Gram-positive DsbE Proteins Function Differently from Gram-negative DsbE Homologs

A STRUCTURE TO FUNCTION ANALYSIS OF DsbE FROM MYCOBACTERIUM TUBERCULOSIS

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*Mycobacterium tuberculosis*, a Gram-positive bacterium, encodes a secreted Dsb-like protein annotated as *Mtb* DsbE (Rv2878c, also known as MP753). Because Dsb proteins in *Escherichia coli* and other bacteria seem to catalyze proper folding during protein secretion and because folding of secreted proteins is thought to be coupled to disulfide oxidoreduction, the function of *Mtb* DsbE may be to ensure that secreted proteins are in their correctly folded states. We have determined the crystal structure of *Mtb* DsbE to 1.1 Å resolution, which reveals a thioredoxin-like domain with a typical CXXC active site. These cysteines are in their reduced state. Biochemical characterization of *Mtb* DsbE reveals that this disulfide oxidoreductase is an oxidant, unlike Gram-negative bacteria DsbE proteins, which have been shown to be weak reductants. In addition, the pH value of the active site, solvent-exposed cysteine is ~2 pH units lower than that of Gram-negative DsbE homologs. Finally, the reduced form of *Mtb* DsbE is more stable than the oxidized form, and *Mtb* DsbE is able to oxidatively fold hirudin. Structural and biochemical analysis implies that *Mtb* DsbE functions differently from Gram-negative DsbE homologs, and we discuss its possible functional role in the bacterium.

Protein disulfide bonds are often buried and serve to stabilize protein structures. However, partially exposed disulfide bonds in the active sites of protein-disulfide oxidoreductases have a variety of mechanistic roles in protein folding, electron transport, and bioenergetics in organisms ranging from prokaryotes to humans. Most disulfide oxidoreductases contain a conserved thioredoxin-like domain such as in thioredoxin, eukaryotic protein disulfide bond isomerase, glutaredoxin (1), peroxiredoxins (2), and disulfide bond-forming proteins (Dbp).1 Except for peroxiredoxins, all of these proteins share a common sequence motif (CXXC) at their active sites.

One such family of disulfide oxidoreductases is the Dsb proteins, which are best characterized in *Escherichia coli*. These proteins reside in the periplasmic space of Gram-negative bacteria (Fig. 1A) and are necessary for the correct folding of many cell envelope proteins (3). *E. coli* DsbE is a thioredoxin-like protein, involved in cytochrome c maturation (4). DsbE has been implicated in the reduction of the thiol ether linkers to the heme of apocytochrome c (5), prior to heme ligation by CcmF and CcmH (4, 6). *E. coli* DsbD is a cytoplasmic transmembrane protein responsible for maintaining DsbE in its reduced state in the periplasm (7). *E. coli* DsbC is a homodimer with disulfide bond isomerase activity that is also kept reduced by the transmembrane protein DsbD (8, 9). In contrast, *E. coli* DsbA is a monomer that catalyzes the oxidation of reduced, unfolded proteins (10, 11). DsbA is oxidized by the transmembrane protein DsbB, which is in turn oxidized by components of the electron transport pathway (12, 13). DsbA proteins, in particular DsaA, have been shown to be involved in virulence in toxin-secreting Gram-negative bacteria such as *Vibrio cholerae* (14, 15), *Yersinia pestis* (16), *Shigella sp.* (17), and *E. coli* (18).

Gram-positive bacteria do not have a periplasm, and proteins that are secreted from the cytoplasm are either cell wall-associated or extracellular. In *Mycobacterium tuberculosis*, the only Dsb proteins present are homologs to *E. coli* DsbE (*Mtb* DsbE or Rv2878c, also known as MPT53) and its redox, transmembrane protein partner, *E. coli* DsbD (*Mtb* DsbD or Rv2874), which are depicted in Fig. 1B. The presence of Dsb proteins in Gram-positive bacteria, such as *M. tuberculosis*, suggests that these proteins are necessary for the correct folding of cell wall-associated and extracellular secreted proteins. Hence, studies of *Mtb* DsbE may give some insights into the virulence of mycobacteria.

