

## Forum Review

# Conservation and Diversity of the Cellular Disulfide Bond Formation Pathways

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### ABSTRACT

Two pathways for the formation of biosynthetic protein disulfide bonds have been characterized in the endoplasmic reticulum (ER) of eukaryotes. In the major pathway, the membrane-associated flavoprotein Ero1 generates disulfide bonds for transfer to protein disulfide isomerase (PDI), which is responsible for directly introducing disulfide bonds into secretory proteins. In a minor fungal-specific protein oxidation pathway, the membrane-associated flavoprotein Erv2 can catalyze disulfide bond formation via the transfer of oxidizing equivalents to PDI. Genomic sequencing has revealed an abundance of enzymes sharing homology with Ero1, Erv2, or PDI. Herein the authors discuss the functional, mechanistic, and potential structural similarities between these homologs and the core enzymes of the characterized ER oxidation pathways. In addition they speculate about the possible differences between these enzymes that may explain why the cell contains multiple proteins dedicated to a single process. Finally, the eukaryotic ER protein oxidation and reduction pathways are compared to the corresponding prokaryotic periplasmic pathways, to highlight the functional, mechanistic, and structural similarities that exist between the pathways in these two kingdoms despite very low primary sequence homology between the protein and small molecule components. *Antioxid. Redox Signal.* 8, 797–811.

### INTRODUCTION

**F**ORMATION OF DISULFIDE BONDS is an essential step in the folding and assembly of the extracellular domains of many membrane and secreted proteins. Combined genetic, biochemical, and structural studies over the last 10 years have contributed significantly to our understanding of how disulfide bonds are formed in cellular proteins. Core pathways for biosynthetic protein disulfide bond formation have been characterized in the lumen of the endoplasmic reticulum (ER) of eukaryotes and the periplasmic space of prokaryotes. These include disulfide bond formation pathways that oxidize cysteine pairs to form protein disulfide bonds, as well as disulfide bond reduction pathways that allow for the reshuffling of mispaired cysteine residues.

Disulfide bond formation pathways in eukaryotes and prokaryotes share a common design that includes the transfer of thiols between two protein groups: membrane-associated

oxidoreductases, which generate disulfide bonds using the oxidizing potential from small molecule redox reactions, and soluble oxidoreductases that carry oxidizing equivalents from the disulfide generating enzymes to substrate proteins. The soluble disulfide carrier proteins, protein disulfide isomerase (PDI) in eukaryotes and DsbA and DsbC in prokaryotes, are members of a structural superfamily based on a conserved thioredoxin fold (56, 57, 74, 78). The disulfide generating enzymes, the membrane-associated oxidoreductases Ero1 and Erv2 in eukaryotes and DsbB in prokaryotes, do not share obvious sequence similarity, but abundant structural, genetic and biochemical data suggest that these three proteins may contain structurally conserved catalytic domains (36, 37, 45).

A wealth of public sequence data, searched with algorithms that recognize potential structural motifs as well as primary sequence homology, have resulted in the identification of multiple orthologs and paralogs of both the disulfide generating and disulfide carrier enzymes from the core disulfide

bond formation pathways. Currently, two Ero1 paralogs (13, 86), three Erv2-like proteins (47, 71, 119), and seventeen PDI homologs (25, 118) have been confirmed in human cells, while one Ero1 protein (29, 88), two Erv2-like proteins (33, 68, 97, 102), and five PDI homologs (94, 104–106, 116) have been identified in yeast. Orthologs and/or paralogs have also been identified for the prokaryotic enzymes DsbB (90, 98), DsbA (100, 110), and DsbC (44, 83, 108). Almost half of these eukaryotic and prokaryotic homologs have been identified in the past 5 years, including ten of the seventeen human PDI homologs.

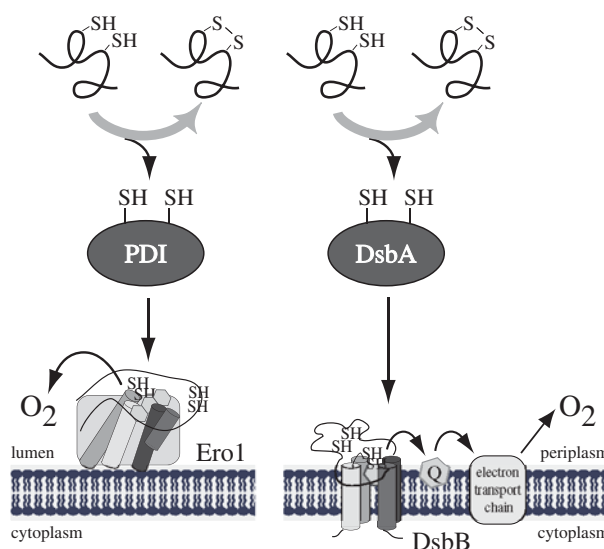
This review focuses on the abundance of eukaryotic and prokaryotic enzymes that have the potential to play a role in disulfide bond formation. We will discuss the functional and potential structural similarities between the recently identified enzymes and the characterized core members of the protein oxidation pathway, as well as the differences between all these proteins that may explain why the cell contains so many enzymes potentially dedicated to a single process. We will also discuss the recent identification of a protein reduction pathway in eukaryotes that uses the small molecule component glutathione and compare it with the protein-based reduction pathway of prokaryotes.

## PROTEIN OXIDATION PATHWAYS IN THE ER AND PERIPLASM

The major disulfide bond formation pathway in eukaryotes is composed of the membrane-associated flavoprotein Ero1 and the soluble thioredoxin-like protein PDI (Fig. 1). Ero1 uses the oxidizing power of molecular oxygen, coupled with its flavin cofactor, to generate disulfide bonds within itself, which are transferred to PDI (11, 30, 81, 111, 112). In turn, PDI transfers its disulfide bonds directly to substrate proteins (30, 81). The transmission of oxidizing equivalents between Ero1, PDI, and substrate proteins, occurs through a series of direct thiol–disulfide exchange reactions between these proteins (30) (Fig. 2).

