Structure analysis of the extracellular domain reveals disulfide bond forming-protein properties of *Mycobacterium tuberculosis* Rv2969c

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ABSTRACT

Disulfide bond-forming (Dsb) protein is a bacterial periplasmic protein that is essential for the correct folding and disulfide bond formation of secreted or cell wall-associated proteins. DsbA introduces disulfide bonds into folding proteins, and is re-oxidized through interaction with its redox partner DsbB. *Mycobacterium tuberculosis*, a Gram-positive bacterium, expresses a DsbA-like protein (Rv2969c), an extracellular protein that has its N-terminus anchored in the cell membrane. Since Rv2969c is an essential gene, crucial for disulfide bond formation, research of DsbA may provide a target of a new class of anti-bacterial drugs for treatment of *M. tuberculosis* infection. In the present work, the crystal structures of the extracellular region of Rv2969c (*Mtb* DsbA) were determined in both its reduced and oxidized states. The overall structure of *Mtb* DsbA can be divided into two domains: a classical thioredoxin-like domain with a typical CXXC active site, and an α-helical domain. It largely resembles its *Escherichia coli* homologue EcDsbA, however, it possesses a truncated binding groove; in addition, its active site is surrounded by an acidic, rather than hydrophobic surface. In our oxidoreductase activity assay, *Mtb* DsbA exhibited a different substrate specificity when compared to EcDsbA. Moreover, structural analysis revealed a second disulfide bond in *Mtb* DsbA, which is rare in the previously reported DsbA structures, and is assumed to contribute to the overall stability of *Mtb* DsbA. To investigate the disulphide formation pathway in *M. tuberculosis*, we modeled *Mtb* Vitamin K epoxide reductase (*Mtb* VKOR), a binding partner of *Mtb* DsbA, to *Mtb* DsbA.

KEYWORDS *Mycobacterium tuberculosis*, disulfide bond forming protein, X-ray crystallography

INTRODUCTION

*Mycobacterium tuberculosis* (*Mtb*) is a Gram-positive bacterium, and the causative agent of Tuberculosis (TB), being the most wide-spread infectious disease on the planet, affecting approximately 30% of the world’s population (Yang et al., 2011). In the year of 2011, about 8.7 million people were infected with TB, resulting in 1.4 million deaths (WHO 2012, Global Tuberculosis Control Report, http://www.who.int/tb/publications/global_report/en/). Currently, the resistance to clinically used drugs, and the co-infection with HIV/HCV present a major challenge to TB therapy (Comas and Gagneux, 2009). The virulence and pathogenicity of *Mtb* is closely related to its secreted proteins, which are either cell wall-associated or extracellular. About 60 percent of the secreted proteins have disulfide bonds (Goulding et al., 2003), the formation of which is crucial for protein folding and function. Thus, the research of the structure and function of disulfide bond forming (Dsb) proteins may provide a way to identify new anti-TB drug targets, and overcome increasingly serious multidrug-resistant TB (Goulding et al., 2002).

Dsb proteins are well-studied in *Escherichia coli* (*E.coli*), which is a Gram-negative bacterium (Messens and Collet, 2006). In its periplasmic space, oxidative *E.coli* DsbA (EcDsbA)...
transfers its disulfide bonds to its substrates, and is re-oxidized by its partner membrane protein \textit{E.coli} DsbB (EcDsbB), which in turn is then re-oxidized by ubiquinone present in the cytoplasm (Inaba and Ito, 2008). \textit{E.coli} DsbC (EcDsbC) (McCarthy et al., 2000) and \textit{E.coli} DsbG (EcDsbG) (Shao et al., 2000) can form homodimers, and both possess disulfide bond isomerase activity. \textit{E.coli} DsbE (EcDsbE) (also known as CcmG, or Cytochrome c biogenesis protein) (Reid et al., 2001) is involved in the maturation of Cytochrome c. \textit{E.coli} DsbD (EcDsbD) is a transmembrane protein, whose function is to transfer reducing equivalents from the cytoplasmic thioredoxin system to the periplasm, to maintain EcDsbC, EcDsbE and EcDsbG in their reduced states (Stewart et al., 1999; Denoncin and Collet, 2012).