*M. tuberculosis* is a pathogenic bacterium that is responsible for tuberculosis, which is the world’s leading cause of adult death by a bacterial infectious disease, with 3 million deaths and 10 million new cases per year (19, 20). The *M. tuberculosis* Structural Genomics Consortium is an international effort focusing on determining protein structures from *M. tuberculosis*, including potential drug targets (21, 22). A promising group of

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1 The abbreviations used are: Dsb, disulfide bond-forming proteins; *Mtb*, *M. tuberculosis*; Rv number, Sanger center notation for each gene in *Mtb*.
potential drug targets is the secreted proteins of *M. tuberculosis*, which are important in the induction and escape from host immune responses and virulence. *Mtb* DsbE is one such uncharacterized secreted protein. This work presents an example of protein structural analysis leading to functional information.

**EXPERIMENTAL PROCEDURES**

**Purification and Crystallization**—A recombinant plasmid producing the mature DsbE (without the signal peptide, residues 30–159) in pQE30 (Qiagen) was constructed as described previously (23). The purification of *Mtb* DsbE has been previously described (24). The purified protein was dialyzed into 0.5M NaCl and 0.1M Tris/HCl, pH 7.4, for crystallization trials. The protein crystallized in 2.2M NH4SO4, 5% isopropyl alcohol, 20% glycerol; the crystals were mounted; and diffraction data were collected under cryoconditions identical to the crystallization conditions. The selenomethionine *Mtb* DsbE protein was prepared as previously described (25) and crystallized under identical conditions to the native protein.

**Data Collection and Structure Determination and Refinement**—A selenomethionine-substituted *Mtb* DsbE crystal diffracted to 1.5 Å, and a native crystal diffracted to 1.1 Å, both having unit cell dimensions of 60.7 × 60.7 × 80.0 Å with one monomer per asymmetric unit in space group P4<sub>3</sub>2<sub>1</sub>2. Data were processed using DENZO and SCALEPACK (26) and multiwavelength anomalous diffraction phasing proceeded by the usual methods of heavy atom location (SHELDX, available on the World Wide Web at shelx.uni-ac.gwdg.de/SHELX/), maximum likelihood phase refinement (27), and density modification (28)). Phase extension
Table I

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<td>Figure of merit*</td>
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Model refinement

| Resolution range (Å) | 20–1.1 |
| No. of reflections (working/free) | 54102/2840 |
| No. of protein atoms | 1018 |
| No. of water molecules | 313 |
| $R_{merge}^{calc}$ (%) | 14.3/20.3 |
| Root mean square deviations | | |
| Bond lengths (Å) | 0.020 |
| Bond angles (degrees) | 1.713 |

* Statistics for the highest resolution shell are given in parentheses.

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The final column refers to the native protein and the preceding columns to the selenomethionine analog.

Structure-Function Analysis of DsbE from M. tuberculosis

The redox potential of Mtb DsbE was determined by the method of protein-protein redox equilibria developed by Aislund (35), as modified by Collet and Bardwell (36). The protein used as a standard was E. coli DsbC, which has a redox potential ($E_{0}^{P+P}$) of $-135$ mV (37). In short, equimolar amounts of oxidized DsbC and reduced Mtb DsbE were incubated in 50 mM Tris/HCl, pH 7.5, 350 mM NaCl, 5 mM EDTA at 25 °C. After different time points, samples were analyzed by reverse phase high pressure liquid chromatography (2695 separations module; Waters). The oxidized and reduced forms of each protein were separated on a Phenomenex Primesphere 5-Å column (buffer A: 0.1% trifluoroacetic acid in water; buffer B: 30% methanol, 60% acetonitrile, 0.1% trifluoroacetic acid). Separation of oxidized and reduced forms of DsbC and Mtb DsbE was achieved using a linear gradient from 50 to 62% B over 450 min at a flow rate of 1 mL/min. For evaluation of the redox potential, peak areas were analyzed using PeakFit (Systat), and the redox potential was calculated as described previously (35).