A second fungal-specific oxidation pathway has been identified that utilizes the membrane-associated flavoprotein Erv2 to catalyze disulfide bond formation (33, 97). *ERV2* was identified as a gene that, when overexpressed, could restore viability to *S. cerevisiae* when *ERO1* function was compromised (97). Like Ero1, Erv2 can reoxidize reduced PDI, while reduced Erv2 is reoxidized by molecular oxygen via its flavin cofactor (97). Unlike yeast *ERO1*, *ERV2* is not an essential gene and deletion of the Erv2 coding sequence has no detectable effect on protein oxidation, suggesting the Erv2 driven pathway is normally either a minor pathway for protein disulfide bond formation or may be a specialized pathway for an as yet unidentified group of nonessential proteins (33, 97, 112).

The core prokaryotic protein oxidation pathway utilizes the cytoplasmic inner-membrane protein DsbB and the soluble thioredoxin-like protein DsbA to drive the formation of disulfide bonds in substrate proteins in the periplasm (reviewed in Refs. 54, 83, 108) (Fig. 1). DsbA, like its homolog PDI, transfers a disulfide bond formed between its active site cysteines



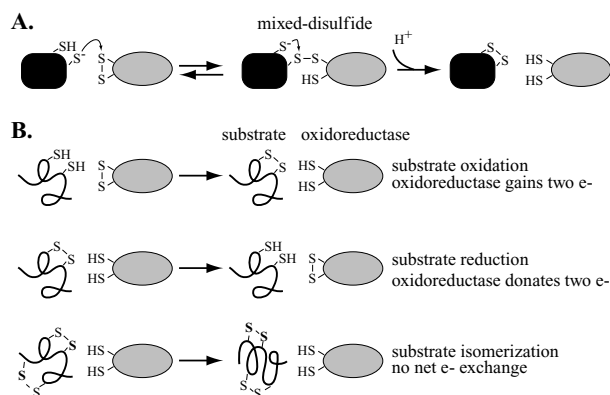
**FIG. 1. Pathways for protein disulfide bond formation.** Substrate proteins in the ER lumen of eukaryotes are oxidized through thiol–disulfide exchange with protein disulfide isomerase (PDI). PDI is reoxidized by the membrane-associated oxidase Ero1, which obtains oxidizing equivalents from molecular oxygen via its flavin cofactor. The catalytic core of Ero1 consists of two redox active cysteine pairs and a bundle of four helices, depicted as cylinders, surrounding a flavin cofactor (see Fig. 3 for details). Oxidizing equivalents are introduced into substrate proteins in the periplasm of prokaryotes through a thiol–disulfide exchange reaction with DsbA. DsbA is reoxidized by the cytoplasmic membrane protein DsbB, which transfers electrons to a quinone cofactor (Q). Electrons are transferred from quinone to oxygen via the electron transport chain. *Thin arrows* depict the flow of electrons.

directly to substrate proteins. DsbB, like its functional homologs Ero1 and Erv2, generates disulfide bonds within itself, which are transferred to DsbA (38, 60, 61). Instead of using a flavin cofactor to mediate the reoxidation of its Cys-x-Cys pair by oxygen, DsbB uses a membrane bound quinone cofactor as an electron acceptor (5, 7). The quinone molecule (ubiquinone in aerobic conditions or menaquinone in anaerobic conditions) serves as a mobile electron carrier, shuttling electrons from DsbB to the terminal oxidases of the electron transport chain, which ultimately transfer electrons to molecular oxygen or other anaerobic electron acceptors (5, 7, 63, 64).

## DISULFIDE GENERATING ENZYMES: SIMILARITIES AND DIFFERENCES AMONG THE PROTEIN SOURCES OF OXIDIZING POWER FOR DISULFIDE BOND FORMATION

### *Inter-cysteine disulfide transfer mechanism*

The eukaryotic and prokaryotic disulfide generating enzymes (Ero1, Erv2, and DsbB) each contain two cysteine pairs that are essential for their oxidative activity. One cys-



**FIG. 2. Thiol–disulfide exchange reactions.** (A) In a thiol–disulfide exchange reaction, a thiolate anion, formed by the deprotonation of a free thiol, attacks a disulfide bond in an oxidized species, leading to the formation of a transient mixed-disulfide bond between the two species. The mixed-disulfide intermediate is resolved by intramolecular attack of the mixed-disulfide bond by a thiolate anion derived from the remaining thiol, resulting in the oxidation of the originally reduced protein and the concomitant reduction of the initially oxidized species. (B) Cellular enzymes called oxidoreductases can catalyze the oxidation, reduction, or isomerization of protein disulfide bonds depending on the redox state of their active site cysteine pair. If the active site pair is oxidized (disulfide form), an oxidoreductase can catalyze the formation of disulfide bonds. If the active site pair is in a reduced (dithiol form) the enzyme can catalyze the reduction or isomerization of mispaired thiol residues, functioning as a reductase or isomerase. Two mechanisms for catalyzed disulfide isomerization have been proposed: an intramolecular mechanism, where disulfide isomerization occurs through an intramolecular thiol–disulfide exchange between the substrate and oxidoreductase, and a reduction/oxidation pathway, which involves repeated cycles of substrate reduction by the oxidoreductase followed with reoxidation by the oxidoreductase (reviewed in Ref. 118).

teine pair is found in a conserved Cys-x-x-Cys motif (where x is any amino acid), which is a hallmark of redox active proteins, while the second pair of cysteines exhibits more variation in spacing (one, four, and twenty-five residues separate the second cysteine pairs in Erv2, Ero1, and DsbB, respectively). A common catalytic mechanism has been proposed for all three enzymes that involves two coupled thiol–disulfide exchange reactions to oxidize the disulfide carrier partner protein PDI or DsbA. The transfer of a disulfide bond from the variably spaced cysteine pair to the reduced thioredoxin-like disulfide carrier protein, and the exchange of a disulfide bond between the Cys-x-x-Cys pair and the reduced variable cysteine pair (12, 31, 37, 99). Transfer of electrons from the Cys-x-x-Cys pair to a small molecule electron acceptor, FAD (for Ero1 and Erv2) or quinone (for DsbB), completes the cycle, returning the Cys-x-x-Cys pair to its active oxidized form (36, 37, 97, 112).

