Structure of the *Acinetobacter baumannii* Dithiol Oxidase DsbA Bound to Elongation Factor EF-Tu Reveals a Novel Protein Interaction Site

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Lakshmanane Premkumar†, Fabian Kurth, Wilko Duprez, Morton K. Grøtstehauge, Gordon J. King, Maria A. Halili, Begona Heras, and Jennifer L. Martin

From the Institute for Molecular Bioscience, Division of Chemistry and Structural Biology, University of Queensland, St. Lucia, Queensland 4067, Australia

The multidrug resistant bacterium *Acinetobacter baumannii* is a significant cause of nosocomial infection. Biofilm formation, that requires both disulfide bond forming and chaperone-usher pathways, is a major virulence trait in this bacterium. Our biochemical characterizations show that the periplasmic *A. baumannii* DsbA (AbDsbA) enzyme has an oxidizing redox potential and dithiol oxidase activity. We found an unexpected non-covalent interaction between AbDsbA and the highly conserved prokaryotic elongation factor, EF-Tu. EF-Tu is a cytoplasmic protein but has been localized extracellularly in many bacterial pathogens. The crystal structure of this complex revealed that the EF-Tu switch I region binds to the non-catalytic surface of AbDsbA. Although the physiological and pathological significance of a DsbA/EF-Tu association is unknown, peptides derived from the EF-Tu switch I region bound to AbDsbA with submicromolar affinity. We also identified a seven-residue DsbB-derived peptide that bound to AbDsbA with low micromolar affinity. Further characterization confirmed that the EF-Tu- and DsbB-derived peptides bind at two distinct sites. These data point to the possibility that the non-catalytic surface of DsbA is a potential substrate or regulatory protein interaction site. The two peptides identified in this work together with the newly characterized interaction site provide a novel starting point for inhibitor design targeting AbDsbA.

The global increase in multidrug- or pandrug-resistant *Acinetobacter baumannii* infections poses a major risk to public health. *A. baumannii* is one of the “ESKAPE” pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Enterobacter* species), so-called because of their ability to “escape” the effects of clinically useful antibiotics by biofilm formation and rapid acquisition of resistance genes (1). Collectively, these ESKAPE pathogens are a major cause of hospital- and community-acquired infections, morbidity, and mortality. New targets and strategies are needed to combat infections caused by these pathogens.

*A. baumannii* (colloquially known as Iraqibacter) is a Gram-negative, opportunistic pathogen that can cause a wide range of diseases, including pneumonia, meningitis, bacteremia, urinary tract infections, and wound infections (2, 3). This microbe has been responsible for multiple outbreaks around the world, including Australia and among United States military personnel deployed in the Middle East (5–9). There is a high incidence of *A. baumannii* infection among immunocompromised patients, especially those who have experienced long stays in hospital (10). Multidrug-resistant *A. baumannii* such as the AYE strain have accumulated a large cluster of resistance genes via horizontal gene transfer. Both its multidrug resistance and its ability to survive on highly desiccated abiotic surfaces (e.g. plastic and glass medical devices) have been linked to its success as a nosocomial pathogen (11, 12). Currently, there is no vaccine against *A. baumannii* (4).

*A. baumannii* can invade host cells, but little is known about its pathogenic mechanism. Biofilm formation is a major virulence trait contributing to bacterial colonization on tissue and persistence in the hospital environment (13). Electron and fluorescence microscopy experiments showed that cell surface fimbriae are necessary for *A. baumannii* attachment and initiation of biofilm formation on abiotic and biotic surfaces (14). Fimbriae are filamentous, multisubunit protein assemblies. Each fimbrial subunit contains an evolutionarily invariant disulfide bridge (15) that requires both the oxidative folding and the chaperone-usher pathways for assembly (13, 14, 16, 17).
**Peptide Interaction Sites of a Class I DsbA Enzyme**

The disulfide bond-forming system of Gram-negative bacteria typically comprises DsbA (a soluble, periplasmic, thiol-disulfide oxidoreductase) and DsbB (an integral inner membrane protein). DsbA catalyzes the formation of disulfide bonds in nascent substrate proteins as they are translocated into the periplasm. Through this reaction, the active site CXXC motif of DsbA becomes reduced and is restored to its functionally competent oxidized state through interaction with DsbB. DsbA is considered a master regulator of virulence and virulence traits (14, 18–21). Deletion or mutation of dsbA attenuates virulence factor maturation in bacterial pathogens including Protein mirabilis (22), uropathogenic Escherichia coli (23), Burkholderia pseudomallei (24), Vibrio cholerae (25), Shigella flexneri (26), and Salmonella enterica serovar typhimurium (27). DsbA inhibitors are potential anti-virulence drugs (28).

Here we characterize DsbA from *A. baumannii* (AbDsbA)\(^6\) and show that it has an unusually basic surface, with redox and catalytic properties that qualify it as a *bona fide* dithiol oxidase. We also report an unexpected interaction between AbDsbA and *E. coli* EF-Tu (EcEF-Tu); the complex can be co-purified from the cytoplasm of the *E. coli* expression system. This serendipitous discovery allowed the crystal structure determination of the AbDsbA-EcEF-Tu complex at 2.15 Å resolution and identification of specific intermolecular interactions.

