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COMPREHENSIVE INVITED REVIEW

Structure and Function of DsbA, a Key Bacterial Oxidative Folding Catalyst

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Abstract

Since its discovery in 1991, the bacterial periplasmic oxidative folding catalyst DsbA has been the focus of intense research. Early studies addressed why it is so oxidizing and how it is maintained in its less stable oxidized state. The crystal structure of *Escherichia coli* DsbA (EcDsbA) revealed that the oxidizing periplasmic enzyme is a distant evolutionary cousin of the reducing cytoplasmic enzyme thioredoxin. Recent significant developments have deepened our understanding of DsbA function, mechanism, and interactions: the structure of the partner membrane protein EcDsbB, including its complex with EcDsbA, proved a landmark in the field. Studies of DsbA machineries from bacteria other than *E. coli* K-12 have highlighted dramatic differences from the model organism, including a striking divergence in redox parameters and surface features. Several DsbA structures have provided the first clues to its interaction with substrates, and finally, evidence for a central role of DsbA in bacterial virulence has been demonstrated in a range of organisms. Here, we review current knowledge on DsbA, a bacterial periplasmic protein that introduces disulfide bonds into diverse substrate proteins and which may one day be the target of a new class of anti-virulence drugs to treat bacterial infection. *Antioxid. Redox Signal.* 14, 1729–1760.

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I. Introduction

The three-dimensional structure of proteins underpins their function. This in turn means that immature proteins may not attain activity if they are not folded properly, and that mature proteins may lose activity prematurely if they become unfolded, or if the underlying polypeptide chain is severed through the actions of proteases. Unfolded proteins also pose a risk of forming toxic aggregates and fibrils (9, 105). Clearly, it is important for a protein's survival—and for the survival of the parent cell and organism—that a protein reaches its full potential by rapidly and efficiently adopting its correct fold.

To overcome possible folding problems, cells encode an assortment of protein factors to help other proteins fold correctly, or to prevent proteins from unfolding under adverse conditions such as an increase in temperature (207). For membrane and secreted proteins that function in the especially unforgiving extracellular environment, a specialized post-translational modification, the protein disulfide bond, is often incorporated to help maintain structure and function.

Disulfide bonds are covalent chemical bonds formed between sulfurs in thiol groups. In proteins, disulfide bonds are formed between the sulfurs in side chains of cysteine residues. The disulfide form of the cysteines is referred to as the oxidized form, and the dithiol form of the cysteines is referred to as the reduced form. A correctly inserted disulfide bond can fortify a protein fold—and hence serve to protect protein function—by providing a structural brace within the polypeptide fold (Fig. 1). Disulfide bonds contribute considerably to the stability of the protein fold, as evidenced by an increase in melting temperature of the oxidized compared with the reduced form of the protein (217, 219). Furthermore, disulfide bonds provide protection against proteolysis (15, 109). Disulfide bond formation may also play a regulatory role in some proteins, by changing the shape of the protein, the surface charge, or reactivity (202). In this way, the reversible nature of the disulfide bond can act as a redox signaling mechanism (38). However, we will not discuss redox signaling here. We focus specifically on the DsbA oxidative folding catalysts that catalyze the formation of structural disulfide bonds and thereby add stability and protease resistance to folding proteins in bacteria.

The importance of disulfide bonds to the function of proteins, and thus to the functioning of cells and organisms, is perhaps best indicated by the finding that almost all organisms encode proteins to catalyze the process of disulfide bond formation, reviewed in (91). In the late 1960s, the first oxidative protein folding catalyst was identified: protein disulfide isomerase (PDI) (65). PDI is a eukaryotic endoplasmic reticulum (ER) protein that introduces disulfide bonds into folding proteins and shuffles incorrect disulfide bonds in misfolded proteins. Surprisingly, it was more than 20 years later that the first bacterial oxidative folding catalyst was discovered in the early 1990s (12, 123). That protein was DsbA, the topic of this review. The first DsbA protein identified was from *Escherichia coli* and was found to have a signal sequence that localized it to the bacterial periplasm. DsbA has a somewhat unsophisticated function in comparison with PDI (77), in that it simply and relatively indiscriminately adds disulfide bonds, via a disulfide exchange reaction, to cysteine-containing proteins in the periplasm.

In this comprehensive review, we summarize the rapidly accumulating body of knowledge on the structure and function of DsbA. In Section II we begin by reviewing the different oxidative folding pathways that have been discovered, covering both eukaryotic and prokaryotic systems. Section III is

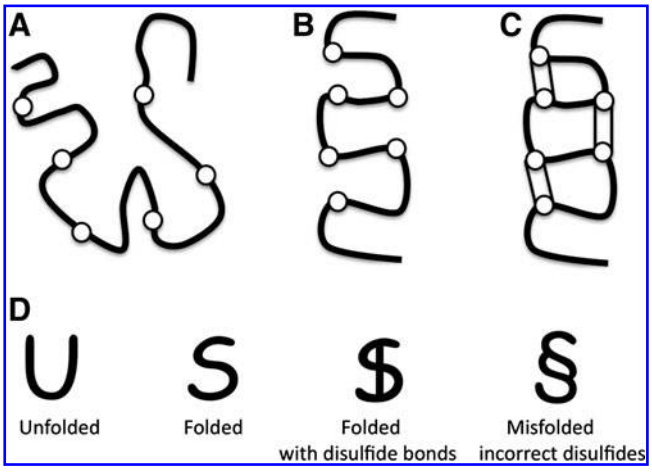

FIG. 1. Disulfide bonds add bracing to protein structure. **(A)** Schematic representation of an unfolded protein having little secondary structure and no tertiary structure. **(B)** Schematic representation of a folded protein structure that in **(C)** is stabilized by disulfide bond formation. In each case, the polypeptide chain is represented by a *black coiled line*, reduced cysteine residues are represented by *white circles with no connecting lines* and disulfide bonds between the cysteines are represented by *white circles connected by white rectangles*. **(D)** The symbols used in figures throughout the manuscript to denote different states of folding protein substrates are shown: unfolded, folded without disulfide bonds, folded with disulfide bonds, and misfolded with incorrect disulfide bonds.

devoted to a description of the archetypal EcDsbA fold and provides a summary of currently available DsbA structures. Section IV describes variations to the archetypal EcDsbA fold that have been identified in the structures of DsbA proteins from different organisms. Section V reviews what is known about the binding of substrates and other ligands by DsbA. Finally, in Section VI, we focus on the function of DsbA as a central mediator of virulence in bacteria. Section VII summarizes briefly the main points and provides perspectives for future DsbA research.

A. Nomenclature

A few words of explanation are required regarding the nomenclature we use for DsbA proteins in this review. For simplicity and clarity, we distinguish DsbA proteins originating from different bacteria by using the initials of the bacteria; for example, *E. coli* DsbA is referred to as EcDsbA. However, as with all nonstandard abbreviations, we refer to the full name of the organism in the first instance, followed by the abbreviation in parentheses [*e.g.*, *Staphylococcus aureus* DsbA (SaDsbA)]. Thereafter, we use the abbreviated name when referring to the specific protein. Where more than one DsbA is encoded by an organism, we use the names that have been assigned to each of the proteins, if these have been published previously. For example, *Neisseria meningitidis* encodes three DsbA proteins, two of which are membrane-anchored and one of which is soluble (203). These have been called NmDsbA1, NmDsbA2, and NmDsbA3 (203); we use this same nomenclature here. Where we make reference to multiple DsbAs from a single organism for which no names have been assigned previously, we provide names as well as the gene coding for each of the specific encoded proteins so that it is clear to which protein we refer.

For figures throughout the article, we use a set of consistent, specific symbols to denote unfolded proteins, folded proteins, folded proteins with correct disulfide bonds, and misfolded proteins with incorrect disulfides (Fig. 1D).

II. Oxidative Protein Folding Pathways

The ability of proteins to fold into their correct three-dimensional structures is vital for cell growth and survival (47). Due to their existence in crowded cellular compartments, nascent polypeptides exposing hydrophobic surfaces are prone to aggregation and in turn misfolding. To overcome this obstacle, a complex machinery of molecular chaperones exists to ensure efficient transport and folding of newly synthesized proteins (83, 226). There are also pathways involving oxidative folding steps present in a range of cellular compartments (*e.g.*, the periplasm in Gram-negative bacteria, and the mitochondria and ER in eukaryotes).

The function of oxidative protein folding pathways is to form disulfide bonds in substrate proteins. Formation of a disulfide bond is a reversible reaction in which the thiol groups of two cysteine residues on the folding protein are oxidized to form a covalent sulfur–sulfur bond. Therefore, oxidative protein folding generally occurs in a cellular compartment separated from the reducing environment of the cytosol where protein synthesis occurs. Disulfide bond formation adds stability to exported and secreted proteins, including membrane proteins, to enable them to withstand the relatively hostile extracellular environment (171).

Oxidative protein folding pathways have been characterized in a number of eukaryotic and prokaryotic cellular compartments. The eukaryotic pathways will be discussed briefly first, so as to illustrate the relationship to the bacterial periplasmic disulfide bond (Dsb) family of proteins. Then we summarize the DsbA–DsbB oxidative folding pathway and the DsbC–DsbD isomerization pathway, from the model organism *E. coli* K-12, before briefly reviewing pathways that have evolved in other bacteria.

We note that selenocysteine (SeCys) containing DsbA-like protein sequences have been identified from the genomes of prokaryotes and lower order eukaryotes (66, 117, 220, 231–233). SeCys residues in proteins can give rise to unique properties in comparison to cysteine-containing variants (3). Furthermore, simple diselenides, but not analagous disulfides, at concentrations approaching catalytic levels can functionally replace DsbA *in vivo* in *dsbA* knockouts (16). However the products of SeCys *dsbA*-like genes have not yet been characterized. Therefore, we will not consider these proteins further in this review. However we, and no doubt many other researchers in this field, are very interested to learn more about the structure and function of this new class of DsbA proteins.

A. Eukaryotic pathways

In contrast to a prokaryotic cell in which the periplasmic space is typically the only cellular compartment separated from the cytoplasm, a eukaryotic cell has a variety of organelles in which oxidative protein folding can occur. Indeed, oxidative protein folding has been identified in the ER, the mitochondria, and in plant chloroplasts. The mechanism of oxidative protein folding in chloroplasts remains largely uncharacterized so this will not be discussed here; instead we refer the reader to Ref. 113. The oxidative protein folding pathway of the ER is unique in that this organelle is responsible for folding and transporting proteins that are targeted to other locations, whereas mitochondria and chloroplast pathways are specific to proteins that remain within those organelles.

For a comprehensive and critical review of ER oxidative protein folding, we refer the reader to Ref. 84. The oxidative protein folding pathway in yeast ER involves two proteins: protein disulfide isomerase (PDI) and a sulfhydryl oxidase (Fig. 2A). The two sulfhydryl oxidase enzymes found in yeast are ER oxidoreductin (Ero1p) and a protein termed "essential for respiration and vegetative growth" (Erv2p) (60, 61, 144, 172). PDI was one of the first identified thiol–disulfide oxidoreductases (70), and there are now at least 20 members of this family in humans (84) and five in yeast (188), each of which is characterized by localization to the ER, and the presence of at least one Thioredoxin-like domain. Individual PDI family members may have tight substrate specificity, suggesting that the wide variety of PDI-like proteins in the ER is necessary to allow folding of a broad range of protein targets (115).

PDI is thought to catalyze several redox reactions, including oxidation, reduction, and isomerization (124, 186). Oxidized PDI becomes reduced when it acts as a disulfide donor in the oxidative folding of substrates (Fig. 2A). Reduced PDI can then function as an isomerase to shuffle incorrectly-formed disulfide bonds in substrates, or it can be re-oxidized

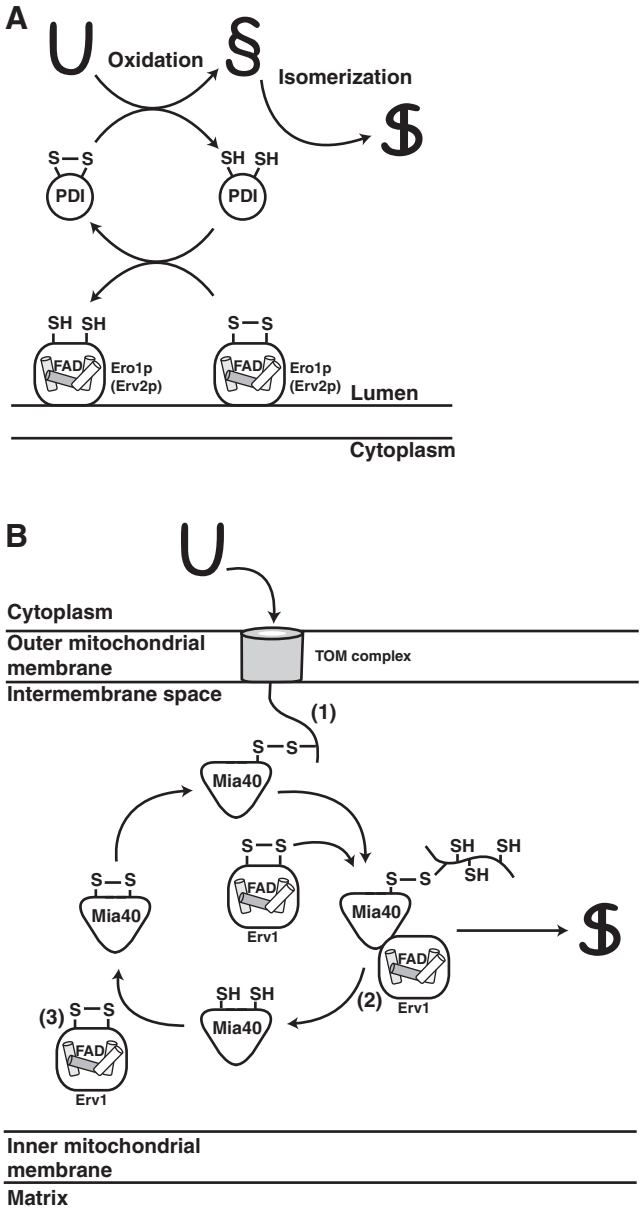

FIG. 2. The oxidative protein folding machinery of the endoplasmic reticulum and of the mitochondria. (A) Oxidized PDI serves as a disulfide donor for reduced substrate proteins. Reduced PDI can also shuffle incorrectly formed disulfide bonds (isomerization). Reduced PDI is in turn re-oxidized by Ero1p (or Erv2p) in yeast to complete the cycle. **(B)** In mitochondria, Mia40 serves as a receptor that directly interacts with twin CX_3C or twin CX_9C proteins which enter the intermembrane space (IMS) through the TOM complex. During the import reaction, Mia40 forms a transient mixed disulfide with the substrate as it exits the TOM pore **(1)**. Importantly, formation of the complex blocks reverse transport of small unfolded cysteine containing proteins, trapping them in the IMS. Erv1 interacts with this complex forming a ternary complex (197) **(2)**. Reduced Mia40 is then re-oxidized by Erv1 to complete the cycle **(3)**.

by the membrane-associated protein Ero1p or the second sulfhydryl oxidase Erv2p in yeast. Ero1p and Erv2p utilize molecular oxygen, coupled with a flavin adenine dinucleotide (FAD) cofactor to generate disulfide bonds, which are transferred to PDI (61, 75, 76). The major ER oxidase, Ero1p, does not share obvious sequence similarity with Erv2p, however the two proteins do share a core four-helix bundle architecture surrounding the FAD cofactor (Fig. 2A) and both require two redox-active cysteine pairs for activity (62, 74, 76).

The discovery of the mitochondrial machinery for import and assembly (MIA pathway) in the intermembrane space (IMS) of mitochondria was a major breakthrough in the field of oxidative protein folding (29, 156). For a detailed description we refer the reader to several excellent reviews (44, 55, 193). The essential MIA pathway comprises Mia40 and the FAD-dependent sulfhydryl oxidase Erv1 (87, 156, 198). Only reduced and unfolded precursors can pass through the translocase of the outer membrane (TOM) complex, so that oxidation of free thiols by Mia40 traps newly imported proteins in the IMS, and therefore contributes to the directionality of the MIA pathway (Fig. 2B).

Mia40 proteins have a divergent N-terminal domain and a conserved C-terminal domain of approximately 60 amino acids, containing six invariant cysteine residues (29, 201). These six cysteines comprise a signature motif: CPC–CX_9C–CX_9C. Curiously, the twin CX_9C motif resembles that of many Mia40 substrates; an alternative twin CX_3C motif has also been identified in some Mia40 substrates (145). Recently, the NMR structure of human Mia40 (10) and crystal structure of yeast Mia40 (fused to maltose binding protein) (128) were reported. These structures confirm that the conserved domain contains the helix–loop–helix organization of the structurally characterized twin CX_9C proteins. Importantly, the structures show that Mia40 is not related to thioredoxin, unlike many other oxidative folding proteins. Additionally, the Mia40 structures reveal a hydrophobic region on the Mia40 surface proposed to be involved in substrate binding.

In the IMS, Erv1 maintains Mia40 in the oxidized state. Erv1 is structurally similar to the Ero1p and Erv2p proteins associated with PDI oxidation in the ER (180) and to the integral inner membrane protein, DsbB, of the bacterial pathway (187) (see Section IIB). The FAD domain of Erv1 can interact with cytochrome *c*, thereby coupling Erv1 directly to the respiratory chain, leading eventually to the production of water. This coupling prevents the formation of H_2O_2, and makes re-oxidation of Mia40 very efficient, even at low oxygen concentrations (19, 39).