Gram-positive bacteria do not have the periplasmic space of Gram-negative bacteria, which requires Gram-positive bacteria to utilize a different mechanism for protein disulfide bond formation (Daniels et al., 2010). For example, the genome of \textit{Bacillus subtilis} encodes a set of proteins corresponding to the EcDsb proteins. BdbD (bacillus disulfide bond protein), a membrane-anchored DsbA-like protein, introduces disulfide bonds to the proteins on the outside of the cytoplasmic membrane (Meima et al., 2002). BdbD is re-oxidized by BdbC, which has a sequence identity of 40% with DsbB, and which plays a similar role (Dorenbos et al., 2002). Another two membrane-anchored proteins, ResA (Erlendsson et al., 2003) and StoA (Crow et al., 2009b), which are involved in Cytochrome c maturation and endospor biogenesis respectively, are reduced by CcdA, a membrane protein that functions as EcDsbD (Erlendsson and Hederstedt, 2002). Some other bacteria, including \textit{M. tuberculosis} and \textit{Synechococcus sp.}, contain DsbA homologs but lack a homologue of DsbB (Dumoulin et al., 2005). Meanwhile, their genomes encode a membrane protein VKOR, which is a homologue of vertebrate Vitamin K epoxide reductase, to functionally replace EcDsbB (Dutton et al., 2008). The overall structure of \textit{Synechococcus sp.} VKOR (SpVKOR) (Li et al., 2010), which contains both a VKOR and a Trx-like domain, shows some distinct features compared to the structure of EcDsbB.

To date, the Dsb proteins identified in \textit{M.tuberculosis} are \textit{Mtb} DsbB, a DsbA-like protein (Goulding et al., 2004), its homologue named \textit{Mtb} DsbF (Chim et al., 2010), its potential redox membrane protein partner \textit{Mtb} DsbD (Goldstone et al., 2005) and \textit{Mtb} VKOR (Wang et al., 2011). In the genome of \textit{M. tuberculosis}, the gene directly adjacent to VKOR encodes an apparent DsbA-like protein (Rv2969c), which was predicted to have an N-terminal transmembrane helix. Because of the difficulties in obtaining crystals of single transmembrane protein, together with the fact that sequence analysis suggested that the extracellular part of Rv2969c is the functional domain, we chose residues 53–255 (\textit{Mtb} DsbA) for our structural and functional studies.

In the present work, we solved the high-resolution crystal structure of the functional domain of Rv2969c, in both its reduced and oxidized states. The overall structure shares similarity with that of BdbD; however, it contains a second disulfide bond, which is likely to play a structural role, and which lacks a Ca$^{2+}$-binding site. Moreover, there are several differences between the structures of \textit{Mtb} DsbA and EcDsbA, with the latter thoroughly characterized by now. \textit{In vitro} oxidoreductase activity assays, which use RNase and insulin as substrates, show different substrate specificities for \textit{Mtb} DsbA and EcDsbA. Based on the structure of SpVKOR, and the local sequence identity of 30\% (28/94) between \textit{Mtb} VKOR and SpVKOR, we decided to use computational structural biology to construct the model of \textit{Mtb} DsbA-VKOR complex, which will help explain their interaction mode, and reveal the disulfide formation pathway in \textit{M.tuberculosis}. According to this model and previous analysis of \textit{Mtb} VKOR, we propose a disulfide formation pathway for \textit{M.tuberculosis}.

**RESULTS**

**Full-length Rv2969c is a single transmembrane protein**

Rv2969c contains 255 amino acid residues with an N-terminal transmembrane sequence. Sequence analysis suggested that one transmembrane helix locates at residues 20–42, and that the extracellular part consisted of residues 43–255. When we expressed the full-length gene of Rv2969c in \textit{E.coli}, the target protein was detected in the membrane fraction and only soluble in solution with detergent, which is consistent with the location of the transmembrane helix at the N-terminus. Due to the low yield of full-length protein and the difficulties in crystallization, we crystallized residues 53–255, to investigate how Rv2969c catalyzes disulfide bond formation.

