Crystal Structure of the Dithiol Oxidase DsbA Enzyme from *Proteus Mirabilis* Bound Non-covalently to an Active Site Peptide Ligand

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Background: DsbA enzymes assemble bacterial virulence factors and are targets for an entirely new drug class.

**Results:** *Proteus mirabilis* DsbA was characterized and its structure determined with a peptide bound non-covalently at the active site.

**Conclusion:** The structure provides an important basis for future inhibitor design.

**Significance:** New drugs to treat superbugs are urgently needed. DsbA inhibitors could have antivirulence activity against bacterial pathogens.

The disulfide bond forming DsbA enzymes and their DsbB interaction partners are attractive targets for development of antivirulence drugs because both are essential for virulence factor assembly in Gram-negative pathogens. Here we characterize PmDsbA from *Proteus mirabilis*, a bacterial pathogen increasingly associated with multidrug resistance. PmDsbA exhibits the characteristic properties of a DsbA, including an oxidizing potential, destabilizing disulfide, acidic active site cysteine, and dithiol oxidase catalytic activity. We evaluated a peptide, PWATCDS, derived from the partner protein DsbB and showed by thermal shift and isothermal titration calorimetry that it binds to PmDsbA. The crystal structures of PmDsbA, and the active site variant PmDsbAC30S were determined to high resolution. Analysis of these structures allows categorization of PmDsbA into the DsbA class exemplified by the archetypal *Escherichia coli* DsbA enzyme. We also present a crystal structure of PmDsbAC30S in complex with the peptide PWATCDS. The structure shows that the peptide binds non-covalently to the active site CX2C motif, the cis-Pro loop, and the hydrophobic groove adjacent to the active site of the enzyme. This high-resolution structural data provides a critical advance for future structure-based design of non-covalent peptidomimetic inhibitors. Such inhibitors would represent an entirely new antibacterial class that work by switching off the DSB virulence assembly machinery.

*Proteus mirabilis* is a significant Gram-negative extra-intestinal human pathogen that belongs to the *Enterobacteriaceae* family, which also includes other important pathogens such as *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter cloacae*. Together, the *Enterobacteriaceae* account for the vast majority of community-acquired and nosocomial urinary tract infections (1, 2). *P. mirabilis* is a frequent cause of both complicated and catheter-associated urinary tract infections (3, 4). Multidrug-resistant strains of clinical pathogenic *P. mirabilis* have been reported for several decades (1). For example, clinical isolates of *P. mirabilis* resistant to streptomycin, tetracycline, kanamycin, chloramphenicol, and polymyxin B were described in the 1960s (5, 6) and since then reports of antibiotic resistance have increased (7–16).

DsbA enzymes from Gram-negative bacteria are targets for the development of anti-virulence drugs that could represent an entirely new class of antimicrobial agents (17, 18). Drugs that target bacterial virulence could minimize the selective pressure that generates antibiotic resistance and simultaneously preserve the endogenous host microbiome (19). DsbA is a key target for such drugs because it is essential for the correct folding or assembly of multiple virulence factors, including toxins, fibrillar adhesins, flagella, and type II and type III secretion systems (17). The mutation of *dsbA* results in the attenuation of virulence factor production in multiple pathogens (17), some examples, include *P. mirabilis* (20), uropathogenic *E. coli* (21), and *Burkholderia pseudomallei* (21, 22), *Vibrio cholerae* (23), *Shigella flexneri* (24), and *Salmonella enterica* serovar *Typhimurium* (25).

DsbA enzymes are thioredoxin-fold proteins (26) localized to the periplasm of Gram-negative bacteria where they catalyze oxidative folding (27). This reaction involves the transfer of a disulfide bond from the active site 30CXXC motif of DsbA to cysteine thiols in newly translocated proteins (28). Through this disulfide-exchange reaction, the active site cysteines of DsbA become reduced. The enzymatic cycle is completed through oxidation of DsbA by the inner membrane partner.
protein, DsbB (29). DsbA from *E. coli* (EcDsbA) is a highly pro-
miscuous enzyme that catalyzes disulfide bond formation in
many cysteine-containing proteins (28). In contrast, EcDsbB has a
very strict binding specificity for EcDsbA (29). The inter-
action between EcDsbA and EcDsbB involves the formation of
a mixed disulfide between Cys30 of the 30CXXC3 motif of
EcDsbA and Cys104 of EcDsbB in the P2 periplasmic loop,
which has the sequence 99PSPFATCDE106 (30). The crystal
structure of the complex between EcDsbA and EcDsbB also
revealed non-covalent binding of EcDsbB P2 loop residues to
the EcDsbA hydrophobic groove (30). The P2 periplasmic loop
sequence of *P. mirabilis* DsbB (PmDsbB) is identical to that of
EcDsbB, suggesting a similar interaction occurs between
PmDsbA and PmDsbB.

Here we characterize PmDsbA from *P. mirabilis*, which
shares 59% sequence identity with EcDsbA, and confirm its
activity as a DsbA enzyme. We demonstrate that the heptapep-
tide PWATCDS, derived from the DsbB P2 loop sequence,
binds to wild type PmDsbA and to a C30S active site variant
(PmDsbAC30S). We report three high-resolution crystal
structures including the PmDsbAC30S/PWATCDS complex, which
represents to our knowledge the first reported example of a
peptide bound non-covalently to a DsbA structure. This non-
covalent complex provides the critical starting point for the
design of non-covalent drugs that act as anti-virulence agents
targeting *Enterobacteriaceae* DsbAs.