B. The classical bacterial dithiol oxidizing pathway: DsbA and DsbB

The bacterial oxidative protein folding pathway was first characterized, and is also best characterized, for *E. coli* K-12. This organism is therefore often used as the archetype for bacterial oxidative protein folding. The oxidative pathway of *E. coli* K-12 involves two proteins, *E. coli* DsbA (EcDsbA) and *E. coli* DsbB (EcDsbB), acting together to introduce disulfide bonds into folding protein substrates (110) (Fig. 3A). EcDsbA is the key player (12) because it interacts directly with folding protein substrates and transfers its disulfide to them. With a redox-potential of −122 mV (100), it is a highly oxidizing protein, though as we will see below, other more recently characterized DsbA proteins are even stronger oxidants. After a disulfide transfer reaction with a folding protein, EcDsbA becomes reduced. The second protein, EcDsbB, reoxidizes

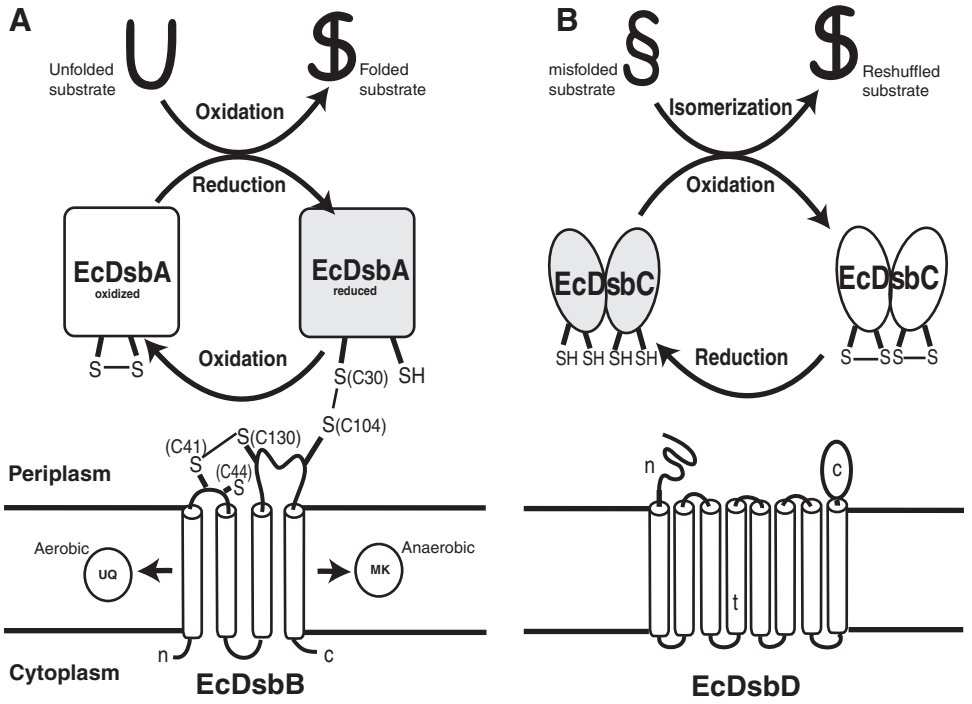

FIG. 3. The oxidative and isomerization pathways in *E. coli* K-12. (A) Oxidized EcDsbA transfers its active site disulfide to an unfolded protein substrate; the substrate cysteines are thus oxidized, and the protein becomes folded. The active site cysteines C30 and C33 of EcDsbA become reduced and are re-oxidized by C104 and C130 of EcDsbB. This cycle is repeated to maintain EcDsbA in the active oxidized form. Ubiquinone (UQ) is the recipient of electrons from EcDsbA and oxidizes EcDsbB under aerobic conditions. Alternatively, menaquinone (MK) can oxidize EcDsbB under anaerobic conditions. **(B)** When EcDsbA catalyzes incorrect disulfide bond formation in substrates, EcDsbC proofreads and shuffles the disulfide bonds until the correct connections are made. After introducing disulfides into the substrate, EcDsbC becomes oxidized. The disulfide at the active site of EcDsbC is reduced by the N-terminal domain of EcDsbD (nDsbD for N-terminal domain of DsbD) so that EcDsbC is converted into the active reduced form. The other two domains of EcDsbB are labeled c and t for the C-terminal and transmembrane domains, respectively.

reduced EcDsbA so that EcDsbA is able to catalyze further oxidative folding reactions (177) (Fig. 3A).

The active site of EcDsbA comprises the classic thioredoxin CXXC motif, encompassing two redox-active cysteines. The specific motif in the case of EcDsbA is Cys30–Pro31–His32–Cys33 (CPHC). The highly oxidizing nature of the EcDsbA active site is unusual for a disulfide and originates from the surprisingly low pK_a of approximately 3.5 of the more N-terminal Cys (Cys30) in the CXXC motif (72, 163, 223, 229). This exceedingly low pK_a means that in the reduced form at physiological pH, the Cys30 residue of EcDsbA exists in the thiolate anion form. Unusually for proteins, the reduced dithiol form of EcDsbA is more stable than the oxidized disulfide-bonded form of the enzyme by 3.6 ± 1.4 kcal/mol (229). This energetic difference between the oxidized and reduced forms of the active site drives disulfide transfer by EcDsbA and thus results in oxidation of its substrates. Stabilization of the reduced form over the oxidized form is thought to occur via a network of interactions with the Cys30 thiolate side chain of EcDsbA (see Section III E below) (79).

The disulfide exchange reaction catalyzed by EcDsbA is a bimolecular nucleophilic substitution reaction (S_N2) (56, 213). Similar to other proteins with a catalytic Thioredoxin fold, thiol–disulfide exchange by EcDsbA is thought to proceed via two steps (64, 119) (Fig. 4). The reaction begins with nucleophilic attack on the surface exposed Cys30 sulfur of oxidized EcDsbA by a cysteine from the reduced substrate protein, resulting in the formation of a mixed disulfide intermediate between the substrate and EcDsbA. In the second step, another substrate cysteine attacks the substrate sulfur participating in the mixed disulfide. The result of this second

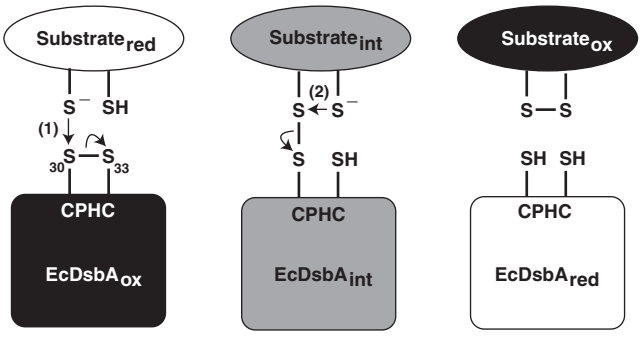

FIG. 4. EcDsbA catalyzed disulfide transfer. The reaction (*left panel*) begins with nucleophilic attack from a substrate protein on the surface exposed N-terminal cysteine (Cys30) of the disulfide bond formed at the CXXC active site of EcDsbA (1). This attack results in an intermediate mixed disulfide complex between EcDsbA and the substrate protein (*center panel*), which in turn is reduced by nucleophilic attack of another substrate thiolate (2), leaving the substrate oxidized and EcDsbA reduced (*right panel*). The reaction proceeds as a bimolecular nucleophilic substitution reaction.

reaction is the formation of a disulfide bond in the substrate (oxidized substrate) and production of reduced EcDsbA. To complete the catalytic cycle, EcDsbB then re-oxidizes reduced EcDsbA.

The discovery that EcDsbA acts by transferring its disulfide bond to folding proteins, and as a result becomes reduced, led to the question of how EcDsbA is re-oxidized in the bacterial periplasm. Mutational analysis identified a second gene, *dsbB*, which encodes a protein that oxidizes reduced EcDsbA (11, 159). The EcDsbB protein is located in the bacterial inner membrane between the cytoplasm and the periplasm and possesses four transmembrane helices connected by two periplasmic domains (114, 234) (Fig. 5). The four-helix topology of EcDsbB is reminiscent of the eukaryotic counterparts Erv2p and Ero1p, though Erv2p and Ero1p are soluble rather than membrane embedded.

EcDsbB contains four essential cysteine residues (118) in two periplasmic loops (114): Cys41 and Cys44 in the more N-terminal periplasmic loop (P1) and Cys104 and Cys130 in the more C-terminal periplasmic loop (P2) (131). Experiments with a Cys33 mutant of EcDsbA led to the accumulation of a disulfide-linked complex between EcDsbA and EcDsbB (81, 130), indicating that the surface exposed Cys30 of EcDsbA interacts with Cys104 of EcDsbB to form the mixed disulfide.

Electrons from EcDsbA are presumed to flow first from EcDsbA to Cys104–Cys130 of EcDsbB and from there to EcDsbB Cys41–Cys44, and then finally to quinones and the respiratory chain (6, 43, 200). The quinone cofactor of EcDsbB is ubiquinone (UQ) in the aerobic oxidative folding cycle and menaquinone (MK) in the anaerobic oxidative folding cycle (6); quinone binding is essential for the EcDsbA oxidizing function of EcDsbB (71). Mutagenesis and kinetics data from Kadokura and Beckwith (118) and Tapley *et al.* (200) had suggested that the oxidation of EcDsbA by EcDsbB occurs through a series of highly concerted disulfide exchange reactions. This idea is strongly supported by the structural model of the EcDsbB:EcDsbA inter-loop complex (generated from NMR data of EcDsbB and of the EcDsbB interaction with the EcDsbA, as well as the crystal structure of EcDsbB:

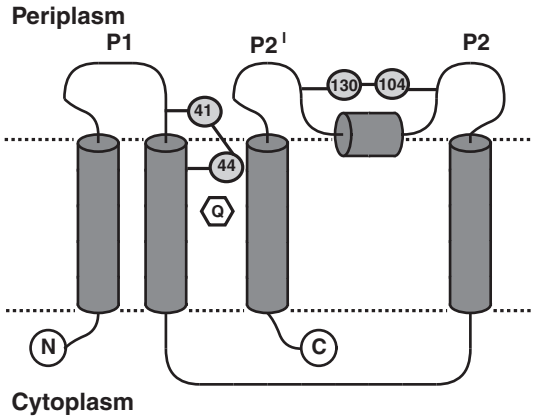

FIG. 5. Topology of EcDsbB. The topology of EcDsbB in the inner bacterial membrane is shown with the four conserved cysteine residues represented as *gray spheres,* indicating the corresponding residue numbers. The quinone is shown as a hexagon labeled Q. Periplasmic loops P1, P2′, and P2 as well as N and C-termini are indicated.

EcDsbA) that reveals a neat straight-line arrangement of the six redox-active cysteines (234).

EcDsbB has strict binding specificity for EcDsbA and does not directly interact with the reduced folding protein substrates of EcDsbA. The standard redox potential values for the EcDsbB cysteine pairs are much lower than that of EcDsbA (–210 mV for Cys41–Cys44 and −220 mV for Cys104–Cys130 of EcDsbB compared with −122 mV for Cys30–Cys33 of EcDsbA) (100, 106). Characterization of EcDsbB in the oxidative pathway revealed that the second periplasmic loop, encompassing Cys104 and Cys130, undergoes considerable conformational changes during the enzymatic reaction, induced by EcDsbA, and that these changes and consequent disulfide rearrangements may facilitate the energetically unfavorable electron transfer from EcDsbA to EcDsbB (107, 118, 200).

C. The classical bacterial disulfide isomerization pathway

When protein substrates with multiple cysteines are being folded, the oxidative system of EcDsbA and EcDsbB can pair cysteine residues in combinations that are not present in the native structure, trapping the proteins in a non-native conformation. Consequently, *E. coli* K-12 has evolved a pathway for disulfide isomerization, to ensure the formation of correct, native disulfide bonds (Fig. 3B). This system is comprised of two proteins, EcDsbC and EcDsbD, which proofread and reshuffle non-native disulfides to aid correct oxidative protein folding. The soluble periplasmic protein EcDsbC mediates the isomerization of disulfide bonds in substrate proteins (17) and it also possesses chaperone activity (31). The second component of the isomerization pathway is EcDsbD, an integral membrane protein that transfers electrons to EcDsbC to maintain the isomerase in the catalytically active reduced form (181). Thus, EcDsbC and EcDsbD in this classical bacterial disulfide isomerizing pathway are the counterparts to EcDsbA and EcDsbB of the classical bacterial dithiol oxidizing pathway.

The crystal structure of EcDsbC reveals a V-shaped homodimer in which two subunits (2×23 kDa) are linked at the base of the V by an N-terminal dimerization domain and the two catalytic Thioredoxin domains are located at the ends of the arms (153). The CXXC motifs of the Thioredoxin fold in the dimeric EcDsbC have the sequence Cys98–Gly99–Tyr100–Cys101. In the DsbC crystal structure, these two active sites face each other on the inside of the V-cleft (153). Dimerization is essential for the activity of EcDsbC; it protects the enzyme from being oxidized by EcDsbB (7), thus preventing a futile electron cycle, and it also generates a molecular cleft lined with hydrophobic residues that is required for substrate binding (4, 153). This cleft is necessary for both isomerase and chaperone activities (7). A recent report showed that it is not necessary for both catalytic domains of EcDsbC to be present for disulfide shuffling to occur in *E. coli* K-12; just one catalytic active site motif and a substrate binding region are sufficient for the reaction to proceed (4). However, this work also showed that engineered EcDsbC variants lacking one of the catalytic domains were susceptible to oxidation by EcDsbB (4).

In the periplasm of *E. coli* K-12 is also found an EcDsbC homologue called EcDsbG. Like EcDsbC, this protein is a homodimer (2×27 kDa) with disulfide bond isomerase and

molecular chaperone activity (18, 189). EcDsbG and EcDsbC share 24% sequence identity, though EcDsbG is expressed at lower levels than EcDsbC, and exhibits a more narrow substrate specificity (18). For example, unlike EcDsbC, EcDsbG does not catalyze the reduction of insulin or the oxidative refolding of RNase (18) (these assays are typically used to characterize the redox activity of Thioredoxin fold proteins). The crystal structure of EcDsbG reveals a similar three-dimensional architecture to that of EcDsbC; thus, the enzyme is V-shaped and each arm of the V comprises an N-terminal dimerization domain linked by an α-helix to a C-terminal Thioredoxin fold catalytic domain (89). Each catalytic domain of EcDsbG contains the characteristic CXXC redox center (in this case Cys109–Pro110–Tyr111–Cys112), and these both face the inside of the V-cleft. As with EcDsbC, the two redox centers are kept in the reduced form by the membrane protein EcDsbD (18). Despite the overall structural and topological similarities between EcDsbG and EcDsbC, there are some striking differences between the two paralogues. For example, the dimensions of the V-cleft are much larger in EcDsbG and it contains many acidic residues that generate distinct negatively charged regions (89), which are absent in EcDsbC. These properties could indicate that EcDsbG preferentially binds large folded or partially folded substrates (89). For several years, the functional role in *E. coli* K-12 of this second isomerase-like protein EcDsbG remained unknown because attempts to identify *in vivo* substrates for this protein had been unsuccessful. However, a recent elegant study by Depuydt and co-workers showed that EcDsbG principally interacts with three L,D-transpeptidases, YbiS, ErfK, and YnhG (45), which catalyze the cross-linking of peptidoglycan in the bacterial cell wall. These three proteins each incorporate a single cysteine, which is required for their activity (148). Depuydt *et al.* discovered that both EcDsbC and EcDsbG protect this cysteine from oxidation to sulfenic acid (45). They also reported that EcDsbG, which has surface properties better suited for interacting with folded proteins, is much more efficient in protecting these folded substrates from cysteine sulfenylation than EcDsbC (45), which is presumably better suited to interacting with unfolded or mis-folded substrates.

In the oxidative environment of the periplasm, both EcDsbC and EcDsbG are maintained in their active reduced form *via* interaction with EcDsbD (181). This 59 kDa enzyme comprises three domains, an N-terminal periplasmic domain (nDsbD) which has an immunoglobulin-like fold (82), a transmembrane domain (tDsbD) consisting of eight transmembrane helices (32), and a C-terminal periplasmic domain comprising a Thioredoxin fold with a CXXC motif (cDsbD) (183). EcDsbD obtains its reducing power from cytoplasmic NADPH. The flow of electrons is via thioredoxin reductase, which reduces thioredoxin, and thioredoxin in turn reduces EcDsbD (electrons are transferred from tDsbD, to cDsbD and then to nDsbD); EcDsbD ultimately reduces the two isomerase proteins EcDsbC and EcDsbG (32, 126). A disulfide exchange cascade mediates all the electron transfer reactions in this system. Analysis of the redox potentials of the proteins involved in the cascade indicates that this process is thermodynamically favored, as reviewed in Ref. 120. It is interesting to note that the EcDsbD membrane-bound domain tDsbD, which contains only one cysteine pair, is capable of transferring electrons from cytoplasmic thioredoxin to periplasmic cDsbD (126). Although there is evidence that these cysteines are accessible from either side of the membrane (127), the precise mechanism of how this occurs remains to be determined.

D. Alternatives to the classical oxidative protein folding pathway

Oxidative protein folding pathways in bacteria other than *E. coli* K-12 have been the focus of considerable recent research. Bioinformatic screening of DsbA homologues (49, 92) has revealed that DsbA proteins are present in many, but not all, bacterial classes and show that some bacteria encode more than one DsbA homologue. The results of these analyses suggest that within the bacterial kingdom a number of alternate oxidative protein folding pathways exist, reviewed in Ref. 92. Some prokaryotes contain multiple DsbA proteins that likely perform specific oxidative folding roles within the bacterium. Structures of multiple DsbAs from representative species are now available and are discussed in detail in Section IV. Additionally, some organisms appear to encode only a single member of the oxidative pathway—for example, *S. aureus* (92) and *Staphylococcus epidermidis* (49) encode only a DsbA and *Helicobacter pylori* encodes only a DsbB (49). These specific organisms may rely on environmental redox conditions to regulate oxidative protein folding.