Determination of $pK_a$ of Cys$^{36}$—The pH-dependent ionization of the Cys$^{36}$ thiol (solvent-exposed) was followed by the specific absorbance of the thiolate anion at 240 nm as described earlier (9). As a control, the pH-dependent absorbance for the oxidized form of Mtb DsbE was recorded. To avoid precipitation artifacts and to minimize buffer absorbance, a buffer system consisting of 10 mM K2PO4, 10 mM boric acid, 10 mM sodium succinate, 1 mM EDTA, and 200 mM KCl (containing 100 μM dithiothreitol for the reduced protein) was used. The pH (initial value of 8.5) was lowered to 2.2 by the stepwise addition of aliquots of 0.1 M HCl, and the absorbance at 240 and 280 nm was recorded and corrected for
the volume increase. Samples had an average initial protein concentration of ~30 μM. The pH dependence of the thiold-specific absorbance signal (S = (A_{295}/A_{230})_{initial}/(A_{295}/A_{230})_{final}) was fitted according to the Henderson-Hasselbalch equation (Equation 6), in which S_{final} represents the corrected absorption intensity of the fully protonated form, and S_{initial} is that of the fully deprotonated form.

\[
S = S_{final}S_{initial}/(1 + 10^{pH - pH_{p}})
\]

(Determination of Unfolding/Folding Equilibrium)—The reversible guanidine hydrochloride (GdnHCl)-induced unfolding/folding of Mtb DsbE was performed by measuring the CD ellipticities at 222 nm (33). The spectrum of the reduced form was recorded in the presence of 0.5 mM dithiothreitol. For unfolding equilibrium, Mtb DsbE (final concentration of 7 μM) was dissolved in difference concentrations of GdnHCl and incubated for 3 h at 25°C. Data were analyzed according to the two-state assumption (39, 40). The standard changes of folding free energy were calculated according to Equation 7.

\[
\Delta\delta_{G}^{o} = -RT\ln K_{eq}
\]

The difference in stability between the oxidized and reduced forms of DsbE protein was calculated as in Equation 8.

\[
\Delta\delta_{G}^{o} = \Delta\delta_{G,red}^{o} - \Delta\delta_{G,ox}^{o}
\]

(Oxidase activity of Mtb DsbE—Refolding of Hirudin—Hirudin (Sigma) refolding was performed as described (41). Reduced, unfolded hirudin (28 μM) was incubated with 84 μM oxidized Mtb DsbE in 10 mM sodium phosphate, pH 7.0, 1 mM EDTA at 25°C. Buffer C was 0.1% trifluoroacetic acid in an aqueous solution, and incubated for 3 h at 25°C. Data were analyzed according to the two-state assumption (39, 40). The standard changes of folding free energy were calculated according to Equation 7.

\[
\Delta\delta_{G}^{o} = -RT\ln K_{eq}
\]

The difference in stability between the oxidized and reduced forms of DsbE protein was calculated as in Equation 8.

\[
\Delta\delta_{G}^{o} = \Delta\delta_{G,red}^{o} - \Delta\delta_{G,ox}^{o}
\]

(Structure-Function Analysis of DsbE from M. tuberculosis—Overall Structure of Mtb DsbE—The crystal structure of Mtb DsbE consists of a main domain that contains a thioredoxin fold domain, a short 3 10-helix (128 Å) and to the amide nitrogen atom of Pro37 (3.34 Å) and to the oxygen atom of both Cys36 (the prime on position 35 indicates that this residue is on the neighboring molecule). Arg64 from one monomer buries itself into the other monomer, forming hydrogen bonds with symmetry-related residues. The NH1 and NH2 atoms of Arg64 both hydrogen-bond to atom Ne1 of Trp269 and the oxygen atom of Asn157, respectively, via a water molecule, and both hydrogen-bond to the oxygen atom of Gln100. Furthermore, the Ne atom of Arg64 interacts with the oxygen atom of Pro118. Other notable homodimer interface hydrogen bonds are atom Ne1 of Trp269 to atoms Oe1 of Gln100 and to the oxygen atom of both Val97 and Pro99. There are also hydrophobic interactions seen between the rings of Trp269 and Trp92.