**The overall structure of Mtb DsbA**

The crystal structure of the reduced \textit{Mtb} DsbA was determined using the Selenomethionine single-wavelength Anomalous Diffraction (SAD) method, and was refined to 1.90 Å resolution, with R-work and R-free values of 16.6\% and 20.6\%, respectively. The structure of the oxidized form was solved by molecular replacement, which used the reduced structure as a model, and was refined to 2.50 Å resolution, with R-work and R-free values of 19.8\% and 22.3\%, respectively. The reduced \textit{Mtb} DsbA crystal belongs to space group \textit{P2}_1\textit{2}_1\textit{2}_1, with unit cell parameters \textit{a} = 46.9 Å, \textit{b} = 74.9 Å, \textit{c} = 119.9 Å (Table 1). In one asymmetric unit, there is a SO$_4^{2–}$ molecule, which forms salt bridges with Lys$^{159}$ and Arg$^{97}$ in chain A and Lys$^{235}$ in the other protomer and contributes to the crystallization packing. Due to the high resolution of the crystal structure, we were able to observe alternative conformations of the side chains of several amino acid residues in the structure.

According to Matthews coefficient analysis (Matthews, 1968), there are two molecules in one asymmetric unit, at a solvent content of 51\% (VM = 2.5 Å$^3$Da$^{-1}$). PISA analysis (Krissinel and Henrick, 2007) indicated that the largest interface in the lattice buries only 480 Å$^2$ of surface area. Also, the complex significance score is 0.0, which indicates that the protein cannot form stable dimers in solution. The result of an-
Protein & Cell

Protein analytical ultracentrifugation indicated that in solution, Mtb DsbA predominantly exists in its monomeric form. The dimers we observed in crystals may have resulted from the crystallographic packing, likely to be of no biological significance. Furthermore, their structures are nearly identical, with a root mean square deviation (r.m.s.d.) of 0.6 Å between 196 Cα atom pairs. Thus, our discussion below is predominantly based on the structure of one arbitrarily chosen protein protomer, unless stated otherwise. The overall structure of Mtb DsbA can be divided into two domains: a classical thioredoxin-like domain (Trx domain) and an α-helical domain (helical domain) (Fig. 1). The Trx domain has a typical thioredoxin fold, and is composed of a βαβ motif (residues 79–118) and a βαα motif (residues 210–255). At the N-terminus of the first helix of the Trx domain is a CXXC motif (Cys89-Pro90-Ala91-Cys92), suggesting that Rv2969c is a redox-active Trx-like protein. The helical domain consists of five α-helices, three of which (α2–α4) form an antiparallel helical bundle and the other α-helix (α6) connects the βαα motif.

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Values in parentheses are for highest-resolution shell.  

\[ R_{merge} = \frac{\sum hkl \|I(hkl)i \| - \langle I(hkl)\rangle}{\sum hkl \sum i I(hkl)i}. \]

\[ R_{work} = \frac{\sum hkl |F_o(hkl)| - F_c(hkl)|}{\sum hkl |F_o(hkl)|}. \]

\[ R_{free} \] was calculated for a test set of reflections (5%) omitted from the refinement.

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and location of N terminal strand β1 is topologically different from the corresponding region in EcDsbA (Fig. 3A). In Mtb DsbA, strand β1 is anti-parallel to strand β3, which approaches the helical domain, while in EcDsbA, strand β1 is anti-parallel to strand β5, which approaches the C terminal helix. Therefore, the position of the loop1, which connects β1 and β2, is considerably different from the equivalent loop1 observed in EcDsbA. Loop1 is approaching the α7 of the Trx domain in Mtb DsbA, while loop1 is approaching the α6 helix of the helical domain in EcDsbA. Secondly, loop2 connecting β3 and α2 has four more residues than the equivalent loop2 observed in EcDsbA. Compared with EcDsbA, loop3 linking α3 and α4 has three extra residues in Mtb DsbA and turns towards the CXXC active site, positioning the acidic side chain of Glu165 near the nucleophilic Cys69 (6.26 Å) (Fig. 3B). According to the sequence alignment, acidic residues are primarily in this loop3 in Gram-positive DsbAs, but are absent in EcDsbA (Fig. 2). Loop3 forms one edge of an acidic groove in Mtb DsbA. The proximity of Glu165 to the redox active site of Mtb DsbA suggests a possible role in substrate specificity, or in modulating the redox characteristics. Thirdly, the motif of β4a7a8 and loop4 (between β4 and α7) at the C-terminal end of the polypeptide chain produces a considerably difference between Mtb DsbA and EcDsbA (Fig. 3C). This region forms one edge of a hydrophobic peptide-binding groove in EcDsbA (Paxman et al., 2009) that binds to DsbB (Inaba et al., 2006) and is supposed to interact with unfolded protein substrates. In Mtb DsbA, this region is not hydrophobic, and is 9 residues shorter, resulting in a shorter loop connecting β4 and α7, a shorter α7, and an extra α8 helix. Sequence alignment shows that this deletion appears to be conserved in DsbAs from Gram-positive organisms (Fig. 2). Finally, there is a second disulfide bond between helices α2 and α5 in Mtb DsbA, which is highly conserved in α-proteobacterial DsbAs, but which is absent in EcDsbA and other DsbAs (Kurz et al., 2009). Thermal stability test (thermally-induced protein melting) (Ericsson et al., 2006) shows that the double mutant form of Mtb DsbA (C140,192K) is less stable. Thus, we predicted that this disulfide bond is associated with the stability and integrity of the overall protein structure.