**Experimental Procedures**

**Protein Production and Molecular Biology—Codon-opti-
mized wild type *P. mirabilis* dsbA (GenBank® accession num-
ber CA45574), lacking the sequence coding for the predicted
signal peptide (amino acids 1–19), was cloned into a modified
pMCSG7 (31) vector using ligation-independent cloning. The
cytoplasmic expressed PmDsbA contained an N-terminal
His6 affinity tag followed by a linker region including a
tobacco etch virus (TEV)° protease cleavage site. The
PmDsbAC30S variant was generated from the wild type con-
struct using QuikChange® (Agilent Technologies). The fol-
lowing primers were used to introduce the point mutation,
forward, GAATTTCCTCATTTATCCTCGATTTGT
TACC and reverse, GGTTAACATGCCAGATAAATGG
AGAAAAATT. Transformed BL21(DE3)pLys cells containing
plasmids for either PmDsbA or PmDsbAC30S were grown
(1 liter) at 30 °C in 2.5-liter baffled shaker flasks for 16–20 h
using autoinduction media (32). Cells were resuspended in 25
mM Tris, 150 mM NaCl (10 g of cells/100 ml of buffer), and
protease inhibitor mixture (diluted 1 in 1000 in the lysate)
(BioPioneer Inc., San Diego, CA) and DNase (1300 units/100 ml
of lysate) (Roche Applied Science) were added. Lysis was per-
formed in a Cell Disruptor (TS-Series, Constant Systems LTD.,
UK) applying a single run with a constant pressure of 25 Kpsi.
Cell debris was removed by centrifugation (18,500 rpm, 30 min,
4 °C, rotor JM-25.5, Beckman Coulter, Brea, CA). The proteins

°The abbreviations used are: TEV, tobacco etch virus; BisTris, 2-[bis(2-hy-
droxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; HbtU, O-benzo-
triazole-N,N,N’,N’-tetramethyluronium hexafluorophosphate; ITLC, isothe-
rmal titration calorimetry; PDB, Protein Data Bank; r.m.s., root mean square.

cytosolic two non-native residues (Ser−2 × Asn−1) at the N termi-

Total protein was determined at 280 nm with a NanoDropTM
2000c (Thermo Fisher Scientific). The His6 affinity tag was
removed by TEV cleavage using a 50:1 (w/w) ratio (protein/ TEV-
protease) incubated in a 50-ml Falcon tube including 1
mM β-mercaptoethanol for 2 h on a rotary mixer at room
temperature. After cleavage, the PmDsbA proteins had an addi-
tional two non-native residues (Ser−2 × Asn−1) at the N termi-

To remove imidazole, the mixture was rapidly buffer
swapped into 25 mM Tris, 150 mM NaCl using a Sephadex
G-25 fine 16/60 column connected to an ÄKTA system (GE
Healthcare). The His6-tagged TEV protease was removed by
reverse metal ion affinity chromatography using Talon resin
(0.5 ml of resin/2 mg of TEV protease) (Clontech, Australia)
and the PmDsbA proteins were recovered in the flow through.

As a positive control, EcDsbA was expressed within the same
operable promoter with an N-terminal EcDsbA signal sequence
(36). The protein was concentrated to 100 mg/ml using Ami-
con Ultra filter devices with a 10-kDa cutoff (Millipore). Yield
was generally 120–150 mg/liter of culture. Protein quality was
assessed by SDS-PAGE (NuPAGE® system, 4–12% BisTris gel,
Invitrogen, Australia). Molar protein concentrations were
determined from calculated extinction coefficients derived
from ProtParam (33).

EcDsbA Complementation—*E. coli* ΔdsbA (JC8817) and
ΔdsbA/ΔdsbB (JC8818) non-motile strains were used for motil-
ity assays as described previously (35). The gene code-
ing for mature PmDsbA (lacking the periplasmic signal
sequence) was cloned into pBAD33 under an arabinose-induc-
ible promoter with an N-terminal EcDsbA signal sequence (36).

As a positive control, EcDsbA was expressed within the same
pBAD33 vector background. 2 × 106 non-motile *E. coli* ΔdsbA
(JC8817) and ΔdsbA/ΔdsbB (JC8818) double-mutant (JC8818
(27) cells harboring pBAD33(EcDsbA) or pBAD33(PmDsbA)
were spotted onto the center of a soft M63 minimal agar plate
containing 40 mg/ml of each amino acid (except l-cysteine)
in the absence or presence of 0.1% arabinose (negative control).
Plates were incubated at 37 °C and cell motility was monitored
after 4–5 h using a Molecular Imager® Gel Doc TM system from
Bio-Rad. Complementation experiments were performed as
biological triplicates.
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Cysteine Thiol Oxidation Assay—A synthetic peptide substrate of EcDsbA (CQQGFDGTQNSCK) with a europium 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid group amide coupled to the N terminus and a methylcoumarin amide coupled to the ε-amino group of the C-terminal lysine was purchased from AnaSpec (Fremont, CA) and prepared as previously reported (37).

Assays were performed using a Synergy H1 multimode plate reader (BioTek) as described previously (38). In brief, fluorescence (excitation λ = 340 nm and emission λ = 615 nm) corresponding to disulfide formation in the substrate peptide was measured in a white 384-well plate (PerkinElmer Life Sciences OptiPlate-384, number 6007290) in 50 mM MES, 50 mM NaCl, and 2 mM EDTA at pH 5.5 buffer. The total reaction volume in each well was 50 μL, containing 40, 80, or 160 nM PmDsbA or EcDsbA, 1.6 μM EcDsbB membrane, and 8 μM peptide substrate (added last to initiate the reaction). Controls used were: PmDsbA or EcDsbA + buffer + substrate (no DsbA); EcDsbB + buffer + substrate (no DsbA or DsbB); and no components (fluorescence background of the plate, which was subtracted from all other measurements). Three independent experiments (biological replicates) were performed, using three technical replicates for each measurement. The reaction rate was estimated by calculating the increase in fluorescence over the first 8 min of reaction. The fluorescence rates were fitted to a two-state unfolding model and errors were calculated using Prism 6 (GraphPad) as described previously (41).