Although the highly conserved EF-Tu is generally considered to be a cytoplasmic protein, extracellular localization has been reported in bacterial pathogens including *A. baumannii* (29), *P. aeruginosa* (30, 31), and *Mycobacterium tuberculosis* (32). Moreover, EF-Tu has been found to bind human fibronectin, platelet-activating factor receptor, plasminogen, and factor H for adhesion, tissue infiltration, and complement system inactivation. Intracellularly, EF-Tu has functions additional to its central role in protein translation. For example, in *Bacillus subtilis* EF-Tu plays a role in cell shape by interacting with actin-like proteins (33). Bacteriophages Qβ and T4 both recruit EF-Tu to perform essential functions (34, 35). We investigated the interaction by generating peptides derived from EF-Tu and showed that these bind tightly to AbDsbA, providing a platform for the future design of peptidomimetic inhibitors targeting AbDsbA.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—A codon-optimized synthetic gene coding for *A. baumannii* (AYE strain) DsbA (amino acids 27–205; Uniprot: B0V5X3) lacking the predicted periplasmic leader sequence (amino acids 1–22) was cloned into the pMCSG7 (36) plasmid to enable expression in the bacterial cytoplasm. This expression construct was also mutated to the *A. baumannii* (SDF strain) DsbA using the QuiChange™ (Agilent) procedure. These two new DsbA variants differ by a single amino acid at the CXXC catalytic motif (CFHC in AYE strain and CPLC in SDF strain).

AbDsbA proteins were expressed in BL21(DE3) cells using autoinduction (37). Cell pellets were resuspended in 50 mm Tris, pH 8.0, 100 mm NaCl and lysed in a Cell Disruptor (TS-Series, Constant Systems LTD.). Cell debris was removed by centrifugation. The supernatant was loaded onto Talon resin (Clontech), packed in a disposable gravity column, and equilibrated with 50 mm Tris, pH 8.0, 100 mm NaCl. Unbound proteins were removed with washing buffer (50 mm Tris, pH 8.0, 100 mm NaCl, 10% glucose, and 10% sucrose). Elution was achieved using wash buffer containing 300 mm imidazole. SDS-PAGE analysis of eluent fractions revealed that AbDsbA co- eluted with a second protein, identified as *E. coli* EF-Tu using mass spectrometry analysis (see below). The His\(_6\) affinity tag was removed by tobacco etch virus protease, leaving three vector-borne residues (Ser-Asn-Ala) at the N terminus of AbDsbA. The AbDsbA-EF-Tu complex was further purified using a Superdex S200 gel-filtration column (GE Healthcare). For purification of AbDsbA free from EF-Tu, the above procedure was modified to a batch purification step with Talon resin and repeated high salt wash (50 mm Tris, pH 8.0, 1.0 m NaCl). EcDsbA and *E. coli* membrane extracts containing overexpressed *E. coli* DsbB (EcDsbB) were prepared as described previously (38) and resuspended in phosphate-buffered saline containing 10% glycerol.

**Protein Identification by Mass Spectrometry**—Coomassie-stained bands corresponding to AbDsbA (21 kDa) and a ~40-kDa endogenous *E. coli* protein were cut from SDS-PAGE and subjected to in-gel tryptic digestion as described previously (39). The extracted peptides were applied to the MALDI target plate with α-cyano-4-hydroxycinnamic acid matrix. MS spectra were recorded in positive reflector mode at a laser energy of 3200 using a 4700 Proteomics Analyzer MALDI TOF-TOF (Applied Biosystems, CA). All MS/MS data from the TOF-TOF were acquired using the default positive ion, 1-kV collision energy, at a laser energy of 4400. Thirteen dominant species from the MALDI-MS data for the *E. coli* protein were queried against the SwissProt *E. coli* database with a tolerance of 0.2 Da using the MASCOT Peptide Mass Fingerprint server (Matrix Science). The top hit from the search indicated that the unknown protein was EF-Tu. In addition, MALDI-TOF-TOF data were used to identify a 2117.2 m/z species as the peptide AIDKPFLLPIEDVFSIGR, an EF-Tu tryptic peptide. The band corresponding to 21 kDa was confirmed as AbDsbA by comparison of the MALDI-MS species detected in its tryptic digest with those expected to arise from the tryptic digestion of AbDsbA.

**Crystallization and Structure Determination**—AbDsbA-EF-Tu complex crystals were grown at 20 °C by setting drops with 250 nl of protein solution (10 mg/ml in 25 mm HEPES, pH 7.4, and 100 mm NaCl) and 250 nl of precipitant solution (20–300 mm KCl and 4.5–6% PEG 3350) using a Mosquito robot (TTP Labtech) at the University of Queensland Remote Operation Crystallization and X-ray (ROCX) diffraction facility. Crystals were cryoprotected (in 250 mm KCl, 30 mm HEPES, pH 7.4, 15% PEG3350, and 25% glycerol) and flash-frozen in liquid nitrogen. Diffraction data from the cryo-cooled crystal were collected at the Australian Synchrotron MX2 beamline with an ADSC Quantum 315r detector controlled by BLU-ICE (40). Reflections were indexed and integrated in XDS (41), analyzed in
TABLE 1