#### Four-helix catalytic core

Despite their functional and mechanistic similarities, Ero1, Erv2, and DsbB share no sequence similarity other than the presence of two redox active cysteine pairs. However, the

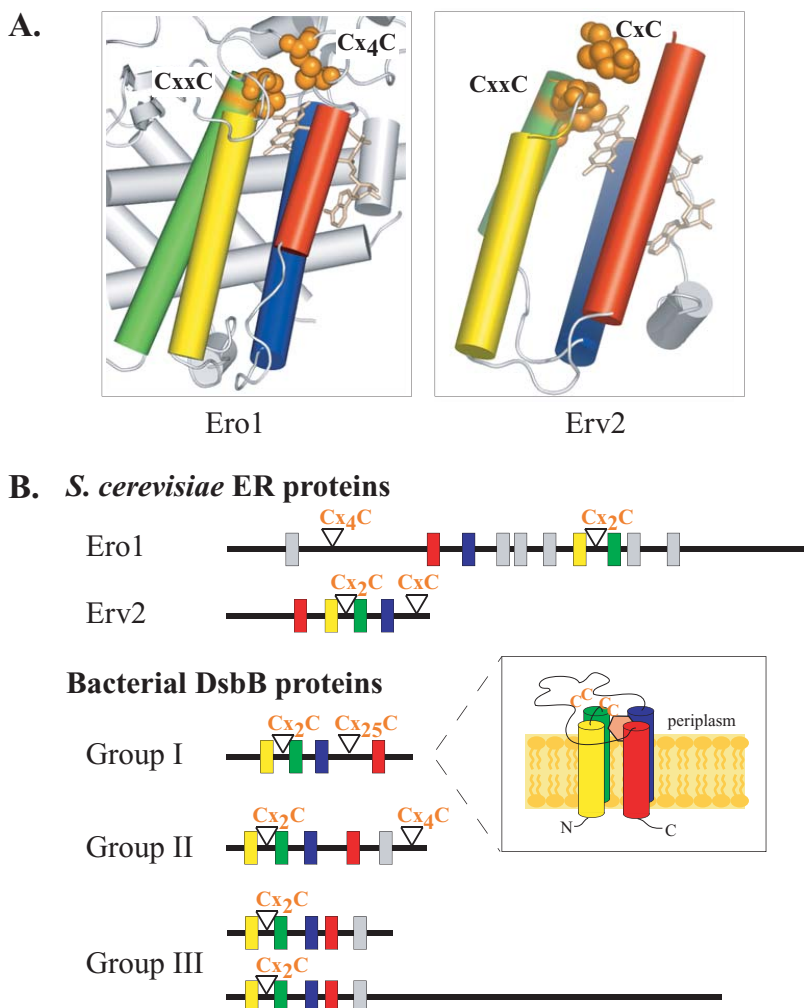
crystal structures of Ero1 and Erv2 revealed these two proteins share fundamental structural features within their catalytic domains (36, 37) and, although a structure for DsbB has not been obtained, sequence analysis has revealed DsbB has the potential to form a similar structural fold (98).

The catalytic core of Ero1 and Erv2 is formed by a bundle of four anti-parallel  $\alpha$ -helices that create the flavin-binding pocket (36, 37) (Fig. 3). The active site Cys-x-x-Cys pair of Ero1 and Erv2 is situated on a tight turn between two of the bundled helices, next to the redox active isoalloxazine moiety of the flavin. The second cysteine pair is found in close proximity to the Cys-x-x-Cys pair within a polypeptide domain that lacks significant secondary structure. In Ero1, the second cysteine pair is localized to a polypeptide loop that rests on the surface of the protein (36), while the second cysteine pair in Erv2 is found in a C-terminal tail (37). The domain containing the second cysteine pair can adopt at least two different conformations that have been observed in the crystal forms of both proteins (36, 37). In one conformation, the flexible region is displaced from the catalytic core, whereas in the second conformation the cysteine pair in the mobile domain approaches the flavin-proximal cysteine pair. The conformation of the domain containing the cysteine pair likely facilitates the two thiol-exchange reactions of Ero1 and Erv2 by making the second cysteine pair more accessible to soluble PDI, or by bringing the cysteine pair close enough to the active site to facilitate the exchange of electrons.

The DsbB protein contains several features reminiscent of the catalytic domains of Ero1 and Erv2 (Fig. 3). DsbB contains four transmembrane domains that have the potential to form a bundle of four anti-parallel helices with a Cys-x-x-Cys pair at the end of one of the four transmembrane helices and a second cysteine pair in a polypeptide loop that is predicted to lack significant  $\alpha$ -helical or  $\beta$ -sheet content (98). A charge-transfer complex between the DsbB Cys-x-x-Cys pair and its quinone cofactor has been detected spectroscopically (51, 107), which places the quinone cofactor next to the active site pair in a position analogous to the flavin cofactor and active site in the catalytic center of Ero1 and Erv2 (36, 37). The four helices forming the catalytic cores of Ero1 and Erv2 occur in a different order in the primary sequences of these proteins, indicating convergent evolution of the same four-helix catalytic core structure of the two enzymes (36) (Fig. 3). Notably, the four transmembrane helices in DsbB occur in an order different from either the Ero1 and Erv2 proteins (see Ref. 98 for details) (Fig. 3).

