extent of cell survival by determining the colony forming units as previously described (*28*). The in vivo expression of ShuS was confirmed by Western blot analysis using a polyclonal antibody against the ShuS protein (*30*).

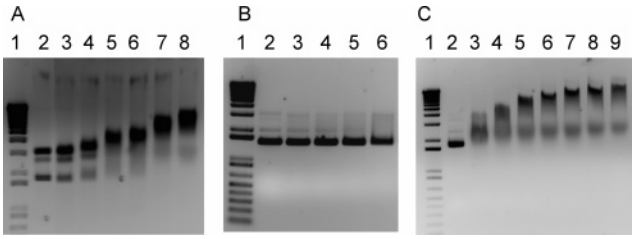

FIGURE 1: Gel retardation analysis of plasmid DNA in the presence of ShuS. All experiments contained 300 ng of pUC19 plasmid DNA. (A) Lane 1, molecular mass markers; lanes 2−8, pUC19 incubated with 0, 25, 50, 75, 100, 200, and 300 μg of apo-ShuS, respectively. (B) Lane 1, molecular mass markers; lanes 2−6, pUC19 incubated with 0, 50, 75, 100, and 200 μg of holo-ShuS, respectively. (C) A total of 300 μg of protein containing varying ratios of apo- to holo-ShuS: lane 1, molecular mass markers; lane 2, pUC19; lanes 3−9, pUC19 incubated with holo-ShuS:apo-ShuS microgram ratios of 300:0, 250:50, 200:100, 150:150, 100:200, 50:250, and 0:300, respectively.

RESULTS

DNA Binding Ability of ShuS. The ability of the apo- and holo-ShuS protein to bind DNA was analyzed by gel shift assay. A significant shift in the mobility of supercoiled pUC19 plasmid DNA was observed following incubation with apo-ShuS (Figure 1A). However, holo-ShuS failed to produce a gel shift under identical conditions (Figure 1B), indicating that heme inhibits the ability of ShuS to bind DNA. These results were further confirmed by incubating supercoiled pUC19 DNA with a mixture of apo- and holo-ShuS, at varying ratios, keeping the total protein concentration constant. As expected, the observed gel shifts were proportional to the concentration of apo-ShuS in the total reaction mixture (Figure 1C). Although not clear at the present time, the DNA binding properties of apo-ShuS may play an as yet unidentified role in heme uptake and/or regulation.

Binding Interactions between Apo-ShuS and the Fluorescein-Labeled Oligonucleotide. When the apo-ShuS protein was titrated into a cuvette containing the fluorescently labeled oligonucleotide and the change in anisotropy was monitored, a binding affinity of apo-ShuS for the DNA was determined. A significant change in anisotropy was observed upon addition of apo-ShuS to the fluorescently labeled oligonucleotide (Figure 2). The results when fit to a one-binding site model gave a dissociation constant of 3.6 ± 0.4 μM.

Determination of the Size of the ShuS−DNA Complex by Size Exclusion Chromatography. Size exclusion chromatog-

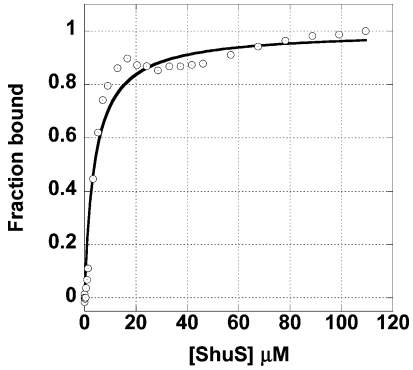

FIGURE 2: Change in anisotropy, *r*, upon addition of the apo-ShuS protein to a fluorescently labeled oligonucleotide. The experiment was carried out as described in Materials and Methods and could be fit to yield a dissociation constant of 3.6 ± 0.4 μM.