Data collection and refinement statistics

| Data collection                              | Value       |
|----------------------------------------------|-------------|
| Wavelength (Å)                              | 0.9537      |
| Resolution range (Å)                        | 74.32–2.15  |
| Higher resolution shell (Å)                 | 2.27-2.15   |
| Space group                                 | P 2 1 2 1   |
| Unit cell                                   |             |
| a, b, c (Å)                                 | 74.3, 78.2, 122.8 |
| α, β, γ (°)                                 | 90, 90, 90  |
| Total reflections                           | 140,846     |
| Unique reflections                          | 39,200 (5,568) |
| Multiplicity                                | 3.6 (3.6)   |
| Completeness (%)                            | 99.4 (98.2) |
| Mean / σ(θ)                                 | 13.7 (2.5)  |
| R-merge                                     | 0.065 (0.596) |
| R-pim                                       | 0.040 (0.366) |

Refinement statistics

| Statistic                           | Value       |
|-------------------------------------|-------------|
| R-work (%)                          | 17.2 (24.0) |
| R-free (%)                          | 21.5 (28.0) |
| No. of non-H atoms                  |             |
| Protein                             | 4,454       |
| Ligands                             | 35          |
| Water                               | 245         |
| Protein residues                    | 567         |
| Root mean square bond lengths (Å)   | 0.008       |
| Root mean square bond angles (°)    | 1.12        |
| Ramachandran                        |             |
| Favored (%)                         | 97.0        |
| Outliers (%)                        | 0.3         |
| Average B-factor (Å²)               | 46.3        |
| Wilson B-factor (Å²)                | 36.1        |
| Molprobity (percentile)             |             |
| Score (percentile)                  | 2.0 [100th (843)] |
|                                   | 1.16 [100th (10,544)]  |

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**EcDsbA Complementation**—A chimera gene encoding for a mature AbDsba with an E. coli DsbA signal peptide or a wild-type EcDsbA was cloned under an arabinose-inducible promoter in the pBAD33 plasmid (50). DsbA null E. coli cells (ICB817; Ref. 51) harboring the EcDsba/pBAD33 or AbDsba/pBAD33 plasmid were spotted onto a soft M63 minimal agar plate containing 40 mg/ml concentrations of each amino acid (except l-cysteine) and 0.1% arabinose. Cell swarming was analyzed after incubating the plates for 4–5 h at 30 °C. The experiment was repeated two times, and the agar plates were photographed.

**Determination of Standard Redox Potential—Oxidized AbDsba (2 μM) in degassed 100 mM phosphate buffer, pH 7.0, containing 1 mM EDTA and 1 mM oxidized glutathione (GSSG) was incubated with a range of reduced glutathione (GSH) concentrations (0.01 μM–1 mM) for 24 h at 25 °C. AbDsba was precipitated with 10% trichloroacetic acid, and the pellet was washed with 100% ice-cold acetone. Free thiols in AbDsba were labeled with 4 mM 4-acetamido-4’-maleimidystilbene-2’-2’-disulfonate in 50 mM Tris, pH 7.0, and 1% SDS. Reduced and oxidized forms of AbDsba were separated on a NuPAGE 12% Bis-Tris gel (Invitrogen) and stained with Coomassie. Intensities of the reduced protein were analyzed using ImageJ (Version 1.44) (52). The fraction of the reduced protein was plotted against the ratio of [GSH]²/[GSSG], and the equilibrium constant K_eq was calculated using a binding equation, Z = ([GSH]²/[GSSG])/(K_eq + ([GSH]²/[GSSG])), where Z is the fraction of reduced protein at equilibrium. The redox potential was calculated using the Nernst equation: E° = E°_GSH/GSSG – (RT/nF)ln K_eq, where E°_GSH/GSSG is the standard potential of −240 mV (53), R is the universal gas constant 8.314 JK⁻¹mol⁻¹, T is the absolute temperature in K, n is the number of electrons transferred, F is the Faraday constant 9.648 × 10⁴ Cmol⁻¹, and K_eq is the equilibrium constant. The redox equilibria measurements were repeated two times, and the resulting mean values are plotted including S.D. for each measurement.

**Disulfide Reductase Assay**—Insulin turbidity as a result of disulfide bond reduction was monitored using a spectrophotometer at 650 nm for 60 min (54). The reaction mixture contained 131 μM insulin, 10 μM AbDsba or EcDsba or EcDsbC in 100 mM phosphate buffer, pH 7.0, containing 0.33 mM DTT and 2 mM EDTA. The mean values obtained for each data point from three repeats are plotted, and the standard deviations are displayed as error bars.