#### Ero1, Quiescin, and DsbB paralogs

Paralogs of Ero1, DsbB, and quiescin (a secreted human Erv2-like protein; discussed in detail within the next section) have been described that contain two identically positioned cysteine pairs (relative to Ero1, DsbB, and quiescin), and are predicted to share a four-helix catalytic domain. Of the five eukaryotes containing characterized Ero1 proteins, three contain two distinct, yet highly homologous *ERO1* (*H. sapiens*, *S. pombe*, and *A. thaliana*) (13, 23, 58, 86) while two possess a single *ERO1* (*S. cerevisiae* and *C. elegans*) (29, 40, 88). A quiescin paralog, QSOXN, was recently characterized in neuroblastoma cells (119), while at least fourteen bacterial organisms with two DsbB paralogs have been identified (98).



**FIG. 3. Catalytic four-helix core of Ero1, Erv2, and DsbB.** (A) Structure of the catalytic core of Ero1 and Erv2 (PDB files 1RP4 and 1JR8) (36, 37). The four bundled helices forming the catalytic core are colored to reflect their relative orientation in the structure. The two catalytic cysteine pairs in each protein are represented as *orange spheres*, while the flavin cofactor is shown in a *brown stick* form. To facilitate viewing of the Erv2 core domain, only one subunit of the homodimer plus the tail cysteine pair from the second subunit is shown. (B) Diagram of the secondary structure for the ER and periplasmic oxidoreductases. The  $\alpha$ -helices from the Ero1 and Erv2 structure, and those predicted to form in the DsbB proteins, are represented as *rectangles* while the four helices forming the catalytic core domain are highlighted in the *colors* shown in part A (see Ref. 98 for DsbB details). The Cys-x-x-Cys active site pair, present at the end of the *green helix*, and the location of the secondary cysteine pair are noted in *orange text*.

Currently no major differences in the redox activities or substrate interactions of these paralogs have been described. Mixed-disulfide intermediates of the thiol-transfer reactions have been captured between both human Ero1 proteins (Ero1-L $\alpha$  and Ero1-L $\beta$ ) and PDI (81). Ero1-L $\alpha$ , Ero1-L $\beta$ , and the *S. pombe* Ero1 proteins (Ero1a and Ero1b) can restore viability to the mutant *S. cerevisiae ero1-1* strain, demonstrating that the Ero1 paralogs from these organisms are both functional homologs of the *S. cerevisiae* Ero1 protein (13, 58, 86).

Striking differences in the transcriptional regulation of the human and plant Ero1 paralogs have been reported, which may reflect the need to modulate the oxidizing power of the ER under specific stress conditions (23, 34, 77, 86). Ero1-L $\alpha$  and Ero1-L $\beta$ , and quiescin and QSOXN, also display different tissue distributions, suggesting these paralogs may participate in tissue specific oxidation pathways (16, 86, 119).

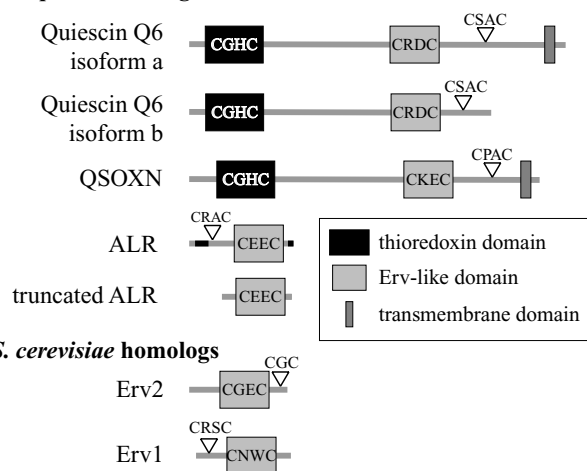
#### Erv-like protein family

A unique family of eukaryotic sulfhydryl oxidases have been described that share sequence homology with the 100-residues that encode the four-helix catalytic core of Erv2, in-

cluding the Cys-x-x-Cys active site, yet contain varying auxiliary polypeptide domains with a secondary cysteine pair (Fig. 4). Erv-like proteins fall into two general subfamilies: the ERV/ALR group, which contains small proteins (~15,000–20,000 Da) with a single Erv-like domain, and the QSOX group, which includes larger proteins that have a carboxy-terminal Erv-like domain and an amino-terminal thioredoxin domain (reviewed in Ref. 109). All the characterized Erv family members are flavoproteins that have the ability to catalyze the oxidation of thiol-containing compounds using oxygen (48, 85, 97), or alternate compounds such as cytochrome *c* (26), as a final electron acceptors. Like the disulfide generating enzymes Ero1, Erv2, and DsbB, the characterized ERV/ALR proteins use a partner protein to mediate the transfer of disulfides to substrate proteins (70, 80, 91, 96, 97, 117). For the QSOX proteins, oxidizing equivalents likely flow from the Erv-like domain to substrate proteins via the fused thioredoxin-like domain (109).

All Erv-like family members contain at least one pair of cysteine residues in a Cys-x<sub>n</sub>-Cys motif (where *n* is 1–4 residues) in addition to the Cys-x-x-Cys motifs in the Erv-like and thioredoxin-like domains. The only characterized ex-

*H. sapiens* homologs



**FIG. 4. Human and yeast Erv-like proteins.** Thioredoxin-like domains and Erv-like domains are represented as *black* and *grey rectangles*, respectively, containing the active site cysteine sequence. The secondary cysteine pair that is part of the catalytic mechanism for the Erv-like domain is also noted. For ALR, at least two splice variants are reported in the sequence databases that alter the regions highlighted in *black*.

ception is the viral Erv-like protein E10R, which contains only a single cysteine residue in addition to its active site (95). However, E10R associates with another viral protein A2.5L that contains a cysteine pair in a Cys-x-x-x-Cys motif (96). Conservation of an auxiliary cysteine pair in addition to the Cys-x-x-Cys active site suggests the Erv-like family of proteins share a common internal thiol transfer relay that, as discussed previously, is a conserved mechanism for the disulfide generating enzymes. Disulfide-linked homodimers dependent on the presence of the second cysteine pair have been captured for several of the ERV/ALR subfamily members (33, 37, 46, 69). These intermediates likely reflect an electron exchange event between the second cysteine pair and the Cys-x-x-Cys active site (37).