Surface characteristics

There are a hydrophobic peptide-binding groove and an acidic groove surrounding the CXXC motif on the surface of Mtb DsbA (Fig. 3D). By contrast, there are a hydrophobic patch and a peptide-binding groove composing a hydrophobic pocket in EcDsbA, which is thought to be vital for catalytic activity (Inaba et al., 2006). Comparing with the peptide-binding groove in EcDsbA, the peptide-binding groove in Mtb DsbA is shallower as well as smaller. Moreover, the hydrophobic patch adjacent to the active site in EcDsbA is absent in Mtb DsbA, but contains an acidic groove instead. Several residues that contribute to this patch in EcDsbA (Phe63-Met64-Gly65-Gly66 in the loop connecting the Trx and helical domains) are replaced or covered with charged residues in Mtb DsbA. Thus, the differ-
ent conformation of the loop3 in Mtb DsbA results in charged residues (Glu165) covering this area, which flanks the active site. The different position of the loop4 in Mtb DsbA also results in an acidic protrusion (generated by Glu225) above the redox active site (left panel of Fig. 3D). The 9-residue deficiency and an extra α8 helix at the C terminus of Mtb DsbA influence the surface properties in which the peptide-binding groove is truncated, and its position is changed when compared to EcDsbA. This groove was considered to be the interface of DsbA and DsbB, and has been supposed to be involved in substrate interactions (Kadokura et al., 2004). The active site thioredoxin-like CXXC motif of Mtb DsbA results in charged residues (Glu165) covering this area, which flanks the active site. The different position of the loop4 in Mtb DsbA also results in an acidic protrusion (generated by Glu225) above the redox active site (left panel of Fig. 3D). 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and the superposition of them gave an r.m.s.d. of 0.178 Å (calculated for all atoms of residues 59–255).

Oxidoreductase activity of Mtb DsbA

In order to investigate the oxidative protein-folding activity of Mtb DsbA, we tested its ability to oxidize reduced and denatured RNase. The regeneration of native RNase from the reduced unfolded form to the fully oxidized native state was carried out in the presence and absence of Mtb DsbA, with the PDI (Protein disulfide isomerase) used as positive control. Native RNase can catalyze the hydrolysis of its cCMP substrate, which leads to change of absorbance at 296 nm. In this assay, PDI catalyzed the refolding of RNase at a high rate, whereas refolding of RNase occurred at almost the same rate, regardless of Mtb DsbA being present or not (Fig. 5A).

To test Mtb DsbA’s disulfide reductase activity, we performed a disulfide reductase activity assay with insulin as a substrate, which has two intramolecular disulfide bonds linking the A and B chains. When these disulfides are in their reduced state, the two chains will dissociate and the insoluble B chain will precipitate. Thus, we can test disulfide reductase activity by measuring the increase in turbidity of insulin solution. We measured the rate of insulin reduction by dithiothreitol (DTT) catalyzed by Mtb DsbA. Mtb DsbA did not show any activity, indicating that its surface features, which are less hydrophobic than those of EcDsbA, do not provide sufficient interaction with insulin (Fig. 5B).