**Cysteine Thiol Oxidation Assay**—Labeled synthetic peptide substrate (CQQGFDGTQNSCK) with a europium DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) at the N terminus and a methylcoumarin at the C-terminal lysine side chain was prepared as previously described (55). The assay was performed in a 50-μl reaction volume in the presence of 2 mM oxidized glutathione or 1.6 μM membrane-solubilized E. coli DsbB (EcDsbB) in a 384-well plate (PerkinElmer Life Sciences OptiPlate). The reaction mixture contained 50 mM MES, pH 5.5, 50 mM NaCl, 2 mM EDTA, and 80 or 120 nM AbDsba or AbDsba-EF-Tu complex. Disulfide bond formation of the peptide (7 μM), which was added at the end to initiate the reaction, was followed using time-resolved fluorescence with excitation at 340 nm and emission at 615 nm, with a delay of 150
**Peptide Interaction Sites of a Class I DsbA Enzyme**

**FIGURE 1. Oxidoreductase properties of AbDsbA.** A and B, Redox equilibria with glutathione at pH 7.0 and 25 °C for AbDsbAH36 and AbDsbAL36, respectively. AbDsbA was equilibrated with redox buffer containing various ratios of glutathione to glutathione disulfide. The fraction of reduced DsbA was fit to a one-site binding equation using a non-linear regression method to obtain the equilibrium constant $K_{eq}$ as described under “Experimental Procedures.” C, in vivo disulfide oxidase activity. The ability of DsbA to recognize EcDsbB and EcDsbA substrates was assessed in vivo using non-motile dsbA null E. coli (72). A representative agar plate shows that expression of AbDsbA (right panel) weakly restores motility of dsbA null E. coli compared with the control EcDsbA (left panel). The motility zone, indicated by the white bar, was imaged 7 h after inoculating with $\sim 2 \times 10^7$ cells. In vitro dithiol oxidase activity in the presence of EcDsbB (D) or oxidized glutathione (E) is shown. Fluorescence increase as a consequence of disulfide bond formation in peptide substrate is monitored. F, disulfide reductase activity. Enzyme catalyzed or DTT-mediated disulfide reduction causes insulin precipitation, which was monitored spectrophotometrically at 650 nm. A mean of two (panels A and B) or three (panels D–F) measurements for each data point are shown. Error bars indicate the S.D. (A, B, and F) or S.E. (D–E).

Filled circle, AbDsbAH36; empty circle, AbDsbAL36; diamond, EcDsbA; star, EcDsbC; cross, control in the absence of DsbA/DsbC.

## RESULTS

**AbDsbA Exhibits the Typical Characteristics of a Dithiol Oxidase—** *P. aeruginosa* exhibits extensive genetic variation even among closely related strains due to gene transfer, mobilization of insertion sequences, recombination, gene loss, and mutation (57). Nevertheless, DsbA is highly conserved with sequence identities of $\geq 99\%$ among 122 *A. baumannii* strains analyzed using BLAST against the Uniprot *Acinetobacter* Knowledgebase. One prominent variation in AbDsbA sequences is located in the active site CXXC motif of an avirulent (SDF) strain. Specifically, the active site motif $^{34}$CPHC$_{37}$ in virulent AYE strain (AbDsbAH36) is substituted for $^{34}$CPLC$_{37}$ in the SDF strain (AbDsbA136). Because variations in the active site motif can directly influence redox potential and disulfide-bond forming activity of DsbA enzymes (58, 59), we measured the redox potentials of both AbDsbA natural variants (Fig. 1, A and B). We found that the redox potential of AbDsbA136 is more oxidizing ($-101 \pm 1.4$ mV) than that of AbDsbA136 (134 mV ± 1.2 mV), comparable to the reported value of $-94$ mV for *P. aeruginosa* DsbA (PaDsbA) (60). Conversely, the redox potential of AbDsbA136 was more similar to the *Enterobacteriaceae* DsbAs ($-116$ to $-129$ mV) (61).

Recombinant expression of a DsbA can rescue the motility of DsbA null *E. coli* cells because DsbAs typically have a broad protein substrate specificity (62, 63). However, we found that...
AbDsbA only weakly restores the motility of *E. coli* DsbA-null cells in the cell-swarming assay (Fig. 1C).

To assess whether AbDsbA has *in vitro* disulfide-bond-forming activity, we used a time-resolved fluorescence assay using a substrate peptide labeled with a europium chelate and a coumarin donor (see “Experimental Procedures.”). In the presence of EcDsbB as a source of DsbA oxidant, we found that AbDsbA enzymes were only marginally active compared with EcDsbA (Fig. 1D). However, using oxidized glutathione as the DsbA oxidant (Fig. 1E), we found that AbDsbA<sup>1-36</sup> and EcDsbA dithiol oxidase had comparable activity and that AbDsbA<sup>1436</sup> was more active again, under the same conditions. These results suggest that AbDsbA is an oxidase, but it does not interact with EcDsbB.

We then assessed for the ability of AbDsbA to catalyze disulfide reduction using insulin as the general substrate in the presence of dithiothreitol as a mild reducing agent (51). In this experiment AbDsbA<sup>1-36</sup> reduced the intermolecular disulfide bonds of insulin more rapidly than AbDsbA<sup>1436</sup> but much more slowly than the dedicated disulfide isomerase EcDsbC (Fig. 1F).

The results of these experiments demonstrated that both variants of AbDsbA are *bona fide* dithiol oxidases. The data also indicated that the interaction surface for DsbB may be different from that of EcDsbA. The lack of AbDsbA interaction with EcDsbB was somewhat surprising given that PaDsbA, which shares 44% sequence identity to AbDsbA, does interact with EcDsbB (60). However, AbDsbA has a calculated pl of 9.5 compared with 5.8 for PaDsbA, and this may contribute to differing interaction propensities.