Location of the auxiliary cysteine pairs relative to the Erv-like domain varies. For example, quiescin and Erv2 have a second cysteine pair C-terminal to the Erv-like domain, whereas the ERV/ALR proteins Erv1, a mitochondrial yeast protein, and ALR, a human hepatic growth factor, contain cysteine pairs N-terminal to the Erv-like core. Intriguingly, the protein domains containing the second cysteine pair are poorly conserved, even between orthologs. It seems likely local diversity in the protein context of the cysteine pair may direct the interaction of each Erv-like protein with a specific substrate protein.

Redox reactions driven by the Erv-like proteins are apparently not limited to the oxidizing environment of the ER and can take place at different locations within the cell (109). Mitochondrial yeast Erv1 is important for cellular iron homeostasis, the maturation of cytosolic Fe/S protein clusters, protein import into the mitochondrial intermembrane space, and mitochondrial biogenesis (67, 80), while its ortholog ALR plays a role in liver regeneration (28). QSOX proteins have

been suggested to play a role in a range of developmental processes ranging from spermatogenesis to cell death (10, 85, 119), and have been implicated in the secretion of many different disulfide-containing secretory proteins (109).

*DsbB* homologs

Similar to the Erv-like protein family, DsbB homologs have been identified that share a core structural unit consisting of four transmembrane domains and a Cys-x-x-Cys motif, yet contain unique auxiliary polypeptide domains with a secondary cysteine pair (90, 98) (Fig. 3). In the recently identified group II DsbB proteins, the second cysteine pair is positioned within a periplasmic C-terminal tail, similar to the location of the second cysteine pair in Erv2, whereas the second cysteine pair in the classical DsbB proteins is found within a loop of sequence between two transmembrane domains, reminiscent of the loop surrounding the second cysteine pair in Ero1 (36, 37, 90, 98) (Fig. 3).

At least five bacterial species contain a five transmembrane domain DsbB-like protein with a Cys-x-x-Cys active site, but lacking a second cysteine pair, alternately referred to as a group III DsbB (98) or DsbI (90) protein (Fig. 3). This interesting group of DsbB proteins must use an alternative catalytic mechanism to oxidize substrate proteins, possibly including the use of a cysteine containing partner protein like the viral Erv protein E10R and its partner A2.5L (96). Organisms that contain DsbI and lack a DsbB group I or II protein, do not contain *dsbA*, suggesting DsbI oxidizes a yet uncharacterized protein (90, 98).

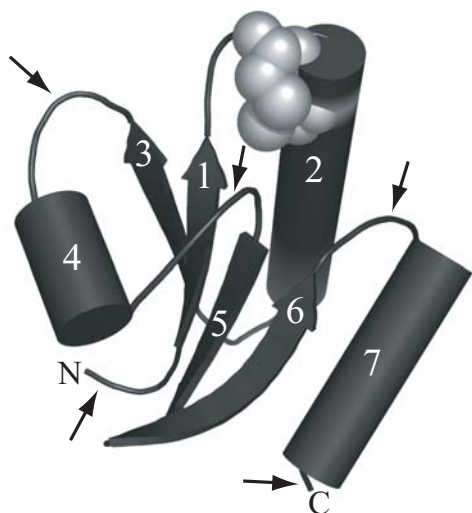
**DISULFIDE CARRIER PROTEINS: SOLUBLE THIOREDOXIN-LIKE PROTEINS**

*The thioredoxin fold*

PDI serves as the major carrier of oxidizing equivalents between the membrane-associated oxidoreductases Ero1 and Erv2, and substrate proteins (reviewed in Refs. 25, 32, 118). Structural studies of PDI (56) and its prokaryotic functional homolog DsbA (74) reveal both proteins are members of the thioredoxin-like protein family, which is a structural family named after the protein the fold was first observed in, *E. coli* thioredoxin (reviewed in Refs. 15, 73). The thioredoxin fold is a substructure of thioredoxin itself, having one less  $\beta$ -strand and one less  $\alpha$ -helix, and can be described as a N-terminal  $\beta\alpha\beta$  motif, a connection loop containing an  $\alpha$ -helix, and a C-terminal  $\beta\beta\alpha$  motif (15, 73) (Fig. 5). The classical thioredoxin fold, a-type fold, contains an active site Cys-x-x-Cys motif on the exposed turn linking the first  $\beta$ -strand and  $\alpha$ -helix, while noncatalytic thioredoxin folds, b-type folds, have been described that lack an active site. Primary sequence identity between the proteins within this superfamily is often very low; for example, PDI and thioredoxin share only 10% sequence homology.

*The PDI family*

Both human and yeast cells contain multiple ER-localized PDI-like proteins; to date seventeen human and five *S. cere-*