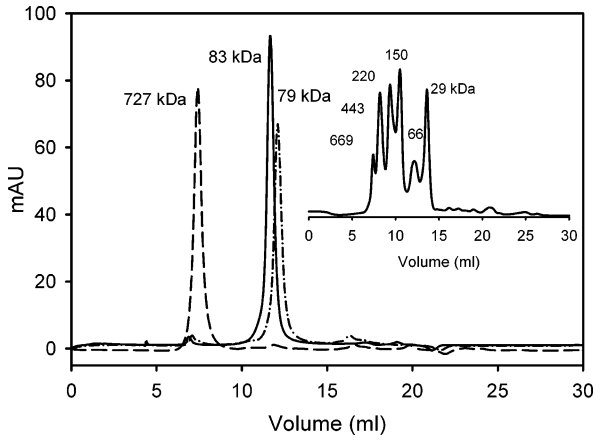

FIGURE 3: Size exclusion chromatography of ShuS and the ShuS−DNA complexes: apo-ShuS (−•−), apo-ShuS complexed with pUC19 (− − −), and holo-ShuS (—). A representative trace of the molecular mass markers is shown in the inset.

raphy revealed that whereas the apo- and holo-ShuS proteins exist as dimers with estimated molecular masses of ~79 and ~83 kDa, respectively, apo-ShuS complexed with DNA exists as an oligomer with an estimated molecular mass of ~700 kDa (Figure 3). Therefore, on the basis of the molecular mass of 37 kDa of one monomer of apo-ShuS, it is estimated that as many as 19 molecules of apo-ShuS may be present in a single protein−DNA aggregate. Holo-ShuS upon incubation with DNA shows no change in elution pattern (data not shown). In this study, the estimated size of apo- and holo-ShuS in the absence of DNA differs from the previously reported size of ~650 kDa (*23*). This inconsistency could be explained by the difference in the protein purification procedures in the two studies. This purification protocol, in contrast to that previously reported, involved extensive DNase treatment for removal of any contaminating DNA from the protein preparation.

Morphology of the ShuS−DNA Complex by Atomic Force Microscopy. The oligomeric complex of apo-ShuS and supercoiled pUC19 was visualized using AFM to determine the biophysical nature of the apo-ShuS−DNA complexes. The plasmid DNA was observed to exist in a supercoiled configuration both in the absence (Figure 4A) and in the presence of holo-ShuS (Figure 4C). In addition, the holo-ShuS protein which does not bind the plasmid DNA can be

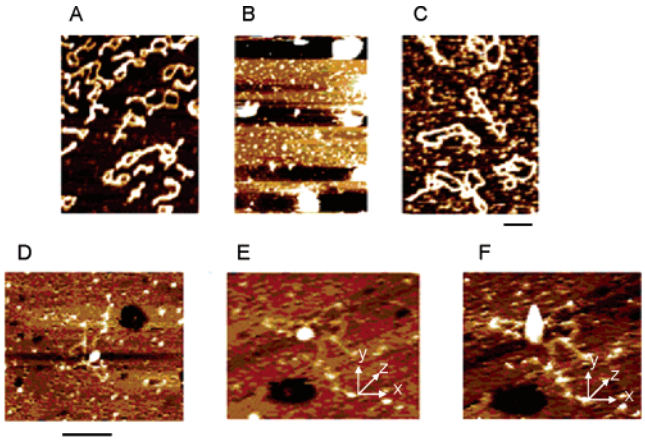

FIGURE 4: Atomic force microscopy images: (A) supercoiled pUC19, (B) pUC19 incubated with apo-ShuS, (C) pUC19 incubated with holo-ShuS, (D) top view of a single oligomeric apo-ShuS−DNA aggregate as seen in panel C, (E) three-dimensional projection of a single oligomeric apo-ShuS−DNA aggregate as seen in panel C from an angle (*z*-axis in micrometers), and (F) same as panel E except the *z*-axis is in angstroms. The height of the oligomeric complex shown is approximately 105 Å. Horizontal bars represent 200 nm. Apo-ShuS−DNA complexes in panel B may appear enlarged with respect to those in panels D−F as a result of tip broadening.

seen as uniformly spread over the mica surface (Figure 4C). However, when incubated with apo-ShuS, the DNA binds to the protein in oligomeric complexes that are large enough that the DNA cannot be readily visualized (Figure 4B). The apo- or holo-ShuS proteins in the absence of DNA were not found to exist in this aggregated form (data not shown). Magnification (Figure 4D) and three-dimensional projection of the apo-ShuS−DNA aggregates (Figure 4E,F) revealed that loops of DNA emerge out from a central protein scaffold.