**AbDsbA Directly Interacts with EF-Tu**—Recombinant His-tagged AbDsbA (AbDsbA<sup>1436</sup>, hereafter referred to as AbDsbA, for simplicity) expressed in the cytoplasm of *E. coli* and purified by His-affinity chromatography consistently co-purified with a 40-kDa contaminant that we identified as an endogenous *E. coli* protein (Fig. 2A). Binding of the *E. coli* protein by His-immobilized AbDsbA was unchanged up to 1.1 M NaCl or 0.5% v/v Triton X-100. Even after metal affinity purification, AbDsbA and the *E. coli* protein co-eluted as a major fraction in size exclusion chromatography (Fig. 2B). Analysis of samples in SDS-PAGE run under non-reducing conditions indicated that the interaction between AbDsbA and EF-Tu was not thiol-mediated (Fig. 2A). Using in-gel digestion and mass spectrometric analysis, we identified the co-eluant as the *E. coli* elongation factor Tu (EF-Tu). Notably, the dithiol oxidase activity of the AbDsbA-EF-Tu 1:1 complex was reduced by ~2/3 compared with the activity of AbDsbA alone (Fig. 2C).

**Structure of the AbDsbA-EF-Tu GDP Complex**—To elucidate the structural basis for the recognition of EF-Tu by AbDsbA, we determined the structure of AbDsbA-EF-Tu complex. Before crystallization the protein complex was purified by size exclusion chromatography. The crystal structure of the complex was phased by molecular replacement using the coordinates of *E. coli* EF-Tu (PDB ID 1EFC) as the search model (Fig. 3A) and refined to 2.15 Å resolution.

The crystal structure revealed a 1:1 complex and showed that EF-Tu is captured in its guanosine diphosphate (GDP)-bound state (Fig. 3, A and E). EF-Tu-GDP folds into three distinct domains: the N-terminal guanine nucleotide binding catalytic domain (Domain 1, also known as the G domain) connected to 7-stranded and 6-stranded β-barrel domains (Domains 2 and 3). The global architecture of EF-Tu-GDP in complex with AbDsbA is similar to that of the previously reported EF-Tu-GDP structure except for a minor movement of Domains 2 and 3 and a major local conformational rearrangement in the catalytic Domain 1 (Fig. 3, B–C).

Previous structural studies showed that the EF-Tu catalytic domain can undergo nucleotide-dependent rearrangements primarily involving two flexible regions, known as switch I (residues 51–65) and switch II (residues 84–100). In the GTP-
bound form, switch I forms an α-helix (PDB ID 1OB2), which rearranges in the GDP-bound form to a β-hairpin (PDB ID 1EFC) or a disordered segment (PDB ID 1EFU). The switch II helix alters both its orientation and length between these two nucleotide binding states. In contrast, in the present complex, switch I is extended (Fig. 3, B and D), whereas the switch II conformation and the GDP binding mode are as described previously for EF-Tu (PDB ID 1EFC). In this complex with EF-Tu, AbDsbA adopts the canonical DsbA fold comprising a non-contiguous thioredoxin fold with a helical domain insertion (Fig. 4, A). The AbDsbA structure is similar to that of PaDsbA with a root mean square deviation of 1.28 Å for 164 Cα atoms. The AbDsbA structure encompasses a five-stranded mixed β-sheet surrounded by 7 helices (H1-H7) with the crucial CXXC motif located at the N terminus of helix H1. Structural comparison of AbDsbA and PaDsbA shows that the catalytic residues formed by the CXXC (34CPHC37) motif and the adjacent essential cis-Pro motif (Val150–Pro151) are identical (Fig. 4B). In AbDsbA, the distance between sulfur atoms of the first and the second of the two active site cysteines measures 3.6 Å, which falls within the range observed in other reduced DsbA structures (3.3–3.8 Å). The interatomic distances of key atoms of the active site residues of AbDsbA are similar to those of PaDsbA, suggesting that EF-Tu binding does not induce changes at the AbDsbA active site. Catalytic residues of DsbA enzymes are surrounded by three surface loops (Fig. 4, L1, the well characterized cis-Pro loop), and the β5-H7 connecting loop (L3). In E. coli DsbA these three loops create a catalytic surface targeted by substrate and partner proteins (63, 66, 67). In the present structure, however, the switch I-mediated EF-Tu interaction does not engage the catalytic surface but instead interacts with the solvent-exposed non-catalytic surface of AbDsbA (see below).