A recent bioinformatic and functional study by Daniels *et al.* (42) has examined disulfide bond formation in Gram-positive bacteria, which lack an outer membrane, and therefore do not possess the protected periplasmic compartment where oxidative protein folding occurs in Gram-negative bacteria. The cysteine incorporation patterns were analyzed for different types of proteins from both Gram-positive and Gram-negative organisms. In agreement with a previous study (49), Daniels *et al.* identified that of the two major phyla of Gram-positive bacteria, Actinobacteria and Firmicutes, oxidative protein folding is only likely to occur in the first. Indeed, their evidence suggests that Firmicutes exclude cysteine residues from protein sequences to compensate for a lack of disulfide folding machinery. To support the observation that disulfide bonds are formed in substrate proteins of Actinobacteria, Daniels *et al.* also identified and characterized three putative secreted soluble Dsb-like proteins with CXXC motifs (42). They concluded that Actinobacteria encode a DsbA-like homolog that may exist as a homodimer and function to oxidize exported substrates. However, as this species does not encode a DsbB protein, it is unclear how this DsbA-like protein might be re-oxidized *in vivo*.

One possible explanation is that such DsbA-like proteins could be re-oxidized by the bacterial homolog of the eukaryotic membrane enzyme vitamin K epoxide reductase (VKOR) (49). Experiments involving a VKOR homologue present in *Mycobacterium tuberculosis* H37Rv illustrates how re-oxidation may occur in Actinobacteria (49). *M. tuberculosis* VKOR (MtbVKOR) was found to restore disulfide bond formation in an *E. coli dsbB* mutant. This complementation of EcDsbB activity depended on the presence of Ec*dsbA*, indicating that MtbVKOR does not oxidize folding substrate proteins itself, but apparently functions in a manner analogous to EcDsbB (49). Therefore VKOR likely re-oxidizes DsbA-like redox proteins in prokaryotic organisms that require disulfide bonds in substrate proteins, and may take the place of DsbB in organisms that do not encode a DsbB protein.

Furthermore, Dutton and co-workers showed that *in vivo* oxidation of EcDsbA was promoted when expression levels of *Mtb*VKOR in *E. coli* were increased (50).

Further investigation into the bacterial homologues of VKOR has revealed that in some species a single polypeptide chain incorporates both a VKOR and a DsbA-like enzyme. The crystal structure of one such natural fusion protein from *Synechococcus spp*. was recently reported (142). The VKOR domain within this structure shares some surprising similarities with the structure of EcDsbB. For instance, the catalytic core of the VKOR membrane domain incorporates a four-helix bundle architecture that binds a quinone. A cysteine is located in the VKOR structure on one side of the quinone ring, a geometry that likely facilitates electron transfer to the aromatic systems and which has been observed for the soluble eukaryotic FAD-containing thiol oxidases Ero1p (74), Erv2p (76), and Erv1 (221) described above. Furthermore, *Mtb*VKOR shares many features with human VKOR: both use similar substrates; both are inhibited by warfarin (whereas EcDsbB is unaffected), and warfarin-resistant mutations are located in similar regions in both proteins (50). The key role of MtbVKOR in *M. tuberculosis* pathology and the finding that MtbVKOR can be inhibited by warfarin highlights the possibility of targeting MtbVKOR for the development of new therapeutics to treat tuberculosis, or potential new anticoagulants (50).

The DsbA-like domain in the structurally characterized VKOR-DsbA fusion incorporates a CPHC catalytic active site, like that found in EcDsbA. However, the domain is smaller than EcDsbA and more like thioredoxin ($\sim$100 residues) and it lacks the EcDsbA surface features that interact with EcDsbB (see Section IIB above). Presumably these features are not necessary in the fused complex because of the high local concentration of the two components.

Extensive bioinformatic analyses of Dsb proteins have also identified that some bacterial species exhibit an unusually high number of unpaired cysteines in their secreted proteins compared to *E. coli* (49). These species were in turn predicted to have reducing periplasms or lack disulfide bond formation altogether. The bacteria of one such phylum, Chlamydiales, all encode *dsbA* homologs (49, 92), and express a disulfide bonded major outer membrane protein (225). However, the identification and characterization of a periplasmic reductase, DsbH, from *Chlamydia pneumoniae* TW183 suggests that these obligate intracellular bacteria may actively reduce proteins in the periplasm, and therefore use an alternate mechanism for oxidative protein folding for their limited number of predicted substrates (146). Further support for this conclusion comes from a recent study on *Bacteroides fragilis* (191). In contrast to *C. pneumoniae, B. fragilis* encodes neither *dsbA* nor *dsbB* homologs (49, 92). Structural and functional characterization of a periplasmic thioredoxin-like protein, TrxP, from *B. fragilis* revealed that this organism, like *C. pneumoniae,* maintains a reduced cell envelope by expressing a periplasmic reductase (191).

III. Structure of the Archetypal DsbA

The first structural details of bacterial oxidative folding pathways were revealed with the determination of the EcDsbA crystal structure in the early 1990s (Fig. 6) (2, 151). Prior to the report of the EcDsbA crystal structure, predictions

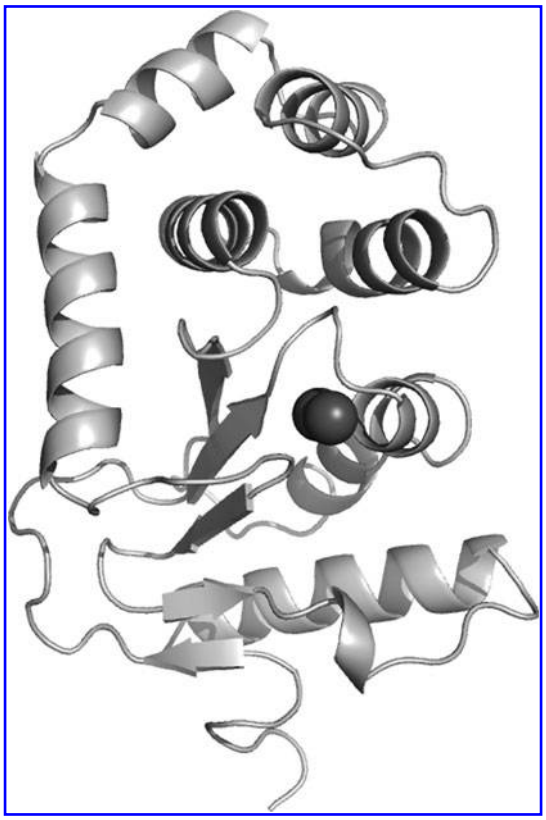

FIG. 6. Structure of EcDsbA. Ribbon diagram of EcDsbA (from PDB 1FVK (80)). The sulfur atoms of the catalytic cysteines are shown as *spheres*.

of the DsbA three-dimensional structure utilized primary sequence and secondary structure data. This information was used to suggest that EcDsbA would have a fold similar to thioredoxin, and was likely to be structurally similar to the N-terminal thioredoxin-like domain of PDI (53). Although the prediction of a Thioredoxin fold turned out to be correct, the overall sequence alignment and secondary structure assignment proved inaccurate because the EcDsbA structure includes an α-helical domain insertion compared to the canonical Thioredoxin protein fold. Structure determination of EcDsbA was not trivial. It was crucial to maintain the protein in the oxidized state to form crystals suitable for diffraction studies and the structure was only elucidated by a combination of multiple isomorphous replacement and selenomethionine multi-wavelength anomalous diffraction (SeMet MAD) phasing methods (151, 152). The structure of EcDsbA was one of the first to be solved using SeMet MAD methods.

The *E. coli* K-12 DSB pathway remains the best characterized bacterial oxidative protein-folding system to date. As such, the 21 kDa EcDsbA protein is regarded as the archetypal member of the bacterial DsbA family. The structure of EcDsbA reveals a protein with a core consisting of a five-stranded twisted β-sheet, sandwiched between α-helices (Fig. 6). Pfam classification places EcDsbA into the family PF01323 (57). This family now consists of more than 3400 sequences and greater than 25 domain architectures. In addition, structurally characterized DsbA proteins have been grouped into the α and β (a/b) protein class according to the structural classification of

proteins (SCOP) database (162). This classification is based on hierarchical levels that embody the evolutionary and structural relationships of proteins. Therefore, proteins with lower sequence identity but whose structures share similar topologies are placed into the same class. Due to the fact that EcDsbA contains a fold similar to the previously characterized thioredoxin, EcDsbA belongs to the Thioredoxin-like superfamily in the SCOP database. However, as a result of the large α-helical domain insertion into the Thioredoxin fold (located above the β sheet in the orientation shown in Fig. 6), EcDsbA and similar proteins have been classified into the DsbA-like family in the SCOP database.

Recently, there has been a rapid growth in the number of reported DsbA structures from species other than *E. coli*. Through comparative structural biology, this collection of DsbA structures has the potential to greatly enhance our understanding of bacterial oxidative protein folding pathways. Beginning with the report of the *Haemophilus influenzae* genome, the advent of genomic sequencing projects at the end of the last century have had an astonishing affect on the field of structural biology (58). In regards to bacterial oxidative folding, these sequencing projects have led to the study and characterization of an ever-increasing number of important bacterial species. In the past 5 years alone, the number of unique DsbA structures from different bacterial species has grown from two to seventeen (Table 1). It is now evident that although sequence identity across DsbA proteins from different species is often relatively low, the structural architecture first described for the EcDsbA structure serves as a valuable reference when attempting to decipher the structure-function relationship of a new DsbA.

A. The Thioredoxin fold

As previously mentioned, the DsbA proteins structurally characterized to date are all classified as members of the Thioredoxin-like superfamily of a/b proteins in the SCOP database. The Thioredoxin-like superfamily is currently further divided into 23 families, some with multiple subfamilies, based on additions to the Thioredoxin fold. The Thioredoxin fold is the core structural element of this superfamily (Fig. 7A), which is common to numerous proteins across different organisms. This fold, named after the protein in which it was first observed, is a distinct structural motif, and encompasses the majority but not all of the secondary structure features of *E. coli* thioredoxin (98, 125, 150) (Fig. 7B). Publication of two glutaredoxin structures later revealed that compared with thioredoxin, the shorter glutaredoxin actually incorporates all the features of the Thioredoxin fold (52, 224); thus the secondary structure features of glutaredoxin and the Thioredoxin fold are virtually equivalent.

The Thioredoxin fold has been described as having two folding units (51). The N-terminal motif consists of a $\beta/\alpha/\beta$ structural unit connected by a loop of residues containing an α-helix to a C-terminal motif comprising $\beta/\beta/\alpha$ elements (Fig. 7A). The two β-strands of the N-terminal structural unit are parallel to one another, whereas the two β-strands of the C-terminal motif run antiparallel to each other. Therefore, the β-sheet formed by these structural elements in the Thioredoxin fold forms a mixed β-sheet. The β-sheet forms the core of the Thioredoxin fold and is twisted relative to a central axis perpendicular to the β-sheet. Furthermore, the β-sheet is

TABLE 1. SUMMARY OF DSBA STRUCTURES DEPOSITED IN THE PDB

Organism	*Protein name*	*Gene Id*	*PDB Code*	*Year of Deposit*	*Resolution (Å)*	*Ref.*	*$E^{o'}$ (mV)*	*CXXC*	*XX cis Pro*	*Seq. id. (%)*[a]	*Rmsd (Å, #Cα)*[a]
E. coli K-12	EcDsbA	948353	1DSB	1993	2.00	(151)	−122 (100)	CPHC	GVP	100	–, –
V. cholerae	VcDsbA (TcpG)	2614468	1BED	1996	2.00	(99)	—	CPHC	GVP	40	1.3, 167
S. aureus	SaDsbA	11935157	3BCI	2007	1.81	(90)	−132	CPYC	TTP	16	2.6, 122
X. fastidiosa	XfDsbA	1126982	2REM	2007	1.90	(182)	−94	CPHC	GTP	19	2.1, 167
E. coli CFT073	EcDsbL	1039926	3C7M	2008	1.55	(73)	−95	CPFC	GVP	24	2.1, 162
*W. pipientis w*Mel	WpDsbA1	2738597	3F4R	2008	1.60	(135)	−163	CYHC	ATP	10	2.9, 137
N. meningitidis MC58	NmDsbA3	902521	3DVX 2ZNM	2008	2.80	(137,212)	−87	CVHC	STP	22	2.2, 152
	NmDsbA1	902389	3A3T 3DVW	2009		(137,211)	−89	CPHC	GTP	23	2.2, 157
B. subtilis 168	BsDsbA (BdbD)	936031	3GH9	2009	1.69	(37)	−80	CPSC	ATP	15	2.7, 137
P. aeruginosa PAO1	PaDsbA	877731	3H93	2009	1.50	(192)	−94	CPHC	GVP	30	2.2, 164
S.enterica	SeSrgA	1256229	3L9V	2010	2.15	(93)	−154	CPPC	GTP	35	1.4, 172
Typhimurium	SeDsbA	256229	3L9S		1.58		−126	CPHC	GVP	85	0.7, 178
SL1344	SeDsbL	1256229	3L9U		1.57		−97	CPFC	GVP	26	2.1, 161
Structural Genomics											
N. europaea	NeDsbA	1083521	2IN3	2006	1.85	—	—	CSWC	GFP	11	2.9, 137
V. fischeri es114	VfDsbA	3279886	3FEU	2008	1.76	—	—	CGHC	SVP	23	2.1, 158
ATCC 700601/ ES114											
S. coelicolor A3	ScDsbA	1097303	3GL5	2009	2.15	—	—	CPWC	GVP	17	2.5, 140
B. parapertussis	BpDsbA	1665561	3HD5	2009	2.35	—	—	CPHC	GTP	23	2.0, 145

[a]Compared with EcDsbA (PDB code 1FVK, molecule B) and calculated using SSM (133) within COOT (54).

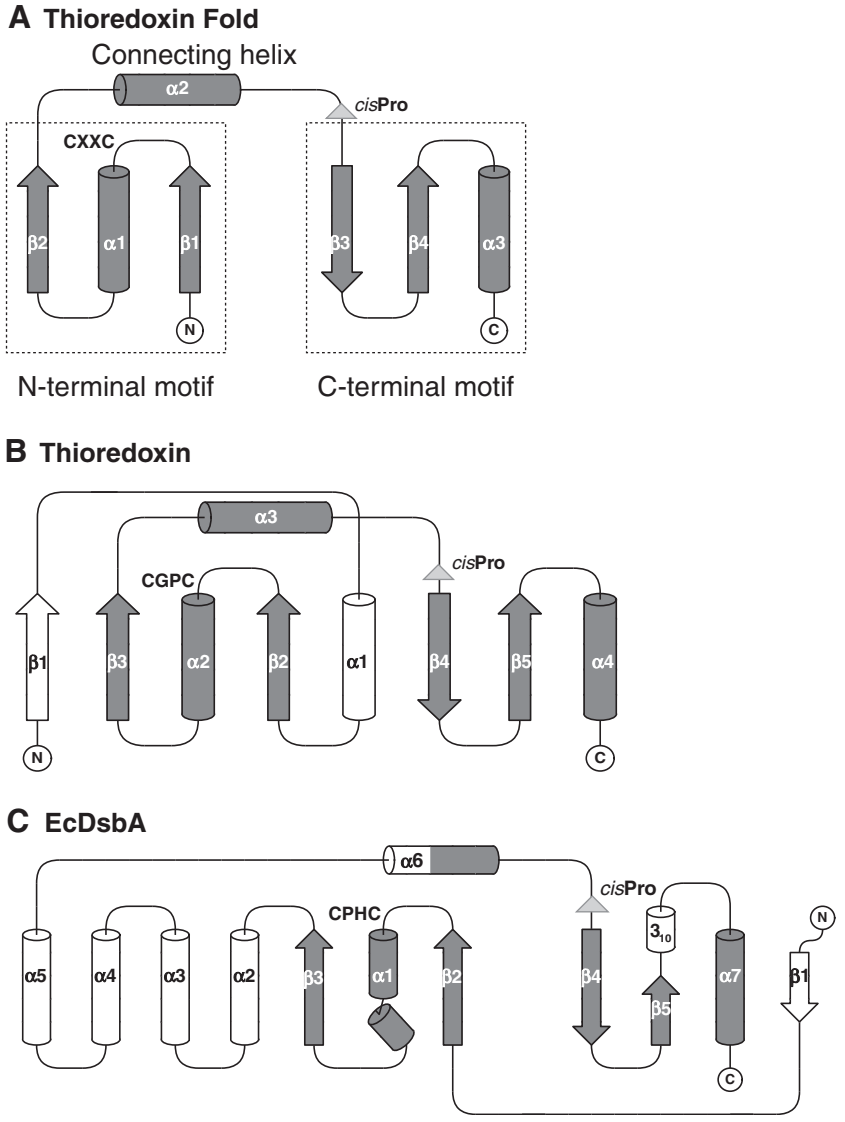

FIG. 7. The Thioredoxin fold and the classical DsbA fold. (A) The structural elements of the Thioredoxin fold are illustrated in *gray*. **(B)** The topology of the *E. coli* thioredoxin A structure is shown with elements of the Thioredoxin fold in *gray* and additional features in *white*. **(C)** The topology of EcDsbA is shown with structural elements of the Thioredoxin fold in *gray* and additional elements in *white*. In each case, the location of the CXXC and *cis*Pro loop and N- and C- termini are indicated. *Arrows* represent β-strands, and α-helices are displayed as *cylinders*. This figure was made using TopDraw (23).

sandwiched between the three α-helices of the fold. The α-helices of the N- and C-terminal folding units are arranged in parallel on one face of the β-sheet, and the α-helix that connects the two folding motifs runs along the opposite face of the β-sheet, and perpendicular to the other two helices (Fig. 7A). Although the Thioredoxin fold was initially identified and is present in many redox active proteins, nature has also used this fold to engineer proteins for a range of other biological tasks. For instance, it has been incorporated into the metal binding protein calsequestrin (216), which binds and releases Ca^{2+} ions during the muscle contraction-relaxation cycle, and in the *E. coli* protein, HyaE, responsible for binding to the arginine rich Tat signal peptide that regulates hydrogenase complex assembly and export (165).