(Structural Comparisons—The crystal structure with the highest structural similarity to Mtb DsbE is Bradyrhizobium japonicum CcmG/DsbE (B. japonicum DsbE) (44), a Gram-negative bacterial DsbE periplasmic protein. The alignment of the two structures gives a root mean square deviation over backbone atoms of 1.8 Å. The structure with the second highest similarity is B. japonicum TlpA (45), which is involved in the maturation of cytochrome aa3 (root mean square deviation of 2.1 Å). Fig. 3, a–c, shows the structural similarity between the three proteins, Mtb DsbE, B. japonicum DsbE, and B. japonicum TlpA, respectively. The most obvious difference between these three proteins is that the active site cysteines in the B. japonicum DsbE and B. japonicum TlpA structures are in their oxidized states but are in their reduced states in the Mtb DsbE structure.

(Gram-positive Mtb DsbE Protein Functions Differently from Gram-negative DsbE Proteins—The function of DsbE homologs in Gram-positive bacteria has not previously been characterized. A phylogenetic tree derived from prokaryotic homologs to E. coli DsbE shows that the Gram-positive and Gram-negative DsbE homolog proteins cluster into two groups with the extremophile Deinococcus radiouardus separating the two groups (data not shown). The divergence of Gram-negative and Gram-positive DsbE homologs and the difference in redox state of Mtb DsbE and B. japonicum DsbE crystal structural suggest that Gram-positive DsbE and Gram-negative DsbE homologs may function differently.

(Determination of the Redox Potential of Mtb DsbE—To further characterize Mtb DsbE, the redox potential relative to that of glutathione was determined, which compares the ability of reduced glutathione to transfer electrons to a protein. The K_{eq} of Mtb DsbE is ~0.25 ± 0.2 mV (Fig. 4a). The corresponding standard redox potential (E_{0}^{\prime}) calculated for Mtb DsbE is ~128 ± 12 mV. In comparison with the standard redox potential for E. coli DsbA (~124 mV) (46), which is an oxidant, and E. coli thioredoxin (~269 mV) (35), which is a reductant, the standard redox potential for Mtb DsbE (~128 mV) suggests that DsbE is an oxidant. In contrast, the standard redox potentials for Gram-negative DsbE proteins (~217 to ~175 mV) (47–49) correspond to these proteins being weak reductants. Since Mtb DsbE is an oxidant and Gram-negative DsbE proteins are weak reductants, this reinforces the hypothesis that Gram-negative and Gram-positive DsbE proteins function differently.

To confirm the redox potential that was determined by equilibrium incubation using GSH/GSSG as a reference, we applied a method based on the analysis of the direct protein-protein redox equilibrium between Mtb DsbE and the protein E. coli DsbC, which has a well established redox potential of E_{0}^{\prime} is ~135 mV (37). By analyzing the equilibrium between E. coli DsbC and Mtb DsbE, we determined the redox potential of Mtb.
DsbE ($E_0'$) to be $-128 \pm 3$ mV. This is in very good accordance with the value determined using GSH/GSSG as a reference. This makes Mtb DsbE one of the most oxidizing proteins known.

**Determination of the pK_a Value of Mtb DsbE**—Determination of the pK_a value of the Mtb DsbE solvent-exposed active site cysteine provides further evidence that Mtb DsbE is functionally divergent from Gram-negative DsbE proteins. The pK_a value of the active site cysteine of Mtb DsbE (Cys^{36}) was measured by observing the change in absorption of the cysteines at 240 nm over a pH range of pH 2–9 (Fig. 4b). The pK_a value of the solvent-exposed active site cysteine
(Cys36) is 5.0 ± 0.2. This is relatively acidic compared with the solvent-exposed active site cysteine of *E. coli* thioredoxin (pKₐ of 7.5) (50) and *E. coli* Δ57DsbE (pKₐ of 6.8) (47) and that of reduced glutathione (pKₐ of 8.7), although not as acidic as *E. coli* DsbA, which is a known oxidant whose pKₐ of the solvent-exposed active site cysteine is 3.5 (51). The acidic nature of the solvent-exposed Cys 36 provides further evidence that *Mtb* DsbE has different biochemical characteristics than its Gram-negative DsbE homologs.