Redox Properties of PmDsbA—The standard redox potential of PmDsbA was measured utilizing the intrinsic fluorescence of tryptophan residues in PmDsbA, similar to the method used for EcDsbA (42). In brief, oxidized PmDsbA was equilibrated for 3 h at 25 °C in degassed 100 mM NaH₂PO₄/Na₂HPO₄, pH 7.0, 1 mM EDTA, containing 1 mM oxidized glutathione (GSSG) and a range of reduced glutathione (GSH) concentrations (0–2000 μM). 200 μL of PmDsbA from each redox condition was dispensed into a 96-well plate (TPP AG, Switzerland, number 92096) and tryptophan fluorescence was measured (excitation wavelength 280 nm, emission 332 nm) using a Synergy H1 microplate reader and Gen5 2.0 software (Biotek). Data were analyzed in Prism 6 (GraphPad) and the redox potential was calculated as described previously for EcDsbA (42).

Determination of Cys₃₀ pKₐ—Absorbance at 240 nm of the catalytic thiolate anion is pH-dependent allowing the equilibrium between protonated and deprotonated Cys₃₀ to be measured (43) using a UV-visible spectrophotometer (CARY 50, Agilent Technologies). Absorbance at 240 and 280 nm were measured over pH values starting at 6.5 and decreasing to 2.0, in 0.25 pH unit increments. Samples contained either oxidized or reduced PmDsbA (40 μM) in composite buffer (10 mM Tris, 10 mM sodium citrate, 10 mM K₂HPO₄, 10 mM KH₂PO₄, 200 mM KCl, and 1 mM EDTA) at 22 °C. The pKₐ value was calculated from the fitted curves using the Henderson Hasselbalch equation (pH = pKₐ – log ([A₂₄₀]/[A₂₈₀])red/[A₂₄₀]/[A₂₈₀])red). Average and mean ± S.D. from triplicate measurements are plotted.

Disulfide Reductase Activity—DsbA enzymes can reduce the intermolecular disulfide bonds between insulin chains A and B under mild reducing conditions (27). Disulfide bond reduction of insulin can be followed spectrophotometrically at λ = 650 nm (A₆₅₀). The A₆₅₀ value gives a measure of turbidity, which occurs as a result of the increase in production of insoluble B chain of insulin (44). Samples were prepared in 1-cm cuvettes containing 10 μM protein (PmDsbA, EcDsbA, or EcDsbC), 0.33 mM DTT, and 2 mM EDTA in 100 mM NaH₂PO₄/Na₂HPO₄, pH 7.0. Catalysis was initiated by the addition of 0.131 mM insulin (I0516, Sigma) to the mixture. The assay was repeated three times and the mean ± S.D. of the measurement at each time point was calculated.

Peptide Synthesis—Peptides were synthesized using solid-phase peptide synthesis on rink-amide (4-methyl)benzhydrylamine resin (ChemLinx International, Wood Dale, IL) with a loading of 0.65 mmol/g. De-protection of the resin and amino acids was performed using an 80:20 (v/v) mixture of dimethylformamide/piperidine (Rci Labscan, Bangkok Thailand/Auspep, Australia) for 2 × 5 min. 1-Amino acids (ChemLinx International) were activated using 4 resin equivalents (eq) and 4 eq of HbtU (O-benzotriazolo-N,N,N’,N’-tetramethyluronium hexafluorophosphosphate, ChemLinx International) (500 mM) and 5 eq of N,N-diisopropylphosphorylamine (Auspep, Australia) for 5 min before coupling to the de-protected resin for 60 min. After the final coupling, all peptides were acetylated at the N-terminus using 4 eq of acetic acid (Chem Supply, Australia), 4 eq of HbtU, and 5 eq of N,N-diisopropylphosphorylamine for 30 min. Cleaving was executed using a 95:2.5:1.25:1.25 (v/v) mixture of trifluoroacetic acid (TFA)/ethanedithiol/triisopropylsilane/water (H₂O) (chemicals from Sigma). Cleaved peptides resulted in an amidated C-terminus from the resin rink-amide and dried using N₂ gas, washed with diethyl ether (DEE, Ajax Finechem, Sydney Australia), and dissolved in a 80:20 (v/v) mixture of acetonitrile/H₂O (Rci Labscan, Bangkok, Thailand) before purification on HPLC. Purification was executed on a C₁₈ column (Phenomenex, Torrance CA) using a gradient from 20 to 80% of acetonitrile and TFA 0.1%. Fractions were analyzed by mass spectrometry (Waters Micromass LCT, Milford, CT) and the purified peptide was lyophilized using a freeze drier (Christ, Osterode am Harz, Germany).

Peptide-induced Thermal Shift Measurements—25 μM PmDsbA in phosphate-buffered saline (PBS), pH 7.4, was incubated with peptide PWATCD5 at concentrations ranging from 125 μM to 4 mM for 1 h. Then Sypro Orange (S-6650, Invitro-
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Gen) was added to a 5× final concentration (stock concentration ×5000). Controls contained either no peptide, no DsbA, or the dye alone. Measurements were conducted in a white 384-well plate (Perkin Elmer OptiPlate-384, number 6007290) with 5 replicates. Fluorescence emission from Sypro Orange binding to unfolded protein was measured following a temperature time course increasing from 25 to 95 °C (heat rate = 0.05 °C/s) using a VICTOR 1420 Wall-Reader plate system (Perkin Elmer) with a λ = 585 ± 15 nm wavelength filter. Raw data were analyzed using Prism 6 (GraphPad). Fluorescence emission was fitted to a classic Boltzmann sigmoidal curve, and the inflection point was used as the melting temperature, Tm. To determine ΔTm (the shift in melting temperature in the presence of peptide), the Tm value for the wild type protein was subtracted from the Tm value for PmsDsbA–PWATCDS and the Tm value for the variant PmsDsbAC30S was subtracted from the Tm value for PmsDsbAC30S–PWATCDS. ΔTm values are presented as the mean ± S.D. from 5 replicates. A significant ΔTm is considered to be greater than two times the S.D. of the Tm value for the protein in the absence of ligand (45).