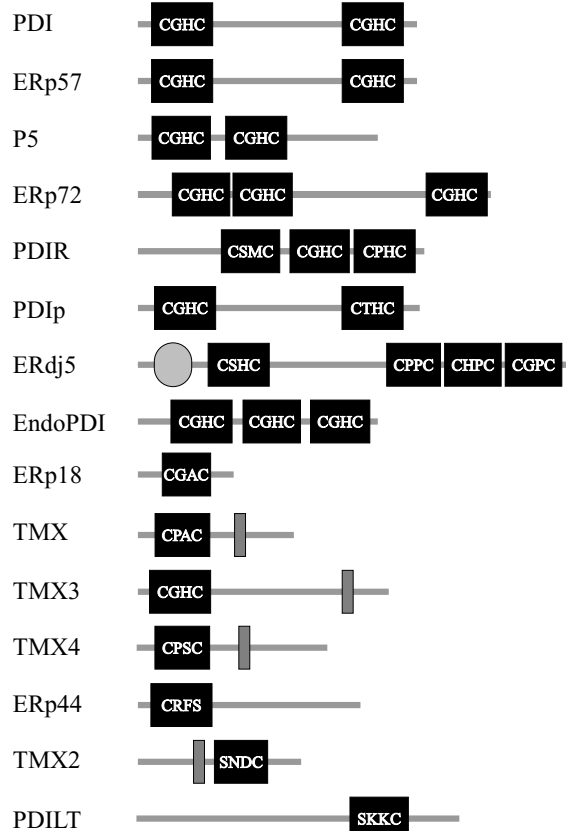


**FIG. 5. Structure of the thioredoxin fold.** Structure of the thioredoxin-fold domain from *E. coli* thioredoxin (PDB file 2TRX) (55). Residues 1–20 in the structure, which form an additional  $\beta$ -sheet and  $\alpha$ -helix that are supplementary to the generic thioredoxin-fold, are not shown. Helices are represented as *cylinders*, sheets are shown as *thick arrows*, and the Cys-x-x-Cys active site is represented as *grey spheres*. Numbers 1–7 follow the connectivity of the thioredoxin fold. *Arrows* indicate regions of the fold that tolerate large polypeptide insertions (for details, see Ref. 73).

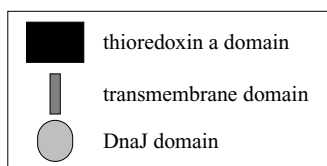
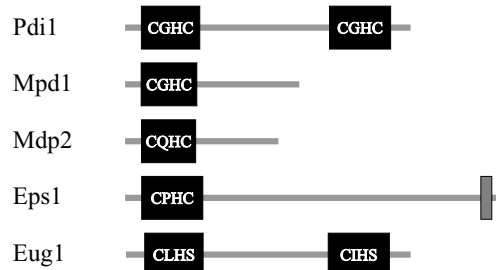
*visiae* ER-localized homologs have been identified (Table 1 and Fig. 6). The PDI superfamily is characterized by the presence of one or more domains with a predicted thioredoxin structural fold, a signal sequence, and an ER localization signal (Table 1 and Fig. 6). PDI itself is a modular protein containing four thioredoxin domains: two catalytic, a-type, domains with a Cys-x-x-Cys motif and two noncatalytic, b-type, domains with a predicted thioredoxin-like fold but no active site. The PDI family contains proteins with zero to four a-type thioredoxin domains and includes three human and one yeast protein containing nontraditional active site sequences, Cys-x-x-Ser and Ser-x-x-Cys, as well as two proteins containing only b-type thioredoxin domains (Table 1 and Fig. 6).

Variations in the ability of the human PDI homologs to ensure viability of *S. cerevisiae* when PDI function is compromised indicate the characterized PDI-like proteins perform distinct functions (Table 1). PDI family members may participate in specialized ER folding pathways, including discrete protein oxidation, reduction, and isomerization pathways. Redox pathways designed for distinct substrates have been well described in prokaryotes, where the membrane protein DsbD promotes disulfide bond isomerization by reducing the thioredoxin-like proteins DsbC and DsbG, and also facilitates cytochrome *c* maturation by reducing the thioredoxin-like protein DsbE (44, 89). Certain prokaryotes may also contain multiple protein oxidation pathways, as a few bacteria, including *Neisseria meningitidis*, *Shewanella oneidensis*, and *Salmonella enterica*, contain three or four DsbA paralogs (100, 110). Interestingly, the presence of multiple DsbA paralogs does not correlate with the number of DsbB proteins; *N. meningitidis*, *S. oneidensis*, and *S. enterica* encode one, two, and three DsbB family members, respectively (98).

#### *H. sapiens* homologs



#### *S. cerevisiae* homologs



**FIG. 6. Eukaryotic PDI-like proteins localized to the ER.** Thioredoxin-like a-domains are represented as *black rectangles* containing the active site sequence. The PDI homologs ERp27 and ERp28 both lack an a-like catalytic domain and are not shown.

Some PDI family members may perform nonredox chaperone functions as suggested for the PDI homolog ERp28, which contains a noncatalytic thioredoxin domain (27). Currently the PDI-like human protein ERp44 has been implicated in inhibiting IP (3) type 1 receptor activity, by binding to a