**Thermodynamic Properties of the Redox Forms**—To compare stabilities of the different redox forms of *Mtb* DsbE, guanidine hydrochloride-induced unfolding and refolding of both oxidized and reduced forms was examined by circular dichroism. The reduced form of *Mtb* DsbE is more stable than that of the oxidized form, given that the reduced form of the protein denatures at a higher concentration of guanidine hydrochloride than the oxidized form (Fig. 4c). Calculation of the free energy change (∆G°_redox) between the reduced and oxidized form of *Mtb* DsbE suggests that the reduced form is 12.4 ± 4 kJ/mol more stable than the oxidized form. *E. coli* DsbA (an oxidant) is
also more stable in its reduced form, with a ΔΔG\text{redox} of 16.3 ± 3.6 kJ/mol (41), whereas E. coli thioredoxin (a reductant) is 16.3 ± 2.4 kJ/mol more stable in its oxidized form (52). Thus, the increased stability of \textit{Mtb} DsbE in its reduced form is consistent with \textit{Mtb} DsbE being energetically more stable as an oxidant.

**Oxidase Activity of \textit{Mtb} DsbE and Hirudin Refolding**—In order to investigate the oxidative protein folding ability of \textit{Mtb} DsbE, we tested its ability to oxidize hirudin from \textit{H. medici-
nalis}. Hirudin is a 6.9-kDa protein that functions as an inhibitor of thrombin. It contains three intramolecular disulfide bridges that connect residues 6–14, 16–28, and 22–29. The regeneration of native recombinant hirudin from the reduced unfolded form to the fully oxidized native state was carried out in the presence and absence of \textit{Mtb} DsbE in stoichiometric quantities. In the absence of added \textit{Mtb} DsbE, a small quantity of spontaneous (presumably air-mediated) oxidation of hirudin occurred to generate randomly oxidized hirudin, as has previously been observed, but negligible native hirudin was generated (Fig. 4d). In contrast, \textit{Mtb} DsbE was able to oxidize hirudin from a denatured reduced state to a completely folded and oxidized state (Fig. 4d). This shows that \textit{Mtb} DsbE is capable of oxidizing substrate proteins.

**DISCUSSION**

**Comparison of \textit{Mtb} DsbE with Gram-negative DsbE Homologs**—Several lines of evidence imply that \textit{Mtb} DsbE does not function in the same way as its Gram-negative homologs. DsbE proteins in Gram-negative bacteria have been shown to be weak reductants, whose solvent-exposed active site cysteines have pK\text{a} values around 6.5–6.8. We have shown that \textit{Mtb} DsbE is an oxidant, and its solvent-exposed, active site cysteine has a lower pK\text{a} (5.0) than those of Gram-negative bacterial DsbE homologs. These differences and the observation that the sequences of Gram-positive DsbE homologs cluster together imply that Gram-positive DsbE proteins, and in particular \textit{Mtb} DsbE, have a different biochemical function than Gram-negative DsbE homologs.