Isothermal Titration Calorimetry—Evaluation of affinity and thermodynamics of binding between PWATCDS and PmsDsbA or PmsDsbAC30S were assessed by isothermal titration calorimetry (ITC) using an Auto-ITC200 instrument (MicroCal, Inc., GE Healthcare). The sample cell was loaded with 200 µl of purified protein (oxidized PmsDsbA or untreated PmsDsbAC30S) at 100 µM concentration in 25 mM HEPES, pH 7.4, 50 mM NaCl, 0.8% dimethyl sulfoxide (ITC buffer). The syringe was filled with purified peptide PWATCDS in ITC buffer at a concentration of 4 mM. Titrations were conducted at 25 °C using 19 consecutive injections of 2 µl each delayed by 180 s with a stirring speed of 1000 rpm. In every experiment an initial 0.5 µl of peptide was injected to avoid slow leakage of titrant and this data point was discarded for binding analysis. As a control for background noise, titration of PWATCDS into a solution containing ITC buffer only was performed. The association constant (Ka = 1/Kd), free energy (ΔG), and enthalpy change (ΔH) were calculated by fitting the data to a single-site binding model using the MicroCal Origin software (Origin 7.0 SR4 version 7.0552β) and correcting peptide concentrations to adjust the stoichiometry parameter close to 1.0. Entropy change (ΔS) was deduced from the standard free energy equation ΔG = ΔH − TΔS. Parameters reported include the mean ± S.D. across three replicates. The calculated c-value for these measurements is 12.

Crystallization, Structure Determination, and Structural Analysis—Crystallization screening was performed at the University of Queensland ROCX diffraction facility. Protein crystallization trials were performed in 96-well plates using the hanging drop vapor diffusion method at 8 or 20 °C. In general, purified protein (200 nl) was mixed with crystallization solution (200 nl) using a Mosquito crystallization robot (TTP Labtech, UK), and trays were incubated and imaged in a RockImager 1000 (Formulatrix). Wild type PmsDsbA was crystallized by mixing a 1:3.3 molar ratio of the protein–peptide complex PmsDsbA–PWATCDSF (3 mM protein, 10 mM peptide, 2% dimethyl sulfoxide incubated on ice for 1 h) with 0.5 µl of 15% PEG 3350 and 0.4 mM sodium malonate, pH 5.0, and incubating the drop over the same solution at 20 °C. The presence of the peptide permitted crystallization but no density was evident for the peptide in the phased data; the final refined structure from this condition is referred to as native PmsDsbA. Crystals were harvested in cryo solution (25% PEG 3350, 0.4 M sodium malonate, pH 5.0, 20% PEG 400) and immediately flash frozen in liquid nitrogen.

PmsDsbAC30S crystals appeared overnight at 8 °C in 0.2 M KSCN, 23% PEG 3350 using a 1:10 molar ratio of the protein–peptide complex (1.5 mM protein and 15 mM PWATCDS, final concentrations of both, incubated on ice for 1 h); again the presence of the peptide permitted crystallization but there was no evidence of peptide binding in the electron density maps. The cryo solution used was 0.2 M KSCN, 25% PEG 3350, and 20% PEG 400. The structure of the protein derived from this condition is referred to as PmsDsbAC30S.

PmsDsbAC30S–PWATCDS co-crystals grew at 20 °C using a 1:10 molar ratio of the protein–peptide complex (3 mM protein, 30 mM peptide, final concentrations of both), with the two components incubated on ice for 1 h prior to crystallization. The protein–peptide solution was then concentrated with an equal volume of crystallization solution, which contained 0.2 M KSCN, 22% PEG 3350, 0.2 M non-detergent sulfobetaine (NDSB-221). Differences in crystallization conditions in comparison to the PmsDsbAC30S crystals that did not yield bound peptide included higher concentrations of protein and peptide (but the same molar ratio), the use of NDSB-221 as an additive and an incubation temperature of 20 °C (rather than 8 °C). Crystals were flash frozen in cryo solution consisting of 0.3 M KSCN, 30% PEG 3350, and 20% PEG 400.

Diffraction data for all three crystal structures were measured at the Australian Synchrotron MX2 beamline at a wavelength of 0.9537 Å, and recorded with an ADSC Quantum 315r detector controlled by BLU-ICE (46). Reflections were indexed and integrated in Mosflm (47) or XDS (48), analyzed in Pointless (49), and scaled in SCALA (49) from the CCP4 suite (50). Phases for PmsDsbA were obtained by molecular replacement using PHASER (51) with EcDsbA as a template (PDB code 1FVK, sequence identity 59%). PmsDsbAC30S–PWATCDS were solved using the wild type PmsDsbA structure as the template. Initial electron density maps from PHASER were improved by cycles of iterative refitting of the model using the program COOT (52) and PHENIX.refine (53). The refinement of the complex structure was stalled at R-factor/R-free: 30/33%. Phenix.xtriage analysis indicated that the diffraction data were twinned with a twinning fraction of 0.49. The twin target function implemented in PHENIX was applied in further refinement cycles with the twinning operator −h+k, k, −l. The final R-factor/R-free was 17.2/19.9%. For PmsDsbAC30S–PWATCDS, the density corresponding to the bound peptide was modeled for all seven residues including N-terminal acetylation and C-terminal amidation. In addition, a malonate anion was modeled into the crystal structure of PmsDsbA and four SCN molecules were modeled into the PmsDsbAC30S–PWATCDS crystal structure with one pair in each protomers A and C. One thiocyanate molecule is buried between α1 and β2 of protomers A/C, whereas the second molecule binds between Pro and PmsDsbAC30S helix α1, possibly forming a weak hydrogen bond with the Pro backbone carbonyl (distance N:O = 3.4 Å). Thiocyanate molecules do not appear to influence peptide
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<tr>
<td>Score (percentile)</td>
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<td>1.01 [100th, (12309)]</td>
<td>1.21 [98th, (6780)]</td>
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</table>

* The values in parentheses refer to the highest resolution shell.