The Positively Charged Non-catalytic Surface of AbDsbA Engages EF-Tu—We previously identified two DsbA classes based on the topological arrangements of strand β1: class I DsbA (3–2–4–5–1) and class II DsbA (1–3–2–4–5) (61). AbDsbA falls into the class I topology, with β1 forming hydrogen bonds to β5, creating a β-sheet topology of 3–2–4–5–1. The structural consequence of the class I DsbA topology (Fig. 4C) is the formation of a large groove between the thioredoxin and helical domains on the non-catalytic surface. This groove is truncated in the class II DsbAs in which β1 interacts with β3. The surface shape and electrostatic features of the non-catalytic surface groove are variable among class I DsbA members (Fig. 4, D–G). In AbDsbA, the non-catalytic surface groove has a distinct positive electrostatic potential, and it is this region that is engaged by the EF-Tu switch I segment (Fig. 4G).
Analysis of the interactions between the two proteins shows that the EF-Tu switch I segment (residues 47–56) adopts an extended conformation, occupying the groove between H1–H9252 and H3 on the non-catalytic surface of AbDsbA (Fig. 5A). The C terminus of switch I (residues 51–56) forms extensive hydrogen bonds and electrostatic interactions with four basic residues (Arg-56, Arg-59, Arg-84, Lys-85) and a polar residue (Gln-45) of AbDsbA (Fig. 5B). Asp-51 at the center of the EF-Tu switch I segment makes contact with five AbDsbA residues in the groove. In addition, EF-Tu residues Phe-47, Gln-49, Ala-53, and Pro-54 form van der Waals contact with Leu-48, Ile-51, His-88, Leu-89, Pro-90, and His-93 of AbDsbA. These polar and hydrophobic interactions bury 40% (683 Å²) of the solvent-accessible surface area of the EF-Tu switch I segment (residues 47–56).

On the basis of this structurally characterized interaction, we designed an 11-residue peptide derived from the E. coli EF-Tu switch I sequence and assessed its binding to AbDsbA using ITC. The binding study showed that the switch I peptide segment (46AFDQIDAPEE56) binds to AbDsbA with an affinity range of 74–162 nM (Table 2). Binding of the switch I peptide is unaffected by the redox status of AbDsbA. In contrast, we did not detect binding of this same switch I peptide to EcDsbA under the same conditions.

We also investigated whether EF-Tu from A. baumannii (AbEF-Tu) might interact with AbDsbA. Sequence comparison of switch I of E. coli EF-Tu with that of AbEF-Tu showed that the two sequences are similar but not identical (Table 2). Positions 1 and 3 of EcEF-Tu switch I are not conserved in AbEF-Tu, but neither of these residues interacts with AbDsbA in the

**FIGURE 4. AbDsbA and other DsbA I enzymes have a large groove on the non-catalytic surface.** A, structural overlay of AbDsbA (blue/violet) and PaDsbA (gold/green). DsbA contains a TRX-domain (bottom) and a helical domain (top) with the domains identified by different colors. Secondary structural elements are annotated, and the catalytic cysteines are shown as green spheres. B, structural superposition of catalytic sites of AbDsbA and PaDsbA. C, AbDsbA shares the same β-sheet topology (3–2–4–5–1) as the DsbA I enzymes (61). The switch I peptide segment of EF-Tu binds to the non-catalytic surface groove created at the interdomain space of AbDsbA. D–G, the non-catalytic groove on the DsbA I enzymes has a charged surface. Electrostatic surface potentials of B. pseudomallei DsbA (D), EcDsbA (E), PaDsbA (F), and AbDsbA (G) are contoured between −6.0 (red) and +6.0 (blue) kTe. The shape of the non-catalytic surface groove (located on the surface opposite to the catalytic site) and the electrostatic potential was not conserved among DsbA I enzymes. The switch I peptide segment of EF-Tu binds to the positively charged non-catalytic surface groove of AbDsbA. Neisseria meningitidis DsbA3 (PDB ID 3DVX) is the only other structurally characterized DsbA that has a positive potential in this region.
crystal structure (Fig. 5). However, Asn at position 7 of EcEF-Tu does interact with Gln-45 of AbDsbA, and this residue is replaced by Ser in AbEF-Tu. Side-chain modeling suggested that substitution of Asn for Ser at position 7 should maintain the hydrogen bond interaction with Gln-45 of AbDsbA. As expected, the switch I peptide of AbEF-Tu did bind to AbDsbA, although with a 3-fold reduced affinity of 500 nM compared with the EcEF-Tu peptide (Table 2). Removal of the 3 residues at the N terminus of the AbEF-Tu switch I peptide reduced binding by a further 3.5-fold, suggesting that the interaction of Phe/Tyr at position 2 with His93 of AbDsbA is very favorable.

**AbDsbA Binds a Peptide Derived from AbDsbB**—Dithiol oxidase activity of AbDsbA relies on interaction with Gln-45 of AbDsbB. As expected, the switch I peptide of AbEF-Tu did bind to AbDsbA, although with a 3-fold reduced affinity of ~500 nM compared with the EcEF-Tu peptide (Table 2). Removal of the 3 residues at the N terminus of the AbEF-Tu switch I peptide reduced binding by a further ~3.5-fold, suggesting that the interaction of Phe/Tyr at position 2 with His93 of AbDsbA is very favorable.

**AbDsbA Binds a Peptide Derived from AbDsbB**—Dithiol oxidase activity of AbDsbA relies on interaction with the redox cycling enzyme DsbB (Fig. 1, D–E). Structural studies of EcDsbA-EcDsbB complex revealed that a transient interaction between the cysteine-containing second periplasmic loop (P2) of the 4-helix bundle protein EcDsbB and the surface surrounding the catalytic cysteine of EcDsbA is responsible for maintaining EcDsbA in a functionally active oxidized state. The interaction of EcDsbA with EcDsbB is mediated in part by three loops, L1, L2, and L3, that define the catalytic surface. In AbDsbA, the L1 loop comprises five residues and is conformationally similar to that of *P. aeruginosa* DsbA (Fig. 4A), whereas the L3 loop is much shorter in AbDsbA, formed from just 3 residues (Gln162–Gly163–Glu164). As a consequence, AbDsbA has small discontinuous pockets (≤50 Å²) on the catalytic surface between the cis-Pro loop (L2) and L3 as opposed to a large continuous groove found in this region in EcDsbA.