Models of the Mtb DsbA and Mtb VKOR complexes

Mtb VKOR is a transmembrane protein with five transmembrane helices, with its N-terminus localized in the cytoplasm, its C-terminus in the periplasm, and four cysteines facing towards the periplasm. Topology analysis indicates that the topology of Mtb VKOR is identical to that of Synechococcus sp. Thus, we used SpVKOR as model and FR-t5 method to construct the 3-D structure model of Mtb VKOR (Fig. 6B). Due to differences in the fifth helix of Mtb VKOR and SpVKOR, the model of the fifth helix in Mtb VKOR is not sufficiently accurate. According to the evaluation criteria of the FR-t5 method (Hu et al., 2011), the model is considered to be reasonable. Moreover, we choose eleven residues (51–61) (called the ‘1/2-segment’) of the loop, located at the periplasmic side, between TM1 and TM2. This loop contains the cysteine whose corresponding residue in SpVKOR forms a disulfide bond with the cysteine of the CXXC motif of the Trx domain. Thus, we propose that the interaction between Mtb DsbA and Mtb VKOR occurs in the region sur-
Protein Cell & rounding the Cys57. Analysis of the autodock result between the 1/2-segment and Mtb DsbA, combined with the surface potential of the interface, we predicted a relatively credible and reasonable model of Mtb DsbA and Mtb VKOR (Fig. 6A). As shown in Fig. 6, the 1/2-segment of Mtb VKOR fitted into the active site groove of the Mtb DsbA Trx domain. In addition, previous structure alignment analyses demonstrated that this hydrophobic groove, which is formed by several loops between
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Besides DsbB orthologue. Until now, only two structures from *M. tuberculosis* had been determined: *Mtb* DsbE (a DsbA-like protein) and its homologue *Mtb* DsbF. Although *Mtb* DsbE is a CcmG (DsbE) homologue, which is a Gram-negative disulfide reductase, it has different functions. *Mtb* DsbE and DsbF can oxidatively refold reduced and unfolded hirudin, which is a small protein with three disulphide bonds in the native state (Hennecke et al., 1999; Goulding et al., 2004; Chim et al., 2010).

In the current study, we have determined two high-resolution crystal structures of *Mtb* DsbA, one in its reduced, and one in its oxidized state. Like *Mtb* DsbE and DsbF, DsbA is also an extracellular protein with its N-terminus anchoring in the mem-

**Figure 6. Complex model of interacting *Mtb* DsbA and *Mtb* VKOR.** (A) Complex structure of *Mtb* DsbA and 1/2-segment of *Mtb* VKOR as modeled using Autodock Vina. *Mtb* DsbA is displayed as electrostatic surfaces, and 1/2-segment is shown as sticks representation (green). (B) The 3-D structure model of *Mtb* VKOR calculated using the FR-I5 threading method. (C) Sequence alignment of *Mtb* VKOR and SpVKOR. Secondary structures of *Mtb* VKOR are shown on the top.

**DISCUSSION**

Disulfide bonds, which are formed by the oxidation of two cysteine thiol groups, are important for the correct folding and stability of many secreted proteins (Depuydt et al., 2011). Cells of all organisms encode a series of proteins to participate in the formation of disulfide bonds. Bacterial Dsb proteins are known to promote the catalysis of disulfide bond formation. *M. tuberculosis* genome encodes several Dsb-like proteins besides DsbB orthologue. Until now, only two structures from *M. tuberculosis* had been determined: *Mtb* DsbE (a DsbA-like protein) and its homologue *Mtb* DsbF. Although *Mtb* DsbE is a CcmG (DsbE) homologue, which is a Gram-negative disulfide reductase, it has different functions. *Mtb* DsbE and DsbF can oxidatively refold reduced and unfolded hirudin, which is a small protein with three disulphide bonds in the native state (Hennecke et al., 1999; Goulding et al., 2004; Chim et al., 2010).

In the current study, we have determined two high-resolution crystal structures of *Mtb* DsbA, one in its reduced, and one in its oxidized state. Like *Mtb* DsbE and DsbF, DsbA is also an extracellular protein with its N-terminus anchoring in the mem-

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brane. Structural differences in the region between \textit{Mtb} DsbA, DsbE and DsbF, which is surrounding the CXXC motif, indicate that they are likely to assist in correct folding of diverse sets of disulfide bonds of secreted or cell wall-associated proteins corresponding to diverse cellular conditions. Because of the absence of a periplasmic space, Gram-positive genomes encode a series of membrane-anchored Dsb proteins, which exhibit different structures and functions when compared to Dsb proteins expressed by Gram-negative bacteria. Thus, the catalytic mechanisms of Dsb proteins from \textit{E.coli} should not be directly extended to these organisms.