100th Molprobity (74) percentile is the best among the structures of comparable resolution. The percentile and the number of structures included in the comparison (N) are given in parentheses within the square bracket.

RESULTS

PmDsbA Catalyzes Disulfide Formation in Vitro and in Vivo—
DsbA enzymes catalyze oxidative folding, or the introduction of disulfide bonds into proteins. To assess whether PmDsbA has dithiol oxidase activity we assessed its activity in an in vitro peptide oxidation assay. A europium-labeled peptide (CQGGFDGTQN-SCK) fluoresces when the two cysteines are oxidized, but not when the cysteines are reduced (37). We found that PmDsbA, like EcDsbA, catalyzed peptide thiol oxidation as evident by an increase in the fluorescent signal over time (Fig. 1A). The fluorescence rate increased with increasing concentrations of PmDsbA and EcDsbA (Fig. 1A). PmDsbA catalysis was a little slower than that of EcDsbA at the same enzyme concentrations. This slightly reduced activity may reflect the use of a peptide derived from an EcDsbA substrate. Nonetheless, the results establish that PmDsbA catalyzes dithiol oxidation in a model substrate.

We also assessed the ability of PmDsbA to complement EcDsbA in vivo. E. coli strains deficient in the P-ring protein FlgI fail to assemble flagella (58). Moreover, E. coli strains lacking EcDsbA, EcDsbB, or both, have the same phenotype (59) because FlgI requires a disulfide bond to function. The non-motile strains E. coli ΔdsbA (JCB817) and ΔdsbA/ΔdsbB (JCB818) were therefore used for in vivo DsbA complementation experiments. When EcDsbA or PmDsbA were expressed in JCB817 following arabinose induction, full rescue of motility was observed in JCB817 (Fig. 1B). However cells from the double knock-out (JCB818) remained non-motile for both PmDsbA and EcDsbA. These results demonstrate that PmDsbA can replace EcDsbA functionally in vivo in the context of flagella assembly. Because EcDsbB was also essential for complementation in this experiment, the results confirm that PmDsbA and EcDsbB can form a functionally competent system. This was expected because the P2 loop sequence from PmDsbB is expected to be the same phenotype (59) because FlgI requires a disulfide bond to function. The non-motile strains E. coli ΔdsbA (JCB817) and ΔdsbA/ΔdsbB (JCB818) were therefore used for in vivo DsbA complementation experiments. When EcDsbA or PmDsbA were expressed in JCB817 following arabinose induction, full rescue of motility was observed in JCB817 (Fig. 1B). However cells from the double knock-out (JCB818) remained non-motile for both PmDsbA and EcDsbA. These results demonstrate that PmDsbA can replace EcDsbA functionally in vivo in the context of flagella assembly. Because EcDsbB was also essential for complementation in this experiment, the results confirm that PmDsbA and EcDsbB can form a functionally competent system. This was expected because the P2 loop sequence from PmDsbB is identical to that of EcDsbB. Furthermore, the P. mirabilis FlgI homologue shares 74% sequence identity with EcFlgI including two conserved cysteine residues.

PmDsbA Shares the Same Characteristic Redox Properties as EcDsbA—DsbA enzymes exhibit unique properties that contribute to their ability to catalyze disulfide bond formation (28,
We next established whether these characteristics are shared by PmDsbA. First, we investigated the relative thermo-stability of the oxidized and reduced forms of the enzyme. In most DsbAs, the reduced form of the CXXC active site is more stable than the oxidized form. For instance, the melting temperature of reduced EcDsbA is almost 10 K higher than that of the oxidized form (39). Similarly, we found that reduced PmDsbA ($T_m^{\text{red}} 348.4 \pm 0.1 \text{ K}$) is 10 K more stable than oxidized PmDsbA ($T_m^{\text{ox}} 338.4 \pm 0.2 \text{ K}$) (Fig. 1C).

DsbAs are also highly oxidizing. The redox potential of EcDsbA is $-122 \text{ mV}$ (42) and the range of values reported for other DsbAs varies from $-80 \text{ mV}$ for NmDsbA1 (37) to $-163 \text{ mV}$ for WpDsbA (41). Nevertheless, closely related (>80% sequence identity) homologues of EcDsbA such as SeDsbA and KpDsbA have redox potential values very similar to that of EcDsbA ($-126$ and $-116 \text{ mV}$, respectively). We determined the redox potential of PmDsbA to be $-129 \text{ mV}$ (Fig. 1D), which is consistent with those of other Enterobacteriaceae DsbAs.

The oxidizing nature of DsbA proteins is thought to be a consequence of the highly acidic cysteine in the CXXC active site motif. The $pK_a$ of 3.3 for this nucleophilic cysteine Cys$^{30}$ for EcDsbA is unusually low for a cysteine ($pK_a 9.0$) (61). Values reported for other DsbAs vary from 3.0 (NmDsbA1) (62) to 5.1 (VcDsbA) (63). We measured the $pK_a$ of the equivalent cysteine in PmDsbA, by pH-dependent specific absorbance at $\lambda = 240 \text{ nm}$, and determined the value to be 4.0 (Fig. 1E). Thus, the nucleophilic
cysteine of PmDsbA, like that of other DsbAs, is likely to be in the thiolate form at physiological pH when the enzyme is reduced.

Although DsbA proteins are dithiol oxidases they can catalyze disulfide reduction in the presence of mild reducing agents such as DTT. Typically, insulin is used as a substrate to study in vitro disulfide bond reduction. The two chains of insulin are linked by three disulfide bonds, which can be rapidly reduced by disulfide reductases such as the disulfide isomerase EcDsbC. This reduction leads to separation of the insulin A and B chains, and precipitation of the insoluble B chain. Reduction can be followed by measuring the increase in turbidity of the solution over time. We found that PmDsbA is able to reduce the disulfide bonds of insulin as readily as EcDsbA (Fig. 1F), but more slowly than the specialist reductant EcDsbC. In summary, the redox properties of PmDsbA reported here place it in the same class as EcDsbA and other Enterobacteriaceae DsbAs such as S. enterica DsbA (SeDsbA) and K. pneumoniae DsbA (KpDsbA) (18).