The *A. baumannii* genome encodes for a protein homologous to EcDsbB (25% sequence identity, Uniprot: B0V9V0). The high secondary structural similarity of the predicted transmembrane helical regions suggested that the putative AbDsbB retains the same four-helix topology as EcDsbB with two loops pointing into the periplasm.

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**FIGURE 5. Analysis of the interaction between EF-Tu and AbDsbA.**

A. location of the bound EF-Tu switch I region on the non-catalytic surface of AbDsbA. Both the EF-Tu switch I residues (Phe-47–Glu-56) and the AdsbA residues (on H1, H3, β3, and the connecting loop between H1 and β3) contributing to the interaction are shown as sticks. B. details of EF-Tu switch I interaction with AbDsbA. The C terminus of EF-Tu switch I (green, Asp-51–Glu-56) forms the key hydrophilic and electrostatic interactions with AbDsbA. EF-Tu residues are labeled in blue; AbDsbA residues are labeled in black (hydrophobic interaction) or red (polar interaction). Interactions of the cis-Pro loop of E. coli DsbA with the P2 loop of E. coli DsbB (C) and similar interactions observed at the AbDsbA cis-Pro loop with a symmetry-related molecule of EF-Tu (domain 2) in the crystal structure (D) are shown. Potential hydrogen-bond interactions are shown as dashed lines. Cysteine thiols are shown as green spheres.
showed that it bound to AbDsbA with a heptapeptide (PSCGPGL) from the AbDsbB P2 sequence and AbDsbA. On the basis of this molecular model, we designed a that 7–8 AbDsbB residues could bind to the catalytic surface of the DsbA Ib sub-class, which also includes DsbAs from Neisseria and Pseudomonas (61). Using ITC, we demonstrated that an AbDsbB-derived peptide binds to AbDsbA via its catalytic cysteine, supporting the idea that DsbA and DsbB form a functional redox pair in A. baumannii. The absence of an interaction between AbDsbA and EcDsbB suggests that the truncated catalytic surface of AbDsbA may engage AbDsbB by a different binding mode to that described for EcDsbB and EcDsbA. In the structure of the EcDsbA-EcDsbB complex, Cys-104 from the EcDsbB P2 loop segment (98PSPFATCDFM107) forms a mixed disulfide bond with catalytic residue Cys-30 of EcDsbA, and other residues form hydrophobic interactions and three hydrogen bonds with the cis-Pro loop (Fig. 5C). The corresponding P2 loop of AbDsbB comprises residues 95PDQVSCPGP104.

In the structure of the EcDsbA-EcDsbB complex, residues 262RKLLD266 from the EcEF-Tu complex showed that residues 262RKLLD266 of a neighboring EF-Tu domain 2 interact with the catalytic surface of AbDsbA, placing the EF-Tu Leu-264’ side chain very close to the catalytic cysteine of AbDsbA (3.8 Å from Cys-34 S). Hydrogen bond interactions are formed between EF-Tu Leu-264’ and Asp-266 and the AbDsbA cis-Pro loop in a manner similar to that observed between the EcDsbA cis-Pro loop and the P2 loop of the EcDsbA-EcDsbB crystal structure (Fig. 5D). Modeling the AbDsbB P2 loop segment onto the observed crystal structure conformation of EF-Tu RKLLD residues suggested that 7–8 AbDsbB residues could bind to the catalytic surface of AbDsbA. On the basis of this molecular model, we designed a heptapeptide (PSCGPGL) from the AbDsbB P2 sequence and showed that it bound to AbDsbA with a $K_D$ of 7.9 μM (Table 2). Similar to a previous study (56), binding of the AbDsbB peptide is cysteine-dependent in that substitution of Cys for Leu in the peptide eliminated binding. Importantly, binding of the AbDsbB P2 peptide to AbDsbA is independent of EF-Tu peptide binding, supporting the notion that EF-Tu and AbDsbB bind to AbDsbA at distinct sites (Table 2).

**DISCUSSION**

Multidrug resistant A. baumannii is emerging as an agent of serious nosocomial and community-acquired infection (68). The current economic loss of preventable hospital-acquired infections ($5.7–6.7$ billion) is now comparable to the costs of stroke ($6.7$ billion), diabetes mellitus ($4.5$ billion), and chronic obstructive lung disease ($4.2$ billion) in the United States (69). Anti-virulence therapy is an attractive strategy to combat rapidly spreading infections caused by multidrug-resistant A. baumannii (28, 70, 71).

Studies from animal models suggested that the absence of DsbA or DsbB attenuates infection and pathogenesis of Gram-negative bacteria (23, 24, 73). Because DsbA/B is not essential for bacterial survival (51) but is required for virulence (17), DsbA/B is a target for the development of novel anti-virulence agents against Gram-negative bacteria (17). For example, biofilm formation is essential for colonization and infection by A. baumannii (13, 74, 75). Interference with this process by inhibiting DsbA could impair the ability of bacteria to establish infection.