Despite these findings, the majority of proteins with a Thioredoxin fold also incorporate the redox-active CXXC catalytic motif at the N-terminal end of the first α-helix of the Thioredoxin fold (Fig. 7). This motif is critical for redox activity in Thioredoxin fold proteins. It has also been shown that two residues located in the loop connecting the α_2-helix and β_3-strand (loop between α_6-helix and β_4-strand in EcDsbA) of the Thioredoxin fold are also important for the proper function, stability, and folding (30, 69, 129) of this family of proteins. This loop contains an isoleucine/valine/threonine residue followed by a proline residue in the *cis* conformation (*cis*Pro). Although this *cis*Pro loop is distant in sequence, it lies adjacent to the CXXC active site in the folded oxidoreductase. The crystal structure of EcDsbA also revealed a feature termed the hydrophobic groove (Fig. 8A) (151). This hydrophobic groove is located below the active site cysteines when viewed in the orientation presented in Figure 6. It is composed of a number of aromatic (Phe36, Phe174, Tyr178) and aliphatic

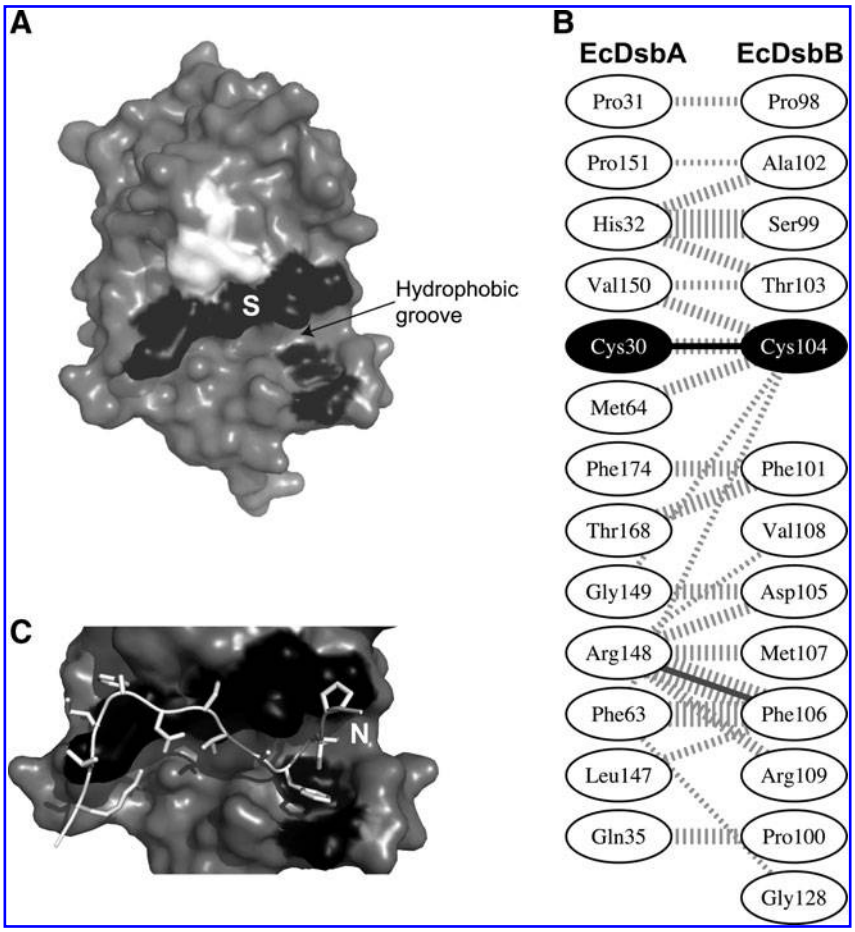

FIG. 8. The binding interface of the EcDsbA and EcDsbB complex. **(A)** Diagram of EcDsbA in an orientation similar to that used in Figure 6, showing the surface features of the protein. Shaded in *black* is the region of EcDsbA that interacts with P2 of EcDsbB, and shaded in *white* is the region that interacts with P2′. The hydrophobic groove is indicated by an *arrow*, and the active site is represented with a *white S*. **(B)** PDBsum (138) analysis of the EcDsbB–EcDsbA complex (from the structure with PDB code 2ZUP) (107) illustrating the residues that form interactions at the protein–protein interface. For nonbonded contacts, the *width of the striped line* is proportional to the number of contacts. Only one hydrogen bond is present, shown as a *solid gray line*, between backbone atoms of Arg148 of EcDsbA and Phe106 of EcDsbB. **(C)** Closeup of the P2 interaction site, with the EcDsbB P2 represented in *white* with the N-terminal end labeled.

(Leu40, Ile42, Leu147) residues and was recently shown to be critical for binding to EcDsbB during the catalytic cycle (108). Even though this groove is vital for the re-oxidation of EcDsbA, as discussed below, other structurally characterized DsbA proteins have differing features in this region.

B. The inserted helical domain

The EcDsbA crystal structure revealed that this 21 kDa monomeric protein not only possesses a Thioredoxin fold comprising about 90 residues, it also contains a large α-helical domain inserted into the middle of the Thioredoxin fold (Fig. 7C). One interesting feature observed in the α-helical domain of EcDsbA is a hydrophobic patch, located just above the active site CXXC motif (151). It is thought that this region may play a role in substrate recognition: recent work showed that residues from this inserted helical domain are involved in binding substrates (see Section IV below for further detail) (167). Additional evidence for such a role has come from the many DsbA structures now available in the Protein Data Bank (PDB). These structures illustrate that DsbA proteins from different species exhibit unique surface characteristics in regards to this α-helical domain. These variations suggest that this region may contribute to substrate specificity. Additional evidence for a function of the α-helical insertion domain is revealed by the study of multiple DsbA paralogues from a single organism. The structural rearrangements and surface variation of this region within DsbAs from a single species illustrates that these different DsbAs may have unique or perhaps limited overlapping substrate specificity.

C. Invariant and highly conserved residues

Although structurally characterized DsbA proteins share a similar architecture, there is very little in common when their primary amino acid sequences are compared. A recent analysis of 421 bacterial genomes identified 330 DsbA homologues of widely varying sequence identities (92). Two residues that are absolutely conserved in all sequences are the catalytic cysteine residues of the CXXC motif. These residues are positioned at the amino-end of the α_1-helix and form the catalytic redox active site of DsbA (Fig. 7C). Studies of the dipeptide residues

connecting the cysteine residues have revealed that, although the EcDsbA sequence (CPHC) is present in a number of other homologues, the identity of these residues can vary amongst the functional DsbAs (78, 160, 175). These studies highlight not only the important role of the CXXC motif in redox proteins, but also the influence of the dipeptide sequence on the redox potential and in turn protein function.

An analysis of the individual residues of the dipeptide reveals that the N-terminal residue is a proline residue in 11 of 13 (85%) characterized DsbA structures. The two variants are the NmDsbA3 (PDB ID 3DVX) (137) that has a valine residue, and the *Wolbachia pipientis* DsbA1 (PDB ID 3F4R) (WpDsbA1) (135) that contains a tyrosine at this position. It is interesting to note that both of these organisms possess multiple putative *dsbA* sequences. *N. meningitidis* contains three *dsbA* genes; both of the other two DsbA sequences (NmDsbA1 and NmDsbA2) contain the EcDsbA dipeptide sequence (CPHC). Analyzing the role of each *N. meningitidis* DsbA via gene deletion has shown that the presence of at least one protein containing the PH dipeptide is sufficient for full virulence (203). In regards to *W. pipientis*, two putative *dsbA* sequences have been identified (134). As indicated above, WpDsbA1 has a tyrosine as the first residue in the dipeptide. The other DsbA, WpDsbA2, (*W. pipientis w*Mel, GeneID: 2737836), has a GH dipeptide in the CXXC motif, which curiously, is the dipeptide sequence typically found in the active sites of eukaryotic PDI (150). *W. pipientis* does not encode a DsbA with a PH dipeptide. It also does not encode sequences for proteins of the isomerase pathway and therefore the *W. pipientis* DsbA proteins likely fulfill differing roles compared to EcDsbA.

The only other invariant residue on the DsbA scaffold of the 330 DsbA sequences is the *cis*Pro residue located in the loop joining the α_2-helix and the β_3-strand of the Thioredoxin fold (α_6-helix and β_4-strand in EcDsbA). This residue is located at position 151 in the EcDsbA sequence and is thus relatively distant from the active-site cysteine residues (Cys30 and Cys33) in the primary sequence. However the *cis*Pro is positioned very close to the active site cysteines in the three-dimensional fold and forms part of the active site. Mutational studies have revealed that the *cis*Pro loop plays an important role in redox function. Functional and biophysical studies have revealed that the *cis*Pro of EcDsbA plays a key role in the stability and function of this protein (30, 161). Mutation of this proline to alanine results in a loop with a different conformation to the wild-type protein, and both redox states of the $P_{151}A$ mutant are less stable than the wild type. Measurements revealed that the $P_{151}A$ substitution results in a reduction of the oxidizing power of EcDsbA (30, 161). Similar changes in activity have been reported for *cis*Pro mutants of *Vibrio cholerae* DsbA (VcDsbA also known as TcpG) (21). Further *in vivo* analysis of EcDsbA *cis*Pro mutants suggests that this residue plays an essential role in substrate release, because site-directed mutants of this residue result in the accumulation of mixed disulfide intermediates with substrates (121, 122) (see also Section VA). This observation is supported by crystal structures of both thioredoxin and EcDsbA bound to substrates, where the *cis*Pro has been shown to be critical for the orientation of loop residues that can interact with substrate molecules (108, 147). Finally, studies on human thioredoxin show that the *cis*Pro residue prevents metal binding to the reactive thiolate-based active site, further supporting a key role in substrate recognition (199).

In addition to the functionally important residues already discussed, there are four highly conserved hydrophobic residues that play critical structural roles. In the structurally characterized DsbA proteins Val22, Phe26, Phe93, and Val155 (numbering is from the EcDsbA sequence) align in almost all structures. The two valine residues are located in the central β-strands of the Thioredoxin fold maintaining the hydrophobic core of the fold and aiding in the positioning of the *cis*Pro loop near the active site. These valine residues can be replaced by leucine, isoleucine, or (in the case of WpDsbA1) methionine. The side chains of the two highly conserved phenylalanine residues are part of a hydrophobic cluster located between the Thioredoxin fold and the inserted α-helical domain. This hydrophobic cluster positions the α_3-helix above the active-site disulfide, and this may be important for substrate recognition. Phe26, located on the β_2-strand, is not strictly conserved in all sequences of the DsbA structures; functionally it can be replaced by a phenylalanine from β_3-strand as seen in the structures of SaDsbA, BsDsbA, and WpDsbA1 [PDB codes 3BCI, 3GH9, and 3F4R, respectively (37, 90, 135)]. Phe93 is not conserved in the two structurally characterized DsbA paralogues, EcDsbL and SeDsbL. These both contain an extended loop at the N-terminal end of the α_3-helix, which replaces the phenylalanine with a tyrosine.

D. Structural features of the interaction with DsbB

A recently updated model of the EcDsbA–EcDsbB crystal structure has enabled the sites of interaction between these important proteins to be better defined (107). The EcDsbA structure has a hydrophobic groove running along the surface of the protein near the active-site cysteine residues (151) (Fig. 8A). This groove is involved in binding to the N-terminal portion of the second EcDsbB periplasmic domain (P2) in all three EcDsbA–EcDsbB crystal structures determined to date (107, 108, 149). An interesting observation from all of the structures is that, in addition to the four transmembrane α-helices and two periplasmic loops (P1 and P2) previously predicted for EcDsbB, an additional short α-helix located at the periplasmic face of the membrane was identified in the C-terminal P2 loop (Fig. 5). This short α-helix, encompassing residues 116 to 120 tethers the second periplasmic loop of EcDsbB to the membrane generating two shorter loops (P2 and P2′), P2 contains Cys104 and P2′ contains Cys130. The short α-helix serves as an anchor to the membrane and likely provides a pivot point enabling the two cysteines of this periplasmic domain to effectively and rapidly oxidize EcDsbA (107).

Analysis using the web-based program PDBsum (138) of the most recent crystal structure of the EcDsbB–EcDsbA mixed disulfide complex reveals that residues other than the cysteines of both molecules are involved in complex formation. A total of 26 residues, 13 from EcDsbA and 13 from EcDsbB, have so far been identified to form the interface (Fig. 8B). In addition to the disulfide bond between EcDsbA Cys30 and EcDsbB Cys104, a hydrogen bond is formed between the backbone oxygen of EcDsbA Arg148 and the backbone nitrogen of EcDsbB Phe106. Other interactions are hydrophobic. The only invariant DsbA residues involved in the interaction between EcDsbA and EcDsbB are Cys30 and *cis*Pro151; there is very little conservation in the remaining 11 residues identified at the protein–protein interface.

Although not definitive given the limited resolution of the available EcDsbA–EcDsbB crystal structures, it is now becoming evident that the P2′ region of EcDsbB that contains Cys130 may also interact weakly with EcDsbA. This interaction may be important for keeping the Cys130 EcDsbB loop separated from the Cys104 loop, allowing the latter to oxidize EcDsbA rapidly (107). The most recently described EcDsbA–EcDsbB structure reveals that Gly128 of P2′ interacts with Phe63 of EcDsbA (Fig. 8B). Phe63 is located on the loop that connects the Thioredoxin fold to the α-helical domain of EcDsbA (between the β_3-strand and α_2-helix) (Fig. 7C). This finding is supported by results from a recent saturation transfer TROSY–HSQC NMR experiments on a sample of ^{2}H, ^{13}C, ^{15}N-labeled EcDsbB and unlabeled EcDsbA (234), where it was shown that Gly128 of P2′ interacts with EcDsbA. Taken together, there is now good evidence that there are two DsbB binding sites on EcDsbA, one which binds P2 (including Cys104) and the other which binds P2′ (containing Cys130). However, many structurally characterized DsbA molecules possess surface features different to those of EcDsbA in these two binding sites, suggesting the possibility of species-specific recognition of DsbB.

E. Structural implications for function

Of the numerous enzymes now recognized to catalyze redox reactions, DsbA is one of the strongest thiol oxidants, with EcDsbA having a redox potential of −122 mV (100). This enzyme catalyzes the formation of disulfide bonds in substrate proteins rapidly both *in vivo* and *in vitro*. In contrast, thioredoxin has a much lower redox potential of approximately −270 mV; it functions as a disulfide reductase in the cytoplasm (5).

As discussed previously, the catalytic cysteines of the EcDsbA CXXC motif, Cys30 and Cys33, are located at the N-terminus of the α_1-helix of the Thioredoxin fold (Fig. 7). The more N-terminal of the two cysteines, Cys30, is surface exposed. The unique structural and biophysical properties of the Cys30 residue impart strong thiol oxidant properties to EcDsbA. As indicated above, Cys30 has an abnormally low pK_a value of approximately 3.5 (72, 163, 223, 229) so that it adopts the thiolate anion state at physiological pH. Furthermore, the reduced form of the protein is stabilized with respect to the oxidized form (229), in contrast to the effect of most protein disulfides.

Structural studies have revealed details of how the Cys30 thiolate anion is stabilized, giving rise to the observed stability of reduced EcDsbA in comparison to the disulfide-bonded form. In the crystal structure of oxidized EcDsbA, Cys30 is located at the N-terminus of the α_1-helix, so that the partial positive charge from the helix dipole could aid in stabilizing the thiolate form of the cysteine (151) (Figs. 6 and 7C). Positioning of the CXXC motif on the helix dipole is a conserved feature of redox active Thioredoxin fold proteins, which all exhibit lowered pK_a values for the N-terminal cysteine residue of ∼7 (compared with typical value for cysteine of ∼9) (46, 72, 85). Mutagenesis studies indicate that His32 is important for the redox stability profile of EcDsbA, suggesting that favorable interactions with the His32 sidechain and Cys30 thiolate anion are likely to be present (72). Indeed, in one of the two monomers observed in the crystal structure of reduced EcDsbA, His32 adopts a conformation that is within hydrogen bonding distance of the Cys30 sulfur (79). Quantum mechanical studies of active site models suggest that differential stabilization of the two cysteines in the CXXC motif may give rise to the differing enzymatic properties of Thioredoxin fold proteins (28). Thus, oxidants like DsbA are characterized as having an active site with two strongly stabilized cysteine thiolates; reductants like thioredoxin as having two poorly stabilized thiolates; and isomerases like DsbC as having only the C-terminal buried cysteine thiolate stabilized (28).

A recent study has examined the role of the residue that precedes the *cis*Pro located near the active site (179). Mutational analysis identified that this residue is at least as important in determining the redox properties of Thioredoxin fold proteins as the dipeptide of the CXXC motif. Of the structurally characterized DsbA proteins, the residue preceding the *cis*Pro is generally either a valine or threonine (Table 1). When present as a valine, as in EcDsbA, a hydrophobic interaction is formed with Cys30 of the active site (80, 151). However, in DsbA enzymes where this residue is a threonine, a hydrogen bond can form between the Thr hydroxyl group and the reactive cysteine sidechain. Similar to the interactions described above, the hydrogen bond formed between these two residues would favor the thiolate or reduced form of the cysteine. This thereby destabilizes the oxidized form of DsbA and has a significant effect on the reactivity of the DsbA disulfide bond. The interaction is therefore predicted to favor oxidase activity of DsbA (179) and is generally supported by the structurally characterized DsbA proteins: those with a Cys–Pro–His–Cys sequence, similar to EcDsbA, but with a Thr residue preceding the *cis*Pro typically have an increased redox potential compared with EcDsbA (Table 1). One exception is *Pseudomonas aeruginosa* DsbA (PaDsbA): it has valine preceding the *cis*Pro and a highly oxidizing redox potential. In this case, Trp34 of PaDsbA (located in the loop preceding the CXXC motif) may help to stabilize the thiolate anion via hydrophobic interactions, giving rise to the increased redox potential.