**PWATCDS Binding to PmDsbA and PmDsbAC30S**—We were interested to understand how peptides interact with PmDsbA, as the basis for future peptidomimetic inhibitor development. The low-resolution crystal structures and NMR characterization of the EcDsbB-EcDsbA complex revealed that the EcDsbB periplasmic loop P2 forms a mixed disulfide with the nucleophilic cysteine of EcDsbA and binds to a hydrophobic groove near the active site (29, 30, 64, 65). The peptide sequence of the EcDsbB P2 loop 98PSPFATCDF106 is conserved in PmDsbB (99PSPFATCDF107) suggesting that a similar interaction occurs between PmDsbA and PmDsbB. From the sequence of this P2 loop peptide, we developed a shorter, modified peptide PWATCDS optimized for solubility, binding affinity, and inhibition of EcDsbA. Briefly, alanine scanning, peptide length scouting, and substitution of specific residues showed that the peptide PWATCDS had a good affinity to mass ratio for binding to EcDsbA. We hypothesized that this peptide would also interact with PmDsbA, and therefore evaluated its binding using thermal shift assay and ITC.

**FIGURE 2.** Peptide PWATCDS interacts with PmDsbA. A, values of ΔTm upon addition of increasing PWATCDS peptide for PmDsbA and PmDsbAC30S. Data are shown as mean ± S.D. from 5 replicates. B, ITC data titrating PWATCDS into PmDsbA. The reaction was exothermic suggesting a dominant enthalpic contribution to binding. C, ITC data titrating PWATCDS into PmDsbAC30S. Panels B and C show a representative example from three replicates.

ΔTm values for PmDsbA and PmDsbAC30S (a variant in which the nucleophilic cysteine was replaced by serine) were measured in the presence and absence of the peptide PWATCDS, with varying peptide concentrations. For PmDsbA, Tm values of +2.0, +4.1, and +5.0 K were observed at peptide concentrations of 1.2, and 4 mM, respectively, suggesting that PWATCDS binds to PmDsbA. These data show that the Tm shift continued with increasing peptide concentration beyond saturation, as is commonly observed in this assay (66). When the variant PmDsbAC30S was used, thermal shifts (ΔTm > +0.3 K) were detectable at concentrations of PWATCDS ranging from 250 μM to 4 mM. However, the maximum ΔTm at 4 mM was significantly lower than that at the same concentration for PmDsbA (ΔTm +1.5 K, compared with 5.0 K, respectively) (Fig. 2A). This difference in ΔTm suggests the possibility of different binding modes.

We also investigated the interaction using ITC. An exothermic reaction was observed when titrating PWATCDS into PmDsbA (Fig. 2B). Analysis of the raw data using a 1:1 binding model revealed a binding affinity Kd of 8.3 ± 0.4 μM with a high enthalpic contribution (ΔH = −13.7 ± 0.3 kcal/mol) and an unfavorable entropy of binding (ΔS = −22.3 ± 1.1 cal/mol/deg). When PmDsbAC30S was used, there was no evidence of PWATCDS binding by ITC under these same conditions (Fig. 2C). Clearly, the C30S mutation reduced the affinity of peptide binding, as evidenced by both thermal shift and ITC. Nevertheless, we were able to generate a crystal structure of this complex as described below. These differing outcomes for the apparently weak complex of PmDsbAC30S-PWATCDS may be a consequence of different experimental design (thermal shift and crystallization used a 1-h preincubation of peptide with PmDsbAC30S, ITC methodology does not allow this; ITC was performed at 25 °C, whereas preincubation was done at 4 °C.

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and crystallization at 20 °C) or different experimental conditions (buffer, pH, and concentration of peptide as outlined under “Experimental Procedures”).

Crystal Structure of PmDsbA—To investigate the different binding modes further, we attempted to determine the crystal structures of PmDsbA and PmDsbAC30S, in the presence of the peptide PWATCDS. We were unable to generate a crystal of a stable complex of PmDsbA with PWATCDS. However, crystals of PmDsbA and PmDsbAC30S without peptide, and PmDsbAC30S with peptide yielded high-resolution diffraction data (PmDsbA, 1.77-Å resolution; PmDsbAC30S, 1.98-Å resolution; and PmDsbAC30S-PWATCDS, 1.6-Å resolution).

PmDsbA crystallized in a tetragonal crystal system and was solved (PDB code 4OCE) by molecular replacement using the EcDsbA structure (PDB code 1FVK) as a template. One protein chain is present in the asymmetric unit, and this has a typical DsbA-like architecture, comprising a TRX core domain (β1, β2, α1, α2, α3, C-terminal region of α6, β4, α5, and α7), interrupted by an α-helical domain (α2-α5 and the N-terminal region of α6). This PmDsbA structure is structurally similar to other Enterobacteriaceae DsbAs including EcDsbA (PDB code 1FVK (67)), KpDsbA (PDB code 4MCU (18)), and SeDsbA (PDB code 3L9S (68)) (Fig. 3A). This is reflected in the r.m.s. deviation of 1.0–1.2 Å comparing 177 Ca atoms from equivalent positions of these enzymes. Fig. 3B shows a structure-based sequence alignment of PmDsbA with homologous DsbA proteins from clinically relevant pathogens that share >59% sequence identity. Sequences are highly conserved, especially at the active site CPHC motif. Sequence identity translates into overall structural similarity between DsbA proteins from these pathogens.