Role of ShuS in Oxidative Stress Protection. The striking resemblance of the non-sequence-specific DNA binding properties of ShuS to that of Dps protein of *E. coli* led us to investigate if ShuS is functionally similar to Dps in protecting cellular DNA against oxidative stress in vitro and in vivo (*28*). Apo-ShuS−DNA complexes were exposed to oxidative stress as described in Materials and Methods, and subsequent DNA damage was analyzed by gel electrophoresis before (Figure 5A) and after extraction of apo-ShuS from the reaction mixture (Figure 5B). Although apo-ShuS remains bound to the DNA after being exposed to the oxidizing agents (Figure 5A), extraction of the protein from the reaction mixture following exposure to the oxidizing agents revealed both linear and nicked DNA (Figure 5B), suggesting that the apoprotein does not confer protection against oxidative damage in vitro. However, more severe damage is observed when the DNA is incubated with holo-ShuS which neither binds DNA nor protects against oxidative damage (Figure 5A).

The lack of a protective response to oxidative stress was further investigated in vivo in an *E. coli dps* knockout strain which was transformed with a plasmid encoding an IPTG inducible copy of the *shuS* gene. The expression of the ShuS protein was confirmed by Western blotting (data not shown). The *E. coli dps* mutant expressing ShuS was then exposed to varying levels of hydrogen peroxide to induce oxidative stress. No differences in colony number, size, or morphology

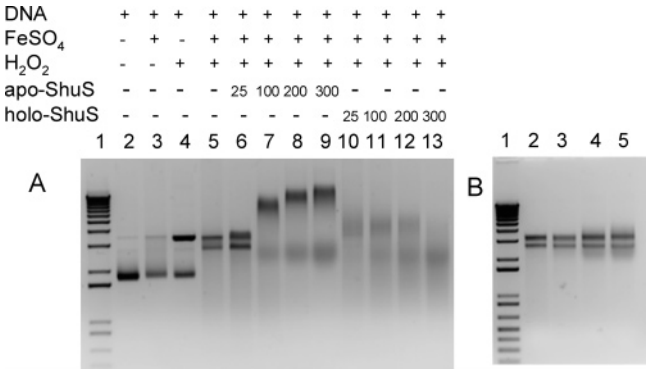

FIGURE 5: In vitro oxidative DNA damage protection assay. (A) Lane 1, molecular mass markers; lane 2, 300 ng of supercoiled pUC19; lanes 3−12, where indicated $FeSO_4$ (0.1 mM), H_2O_2 (10 mM), and purified apo- or holo-ShuS (25, 100, 200, and 300 μg) were added to the reaction mixture. (B) Lanes 2−5 were identical to lanes 6−9, respectively, in panel A, except that reaction mixtures were extracted with phenol and chloroform prior to gel electrophoresis.

were observed between the cells expressing ShuS and those which were transformed with the control vector, indicating that in vivo ShuS does not function in a manner similar to that of the Dps proteins in the global protective response to oxidative stress (data not shown).

DISCUSSION

The cytoplasmic heme binding proteins have previously been shown to be required in preventing heme toxicity at higher heme concentrations (*25*). However, the molecular mechanism by which this family of proteins protects the bacterial cell remains unknown. In this report, we show that that the apo-ShuS protein of *S. dysenteriae* binds DNA in a non-sequence-specific manner with a binding affinity (K_d) of 3.6 μM. The K_d value of apo-ShuS for DNA is far below the values for transcription factors which bind DNA with much greater affinities. This is consistent with the proposed function of ShuS in cellular defense mechanisms against heme toxicity by nonspecific DNA sequestration. The inability of holo-ShuS to bind DNA may be either due to the overlap of the heme and DNA binding sites of ShuS or due to the subtle conformational changes in the protein upon heme binding which mask the DNA binding site. A conformational change upon heme binding is supported by the observed faster migration by size exclusion chromatography of holo-ShuS when compared to the apo-ShuS protein. However, we have previously shown by circular dichroism spectroscopy that there are no significant changes in the secondary structure of ShuS upon binding to heme (*23*). During the preparation of this work, the crystal structures of the ShuS homologue HemS from *Y. entercolitica* in both the apo and holo forms were reported (*34*). Interestingly, the authors report that the protein is characterized by two topological domains that are joined by two stacked β-sheets, resulting in two distinct pockets on either side of the protein at the interface of the two domains. The deeper of the two pockets sequesters the heme in the holo structure. Furthermore, the second pocket or cleft, like the deeper heme pocket, contains a significant number of conserved residues. In the apo form, this pocket may expose a greater surface area or an altered topology for DNA binding that is lost with conformational changes induced by heme binding. Although the binding of heme does not afford any major secondary structure changes within HemS in keeping with previous circular dichroism studies on ShuS (*23*), the N- and C-terminal domains move toward the heme, causing a marked shift in the position of the domains relative to each other. Despite the limited insight gained from the current three-dimensional structure of HemS, targeted mutagenesis of conserved regions, especially those in the non-heme binding cleft, should shed some light on the interaction of ShuS with DNA. It is also hoped that ongoing studies aimed at determining the three-dimensional structure of the apo-ShuS−DNA complex will further improve our understanding of the relationship among heme binding, conformational change, and DNA complexation.