IV. Variations to the Archetypal DsbA Fold

To date, 13 DsbA homologues from 10 organisms have been both structurally and functionally characterized (Table 1). Furthermore, the structures of four other DsbA-like proteins have been deposited into the PDB—without accompanying functional characterization or publications—by two structural genomics consortia, the Midwest Center for Structural Genomics (MCSG) and the New York SGX Research Center for Structural Genomics (NYSGXRC) (Table 1).

DsbA homologues from different organisms share very low sequence identity overall (generally 15%–40%) (92). Nonetheless, the main structural elements present in EcDsbA (151; Fig. 7) are generally conserved across DsbA structures from different bacterial genera. Hence, the DsbA-like protein structures so far elucidated contain a Thioredoxin fold with an inserted helical domain. However, differences in primary sequence give rise to unique structural features that distinguish DsbAs originating from different bacteria. In this section, we summarize the structural characteristics of the DsbA-like proteins deposited in the PDB and compare these to the archetypal EcDsbA structure.

A. DsbA structures from gamma-proteobacteria

So far, most of the DsbA proteins that have been structurally characterized belong to the phylum Proteobacteria, specifically the gamma-Proteobacteria. These DsbAs share the highest sequence identity with EcDsbA and therefore are not surprisingly also the closest structural homologues. One protein that attracted early attention is VcDsbA because of the key role this enzyme plays in the assembly of the co-regulated pilus (Tcp) necessary for *V. cholerae* to colonize its host and establish an infection (169) (see Section VI below). VcDsbA shares ~40% sequence identity with EcDsbA; it has a similar overall architecture (compare Figs. 9A and 9B; root mean squared deviation (rmsd) of 1.3 Å for 167 aligned Cα atoms) and an identical CXXC motif (Cys30–Pro31–His32–Cys33). However, the structure of the *V. cholerae* protein diverges from that of EcDsbA in at least two respects. First, the active site helix α_1, is less bent than that of EcDsbA (Fig. 9C; angle between the two helices is 40° compared with 50° for EcDsbA) (99). The kink is caused by a 3-residue "loop-out" in EcDsbA, whereas in VcDsbA it is caused by a proline. Although a kinked active-site helix appears not to be necessary for DsbA function, this feature is relatively conserved among Thioredoxin fold proteins (88). Second, the length of the C-terminal helix α_7 is much shorter in VcDsbA compared with EcDsbA; this helix forms one of the edges of the hydrophobic groove located immediately adjacent to the CXXC active site (151). As indicated in Section III, the groove is part of the binding site for the redox partner protein EcDsbB (108). Helix α_7 is six residues shorter in VcDsbA compared with EcDsbA (Figs. 9A and 9B). This truncation results in a considerably shortened hydrophobic groove (99).

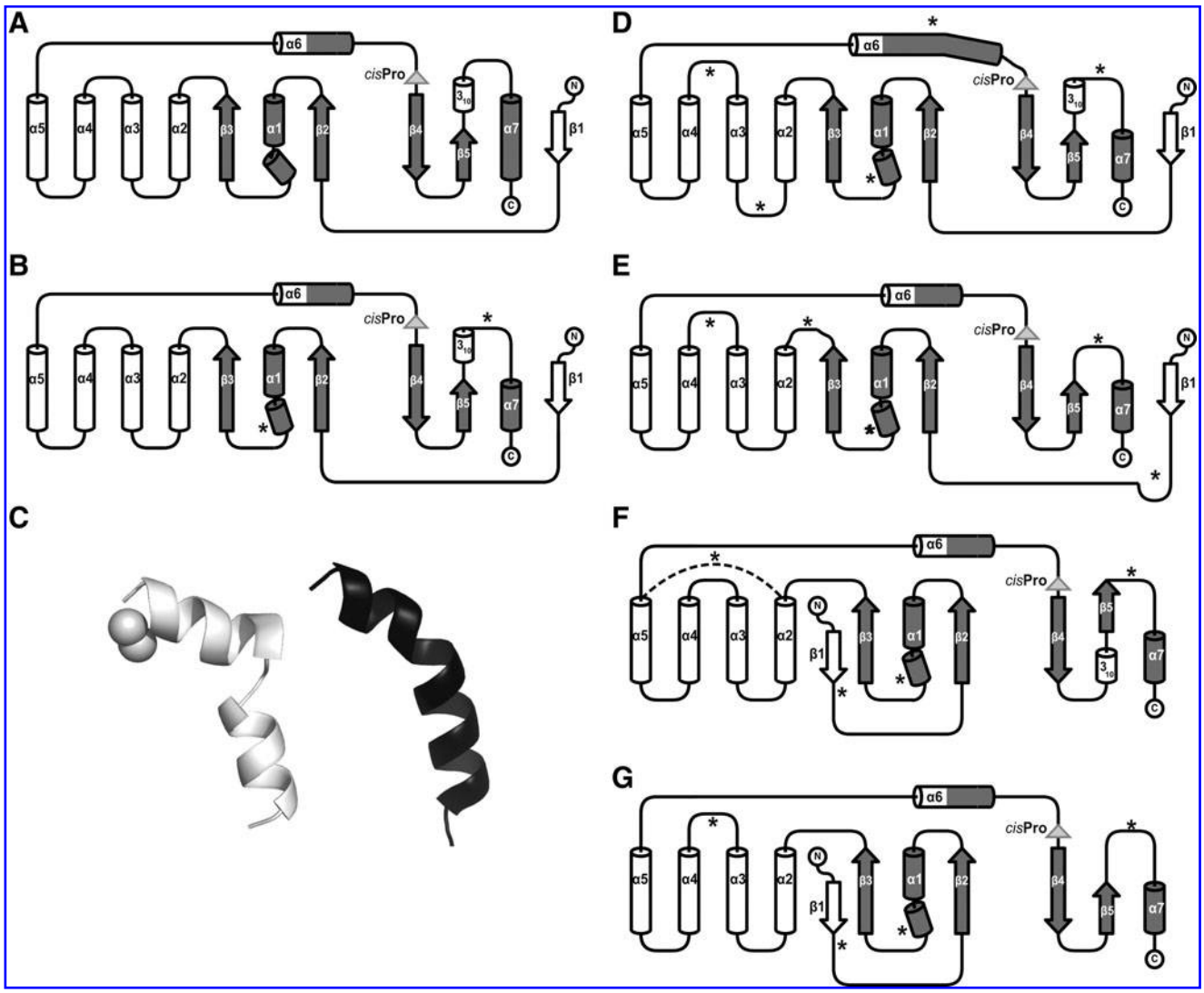

FIG. 9. Topologies of DsbA proteins. Topology of **(A)** EcDsbA; **(B)** VcDsbA(TcpG)/PaDsbA; **(D)** UPEC EcDsbL, **(E)** XfDsbA; **(F)** WpDsbA1; **(G)** BsBdbD. The architecture of the Thioredoxin fold is shown in *dark gray*, and inserted regions are shown in *white*. *Asterisks* show regions of major divergence compared with EcDsbA. *Triangles* indicate the *cis*Pro loop. **(C)** Comparison of the kinked α_1 helix of EcDsbA (*left, white*) and VcDsbA (*right, black*) showing the more marked kink in EcDsbA induced by a 3-residue loop-out of the helix, whereas the helical kink of VcDsbA is caused by a proline. For orientation purposes, the EcDsbA active site cysteine sulfurs at the N-terminus of the α_1 helix are shown as *spheres* (*left panel*).

The structure of a second DsbA from the genus *Vibrio*, *Vibrio fischeri* DsbA (VfDsbA), is now available. Although to date no biochemical or functional characterization of this protein has been reported, VfDsbA contains all the structural hallmarks of DsbA-like proteins, including the Thioredoxin and helical domains and the canonical CXXC active motif (Cys31–Gly32–His33–Cys34). However, VfDsbA shares just 23% sequence identity with EcDsbA and their structures superimpose with an rmsd of 2.1 Å for 158 Cα atoms. In addition to the structural differences identified for VcDsbA, VfDsbA also exhibits differences in the helical domain, including the position of the α_5-helix and the loops connecting the inserted helices, which are shifted relative to both EcDsbA and VcDsbA.

Another gamma-Proteobacterium DsbA structure recently investigated is PaDsbA (192). This protein plays a pivotal role in the pathogenicity of the opportunistic human pathogen *P. aeruginosa* by folding a diverse array of virulence factors required by this organism to infect the host (192) (see Section VI). A structural superposition of PaDsbA and EcDsbA gives an rmsd of 2.2 Å (for 164 Cα atoms). Like the DsbAs from the genus *Vibrio*, PaDsbA also has a less pronounced kink in helix α_1, caused by a proline in the helix sequence, and it also has a truncated α_7-helix groove (Fig. 9B) resulting in a shortened surface groove. Moreover, the relatively low homology between PaDsbA and EcDsbA (they share 27% sequence identity) gives rise to different electrostatic surfaces. Thus, PaDsbA has a smaller hydrophobic patch adjacent to the active site compared with EcDsbA, and overall its surface is more basic (192).

B. DsbA structures from bacteria with multiple DsbAs

Many bacteria encode oxidative protein-folding machinery, but the type and number of DsbA homologues in these pathways varies enormously from one bacterium to another (49, 92). This variability is particularly apparent among Gram-negative pathogens, some of which contain an extended arsenal of thiol-disulfide oxido-reductases (73, 137). Recently, several research groups have carried out comprehensive structural and functional characterizations of DsbA homologues from pathogens encoding a wide repertoire of DsbA proteins. For example, *N. meningitidis*, the causative agent of meningitis and meningococcal septicemia, encodes three DsbA-like proteins; two lipoproteins anchored to the inner membrane (NmDsbA1 and NmDsbA2 that share 78% sequence identity with each other), and a soluble periplasmic protein NmDsbA3 (194, 203). As mentioned in Section IIIC, NmDsbA1 and NmDsbA2 have a CPHC redox active site while NmDsbA3 has a CVHC motif. All three Neisserial enzymes have a Thr-*cis*Pro sequence in the *cis*Pro loop (EcDsbA contains a Val-*cis*Pro), which plays an important role in determining the strong oxidizing power of these three enzymes (137, 179, 211).

Two crystal structures are available for the soluble portions of both NmDsbA1 (3A3T (211) and 3DVW (137)) and NmDsbA3 (2ZNM (212) and 3DVX (137)). The two NmDsbA1 structures are similar to each other (rmsd of 1.0 Å for 189 Cα atoms). However, the two NmDsbA3 structures exhibit a relatively low structural similarity (rmsd of 2.3 Å over 183 Cα atoms). This is principally due to differences between the active site conformations in the two NmDsbA3 crystal structures. In the 3DVX NmDsbA3 structure, the CVHC active site adopts the right-hand hook conformation observed in other Thioredoxin fold proteins (104) with the two sulfur atoms of the cysteines separated by 3.5 Å in the reduced form (137). In the 2ZNM NmDsbA3 structure, the redox catalytic site is not located at the N–terminus of a helix but instead adopts an open looped conformation with the sulfur atoms of the two cysteines separated by 14 Å (212). This CXXC conformation is unprecedented among Thioredoxin fold proteins and it remains to be determined if it is biologically relevant to NmDsbA3 or is an artifact of crystallization or X-ray exposure. For the purposes of our structural comparison with EcDsbA, we have used the 3DVX NmDsbA3 coordinates.

NmDsbA1 and NmDsbA3 share 23% and 22% sequence identity with EcDsbA, respectively, and their atomic structures can be superimposed with the *E. coli* homologue with an rmsd of 2.2 Å (157 Cα atoms aligned) and 2.2 Å (157 Cα atoms aligned), respectively. The structures of the two Neisserial enzymes are similar, superimposing with an rmsd of 1.1 Å over 186 Cα atoms (137). Several structural differences exist in regions surrounding the active site; for example, the α_1-helix is distinctly kinked in EcDsbA but is less so in the Neisserial protein structures. Furthermore, a shorter loop connects β_5 and α_7, and α_7 is shorter by 1.5 turns than the equivalent helix in EcDsbA, resulting in a more shallow groove in NmDsbA1 and NmDsbA3. In these respects the NmDsbA1 and NmDsbA3 structures are similar to those of VcDsbA and PaDsbA (Fig. 9B). Structural differences with EcDsbA also occur in the helical domains of NmDsbA1 and NmDsbA3 with variations in the length and position of the loops connecting the helices and the orientation of helix α_5 (137, 211, 212).

Finally, NmDsbA1 and NmDsbA3 have distinct surface properties; NmDsbA3 retains most of the surface characteristics of EcDsbA including the hydrophobic patch (thought to be important for substrate binding) and the hydrophobic groove (which interacts with DsbB). In NmDsbA1, the hydrophobic patch and a groove are present, albeit the latter is principally composed of acidic rather than hydrophobic residues (137).

Multiple DsbA proteins are also found in uropathogenic *E. coli* (UPEC). In addition to the oxidase and isomerase pathways found in *E. coli* K-12, this organism also has the DsbL/DsbI proteins which form a second functional redox pair (73, 205). UPEC EcDsbL and EcDsbA are 24% identical in sequence (with different catalytic sites, CPFC versus CPHC, respectively) and their structures are divergent. Superposition of the atomic models of UPEC EcDsbL and EcDsbA (rmsd of 2.1 Å over 162 Cα atoms) reveals considerable variability in both the Thioredoxin and helical domains. Two major differences in the Thioredoxin fold of EcDsbL include bending of the connecting helix α_6 towards the $\beta_4\beta_5\alpha_7$ motif and truncation of that motif by 6 residues (73; Fig. 9D). The effect of these two modifications is that UPEC EcDsbL has a considerably shortened groove. Furthermore, this groove is lined with positively charged rather than hydrophobic residues. Structural variations also map to the helical domain of UPEC EcDsbL where helices α_2, α_3, α_4, and α_5 are shifted by about one helical turn relative to EcDsbA (73), and longer loops connect α_2 and α_3, and α_3 and α_4 (Fig. 9D).

Another Gram-negative pathogen with an extended collection of DsbA proteins is the causative agent of acute human gastroenteritis *Salmonella enterica* serovar Typhimurium (93). Like *E. coli* K-12 this organism contains an oxidase and isomerase system; it also has a DsbL/DsbI redox pair similar

to that present in UPEC; and it has a virulence plasmid-encoded DsbA-like protein, called SeSrgA. The three *S. typhimurium* DsbA paralogues, SeDsbA, SeDsbL, and SeSrgA, have been structurally and functionally characterized recently (93). SeDsbA shares 85% sequence identity with EcDsbA and as expected these two share very similar structures (rmsd 0.7 Å over 178 Cα atoms). SeSrgA has 35% sequence identity with EcDsbA and pairwise comparison with EcDsbA gives an rmsd of 1.4 Å (172 Cα atoms) for the two structures. Thus, SeSrgA is one of the closest structural homologues to EcDsbA (93), yet they have very different active site motifs (CPPC and CPHC, respectively) and different residues preceding the *cis*Pro residue (Thr-*cis*Pro and Val-*cis*Pro, respectively). Moreover, the groove is very different in these two proteins. It is truncated in SeSrgA mainly because of an altered orientation of the loop between α_6 and β_4, immediately preceding Thr147-*cis*Pro148, which forms one edge of the groove in EcDsbA (151). This loop points away from the hydrophobic groove in EcDsbA, resulting in a relatively extended cavity, whereas in SeSrgA the same section of the protein is positioned more towards the β_1-strand at the N-terminus of the Thioredoxin fold. This orientation that shortens the groove is possibly secured by a salt bridge between the side chains of Arg145 and Glu7 (3.7 Å) (93). SeDsbL is closely related to UPEC EcDsbL both in sequence (93% sequence identity) and structure (rmsd of 0.4 Å over 195 Cα atoms; Fig. 9D) and consequently shows all the characteristic features distinguishing UPEC EcDsbL from EcDsbA (93). SeDsbL shares 26% identity with EcDsbA and their structures can be superimposed with rmsd of 2.1 Å for 161 Cα atoms.

The Gram-negative plant pathogen *Xylella fastidiosa*, which causes economically important diseases in many crops including grape, citrus, almond, alfalfa, peach, and coffee, contains two adjacent genes coding for two DsbA homologues (182). XfDsbA and XfDsbA2 are 41% identical and despite not sharing the same catalytic site sequence (CPHC and CPAC, respectively), both have similar redox potentials (–94 and −100 mV, respectively (182)) and are stronger oxidants than EcDsbA (–122 mV) (100). To date, only the structure of XfDsbA has been solved (182). Superposition of EcDsbA and its counterpart XfDsbA (19% sequence identity) results in an rmsd of 2.1 Å (167 Cα atoms). Like almost all the DsbA proteins structurally characterized since EcDsbA, the α_1 helix of XfDsbA is less kinked than that of EcDsbA and it has a truncated groove (182). Other structural differences between XfDsbA and EcDsbA include insertions in loops connecting β_1 and β_2, and α_3 and α_4, and a deletion in the loop between β_3 and α_2 (Fig. 9E); the equivalent loop in EcDsbA is involved in the interaction with EcDsbB (108).