Unlike EcDsbA, \textit{Mtb} DsbA does not show any activity in the reduction of insulin or the oxidative refolding of RNase, which is typically used to characterize the redox activity of Thioredoxin fold proteins. Despite the overall structural similarity, \textit{Mtb} DsbA does not appear to function like EcDsbA. The truncated binding groove, as well as an acidic rather than hydrophobic surface surrounding the CXXC motif, suggests that \textit{Mtb} DsbA possesses different substrate specificity to EcDsbA. Furthermore, this region is also associated with the interaction between DsbA and its partner EcDsbB or \textit{Mtb} VKOR. Structural analysis revealed that there are more residues between the two cysteines of loop1 in EcDsbB than present in VKOR. Thus, we speculate that EcDsbA and \textit{Mtb} DsbA possess different surface features, to recognize and bind to their corresponding redox partners.

In the past 20 years, seventeen unique DsbA structures from different bacterial species have been solved (Shoulolce et al., 2011). Amongst these structures, only \textit{Wolbachia pipiens} a-DsbA1 possesses a second disulfide bond, which is highly conserved in a-proteobacterial DsbAs, not however, in other DsbAs. In \textit{Mtb} DsbA, the second disulfide bond, which links α3 and α5 helices, is formed by Cys \(^{140}\) and Cys \(^{192}\). The oxidized forms of \textit{Mtb} DsbA variant (C140,192K) is less stable than its reduced form as well as its native protein. This was measured in two ways, a thermal stability assay (data not shown) and a pKa value determination assay. In the second assay, addition of hydrochloride to the oxidized \textit{Mtb} DsbA variant solution resulted in instant protein precipitation, which resulted in difficulties in determining the pKa of Cys \(^{89}\). In addition, the disulfide bond in the active site of oxidized \textit{Mtb} DsbA is a mixture of oxidized and reduced conformations. Reduction of redox-active disulfides is commonly caused by radiation damage. However, the second disulfide bond of \textit{Mtb} DsbA remains oxidized in the crystal structure, suggesting that it is resistant to reducing conditions and is not redox-active, but contributes to the structural stability of the protein.

To date, the structures of two Gram-positive DsbA proteins have been solved, namely \textit{S.aureus} DsbA, SaDsbA (PDB: 3BCI) and \textit{B.subtilis} BsDsbA (known as BdB, PDB: 3EU3). \textit{Mtb} DsbA shares some structural characteristics of SaDsbA and BdB (Fig. 3E), especially the surface features surrounding the active site. Sequence alignment indicates that these features of \textit{Mtb} DsbA are commonly conserved among Gram-positive DsbAs. Thus, Gram-positive organisms may operate a common mechanism to form disulphide bonds. Moreover, an entire subgroup of Gram-positive bacteria lacks DsbB protein homologues; instead they contain VKOR, which is a functional alternative to DsbB.

VKOR plays an important role in the protein disulfide bond-forming pathway in many bacteria (Dutton et al., 2010). The gene encoding DsbA in \textit{M.tuberculosis} is directly adjacent to VKOR. According to the analysis of \textit{Mtb} DsbA structure and the function of \textit{Mtb} VKOR, we speculate that \textit{Mtb} DsbA catalyzes the disulfide bond formation of substrate proteins, and then it is re-oxidized by \textit{Mtb} VKOR, which in turn is re-oxidized by quinones present in the cytoplasm. Thus, we have attempted to express \textit{Mtb} DsbA and VKOR in the \textit{E.coli} expression system, both separately as well as together. However, we could not obtain satisfactory results from the overexpression studies of \textit{Mtb} VKOR in \textit{E.coli}, which may have been caused by differences in lipid composition of the cell membranes of \textit{E.coli} and \textit{M.tuberculosis}. Therefore, we used FR-t5 threading method in combination with Autodock Vina, to simulate the interaction between \textit{Mtb} DsbA and VKOR. Based on membrane topology and mutational analysis of \textit{Mtb} VKOR, we propose a disulfide formation pathway as presented in Fig. 7. We propose the following sequence of events: Cys \(^{89}\) and Cys \(^{92}\) of oxidized \textit{Mtb} DsbA transfer their disulfide bond to an unfolded protein substrate; then Cys \(^{89}\) of reduced \textit{Mtb} DsbA and Cys \(^{57}\) of \textit{Mtb} VKOR can form a mixed disulfide bond, which is transferred onto \textit{Mtb} DsbA; subsequently Cys \(^{57}\) of \textit{Mtb} VKOR forms a disulfide bond with Cys \(^{139}\), resulting in re-oxidized Cys \(^{57}\) and Cys \(^{65}\), and finally Cys \(^{139}\) and Cys \(^{142}\) are re-oxidized by a quinone in the cytoplasm.