Sequence and structural alignments of Gram-negative and Gram-positive DsbE homologs also suggest different functions. They show two regions that are conserved among Gram-negative DsbE homologs but are significantly different in Gram-positive DsbE proteins (Fig. 2c, yellow boxes). The first N-terminal region, conserved in the Gram-negative DsbE homologs contains an insertion of 15 residues that are absent in the \textit{Mtb} DsbE structure (Fig. 2c). The additional 15 residues, together with alterations in surrounding residues, generate a region that is structurally similar to a β-hairpin. This region forms a distinct groove on the protein’s molecular surface (Fig. 5b), which has been shown to have the required architecture for interaction with the protein partners of \textit{B. japonicum} DsbE (44). Sequence alignments show that this region is well conserved among Gram-negative DsbE proteins (Fig. 2c, N-terminal yellow box). In contrast, the \textit{Mtb} DsbE has a shorter N-terminal region, the 3₁₀-β-β-3₁₀ segment (Fig. 2c), which does not form a groove on the protein’s molecular surface (Fig. 5a). This region is poorly conserved among Gram-positive DsbE proteins. Since this groove is thought to play an important role in protein-protein interactions of \textit{B. japonicum} DsbE, we may conjecture that the protein interaction partners of \textit{Mtb} DsbE differ from that of \textit{B. japonicum} DsbE (44), that \textit{Mtb} DsbE does not use this region for protein-protein interactions, or both. A second region of conserved residues in Gram-negative DsbE homologs contains the Gram-negative DsbE protein motif (53), GVYGXPET₁⁰, which is located in a solvent-exposed loop region between α₁ and β₁ in the \textit{B. japonicum} DsbE structure (Fig. 2c, second yellow box). The side chains of these residues pack tightly around the protein backbone and do not protrude into the solvent. In Gram-positive bacteria, this motif is not conserved, and the corresponding sequence in \textit{Mtb} DsbE, NVPWQPAF₁⁰, contains residues that protrude from the protein backbone to form a potential homodimer interface. The \textit{B. japonicum} DsbE crystal structure contains no crystallographic or potential homodimer interface (44). In summary, the differences in the sequence conservation between the Gram-
negative DsbE proteins and Gram-positive DsbE proteins reinforce our conclusion that MtbpDsbE functions differently from Gram-negative DsbE proteins.

Comparison of the active sites of B. japonicum DsbE and MtbpDsbE structures is also consistent with the hypothesis that Gram-negative and Gram-positive homologs of DsbE function differently. B. japonicum DsbE crystallized in its oxidized form, and MtbpDsbE crystallized in its reduced form (Fig. 3, b and a, respectively), suggesting that the active site environment differs between the two proteins. The active site of B. japonicum DsbE is unusually acidic, due to residues Asp37, Glu38, and Glu396 (which are conserved among the Gram-negative DsbE proteins), which may account for the reducing power of B. japonicum DsbE compared with MtbpDsbE. The corresponding residues in MtbpDsbE (Ala41, Glu42, and Ala102) are not acidic except for Glu42, which is conserved throughout the DsbE homologs (Fig. 2c). Alignment of the two structures in the vicinity of the active site loop (residues Trp30–Glu42 and Asn86–Glu396 in the MtbpDsbE and B. japonicum DsbE, respectively) gives a root mean square deviation over backbone atoms of 0.42 Å (Fig. 5, c and d). Thus, the redox state of DsbE proteins is not dependent on a conformational change near the active site, implying that the redox state of the protein is not determined by the architecture of the active site.

Structural analysis implicates an acidic amino acid pair that contributes to the stability of the reduced and oxidized forms of the Dsb proteins. In the reduced form of MtbpDsbE, the amino acid pair Trp30 and Glu42 are flanking the active site residues in β-strand 3 (β3) and α-helix 3 (α3), respectively (Fig. 5c), and form hydrogen bonds between Ne1 of Trp30 and both Oc1 and Oc2 of Glu42 (2.90 and 2.77 Å). This interaction probably contributes to the stability of the active site loop to form a conformation where the reduced thiold form of the active site cysteines is favored, and therefore MtbpDsbE is an oxidant. This acidic amino acid pair is well conserved throughout the Gram-positive DsbE proteins. In comparison, within the B. japonicum DsbE structure (44), which crystallized in its oxidized form, the corresponding residues are Asn86 and Glu396. These two residues also form a hydrogen bond (3.08 Å) across the β-strand and α-helix (Fig. 5d), possibly maintaining the active site loop in a conformation that favors the disulfide form of the active site cysteines. In all of the Dsb and thioredoxin structures determined thus far, there is a corresponding conserved amino acid pair that forms a hydrogen bond between the β-strand and α-helix containing the active site cysteines in the protein’s most stable form. For example, the structure of E. coli DsbA, an oxidant, shows the amino acid pair to be Glu37 and Lys38. In the reduced form of DsbA (the more stable form), the hydrogen bond between Glu37 and Lys38 has a distance of 2.92 Å, whereas in the oxidized form, the distance is ~0.75 Å greater (54). This implies that the hydrogen bonding flanking the active site in thioredoxin-like proteins may influence their redox state by favoring conformations in which active site cysteines are most stable in their reduced or oxidized states.