The α-Proteobacterium *W. pipientis* is one of the most successful endosymbionts in the biosphere, infecting many species of arthropods and nematodes (94, 116). *W. pipientis* encodes two DsbA-like proteins WpDsbA1 and WpDsbA2 (also called αDsbA1 and αDsbA2) and a homologue of EcDsbB, but lacks an isomerase pathway (135). The recent structural and biochemical characterization of WpDsbA1 showed that this protein diverges considerably from EcDsbA in sequence (10% identity), biochemical properties and structure (rmsd 2.9 Å for 137 Cα atoms) (135). WpDsbA1 has a DsbA-like architecture, yet it also contains distinct structural characteristics, including a different topology (strand β_1 is hydrogen bonded to the opposite edge of the central β-sheet (Fig. 9F); it lacks the groove altogether due to a deletion in the $\beta_4\beta_5\alpha_7$ motif. Additionally, WpDsbA1 does not have the hydrophobic features of EcDsbA; instead its surface is mostly basic. Another peculiarity of this protein is the presence of a second disulfide bond that links helices α_2 and α_5 of the helical domain (135; Fig. 10A). The two cysteines forming the second disulfide are highly conserved in DsbA-like proteins from α-Proteobacteria, and the disulfide adopts an unusual rotamer conformation suggesting a functional role, though this remains to be confirmed. However, the structural discrepancies are also reflected in unique biochemical properties of WpDsbA1. For example, this protein is the most reducing DsbA-like protein so far characterized (redox potential −163 mV). Moreover,

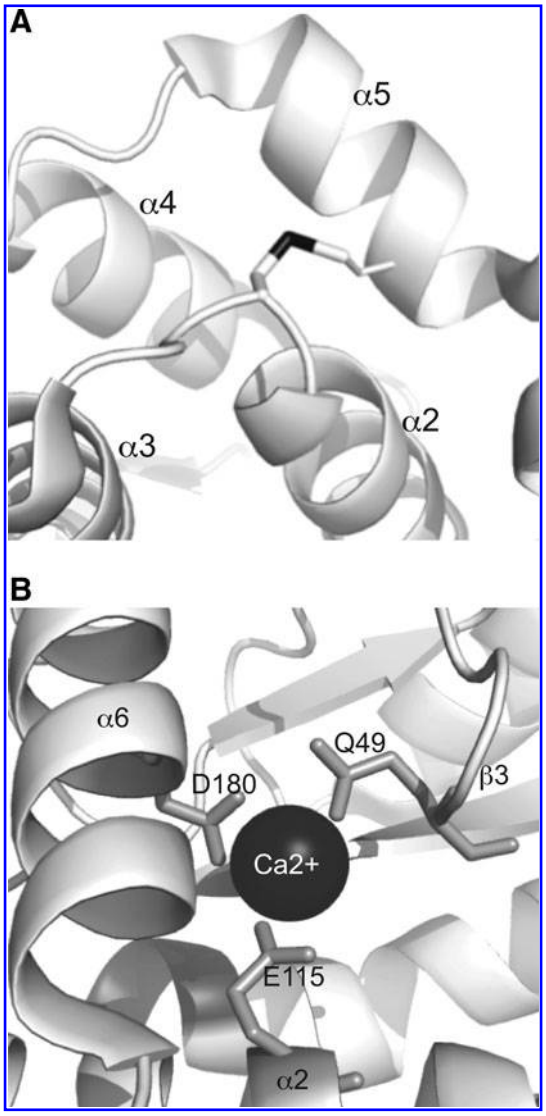

FIG. 10. Unusual features of DsbA structures. (A) The second disulfide bond of WpDsbA1 is shown. This disulfide links helices α_2 and α_5 and is located on the surface of the protein opposite to that revealing the catalytic active site (PDB code 3F4R) (135); **(B)** The calcium binding site of BsDsbA (BdbD) is shown, located between the Thioredoxin and inserted helical domains. The Ca^{2+} is coordinated by Gln49 (Q49), Glu115 (E115), and Asp180 (D180) (PDB code 3GH9) (37).

WpDsbA1 does not complement the *E. coli* K-12 *dsbA*- strain and it is not reoxidized by EcDsbB (135).

C. DsbA structures from Gram-positive bacteria

Gram-positive genomes exhibit great diversity in the number and type of Dsb proteins they encode. For example, *Bacillus subtilis* has one homologue of DsbA and two homologues of DsbB; pathogens like *S. aureus* and *Listeria monocytogenes* encode just a homologue of DsbA and lack other Dsb-like proteins; other Gram-positive bacteria like *Bacillus halodurans and Geobacillus kaustophilus* encode just a DsbB orthologue (132). As described in Section II above, some Gram-positive bacteria including *Streptococcus pyogenes* do not encode Dsb-like proteins.

To date, the structures of two Gram-positive DsbA proteins have been investigated; those of *S. aureus* DsbA, SaDsbA (90), and *B. subtilis* BsDsbA (37) (known as BdbD). On the basis of sequence and structures, these two proteins are only remotely related to EcDsbA (16% and 15% sequence identity, respectively; rmsd 2.6 Å and 2.2 Å for 122 and 152 aligned Cα atoms, respectively) and contain significant structural variations from EcDsbA (37, 90). As with most other DsbA structures, helix α_1 in these two Gram-positive DsbAs is less kinked than in EcDsbA. Second, both proteins include longer and negatively charged loops between helices α_3 and α_4 in the helical domain, which map close to the CXXC active site. Their active sites differ from the catalytic motif in EcDsbA (CPYC and CPSC, respectively, compared with CPHC of EcDsbA). Third, BsDsbA and SaDsbA have truncated grooves compared with EcDsbA due to a deletion in the $\beta\beta\alpha$ domain. Moreover, in BsDsbA the β_1 strand adopts the same topology as WpDsbA1, interacting with β_3 rather than β_5 (Fig. 9G). Finally, BsDsbA has an intrinsic Ca^{2+} binding site, which is absent in other DsbA proteins characterized to date (Fig. 10B). Interestingly, cation binding affects the redox properties of the protein, in that it selectively stabilizes the reduced form of the protein and thereby increases its oxidizing power, despite the fact that the Ca^{2+} ion is located ~14 Å from the redox catalytic centre of BsDsbA (37).

D. Structures of other DsbA homologues

Two structural genomics consortia have deposited into the PDB three other proteins identified as DsbA homologues: *Nitrosomonas europaea* DsbA (NeDsbA); *Streptomyces coelicolor* DsbA (ScDsbA); and *Bordetella parapertussis* DsbA (BpDsbA). Superposition of EcDsbA and its counterparts NeDsbA (11% sequence identity), ScDsbA (17% sequence identity), and BpDsbA (23% sequence identity) resulted in rmsds of 2.9 Å (137 Cα atoms), 2.5 Å (140 Cα atoms) and 2.0 Å (145 Cα atoms), respectively.

The architecture of NeDsbA and ScDsbA structures resembles that of EcDsbA, incorporating a Thioredoxin fold and an inserted helical domain. For NeDsbA, the α_1 helix is less kinked than that of EcDsbA; it has a proline in the sequence rather than the 3-residue loop-out of EcDsbA. By contrast, the ScDsbA structure lacks both the proline and the kink in the α_1 helix. Both NeDsbA and ScDsbA have a deletion in the $\beta\beta\alpha$ motif, resulting in a truncated groove, but overall the Thioredoxin domains compare reasonably well with that of EcDsbA. However, the helical insertion domain in both NeDsbA and ScDsbA differs considerably from that of EcDsbA, comprising seven rather than the usual four α-helices (Fig. 11A),

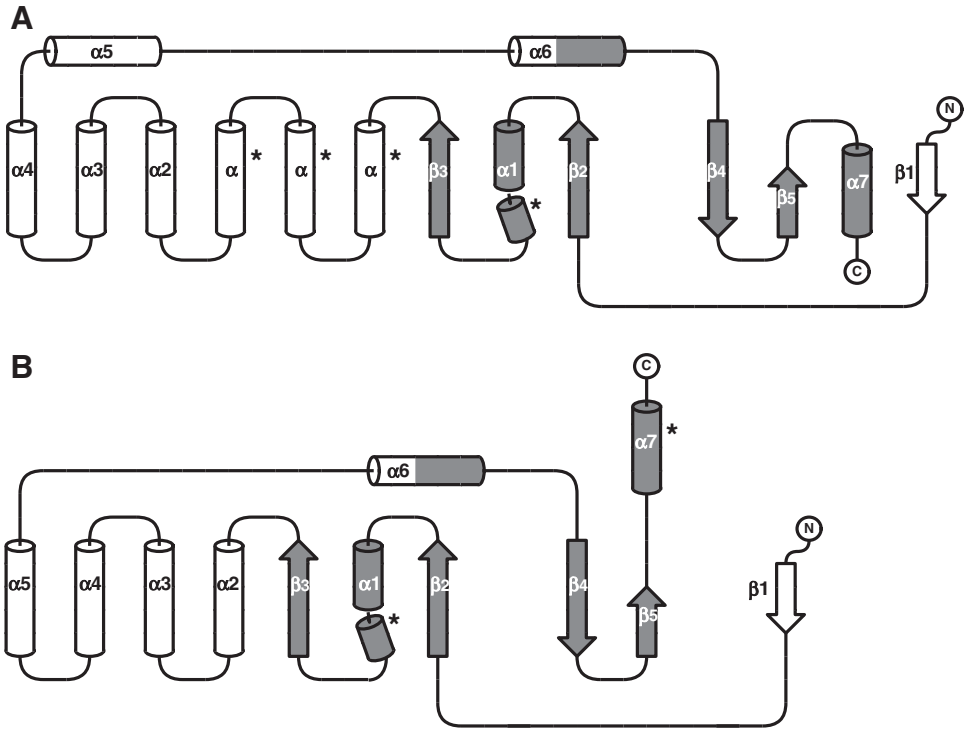

FIG. 11. Structural genomics DsbA structures. Topology diagrams of **(A)** NeDsbA and ScDsbA and **(B)** BpDsbA. The architecture of the Thioredoxin fold is shown in *dark gray*, and insertions are shown in *white*. *Asterisks* show regions of major divergence compared with EcDsbA.

and thus more closely resemble the κ-class of glutathione S-transferases (κ-GSH transferases). Indeed, a Dali search (97) indicated that the closest structural homologues to NeDsbA and ScDsbA are κ-GSH transferases, human kappa class glutathione 2 transferase (15% sequence identity, PDB code 1YZX) and mitochondrial kappa class glutathione transferase (13% sequence identity, PDB code 1R4W), respectively. Nevertheless, NeDsbA and ScDsbA retain the catalytic CXXC motif of DsbA proteins whereas the two κ-GSH transferases so far structurally characterized have instead a SXXS active site (136, 141). Neither the NeDsbA nor the ScDsbA structures have been reported in the literature. Given their structural similarity to both DsbAs and κ-GSH transferases, functional studies would be required to confirm their biochemical function.

The structure of BpDsbA has an unusual conformation of α_7 at the C-terminus of the Thioredoxin fold, which is extended away from the core fold (Fig. 11B). This feature has not been observed in other Thioredoxin fold containing proteins, which all have a relatively compact $\beta\beta\alpha$ domain. The conformation observed in this crystal structure may be an artifact related to the packing of three molecules in the crystal because the extended α_7-helix of one monomer packs against the Thioredoxin fold of another monomer in the asymmetric unit.

V. Ligand Binding by DsbA

In Gram-negative Proteobacteria like *E. coli* the oxidation of substrate proteins is thought to occur in a stepwise manner (106). In the first step, EcDsbA interacts with an unfolded substrate protein that has just been translocated into the periplasm; this involves formation of a mixed disulfide bond between the reactive cysteine of EcDsbA and a cysteine of the substrate. In the second step, a deprotonated cysteine in the substrate helps to resolve the mixed disulfide, thus forming oxidized substrate and leaving EcDsbA reduced. Finally, EcDsbA is rapidly re-oxidized by its partner protein EcDsbB. The transition between EcDsbA-substrate complex and fully oxidized substrate is relatively rapid so that it has been difficult to capture and characterize the EcDsbA-substrate complex (122). To investigate this step, different approaches have been used.

A. Identification of DsbA substrates

One strategy to trap EcDsbA–substrate complexes has been to mutate residues in the *cis*Pro loop (Val150-*cis*Pro151 in EcDsbA). As indicated above, the *cis*Pro loop is highly conserved in proteins with a Thioredoxin fold, so this approach could provide a general method to detect substrates in other DsbA proteins. Three such EcDsbA mutations—$P_{151}T$, $P_{151}S$, and $V_{150}G$—yielded important outcomes (122). The first mutant, $P_{151}T$, was shown to slow the second step of oxidative folding that releases the oxidized substrate protein after interaction with EcDsbA. Hence, the $P_{151}T$ mutation resulted in the accumulation of EcDsbA complexed with substrates (122). By contrast, the $P_{151}S$ mutant exhibited accumulation of another intermediate, the complex between EcDsbA and EcDsbB (71, 118, 130). This mutant therefore showed a defect in the mechanism to release oxidized EcDsbA from EcDsbB. The third mutation, $V_{150}G$, also demonstrated a defect in the step involving EcDsbA–EcDsbB complex formation, though there was much less accumulation of the complex and half of the EcDsbA in the periplasm remained reduced (122). Sixteen other mutations of the *cis*Pro residue were also investigated (121). However, aside from $P_{151}T$, no other mutation was able to accumulate EcDsbA–substrate complexes. Kadokura and co-workers carried out pulse-chase experiments to identify the substrates complexed to the EcDsbA $P_{151}T$ mutant (122). Cellular proteins were labeled, and complexes with EcDsbA $P_{151}T$ were separated by diagonal electrophoresis and the identity of bound partner proteins confirmed by mass spectrometry (122). Putative partners were then verified as EcDsbA substrates by investigation of their redox state in wild-type and Δ*dsbA* strains of *E. coli*.

Kadokura *et al.* investigated the oxidative folding of the EcDsbA substrate alkaline phosphatase (PhoA) (119), a homodimeric periplasmic protein harboring two disulfide bonds in each subunit. The results of this study suggested that disulfide bond formation can occur co-translationally (119). However, it has also been suggested that post-translational export exceeds co-translational export and this may impact on the oxidative folding rate and pathway, reviewed in (120).

In addition to trapping DsbA–substrate complexes, other methods have proven useful for identifying substrates and their abundance, including: two-dimensional gel analysis combined with mass spectrometry (96), differential thiol trapping (140), and differential two-dimensional liquid chromatographic tandem mass spectrometry/mass spectrometry analysis (209). In a recent review, Collet and Messens (33) describe methods for identifying substrates of thioredoxin that could also be applied to DsbA enzymes. These include mutating to alanine the more C-terminal active site cysteine so that mixed disulfide intermediates are not resolved and Tandem Affinity Purification (TAP) where a TAP-tag is used to purify thioredoxin-substrate complexes on affinity columns, followed by identification of the substrate by mass spectrometry (33).

B. DsbA structures with bound peptides

Several DsbA structures have now been described with peptides or small molecules bound (37, 93, 135, 167, 182, 192). The first DsbA–substrate complex to be described was the complex of EcDsbA with its upstream redox partner EcDsbB. This complex revealed that the periplasmic loop of EcDsbB binds in the hydrophobic groove of EcDsbA. Interactions are formed between the periplasmic loop P2 of EcDsbB and residues Arg148–Gly149–Val150–*cis*Pro151 of the EcDsbA *cis*Pro loop and between EcDsbB residues Pro100-Phe101-Ala102 and residues Thr168, Met171, and Phe174 of the hydrophobic groove of EcDsbA (Fig. 8).

Recently, other structurally characterized DsbA complexes have revealed further information about substrate interactions. The structure of EcDsbA in a complex with a nine-residue peptide derived from the autotransporter protein SigA of *Shigella flexneri* revealed that the peptide binds to the hydrophobic patch of EcDsbA via the formation of backbone hydrogen bonds to a loop immediately preceding the *cis*Pro residue (167) (Figs. 12A and 12B). To stabilize the EcDsbA: peptide complex for crystallization, the native cysteine in the nine-residue peptide was replaced by a homoserine (Hse) residue to give the following sequence: Ac–Pro–Iso–Pro–Phe–Leu–Hse–Glu–Lys–Asp-NH_2. The Hse reacted with Cys30 of EcDsbA. The EcDsbA active site residues Phe29, Cys30,

Pro31, and His32, residues Phe63 and Met64 in the loop connecting the Thioredoxin and helical domains, and residues Arg148, Gly149, and Val150 of the *cis*Pro loop, comprise 95% of the binding surface of the SigA peptide with EcDsbA. This DsbA–peptide complex revealed that the binding site of the SigA-peptide differs from the EcDsbB binding site, suggesting that EcDsbB binding may be more specific than that of downstream EcDsbA substrates.