Here, we have presented a structural study of \textit{Mtb} DsbA, which suggests that it functions as a disulfide bond-forming protein. Biochemical analysis using an oxidoreductase activity assay indicates that it possesses different substrate specificities when compared to EcDsbA. The model of interaction between \textit{Mtb} DsbA and VKOR will help in future investigations to elucidate the disulfide formation pathway of \textit{M.tuberculosis}. Our study should provide an important first step in identifying the mechanisms for substrate recognition, and the details of electron transfer and disulfide bonds formation in \textit{M.tuberculosis}. We believe that further structural and functional studies of \textit{Mtb} DsbA and VKOR will contribute to the timely identification of much-needed, novel anti-TB drug targets.

**MATERIAL AND METHODS**

**Protein expression and purification**

The sequence encoding the \textit{M.tuberculosis} Rv2969c’ extracellular domain (residues 53–255 of \textit{Mtb} DsbA, lacking the N-terminal single transmembrane helix) was amplified from \textit{M.tuberculosis} H\textsubscript{3}Rv genomic DNA using two primers (forward, 5’-AGCCAT-ATGGGCGTCGCCGCGCCG-3’; reverse, 5’-CCGCTC-GAGTCAGGATGTCGGTACGAC-3’). The target sequence was inserted into the pET-28a protein expression vector (Novagen) at NdeI and XhoI restriction sites (underscored in the primers). Escherichia
To oxidize and reduce Mtb DsbA, 1.7 mmol/L Dichloro (1,10-phenanthroline) copper (II) and 100 mmol/L dithiothreitol (DTT) were added to Mtb DsbA (approximately 20 mmol/L), respectively, in 150 mmol/L NaCl and 20 mmol/L Tris-HCl, pH 8.0, and incubated overnight at 4°C. The oxidized and reduced proteins were then isolated using a HiTrap® Desalting column (GE Healthcare) equilibrated in their original buffer. Under non-reducing conditions, oxidized proteins migrate faster on an SDS-PAGE gel than reduced proteins. Therefore, samples of the treated Mtb DsbA were run on an SDS-PAGE gel with no DTT present, to ensure that Mtb DsbA was either fully oxidized or fully reduced.

**Crystallization**

Crystallization of the Mtb DsbA was performed at 16°C by the hanging drop vapor diffusion method. Equal volumes (1.5 μL) of protein and crystallization reagent (30%–35% (w/v) PEG 2000, 0.2 mol/L Ammonium Sulfate, 0.1 mol/L Sodium Acetate trihydrate, pH 4.3–4.6) were mixed and equilibrated over a 200 μL reservoir solution. Twined, plate-like crystals were obtained with poor diffraction quality. Using initial crystals as seeds, single plate-like crystals grew in the drops containing lower concentrations of 26%–28% (w/v) PEG 2000. The crystal used for data collection was grown in 28% (w/v) PEG 2000, 0.2 mol/L Ammonium Sulfate, 0.1 mol/L Sodium Acetate trihydrate, pH 4.3. The Se-Mtb DsbA derivative and oxidized crystals were obtained using the reduced protein crystals as seeds under identical conditions.