TABLE 1. ER LOCALIZED PDI FAMILY MEMBERS

Protein (synonyms)	Size (kDa)	NCBI accession number	Thioredoxin motifs ( $\alpha$ -type)	Calsequestrin motifs	ER-localization motif	Redox state in vivo	Mixed-disulfide with Ero1	Complement <i>S. cerevisiae</i> <i>pdi1</i> $\Delta$	Unique characteristics	References
<i>H. sapiens</i> homologs										
PDI (P4HB, P55)	55	AAH71892	2	1	KDEL	Red	Ero1-L	Yes	Binds N-glycosylated proteins through calnexin/calreticulin	39, 53, 81
ERp57 (GRP58, P58, ERp60, ERp61)	54	NP_005304	2	-	QDEL	Red	ND	No		4, 24, 39, 53, 81
P5 (ERp5, CaBP1)	46	NP_005733	2	-	KDEL	Red	ND	Yes	53, 65, 81	53, 65, 81
ERp72 (CaBP2, ERP70)	71	NP_004902	3	1	KEEL	Red	ND	Yes		39, 53, 65, 81
PDIR (PDIr)	57	BAA08451	3	-	KEEL	Red	NR	No*	Highly expressed in pancreas	42, 53, 59
PDIp (PDIP)	55	NP_006840	2	-	KEEL	NR	NR	NR		21, 22
ERdj5 (JPDI)	91	AAN73271	4	-	KDEL	NR	NR	No	DnaJ domain	17, 49
EndoPDI (ERp46)	48	NP_110437	3	-	KDEL	NR	NR	Yes		Highly expressed in endothelia
ERp18 (ERp19, TLP19)	18	NP_056997	1	-	EDEL	NR	NR	No	1, 62, 72	1, 62, 72
TMX	32	NP_110382	1	-	-	NR	NR	NR		75, 76
TMX3	52	NP_061895	1	1	KKKD	Predom red	NR	NR	41	25
TMX4	39	AAQ89363	1	-	-	NR	NR	NR		
ERp44	44	NP_055866	1	1	RDEL	NA	Ero1-L	NR	Cytoplasmic thioredoxin domain	2, 3
TMX2	34	NP_057043	1	-	KKDK	NA	NR	NR		
PDILT	67	AAH44936	1	-	KEEL	NA	Ero1-L	NR	Testis-specific expression	115
ERp27	30	NP_689534	-	-	KVEL	NA	NA	NR		
ERp28	29	P30040	-	-	KEEL	NA	NA	NR	27	
<i>S. cerevisiae</i> homologs										
Pdi1 (Trg1)	55	NP_009887	2	-	HDEL	Ox	Ero1	Yes	Essential for viability	30, 84
Mpd1	36	NP_014931	1	-	HDEL	NR	ND	Yes	30, 84	30, 84
Mpd2	32	NP_014553	1	-	HDEL	Ox	Ero1	Yes		
Eps1	81	NP_012261	1	-	KKNQD	NR	NR	Yes	84, 116	84
Eug1	58	NP_010806	2	-	HDEL	NA	NR	Yes		

\*Under the experimental conditions described in Ref. 59 both PDIR and human P5 were unable to complement the *S. cerevisiae pdi1*  $\Delta$  strain, although complementation by overexpression of rat P5 has been previously observed (65). ND, no interaction detected under the experimental conditions described in the cited literature; NR, no attempts to complete the experiment have been reported in the literature; NA, not applicable; ox, oxidized; red, reduced; predom red, predominantly reduced.

free cysteine residue in a luminal loop of the receptor (43), and in mediating the ER-retention of Ero1-L through the formation of reversible mixed disulfides with free Ero1 cysteine residues (2). If PDI family member TMX2 does affect protein folding in the ER, it will have to be through a novel mechanism as TMX2 is predicted to be a type I membrane protein, placing the Ser-x-x-Cys motif in the cytosol (79).

### *PDI homologs may participate in discrete protein maturation pathways*

It has been proposed that the *S. cerevisiae* ER oxidases Ero1 and Erv2 may have different preferences for the five PDI homologs, which could form distinct ER oxidation pathways. In both yeast and mammalian cells, only a subset of PDI family members appears to be substrates for yeast and human Ero1. In yeast, a mixed-disulfide transfer intermediate has been isolated between Ero1 and PDI or the PDI-like protein Mpd2, yet an interaction between Ero1 and yeast PDI family member Mpd1 could not be detected (30). A mixed-disulfide transfer intermediate has been successfully captured between human Ero1-L and human PDI (11, 81), and the human PDI-like proteins ERp44 (3) and PDILT (115), yet attempts to detect disulfide transfer between Ero1-L and human PDI family members ERp57, P5, and ERp72 have been unsuccessful (81) (Table 1). In addition, overexpression of Ero1 does not alter the reduced redox state of human PDI-like protein ERp57 (81).

Interaction of individual PDI homologs with discrete sets of protein substrates may reflect the presence of substrate specific pathways for protein maturation. Preferential expression of human PDI family members PDIp, EndoPDI, and PDILT in pancreas, endothelia, and testis cells, respectively, suggests these proteins contribute the folding of tissue specific proteins (22, 103, 115). Human PDI family members ERp57 and ERdj5 appear to cooperate with additional protein chaperones to mediate specific substrate interactions. ERp57 interacts with the lectin chaperones calnexin and calreticulin to promote disulfide bond formation in a class of glycoproteins, including MHC class I (reviewed in Ref. 24) while the DnaJ domain of ERdj5 can bind the Hsp70 ER chaperone BiP *in vitro*, which may facilitate the folding of a unique protein group within the ER (17). Different relative substrate interactions for the PDI-like proteins have also been observed. Expression of EndoPDI, a PDI-like protein highly expressed in endothelial tissue, impacts the folding and secretion of a broader range of cell survival factors in hypoxic endothelial cells than affected by PDI levels (103), while the PDI related human protein PDIR, but not human PDI, tightly associates with  $\alpha$ -antitrypsin in *in vitro* binding studies (42). Interestingly, the calnexin and calreticulin binding site in ERp57, and the peptide binding regions of PDI and PDIR, have been mapped to regions outside of the a-type thioredoxin domains, suggesting that interactions between PDI homologs and their substrates are determined in part by domains outside of the redox active thioredoxin fold (24, 25, 42). By fusing a thioredoxin-like domain with an additional protein sequence that confers a unique substrate contact region, the PDI family may allow for an unlimited number of specific substrate interactions.

### *PDI homologs may facilitate protein oxidation and reduction in the ER*

PDI can catalyze the *in vitro* oxidation, reduction and isomerization of disulfide bonds, depending on the redox environment (118). All the PDI homologs with a Cys-x-x-Cys or Cys-x-x-Ser active site that have been tested for oxidoreductase activity (with the exception ERdj5) have demonstrated the ability to oxidize free thiols and/or isomerize disulfides. However, it has been difficult to determine the preferential *in vivo* redox activities of the PDI homologs due to overlapping and complementing functions of the PDI family members (84), and even the essential *in vivo* function for PDI itself remains unclear (66, 101).