As indicated above, the plant pathogen *X. fastidiosa* encodes two oxidoreductase-like proteins, XfDsbA and XfDsbA2 (182). The crystal structure of XfDsbA incorporates density for an 8-mer peptide that was modeled into the structure with the sequence Ala–Ala–Ala–Ala–Ala–Gly–Gly–Ala. This peptide was not included in the crystallization condition and its exact sequence is not known. Interaction between XfDsbA and the peptide occurs in the active site region and the helical domain mainly through hydrophobic contacts. Hydrophobic interactions were found with XfDsbA residues Cys38, His40, Phe68, Trp72, Leu150, Gly156, Asp172, and Phe173, and hydrogen bonds were found with Val155, Gly156, and Thr157 of the *cis*Pro loop (182) (Figs. 12C and 12D). An additional interaction was identified between the Cys38 side chain and residue

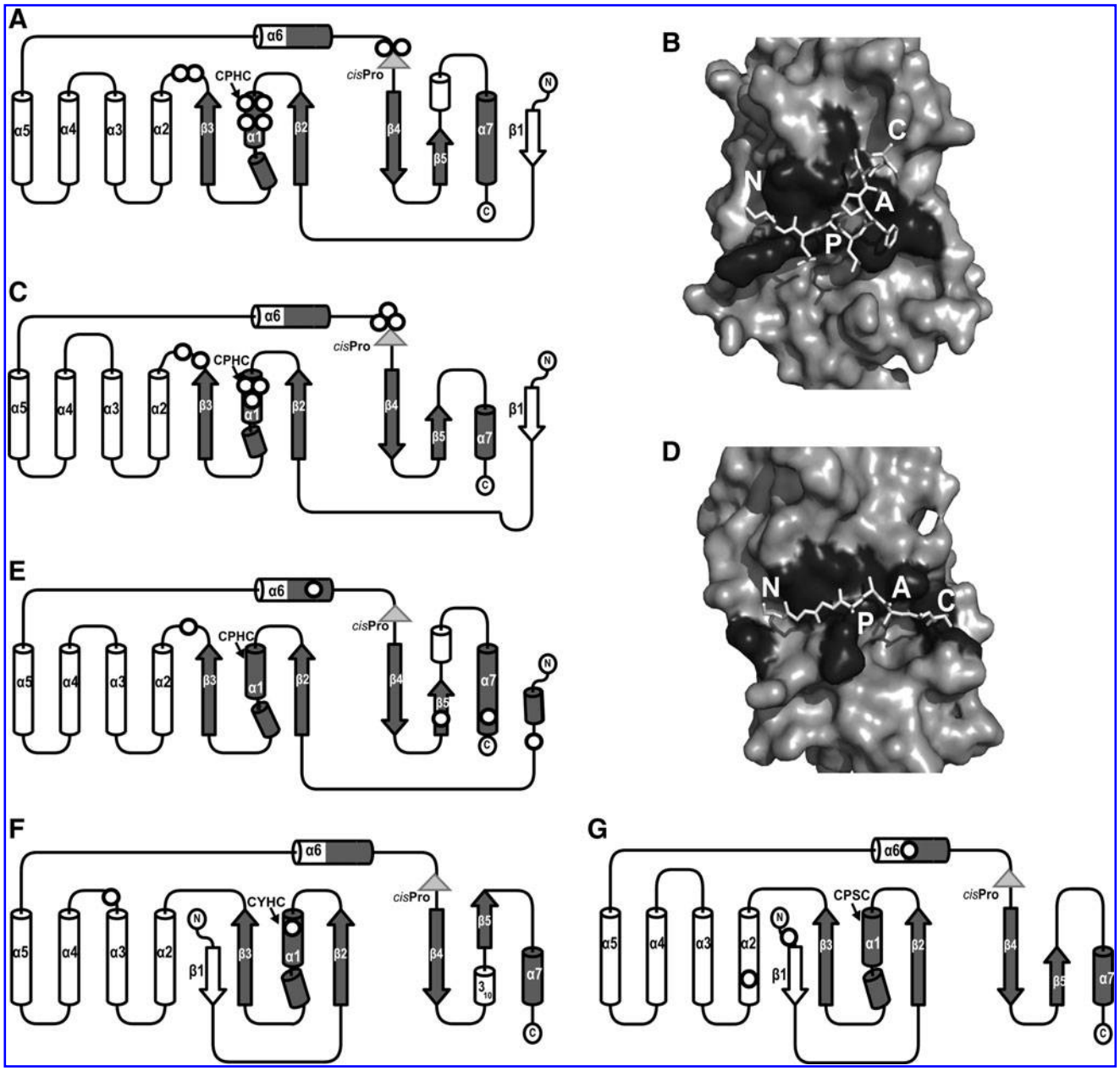

FIG. 12. Ligand binding in DsbA homologues. (A and B) Topology diagram and peptide binding surface, respectively, of EcDsbA. **(C and D)** Topology diagram and peptide binding surface, respectively, of XfDsbA. **(E, F, and G)** Topology diagrams of PaDsbA, WpDsbA1, and BsDsbA (BdbD), respectively. For all topology diagrams, approximate position of residues contributing to ligand binding are shown as *white dots*. For the peptide binding surfaces **(B and D)**, the enzyme is shown in *gray*, with the residues contributing to the interacting surface highlighted in *black* and the peptide shown in *white*. Labels indicate: A, active site cysteine; C, peptide C-terminus; N, peptide N-terminus; P, *cis*Proline.

4 of the peptide. The peptide bound to XfDsbA revealed similar interactions to those described for the P2 loop of EcDsbB interacting with EcDsbA, specifically with regard to anti-parallel interactions with residues of the *cis*Pro loop.

C. DsbA structures with other bound ligands

The closest structural homologue to XfDsbA is PaDsbA with 37% sequence identity (192). Structural investigation of PaDsbA revealed the presence of bound small molecules, specifically four glycerol molecules (Glyc1–Glyc4; glycerol was included in the crystallization condition). Two of the glycerols bind to the active site on one surface of the protein, the other two bind the opposite face of the enzyme (192). Glyc-1 binds close to the active site in two alternate conformations and forms hydrogen bonds to side chains of Lys10 and Arg163 (Fig. 12E). Glyc-2 forms hydrogen bonds with the backbone of Leu66, and to a symmetry-related residue Lys124. Glyc-3 binds in an area rich in acidic and basic residues and forms hydrogen bonds with Asn187. Glyc-4 binds to Gly119 and Asp121.

The structure of WpDsbA1 from *W. pipientis* has PEG molecules bound in two distinct regions (135). The first PEG binding site is near the active site, in a region that often binds a water molecule (135). The second PEG binds close to the second disulfide of this DsbA structure in a region that was described as resembling a peptide-binding groove (135) (Fig. 12F). The recently determined structure of SeSrgA, the DsbA from the Gram-negative bacterium *S. typhimirium,* revealed a PEG molecule bound in the peptide binding groove near the CXXC active site (93).

The *B. subtilis* DsbA homologue BdbD binds Ca^{2+} (37). The Ca^{2+} ion interacts with residues Asp180, Gln49 and Glu115 (Fig. 12G) and is situated $\sim$14 Å from the active site residues. The presence of Ca^{2+} contributes to the catalytic function of BdbD (37).

D. Common features of binding regions in DsbA homologues

DsbA interactions with substrates are relatively weak (36), and even small alterations could lead to divergent substrate specificities. Thus, different DsbA homologues may interact with a very specific range of substrates (90, 99, 135, 192, 211). On the other hand, EcDsbA has been demonstrated to interact with hundreds of substrates, and to have a very wide substrate specificity (49).

One thing that is evident from the DsbA:substrate complexes solved to date is that substrate interactions all involve regions directly surrounding the CXXC catalytic motif (90, 135, 182, 192, 212). Thus, the active site motif not only helps control the redox potential of the enzyme, it also contributes to interactions with redox partners (175). The *cis*Pro loop near the active site is also important for substrate binding (12), including interaction with EcDsbB (108). The hydrogen bonding pattern between bound peptide and *cis*Pro loop is similar for both EcDsbA and XfDsbA (Figs. 12A–12D). Together the CXXC and *cis*Pro loop form the central binding site for substrates, with the nearby groove (if present) and the surface of the helical domain sometimes providing additional interaction surfaces. Additionally, peptides bound to EcDsbA and XfDsbA form interactions with residues in the loop connecting β_3 of the Thioredoxin domain and α_2 of the helical domain (167, 182) and this region has also been found to contribute to binding of small molecules to PaDsbA, WpDsbA1, and BdbD (37, 135, 192).

VI. Virulence-Associated Substrates of DsbA

The DsbA–DsbB oxidative protein folding system plays an important role in bacterial virulence. DsbA catalyzes the correct folding of many bacterial virulence factors, including proteins associated with adhesion (*e.g.,* fimbrial proteins), host cell manipulation (*e.g.,* toxins) and cellular spread (*e.g.,* flagella) (92). In general, mutants defective in the DsbA–DsbB oxidative pathway are either directly or indirectly altered in a range of pathogenicity-associated phenotypes and display reduced fitness or attenuated virulence in animal infection models (12, 14, 26, 27, 35, 139, 143, 154, 169, 204, 227). Here we summarize the role DsbA plays in the correct folding and function of a selection of bacterial virulence factors.

A. Chaperone-usher fimbriae

Fimbriae (also referred to as pili) are long polymeric hair-like organelle structures that typically extend $\sim$1 μm from the bacterial surface. Fimbrial adhesins normally recognize specific molecular motifs in a lock-and-key fashion; they therefore enable the bacterium to be targeted to a specific surface and thus determine tissue tropism. Gram-negative bacteria produce many different classes of fimbriae, with one of the best studied being fimbriae that are assembled by the chaperone–usher-dependent pathway (164, 178, 184, 195, 215). In this pathway, the fimbrial subunits are secreted into the periplasm via the general secretory system. From there, two specific accessory proteins are required for biogenesis: a periplasmic located chaperone and an outer membrane located usher. The chaperone protein binds to individual subunits, protects them from polymerization and targets them to the usher. In turn, the usher protein acts as an assembly platform for organelle biogenesis and coordinates the polymerization of structural components of the fimbriae.

One of the best characterized fimbriae synthesized via the chaperone–usher pathway is P fimbriae from UPEC. P fimbriae are comprised of six subunit proteins arranged into two distinct components: a rod structure comprising more than 1000 copies of the PapA major subunit protein and a tip fibrillum comprising the PapG adhesin as well as other adaptor proteins (101, 102). In UPEC, EcDsbA is required for the PapD chaperone to adopt its native conformation and bind to P fimbrial subunits in the periplasm (Fig. 13); deletion of EcDsbA results in an inability to assemble functional P fimbriae (112). PapD has an immunoglobulin-like structure and contains a single intramolecular disulfide bond that is formed between two cysteine residues located near the carboxyl terminus. In the absence of PapD, the unbound fimbrial subunit proteins do not fold properly and form aggregates that are targeted for degradation by the DegP protease in the periplasm (13). The P fimbrial adhesin PapG, which recognizes carbohydrate moieties on the surface of host cells in the urinary tract (129), also requires EcDsbA for the formation of a disulfide bond in its tertiary structure (112).

PapD is representative of a chaperone subfamily that is responsible for the assembly of rod-like fibers of varying width and helical symmetry (103). The cysteine residues that form the disulfide bond in PapD are also conserved in two other chaperones from this subfamily, both of which are

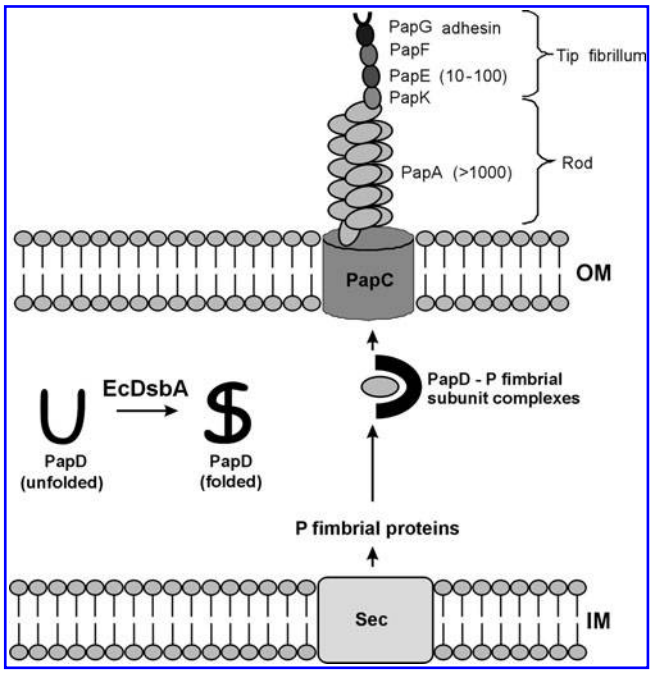

FIG. 13. The role of EcDsbA in the biosynthesis of P fimbriae. The adhesion of UPEC to epithelial cells of the urinary tract is mediated by P fimbriae. EcDsbA catalyzes the formation of a disulfide bond in the PapD chaperone, which is essential for P fimbrial biogenesis. P fimbrial proteins are transported across the inner membrane (IM) via the general secretion pathway (depicted as Sec). The PapC usher protein inserts into the outer membrane (OM) and acts as a scaffold for the synthesis of the fimbrial organelle, which is made up of a rod structure comprising more than 1000 copies of the PapA major subunit protein and a tip fibrillum comprising adaptor proteins (PapK, PapE, and PapF) as well as the tip-located PapG adhesin. The system is depicted in a simplified format to highlight the specific role of EcDsbA and is not drawn to scale.

found in the uropathogen *Proteus mirabilis*. This includes the MrpD chaperone (which coordinates the production of Mannose-resistant *Proteus*-like [MR/P] fimbriae) and the PmfD chaperone (which coordinates the production of *P. mirabilis* fimbriae [PMF]) (103). In *P. mirabilis*, a *dsbA* deficient strain produced by signature-tagged mutagenesis lacked expression of MR/P fimbriae (27), suggesting PmDsbA may be required for the folding of the MrpD chaperone in a manner analogous to that observed for PapD. A second chaperone subfamily has also been described (103). Members of this chaperone subfamily are associated with the production of short adhesins or very thin fibers that coil up as an amorphous mass on the cell surface (*e..,g* CS3 pili, aggregative adherence fimbria I) (103). These chaperones contain two conserved cysteine residues separated by approximately 30 residues in the amino terminal region, and are predicted to form a disulfide bond. Although not yet demonstrated experimentally, it is likely that DsbA proteins are responsible for the correct folding of these chaperones.

B. Type 4 fimbriae

DsbA is associated with the production of fimbriae belonging to the type 4 class. Type 4 fimbriae are long, thin, flexible filaments that mediate bacterial adhesion to target tissues and a type of movement referred to as twitching motility (170). In the case of enteropathogenic *E. coli* (EPEC), EcDsbA is required for the formation of a single intramolecular disulfide bond in the BfpA major subunit of Bundle forming pili (Bfp) (214, 230). Similarly, *N. meningitidis*, the causative agent of cerebrospinal meningitis, utilizes at least one of two membrane-bound NmDsbA paralogues for the biogenesis of functional type 4 pili (203). In *V. cholerae*, the toxin co-regulated pilus (Tcp) is an essential colonization factor and VcDsbA (TcpG) is required for the assembly of functional pili (169). Type 4 pili-mediated twitching motility in *P. aeruginosa* requires PaDsbA as this phenotype is abolished in a *P. aeruginosa dsbA* mutant (14).

C. Intimin, an outer membrane adhesin

Intimins are large (~95 kDa) outer membrane adhesins produced by some pathotypes of *E. coli* that can cause disease in both humans and animals through the formation of attaching and effacing (A/E) intestinal lesions (63). Intimin mediates the intimate attachment of bacteria to the host cell membrane, which is a prerequisite for A/E lesion formation and is thus a key virulence factor of these enteric pathogens (68). Intimins contain two distinct regions that define their structural and functional properties. The highly conserved N region is responsible for anchoring the protein in the outer membrane. The C region is less well conserved and forms the surface-exposed receptor binding part of the protein. In EPEC, it has been demonstrated that EcDsbA catalyzes the formation of a disulfide bond in the D3 lectin-like domain of the C region (22). This suggests that this domain is at least partially folded in the periplasm prior to its translocation across the outer membrane and provides further evidence to support the role of EcDsbA in the correct folding and function of important bacterial virulence factors.

D. Flagella

Flagella are complex cell surface organelles that mediate bacterial motility. The mutation of either *dsbA* or *dsbB* results in a nonmotile phenotype due to the inability to produce functional flagella in many different bacteria including *E. coli* (26, 41, 92), *S. enterica* (1, 77, 206), *P. mirabilis* (27), *Erwinia carotovora* subsp. *atroseptica* (35), *Burkholderia cepacia* (86), and *Campylobacter jejuni* (169). The role of the Dsb system in flagellar biosynthesis has been best characterized in *E. coli*, where EcDsbA catalyzes the formation of an intramolecular disulfide bond between two cysteine residues in the FlgI flagellar P-ring motor protein (41; Fig. 14). The major structural component in the flagella organelle, FliC, is not integrated into the basal apparatus in the absence of correctly folded FlgI (95).

E. Toxins, secreted enzymes, and antimicrobial peptides

Some pathogenic bacteria secrete toxins and enzymes such as proteases to manipulate, damage, and even kill host cells. In some cases, these secreted virulence factors require Dsb enzymes for their correct folding and function, demonstrating a role for the Dsb system in host–pathogen interactions. One well-characterized example is illustrated by the role of DsbA in the correct folding and functional assembly of AB_5 toxins

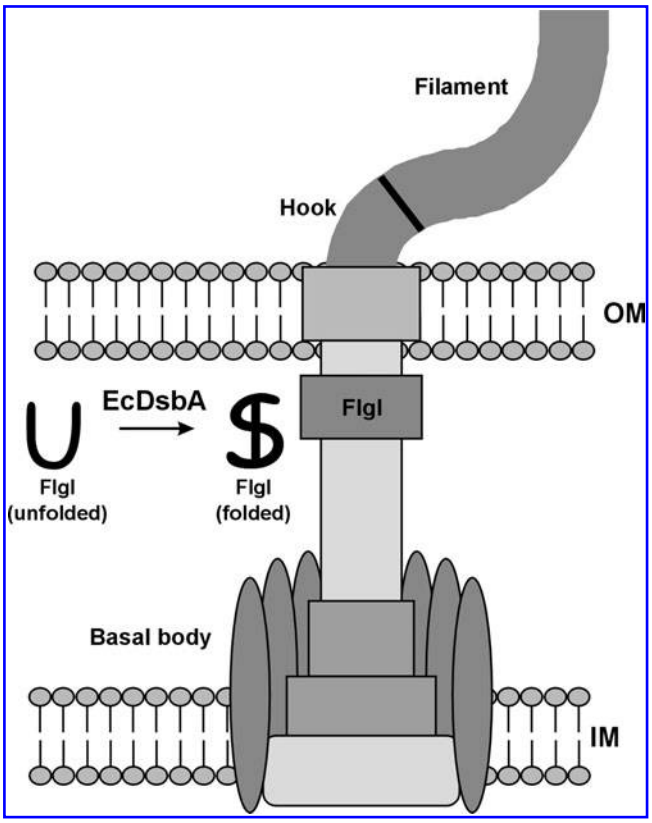

FIG. 14. The role of EcDsbA in the biosynthesis of flagella. EcDsbA catalyzes the formation of a disulfide bond essential for the correct folding of the FlgI protein of bacterial flagella. Indicated are the inner membrane (IM) located basal body component, the FlgI P-ring motor protein, and the external hook and filament components. The system is depicted in a simplified format to highlight the specific role of EcDsbA and is not drawn to scale. OM, outer membrane.

such as cholera toxin from the enteric pathogen *V. cholerae* (169), pertussis toxin from the respiratory pathogen *Bordetella pertussis* (196), and heat-labile enterotoxin from enterotoxigenic *E. coli* (222, 228). Pertussis toxin represents arguably the most complex structure and is assembled from six subunits containing 11 intramolecular disulfide bonds (42). In *V. cholerae*, mutation of *dsbA* affects the secretion of cholera toxin A subunit and other proteases (169). Protease production, and multi-drug resistance, in the opportunistic lung pathogen *B. cepacia* also requires the BcDsbA-DsbB system (86).