**Data collection and processing**

A set of single-wavelength anomalous dispersion data for Se-Met crystals was collected at BL5A of Photon Factory (Japan) at a wavelength of 0.9790 Å. About 15% (w/v) glycerol was added to the reservoir solution as a cryoprotectant for the crystals measured. Native diffraction
data for the oxidized Mtb DsbA crystals were collected at BL17A of Photon Factory (Japan) at a wavelength of 0.980 Å. Data processing for both native and Se-Met crystals was carried out using the HKL2000 program package (Otwonowski and Minor, 1997).

Structure determination and refinement

We used the Phenix program package (Emsley and Cowtan, 2004) to solve the phases of the reduced Mtb DsbA protein utilizing the SAD data, and built ~90% of the structure of the reduced Mtb DsbA protein with the automated program. The missing structure was manually added and adjusted in COOT (Emsley et al., 2010). Phenix was used for structure refinement. Water molecules were then added in cases where there were >3 σ (Fo-Fc) electron density, and where good hydrogen bonds with nearby residues could be formed. The final structure of reduced Mtb DsbA was validated with PROCHECK, indicating that 99% of all residues lie in the favored regions of the Ramachandran Plot, with 1.0% of residues located in allowed regions, and no residues in disallowed regions. The oxidized state structure of Mtb DsbA was solved by the Molecular Replacement method, using the reduced Mtb DsbA structure as model. Protein structures were viewed, and pictures were made using the PyMOL software (DeLano, 2002).

In vitro oxidoreductase activity assays

The preparation of reduced, denatured RNase, and the subsequent refolding assays were performed as described previously by Wang et al. (2010). Briefly, 8 μmol/L reduced and denatured RNase was incubated with 6 μmol/L oxidized Mtb DsbA in glutathione redox buffer (100 mmol/L Tris acetate buffer containing 50 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L GSH (glutathione), and 0.2 mmol/L GSSG (glutathione disulfide) pH 8.0). The assay was performed at 25°C, and samples were recorded every 6 seconds; RNase activity was determined by monitoring the absorbance increase at 296 nm due to cCMP hydrolysis. Insulin was added to a final concentration of 130 μmol/L to 0.1 mol/L potassium phosphate buffer containing 2.5 mmol/L EDTA, 0.1 mmol/L DTT (pH 7.5) in the presence of 6 μmol/L reduced Mtb DsbA to initiate the reaction at 25°C. The measurements for the reaction mixture were performed in a spectrophotometer at 650 nm, detecting light scattering from reduced and precipitated insulin chains, and data were collected every 6 s. One reaction using treated RNase in the presence of PDI was used as positive control (Wang et al., 2010), while treated RNase in the absence of any oxidoreductase served as negative control reaction.

Construction of the protein complex structure

Modeling of protein structures was carried out using experimentally determined structures as structure templates, a process called template based modeling. To this end, we used a known crystal structure of VKOR from Synechococcus sp., which exhibits local sequence identity with Mtb VKOR of about 30% (28/94), in combination with a threading method called FR-I5 (fold recognition by use of 5 terms) (Hu et al., 2011) to construct the model of Mtb VKOR. Analysis of the structures of Sp/VKOR and EcDabA-DabB complex (Inaba et al., 2006) indicates the features and the residues that form interactions at the protein–protein interface. Therefore we chose eleven residues (51–61) from the loop between α1 and α2 of Mtb VKOR, and used Autodock Vina (Trott and Olson, 2010) to construct the model of the interaction between Mtb DsbA and VKOR.

DEPOSITION OF COORDINATES

Coordinates and structure factors for the crystal structure of the Mtb DsbA have been deposited to the Research Collaboratory for Structural Bioinformatics Protein Data Bank (http://www.rcsb.org/pdb/). The entries are 4JR4 for the oxidized form and 4JR6 for the reduced form.

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ABBREVIATIONS

Dsb, disulfide bond-forming proteins; DTT, dithiothreitol; E. coli, Escherichia coli; FR-I5, fold recognition by use of 5 terms; Mtb, Mycobacterium tuberculosis; PDI, Protein disulfide isomerase; r.m.s.d., root mean square deviation function; SAD, single anomalous diffraction; TB, tuberculosis; VKOR, Vitamin K epoxide reductase

COMPLIANCE WITH ETHICS GUIDELINES

Lu Wang, Jun Li, Xiangxi Wang, Wu Liu, Xuejun Zhang, Xuemei Li, Zhe Rao declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by the any of the authors.

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