The AFM images of apo-ShuS complexed to DNA are consistent with the observed molecular mass of the oligomeric apo-ShuS−DNA complex determined by size exclusion chromatography. Interestingly, the three-dimensional topographical analysis of apo-ShuS−DNA aggregates indicates a central protein scaffold from which the DNA is seen to loop out. Similar observations have previously been made for the Dps protein of *E. coli* which binds DNA in a non-sequence-specific manner to form oligomeric complexes in response to nutrient starvation and cellular stresses such as oxidative stress (*35*). This resemblance between apo-ShuS−DNA complexes and Dps−DNA complexes led us to investigate whether ShuS was functionally similar to the Dps protein in protecting DNA against oxidative stress (*28*). In vitro, the apoprotein while retaining the ability to bind the DNA on exposure to reactive oxygen species did not appear to protect DNA from oxidative damage. The lack of a protective response to oxidative stress in vitro was confirmed in vivo by the inability of *shuS* to complement an *E. coli dps* knockout strain when it was subjected to oxidative stress. We, therefore, conclude that although ShuS binds DNA non-sequence-specifically as observed for the Dps protein, this appears to be a specific response to low-iron conditions and active heme uptake rather than a generalized response to oxidative stress. It was further reported that the physical interaction between purified Dps and DNA results in a rapid formation of highly ordered, tightly packed Dps−DNA cocrystals within the cytoplasm of starved bacteria (*29*). Such a crystalline arrangement has not been observed for the apo-ShuS−DNA complexes and may in part explain why apo-ShuS does not appear to protect the cellular DNA against oxidative stress in the same manner as does the Dps−DNA complex.

On the basis of these observations, our current working hypothesis is that under low-iron conditions, the *shu* heme uptake system of *Shigella* is upregulated in a Fur-dependent manner. The cytoplasmic heme binding protein, ShuS, responds to active heme uptake as a preventive measure against potential free heme toxicity and binds cellular DNA non-sequence-specifically. Therefore, the ShuS protein appears to have a dual function in binding DNA as a protective measure during heme uptake and as a potential heme-trafficking protein to as yet unidentified proteins, as previously shown for the ShuS homologue PhuS of *P. aeruginosa* (*28*). It is possible that to maintain appropriate expression levels of apo-ShuS to accomplish the dual task of efficient heme utilization and DNA binding, *shuS* may be transcribed

from two promoters. This is supported by the fact that in addition to the promoter upstream of *shuA*, there is a second potential promoter upstream of *shuS* and downstream of *shuA* (*18*, *24*). Transcription from this promoter could potentially be activated for the ShuS protein to be synthesized in greater quantities than the outer-membrane receptor ShuA. Future studies will be necessary to determine if the promoter upstream of *shuS* is indeed active.

In summary, these preliminary data together with the previous studies identifying a role of ShuS in the prevention of heme toxicity (*24*) suggest that the cytoplasmic heme binding protein plays a critical role in both heme trafficking and intracellular protection during heme uptake via its DNA binding properties. We therefore propose that ShuS may play a role in the global sensing and trafficking of intracellular heme that may be central to the overall iron balance within the cell. It is further hypothesized that the interaction between DNA and apo-ShuS is transitory and reversible (which is supported by the micromolar binding affinity), allowing the pathogen to quickly adapt to changing intracellular levels of heme. Biochemical and genetic studies directed toward understanding the role of the cytoplasmic heme binding proteins in the regulation and maintenance of the iron levels within the cell are currently underway.

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