Many Gram-negative pathogens use the type II secretion pathway to secrete enzymes that contribute to their ability to cause disease. Some of these enzymes represent specific substrates for DsbA. In *P. aeruginosa*, PaDsbA catalyzes the formation of two intramolecular disulfide bonds in the secreted metalloprotease elastase. This imparts stability and enhanced activity to elastase, an important proteolytic enzyme that contributes to the virulence of *P. aeruginosa* during lung infection in cystic fibrosis patients (25). Lipase, another enzyme associated with virulence and secreted by *P. aeruginosa* also requires PaDsbA-catalyzed disulfide bonds for its stability and secretion (208). In some pathogens, DsbA actually mediates an essential step in the structural configuration of the type II secretion apparatus (Fig. 15). For example, secretion of the starch-debranching enzyme pullulanase by *Klebsiella oxytoca*, requires KoDsbA for the correct folding and stabilization of the PulS and PulK type II secretion components (173, 185).

The soft rot erwinias are enterobacterial pathogens that cause economically significant disease in plants. *Erwinia* species phytopathogens secrete multiple virulence factors via the type II secretion system (also referred to as the Out system) that lead to the degradation of plant tissue. Some of these secreted enzymes, such as cellulase, endopolygalacturonase, and multiple pectate lyases, represent specific substrates of DsbA (190, 210). In the potato pathogen *E. carotovora* subsp. *atrosceptica*, DsbA (ErcDsbA) is required for the proper production of almost all secreted virulence factors (35). Furthermore, mutation of the *dsbA* gene compromised several virulence-related phenotypes, including motility, quorum sensing, and degradation of plant tissue. Transcriptome analysis of an *E. carotovora* subsp. *atrosceptica dsbA* mutant revealed that ErcDsbA inactivation alters the expression of multiple genes; the authors postulated this may occur via the

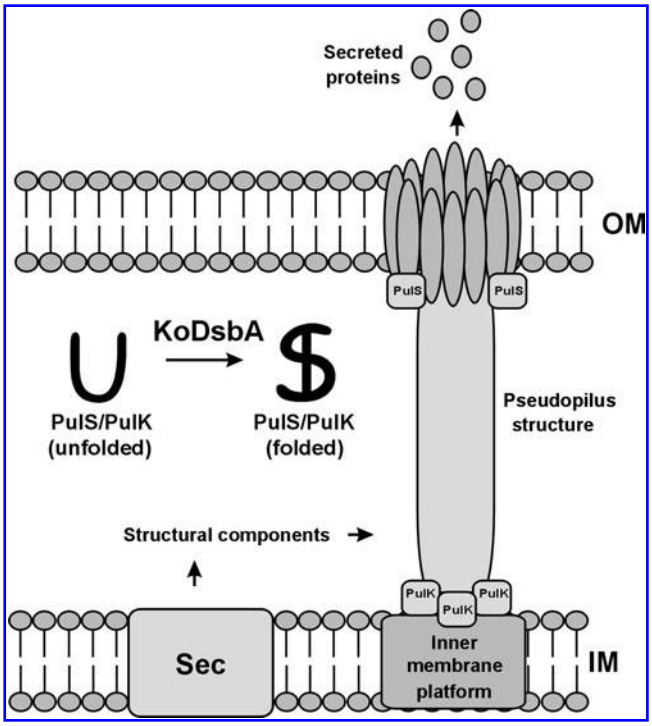

FIG. 15. The role of KoDsbA in type II secretion. The type II secretion system of *K. oxytoca* is depicted schematically with the three major components indicated: the inner membrane (IM) platform, the pseudopilus structure that spans both membranes, and the outer membrane (OM) secretin complex (174). KoDsbA catalyzes the formation of a disulfide bond in the PulS lipoprotein (which is required for the correct insertion of the secretin into the OM) as well as the PulK protein (which is thought to be located at the base of the pseudopilus structure). In type II secretion, proteins are initially translocated across the IM by the general secretory pathway (depicted as Sec) and then across the OM by the type II secretion system. The system is depicted in a simplified format to highlight the specific role of KoDsbA and is not drawn to scale.

pleiotropic effects of a regulator(s) responsive to periplasmic redox status and/or the presence of misfolded proteins (35). Taken together, ErcDsbA therefore exerts its effect on virulence factor production in *E. carotovora* subsp. *atrosceptica* at multiple levels, including protein folding and stability, transcript abundance, and protein secretion.

The Gram-positive organisms *B. subtilis* and *Streptococcus thermophilus* require DsbA to enhance their survival in some environments (48, 59). These bacteria use the DsbA homologues BdbB (from *B. subtilis*) or $BlpG_{ST}$ (from *S. thermophilus*) for the production of functional antimicrobial peptides (lantibiotic sublancin 168 and bacteriocin thermophilin 9, respectively). These antimicrobial peptides enhance the fitness of the producer organism by eliminating competition for nutrients. DsbA-dependent bactericidal activity has also been demonstrated for some strains of *P. aeruginosa* (166).

F. Type III secretion

Some bacterial pathogens employ a Type III secretion system (T3SS) to inject effectors (bacterial virulence proteins that modulate eukaryotic cell pathways) into host cells (34, 67). The T3SS apparatus is comprised of more than 20 proteins and consists of a basal body anchored in the bacterial membrane linked to a needle that extends from the bacterial surface. One key component of the T3SS apparatus is the secretin, an integral outer membrane protein that forms a stable oligomeric structure consisting of 12 to 14 subunits with a ring-like appearance (20). DsbA is required for the correct folding of the T3SS secretin complex in *Yersinia pestis* (111), enteropathogenic *E. coli* (158), *S. flexneri* (218), and *S. typhimurium* (157; Fig. 16).

Yersinia pestis, the causative agent of plague, possesses a plasmid-encoded T3SS that injects a set of effector proteins known as Yops (*Yersinia* outer proteins) into the cytosol of host cells. *Y. pestis* DsbA (YpDsbA) catalyzes the formation of two intramolecular disulfide bonds in the YscC secretin. *Y. pestis dsbA* mutants are defective in Yop effector protein translocation due to the incorrect folding of YscC (111). Similarly, DsbA also catalyzes the formation of an intramolecular disulfide bond in the SpiA outer membrane secretin of the intracellular pathogen *S. typhimurium*. *S. typhimurium dsbA* mutants are affected in effector protein secretion and display attenuated virulence in a mouse infection model (157). *P. aeruginosa dsbA* mutants are also affected in type III secretion (14, 40), however the precise molecular mechanism by which this occurs is unknown.

The intracellular pathogen *S. flexneri* requires DsbA (SfDsbA) for oxidative folding of the T3SS Spa32 secretin (155). *S. flexneri* employs a T3SS to secrete Ipa proteins that trigger a series of signaling pathways that lead to invasion as well as intra- and inter-cellular spread (8). Mutations in *dsbA* result in defective Ipa secretion and thus affect these processes (8, 218).

G. Role of DsbA paralogues in bacterial pathogenesis

In recent years, it has become apparent that there is significant diversity among bacterial Dsb systems (49). Some bacterial species encode multiple DsbA paralogues, often with different substrate specificity. For example, the UPEC strain CFT073 contains two Dsb systems, the prototypic DsbA–DsbB system, and a second DsbL–DsbI system (73).

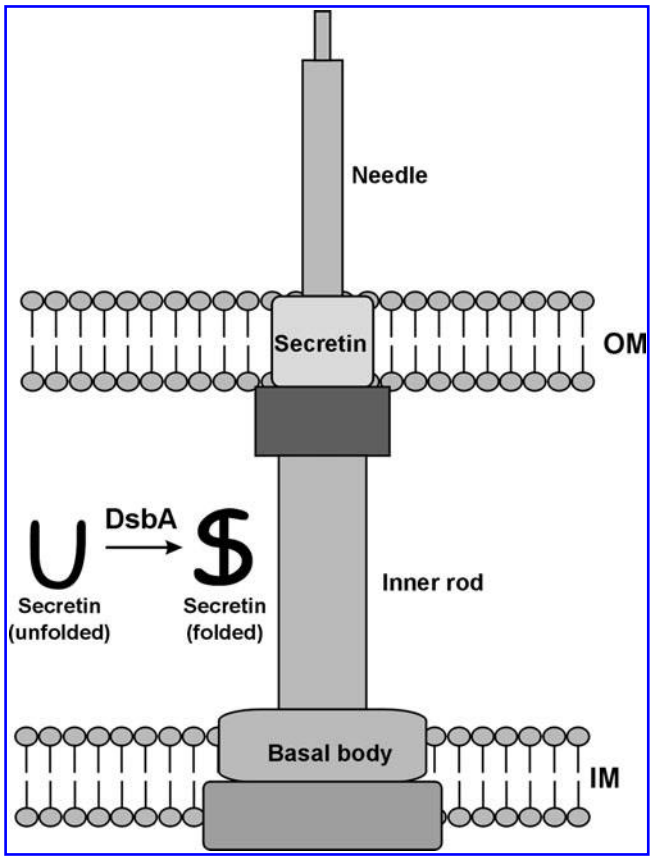

FIG. 16. The role of DsbA in type III secretion. Some bacteria are able to manipulate host cells using effectors secreted by the type III secretion system. DsbA catalyzes the formation of a disulfide bond essential for the correct folding of the outer membrane secretin. Indicated are the inner membrane (IM) located basal body component, the periplasmic spanning inner rod, the outer membrane (OM) located secretin, and the protruding needle component. The system is depicted in a simplified format to highlight the specific role of DsbA and is not drawn to scale.

DsbL is an oxidoreductase that catalyzes the formation of a disulfide bond in the periplasmic located enzyme arylsulfate sulfotransferase (73). DsbL can also catalyze disulfide bond formation in several well-defined DsbA targets when provided *in trans* on a multicopy plasmid (92). However, the role of the DsbL–DsbI system in virulence remains to be determined since a UPEC CFT073 *dsbLI* mutant was not affected in bladder colonization using a mouse urinary tract infection model (92).

Neisseria meningitidis produces three DsbA oxidoreductases, two inner membrane-associated lipoproteins (NmDsbA1 and NmDsbA2) and one periplasmic enzyme (NmDsbA3). NmDsbA1 and NmDsbA2 are required for the formation of functional type IV pili, which mediate bacterial adhesion to human endothelial cells (203). NmDsbA1 and NmDsbA2 also mediate the correct folding of PilQ, a protein involved in DNA uptake. NmDsbA3, on the other hand, is not involved in either of these functions and its specific target remains to be defined. *S. typhimurium* also encodes three DsbA oxidoreductases, namely SeDsbA, SeDsbL, and SrgA. The three proteins share low sequence identity, have different redox properties, and

display some degree of redundancy with regards to their target specificity (77, 143).

VII. Conclusions and Perspectives

Analysis of current literature on DsbA proteins indicates that the prototypical organism *E. coli* seems to be an incomplete model for oxidative protein folding, though it does represent one data point on a spectrum of bacterial oxidative folding systems. For example, the combined oxidative and isomerase machineries of *E. coli* are not standard in bacteria. On the other hand, the DsbA–DsbB system is distributed across many bacteria, and probably does represent a reasonably standard system for oxidative folding in Gram-negative bacteria. Having said that, there is considerable variability in DsbA–DsbB machineries; the Gram-negative γ-Proteobacteria appear to take a somewhat modular approach by sometimes encoding multiple DsbA homologues or on occasion encoding an entire additional paralagous DsbL–DsbI system with a specialized oxidative folding role.

Similarly, although the structure determination of EcDsbA represented an important milestone in the field, in many ways the structural features of EcDsbA may be the exception rather than the rule. DsbA structures all incorporate the Thioredoxin fold with CXXC active site, *cis*Pro loop, and an inserted helical domain. However, the highly kinked active site α-helix and the extended hydrophobic groove of EcDsbA are not typical. All other structurally characterized DsbAs have a reduced angle in the active site helix and a truncated groove, if it is present at all. The surface hydrophobic features of EcDsbA are also modified considerably in other structurally characterized DsbAs, with the surface surrounding the reactive cysteine often being charged rather than hydrophobic. Furthermore, DsbA structures have been reported that incorporate metal binding sites and additional disulfides. It will be interesting to see whether the structures of DsbB proteins from other organisms vary as much as those of the DsbAs, and to investigate variability in the molecular interaction between DsbB and DsbA proteins.

Bacterial oxidative protein folding has moved center-stage recently, with the now overwhelming evidence that the DsbA–DsbB system plays a key role in virulence in many pathogenic Gram-negative organisms. The increase in antibiotic resistance and the dearth of novel antibiotics in the pharmaceutical development pipeline has led to calls to identify and validate new drug targets (24, 168). DsbA and DsbB could represent exciting new targets because blocking their activity will interfere with the assembly of numerous virulence factors. However, it is not yet clear whether blocking virulence is a valid drug development strategy (176). In the case of DsbA and DsbB, experiments aimed at inhibiting these proteins *after infection is established* would need to be performed to demonstrate the validity of this approach for therapeutic development.

Substrate identification has been critical in understanding the role of DsbA in bacterial pathogenicity. The finding that *cis*Pro substitution can lead to accumulation of EcDsbA-substrate complexes is tantalizing: can the same strategy be used to identify substrates of divergent DsbA homologues? If so, this approach might lead to a much better understanding of how the variation in surface charge, groove shape, and size in DsbAs relates to substrate specificity. Nevertheless, the recent structural data showing the binding of peptides and other ligands to DsbAs have identified the *cis*Pro loop, the groove adjacent to the active site and the helical domain surface near the active site as hot spots for substrate interaction. We eagerly await new data on DsbA-substrate interactions.

A final word on nomenclature: We have indicated above that EcDsbA may not represent a complete model for investigating general features of DsbA structure and function. We therefore suggest that in publications referring to *Escherichia coli* DsbA the terminology EcDsbA be used rather than the more general term DsbA. We have provided a possible naming scheme for DsbAs, though we recognize that this may need further development as more bacterial genomes are sequenced.

Acknowledgments

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Abbreviations Used

A/E lesions = attaching and effacing lesions
ATP = adenosine triphosphate
Bdbd = alternative name for BsDsbA
BcDsbA = *Burkholderia cepacia* DsbA
Bfp = bundle forming pili from *E. coli*
BpDsbA = *Bordetella parapertussis* DsbA
BsDsbA = *Bacillus subtilis* DsbA
Dsb = disulfide bond forming protein
EcDsbA = *E. coli* DsbA
EcDsbB = *E. coli* DsbB
EcDsbC = *E. coli* DsbC
EcDsbD = *E. coli* EcDsbD
EcDsbG = *E. coli* DsbG
EcDsbI = *E. coli* DsbL, a paralogue of *E. coli* DsbB
EcDsbL = *E. coli* DsbL, a paralogue of *E. coli* DsbA
EPEC = enteropathogenic *E. coli*
ER = endoplasmic reticulum
ErcDsbA = *Erwinia carotovera* subsp. *atrosceptica* DsbA
Ero1p = yeast protein "endoplasmic reticulum oxidoreductin"
Erv2p = yeast protein "essential for respiration and vegetative growth"
FAD = flavin adenine dinucleotide
Glyc = glycerol
GST = glutathione *S*-transferase
IMS = inter membrane space

Abbreviations Used (cont.)

KoDsbA = *Klebsiella oxytoca* DsbA
MIA = mitochondrial machinery for import and assembly
MK = menaquinone
NeDsbA = *Nitrosomonas europaea* DsbA
NmDsbA1 = NmDsbA2 and NmDsbA3, *Neisseria meningitidis* DsbA1, DsbA2 and DsbA3
P1 = first periplasmic loop of EcDsbB
P2 = second periplasmic loop in EcDsbB
PaDsbA = *Pseudomonas aeruginosa* DsbA
PDB = protein data bank
PDI = protein disulfide isomerase
PEG = polyethylene glycol
PhoA = alkaline phosphatase
PmDsbA = *Proteus mirabilis* DsbA
SaDsbA = *Staphylococcus aureus* DsbA
ScDsbA = *Streptomyces coelicolor* DsbA
SCOP = structural classification of proteins
SeDsbA = *Salmonella enterica* serovar Typhimurium DsbA
SeDsbL = *Salmonella enterica* serovar Typhimurium DsbL
SeMet = selenomethionine
SeSrgA = *Salmonella enterica* serovar Typhimurium virulence plasmid-encoded DsbA
SfDsbA = *Shigella flexneri* DsbA
T3SS = type III secretion system
Tcp = toxin co-regulated pilus from *V. cholerae*
TcpG = alternative name for VcDsbA
TIC = translocon at the inner envelope of chloroplasts
TOC = translocon at the outer envelope of chloroplasts
TrxP = *Bacteroides fragilis* periplasmic thioredoxin
UPEC = uropathogenic *E. coli*; UQ, ubiquinone
VcDsbA = *Vibrio cholerae* DsbA
VfDsbA = *Vibrio fischeri* DsbA
VKOR = vitamin K epoxide reductase
WpDsbA1 and WpDsbA2 = *Wolbachia pipientis* DsbA1 and DsbA2
XfDsbA = *Xylella fastidiosa* DsbA
YpDsbA = *Yersinia pestis* DsbA

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