Our characterization of AbDsbA showed that it is a member of the DsbA Ib sub-class, which also includes DsbAs from Neisseria and Pseudomonas (61). Using ITC, we demonstrated that an AbDsbB-derived peptide binds to AbDsbA via its catalytic cysteine, supporting the idea that DsbA and DsbB form a functional redox pair in A. baumannii. The absence of an interaction between AbDsbA and EcDsbB suggests that the truncated catalytic surface of AbDsbA may engage AbDsbB by a different binding mode to that described for EcDsbB and EcDsbA.

We also reported the detailed interaction between AbDsbA and EF-Tu. EF-Tu is highly conserved (85% sequence identity between AbEF-Tu and EcEF-Tu) and is best known for its catalytic role in the elongation cycle of bacterial protein synthesis. However, EF-Tu also acts as a response protein in abiotic and biotic stresses (76, 77), and it has roles in biofilm development (78) and immunospecific immunity (79). EF-Tu is generally considered to be a cytoplasmic protein, but there is evidence for localization on the surface and outer membrane vesicles of bacteria (29, 80, 81). It is also a relatively ubiquitous interactor with other proteins (actin like MreB (33) and fibronectin (32)). The association between AbDsbA and EF-Tu provides new avenues for understanding the interaction of EF-Tu with other proteins; for example, the Switch I region may interact tightly with cytoplasmic proteins that exhibit similarly strong positive surface

**TABLE 2**

| Peptide derived from: | Protein | $N$ | $K_D$ (nM) | $\Delta H$ (kcal/mol) | $-\Delta S$ (kcal/mol) |
|-----------------------|---------|-----|------------|-----------------------|------------------------|
| EF-Tu                 | AbDsbA (ox) | 0.9 | 146 ± 37 | -6.4 ± 0.1 | -3.0 |
| AFQIQIDNAPEE          | AbDsbA (red) | 0.8 | 74 ± 7  | -5.7 ± 0.0 | -4.0 |
| AFQIQIDNAPEE          | AbDsbA (mix) | 1.6 | 162 ± 9 | -5.5 ± 0.2 | -3.7 |
| AFQIQIDNAPEE          | EcDsbA (ox) | | | | |
| DYSQIDSAPAEE          | AbDsbA (mix) | 1.1 | 476 ± 222 | -7.0 ± 1.4 | -1.6 |
| QIDSAPAEE             | AbDsbA (mix) | 1.3 | 1675 ± 216 | -4.9 ± 0.2 | -3.0 |
| AbDsbB                | AbDsbA (ox) | 1.0 | 7.9 ± 1.7 | -10.3 ± 0.9 | 3.3 |
| PSCGPGL               | AbDsbA (ox or red) | | | | |
| PSLGPGL               | AbDsbA (ox or red) | | | | |
| PSCGPGL               | AbDsbA (ox) with AFQIQIDNAPEE | 1.0 | 9.4 ± 1.9 | -9.5 ± 0.4 | 2.6 |
charge. The switch I region is also the binding site for other proteins including ribosome (82). The PDB entry 4IW3 (yet to be published) shows the EF-Tu switch I region interacts with Pseudomonas putida prolyl-4-hydroxylase, although in a somewhat different conformation. Overall, these data suggest that the switch I region of EF-Tu is a flexible protein interaction loop.

It is tempting to speculate a physiological/pathological role for the association between AbDsbA and EF-Tu that might involve regulation of cell growth in response to nutrient deprivation or oxidative stress in the host environment or chaperoning of EF-Tu by AbDsbA. Both AbDsbA and EF-Tu are important proteins in their own right, and both have been identified as antibacterial targets. Although we showed that AbEF-Tu-derived peptides do bind to AbDsbA, we have no direct evidence for an AbDsbA:AbEF-Tu interaction in A. baumannii. Consequently, we cannot rule out the possibility that the interaction between EcEF-Tu and AbDsbA is simply an artifact.

Nevertheless, the detailed interactions derived from the AbDsbA:EF-Tu structure provides a potential new approach for AbDsbA inhibitor development. The DsbA catalytic surface is widely accepted as the major target surface for substrate/partner protein interactions (63, 66, 67). This interaction is essential for formation of intermediate mixed disulfides with substrate proteins. However, the non-catalytic surface may also contribute to the folding of substrates or to the interaction with as-yet unidentified regulatory proteins.

Members of class I DsbAs all have a large charged non-catalytic surface groove (Fig. 4, D–G). In EcDsbA the equivalent acidic surface was identified several decades ago as a potential interaction site (83). The variable molecular landscape and nature of this surface among DsbAs may reflect the extraordinary variability of substrates (61, 84). The high resolution structure of the AbDsbA:EcEF-Tu complex supports the idea that the pronounced non-catalytic surface groove of class I DsbA enzymes serves as an additional protein interaction site. Whether or not this is the case remains to be elucidated. Nevertheless, the reduced activity of AbDsbA in the presence of bound EcEF-Tu suggests compounds designed to target this non-catalytic site would inhibit AbDsbA enzyme activity.

In summary, we have characterized AbDsbA and identified two peptides that bind to non-overlapping AbDsbA surfaces. These distinct peptides provide the starting point for peptide-based inhibitors targeting AbDsbA activity.

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