The sequence and structure of the active site motif (CPHC) and the cis-Pro loop are identical in these DsbA homologues. However, helix α1 and the loops connecting β1 and β2, and α3 and α4 differ in their relative positions. Most importantly loop L3 that connects β3 and helix α7 varies across the structures. The EcDsbA L3 residues Asp67, Thr168, and Ser69 are positioned closer to the active site helix α1 than the equivalent residues (Ala67, Lys168, and Ser69) in PmDsbA. This region is relatively hydrophobic in both EcDsbA and EcDsbB, although more basic in PmDsbA than in EcDsbA due to the presence of Lys159 in the former and Glu160 in the latter. Overall these differences in structure are relatively minor compared with the structures of other DsbA proteins, such as Pseudomonas aeruginosa DsbA (25% identity with PmDsbA, r.m.s. deviation 2.2 Å, 175 Ca, PDB code 3H93 (35)), and Wolbachia pipientis DsbA1 (16% identity with PmDsbA, r.m.s. deviation 3.6 Å, 151 Ca, PDB code 3FR4 (41)).

Crystal Structure Determination of PmDsbAC30S and PmDsbAC30S-PWATCDS—We solved the structure of the active site mutant PmDsbAC30S (PDB code 4OCF). The crystals grew in the presence of peptide, but no bound peptide was evident in the electron density map. We therefore used this structure of PmDsbAC30S to assess whether replacement of Cys30 with Ser30 induced any structural changes. The mutant crystallized in a monoclinic crystal system containing 4 protomers in the asymmetric unit. The structure was solved by molecular replacement using the coordinates of PmDsbA described above. The average r.m.s. deviation for comparison of the 6 combinations of the 4 protomers range from 0.3 to 0.7 Å for 177 equivalent Ca atoms (residues 6–181).

PmDsbAC30S in complex with peptide PWATCDS crystallized in a trigonal crystal system, containing 3 protomers in the asymmetric unit. The complex was solved by molecular replacement using PmDsbA (PDB code 4OD7) as the template. All three protomers (A, B, and C) reveal strong electron density corresponding to bound PWATCDS. The average r.m.s. deviation for the 3 comparisons of the protomers in this crystal structure range from 0.4 to 0.6 Å for 177 equivalent Ca atoms (residues 6–181). The r.m.s. deviation for comparison of PmDsbAC30S with PmDsbAC30S-PWATCDS range from 0.5 to 0.9 Å (177 Ca) for the 12 combinations. Similarly, comparison of the wild type PmDsbA structure with PmDsbAC30S and PmDsbAC30S-PWATCDS gave r.m.s. deviation values of 0.3–0.9 Å (177 Ca). This result indicates there is no major realignment of the PmDsbAC30S crystal structure upon interaction with PWATCDS. However, there is evidence of rigid body shifts for helix 1 and side chain adjustments for residues on helix 1 (His32, Tyr34, Glu156, Phe36, Ser37) and flexible loop 3 (Ile165 and Ser166) that together form part of the peptide binding site.

Active Site Structure Is Conserved after Peptide Binding—A comparison of the active site motifs (CPHC and CPHS) and the Val150/Pro151 residues of the cis-Pro loop across all the protomers in the PmDsbA, PmDsbAC30S, and PmDsbAC30S-PWATCDS crystal structures reveals a high degree of structural conservation. The active site cysteines Cys30 and Cys33 in the native PmDsbA structure were modeled as a mixture of oxidized and reduced (ratio 0.7:0.3). In the oxidized state the two sulfurs (Fig. 3C) are 2.2 Å apart, reflecting the typical length of a covalent disulfide bond found in other oxidized DsbA structures (e.g. EcDsbA and SeDsbA, S-S distance 2.0 Å) (26, 68). The dithiol form of the cysteines is likely a consequence of radiation-induced disulfide reduction. The distance between the dithiol sulfurs is 3.4 Å. In the PmDsbAC30S and PmDsbAC30S-PWATCDS structures, the distance between the hydroxyl oxygen of Ser30 and the sulfur atom of Cys35 varies between 3.3 and 3.5 Å (Fig. 3D). These distances are in agreement with those of other reduced DsbA structures (e.g. KpDsbA 3.3–3.8 Å) (18). Overall, there is no apparent change in the active site upon binding of the peptide.

Peptide Binding Mode and Interaction with PmDsbAC30S—The binding mode of the peptide (Fig. 4, A and B) is highly conserved across the three protomers (r.m.s. deviation 0.1–0.2 Å over all 82 atoms). The electron density from this high-resolution structure provides strong evidence for both the position and conformation of the bound peptide (Fig. 4C). The binding site for PWATCDS (residues 1 to 7) includes the hydrophobic groove and the active site regions (CXXC and cis-Pro motif) of the enzyme. As expected, the hydrophobic residues Pro1 and Trp2 (italics indicate peptide residues) interact with the hydrophobic groove, and the C-terminal residues interact with the cis-Pro region of the active site.

However, the observed binding mode was not entirely as predicted. On the basis of the EcDsbA-EcDsbB covalent complex,
Cys5 of the peptide should interact with PmDsbAC30S residue 30 (mutated from Cys to Ser). This was not the case. In all three complexes in the asymmetric unit, Asp6 and not Cys5 interacts with PmDsbAC30S Ser30 (Fig. 4D). The acidic side chain of Asp6 is within hydrogen bond contact distance of Ser30 and His32. Moreover, the backbone amide of Asp6 forms hydrogen bonds with the backbone amide of Val149 of the PmDsbAC30S cis-Pro loop (Fig. 4D).

Comparison of Peptide Binding Mode with DsbA-DsbB Complexes—The EcDsbB P2 loop (sequence PSPFATCDF) and the synthetic peptide we used (PWATCDS) share the same binding location on EcDsbA and PmDsbAC30S, respectively. The EcDsbA-EcDsbB complex (3.7-Å resolution (30)), revealed one possible hydrogen bond between P2 and the enzyme (backbone oxygen of Arg148 of the EcDsbA cis-Pro loop with the backbone nitrogen of EcDsbB Phe106). All other interactions with the P2 loop, aside from the covalent disulfide, are hydrophobic (28).