Biological Implications—In Gram-negative bacteria, it has been proposed that DsbE proteins are involved in the maturation of cytochrome c. Cytochrome c maturation converts a linear polypeptide, the apocytochrome, into a three-dimensional structure that contains one or more covalently bound, redox-active heme co-factors. There are at least three systems of cytochrome c maturation of varying complexity (55, 56). Gram-negative bacteria cytochrome c maturation utilizes a well-characterized pathway, System I, which contains periplasmic anchored DsbE (6, 56). Since Gram-negative DsbE has been proposed to play a role as a reductant, and we have shown that MtbpDsbE is an oxidant, the reduction of the cysteines of apocytochrome c would be an unfavorable reaction. This suggests that MtbpDsbE is not involved in cytochrome c maturation via System I. Cytochrome c maturation in Gram-positive bacteria is thought to utilize System II, which is a less well-characterized pathway as compared with System I (55, 57). System II also contains a predicted thioredoxin-like protein (Ces1/ResB), and the M. tuberculosis genome contains all of the known genes in System II cytochrome c maturation. Thus MtbpDsbE may be involved in cytochrome c maturation by the System II pathway.

An alternative role for MtbpDsbE could be to function as a disulfide bond-forming (Dsb) protein. The M. tuberculosis genome contains no genes that encode for other homologs of Dsb proteins, such as E. coli DsbA, DsbC, or DsbG. In Gram-negative bacteria, Dsb proteins function in the periplasm. Because M. tuberculosis is a Gram-positive bacterium and does not contain a periplasmic space, MtbpDsbE might function extracellularly within the cell wall environment. If so, MtbpDsbE might function as a disulfide isomerase to ensure that secreted or surface-associated proteins have correctly formed disulfide bonds. It has been predicted that greater than 60% of the 161 predicted secreted proteins of M. tuberculosis contain at least one disulfide bond.2 However, in vitro, we found no activity for disulfide bond isomerase in MtbpDsbE(data not shown); nor did previous studies (24). Therefore, if MtbpDsbE functions as an isomerase, this suggests a high specificity of MtbpDsbE for its functional protein partners.

The final option and the one that we favor is that MtbpDsbE may have a similar function to E. coli DsbA, which catalyzes the oxidation of reduced, unfolded proteins with disulfide bonds (10, 11). This hypothesis is supported by the observation that MtbpDsbE and E. coli DsbA are both oxidants, and their solvent-exposed active site cysteines both have relatively low pK̂ values, 5.0 and 3.4 (38), respectively. In addition, MtbpDsbE has been shown to have oxidase activity as it reoxidizes reduced hdirudin. In fact, the NCBI conserved domain site selects the first 50 residues of MtbpDsbE in having a DsbA domain. Therefore, MtbpDsbE may catalyze the oxidation and folding of reduced, unfolded proteins as they are secreted into the extracellular space of the M. tuberculosis cell wall environment.

In summary, structural and functional analysis of MtbpDsbE suggests that it has a similar function to E. coli DsbA, which catalyzes the oxidation of reduced, unfolded secreted proteins to form disulfide bonds (10, 11). Hence, MtbpDsbE may be involved in virulence, since many secreted proteins may depend on the oxidase activity of MtbpDsbE to form correctly folded proteins. Interestingly, although the overall structures of Gram-negative and Gram-positive DsbE proteins have similar thioredoxin-like folds, the structural differences between these two proteins imply that they function differently (the N-terminal regions, the potential interaction interfaces, and the redox state of the active sites differ between the two proteins). Biochemical analysis of MtbpDsbE confirms this assumption, since MtbpDsbE is an oxidant probably acting upon secreted proteins, whereas B. japonicum DsbE is a weak reductant acting upon apocytochrome c. Thus, the determination and analysis of the structure of MtbpDsbE along with comparison with homologous sequences and structures has provided an opportunity to make specific predictions about protein function that were confirmed biochemically.

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