As indicated above, no interaction was observed between Cys5 and Ser30 of PmDsbAC30S. Instead, hydrogen bond interactions with Asp6 and hydrophobic interactions with Trp2 appear to dominate the PWATCDS binding mode. Residues between these two anchor points, Cys5, Thr4, and Ala3, protrude out of the binding site relative to the equivalent EcDsbB loop residues (Fig. 4, E and F).

The r.m.s. deviation for comparison of PWATCDS with the EcDsbB P2 loop conformation is relatively high (2.2 Å for 28 backbone atoms), because of the bulge in the peptide confor-
mation. Binding of PWATCDS results in an average buried surface area (i.e., surface that becomes inaccessible to solvent) of 955 ± 12 Å² or 9.0 ± 0.1% of the total surface of PmDsbAC30S (values are mean ± S.D. generated from the three molecules in the asymmetric unit). This is similar in area to the EcDsbA buried surface upon binding of the EcDsbB P2 loop (924 Å²) (30). In both structures a major feature is the binding of an aromatic residue in the hydrophobic groove. EcDsbB P2 loop residue Phe101 forms a T-shaped π-π stacking interaction with EcDsbA Phe174, whereas Trp2 of the PWATCDS peptide forms a parallel π-π stacking interaction with PmDsbAC30S Tyr173 and possible edge interactions with enzyme residues Pro170 and Tyr40.

DISCUSSION

The increasing incidence of infections caused by multidrug-resistant pathogens represents a serious global human health issue. Indeed, the emergence of carbapenem-resistant Enterobacteriaceae threatens to make common infections such as urinary tract infections untreatable (69). Antibiotic resistance is spreading rapidly and treatment options are becoming increasingly limited. One possible approach to address the paucity of

FIGURE 4. Analysis of the interaction between peptide PWATCDS and PmDsbAC30S. A, superposition of the PWATCDS peptides from all three protomer peptide complexes in the asymmetric unit. Note the peptide includes an N-terminal acetyl and a C-terminal amide group. Carbon atoms are shown in cyan, oxygens in red, nitrogens in blue, and sulfurs in yellow. B, location of the bound PWATCDS peptide on the surface of PmDsbAC30S (protomer A shown in gray). Peptide residues are labeled and colored as for panel A. The yellow patch indicates the location of Ser30. C, electron density map of PWATCDS (chain F) at the interface with PmDsbAC30S (chain B). The 2Fo – Fc map was generated in Phenix (53) and is contoured at 1.0 σ. PmDsbAC30S residues forming the binding site are labeled. D, interactions between the peptide (D–F, in cyan) and protein (chains A–C, in gray) are shown: hydrogen bonds are indicated as black dashed lines; a circle highlights the stacking interaction between Trp2 and PmDsbAC30S Tyr173. E, superposition of the EcDsbB P2 periplasmic loop (PSPFATCD, orange and black letters, PDB code 2ZUP) with PmDsbAC30S-PWATCDS (red letters, backbone in cyan). F, schematic representation of the interactions formed between EcDsbB P2 loop binding to EcDsbA (top) in comparison to PWATCDS binding to PmDsbAC30S (bottom), showing the comparative shift in register. Covalent bonds are shown as black lines, hydrogen bonds are indicated with red dashed lines, and hydrophobic interactions with black dotted lines.
new antimicrobials in the developmental pipeline lies in the generation of novel anti-virulence drugs (19, 70).

The Gram-negative DsbA/B system has been proposed as a target for the development of novel anti-virulence drugs (17). Targeting DsbA/B for development of inhibitors could be beneficial in many ways. First, DsbA is not essential for bacterial survival (27) although it plays an essential role in virulence (17). Thus, inhibiting DsbA/B would not kill bacteria and this property may reduce the selective pressure to develop resistance. Second, structures of several DsbA homologues have been solved (18, 28, 71), providing a framework for structure-based drug design. Third, DsbA structures and properties are more highly conserved than the structures and sequences of virulence factors across pathogens (18). Therefore, inhibitors that target one DsbA enzyme within a subclass are likely to block DsbAs within the subclass offering the possibility of medium-spectrum inhibitors (18).

Designing an inhibitor to block a protein-protein interface, such as that of a DsbA, requires a comprehensive knowledge of its binding interactions. Here we have used an innovative approach to define the interaction surface of PmDsbA by using knowledge from the low resolution crystal structure of EcDsbA-EcDsbB. We characterized a peptide derived from the sequence of DsbB, showed that it bound to PmDsbA and co-crystallized it in a non-covalent complex with PmDsbAC30S. This provides the first example of a high-resolution crystal structure of a DsbA in complex with a non-covalently bound peptide. Two previously published structures of DsbA in complex with bound peptides include (i) EcDsbA bound covalently with a substrate SigA-derived peptide; and (ii) Xyfella fastidiosa DsbA also bound covalently with a peptide that co-crystallized fortuitously.

P. mirabilis associated infections are often difficult to treat due to its propensity to form biofilms (4). Two cell surface organelles associated with P. mirabilis biofilm formation, mannose-resistant Proteus-like (MR/P) fimbriae (72) and flagella (which mediate swarming) (4), require DsbA for their correct assembly. Taken together, our data show that PmDsbA exhibits redox, functional, and structural properties typical of the DsbA class Ia enzymes (73), which extends to all DsbAs characterized to date from Enterobacteriaceae (18). We expect that the structural details of the peptide binding mode and the interactions observed will likely hold true for all of these enzymes. Specifically, the hydrophobic groove and the cis-Pro loop provide key points of interaction that could be exploited further. The high-resolution crystal structure of the non-covalent complex between PmDsbAC30S and peptide may provide an important platform for the development of peptidomimetic antivirulence compounds targeting PmDsbA and by extension the DsbAs from all Enterobacteriaceae.

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Crystal Structure of the Dithiol Oxidase DsbA Enzyme from Proteus Mirabilis Bound Non-covalently to an Active Site Peptide Ligand

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