Evaluation of the structural requirements for the reductase, isomerase, or oxidase activities of PDI suggests oxidation of thiols requires one of the two thioredoxin-like a domains containing an active site Cys-x-x-Cys motif, while isomerization activity requires a least one of the thioredoxin-fold b-type domains of PDI as well as an a-type domain (19, 20). Applying this functional categorization to other members of the PDI family suggests human P5 and EndoPDI, or yeast Mpd2, which only contain a-like domains may be dedicated oxidases, while other homologs like human ERp57 and yeast Eogl have the additional sequence motifs that could allow them to serve as isomerases (25, 32).

The redox state of the active site cysteine pairs determines the reaction catalyzed by a given oxidoreductase (Fig. 2). At steady state, the active site of yeast PDI and Mpd2 are in an oxidized (disulfide) form, suggesting they predominantly act as oxidases, while the active sites of most of the human PDI homologs examined are in a reduced (dithiol) form, suggesting these proteins serve predominantly as reductases or isomerases (Table 1). Although human PDI is found in a mostly reduced form in mammalian cells, it is generally accepted that PDI serves as an oxidase in the Ero1-driven disulfide bond formation pathway (81) (Table 1). The discrepancy between human and yeast PDI redox state may reflect a more rapid transfer of oxidizing equivalents to cargo and/or slower reoxidation by Ero1 in human cells than in yeast.

## COMPARISON OF THE EUKARYOTIC AND PROKARYOTIC PATHWAYS FOR PROTEIN REDUCTION

Cells contain disulfide isomerization or reduction pathways to correct misoxidized cysteine pairs formed during the protein folding process. The best characterized cellular pathway for protein isomerization is present in the bacterial periplasm (reviewed in Refs. 44, 89) (Fig. 7). In this pathway, the soluble thioredoxin-like protein DsbC catalyzes disulfide reshuffling by reducing incorrectly paired cysteine residues. The active site cysteines of DsbC are maintained in an active reduced state by the cytoplasmic membrane protein DsbD, which shuttles electrons from the cytoplasm across the inner membrane to DsbC. Reducing equivalents for the DsbD-DsbC pathway are ultimately derived from the small molecule NADPH and thioredoxin reductase.

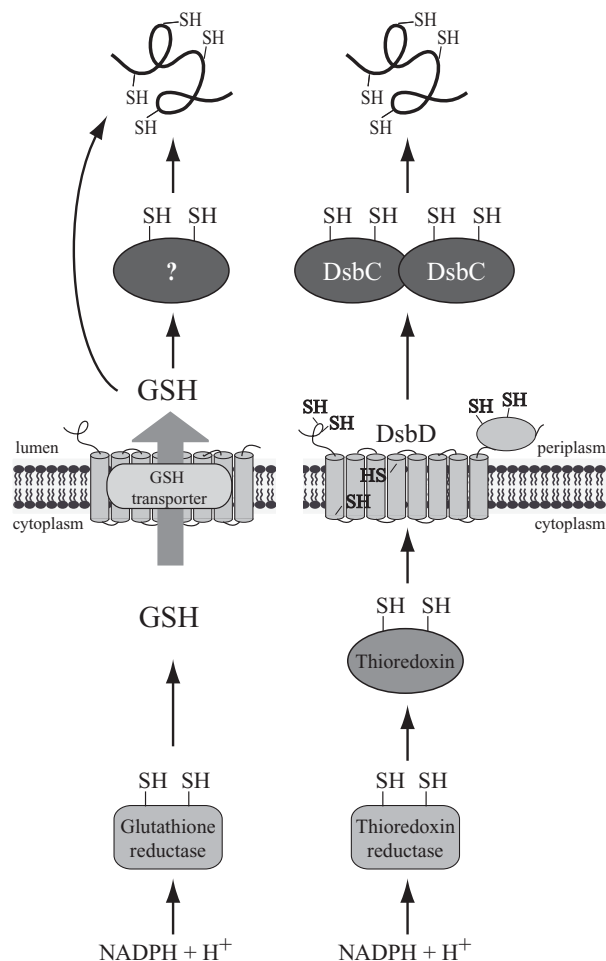
Recently a view of the pathway for disulfide isomerization in the eukaryotic ER has begun to emerge (Fig. 7). In this pathway, reduced glutathione ( $\gamma$ -L-glutamyl-L-cysteinylglycine, GSH) serves as the reducing agent. For many years it was widely accepted that glutathione acted as a protein oxidant in the ER, an idea that emerged from studies of glutathione levels within the secretory pathway, which demonstrated a higher ratio of oxidized to reduced glutathione in the ER lumen relative to the cytosol (50). The observation that

lowering the intracellular glutathione levels in yeast by disrupting the *GSH1* gene (encoding  $\gamma$ -glutamylcysteine synthetase, the enzyme for the first step in glutathione synthesis) not only causes oxidative protein folding to be more readily compromised by the addition of a small molecule oxidant but also restores disulfide bond formation to cells containing a mutant *ERO1* allele, provided the first evidence that glutathione acts as a net reductant in the ER to counteract the Ero1-driven oxidation pathway (18). Experiments in semipermeable mammalian cells demonstrate an increased amount of reduced glutathione is necessary to balance the increased oxidative activity in cells overexpressing Ero1, suggesting glutathione serves as an antagonist of the Ero1-PDI oxidation pathway in all eukaryotes (82).

Glutathione-depleted mammalian cells (treated with an inhibitor of  $\gamma$ -glutamylcysteine synthetase), like *gsh1* mutant yeast, do not exhibit defects in oxidative folding or secretion (14, 29). However, when glutathione-depleted cells are treated with a pulse of the reductant dithiothreitol (DTT), cells accumulate proteins with incomplete disulfide linkages (14) and detergent-insoluble protein aggregates (82), demonstrating glutathione protects against the accumulation of misfolded proteins or folding intermediates under conditions of reductive stress. Considering these data in light of the basic property that glutathione acts as a net reductant in the ER, reduced glutathione may play a role in the reduction of misoxidized substrates or reshuffling of protein intermediates along the folding pathway. Currently it is not known whether reduced glutathione influences native disulfide bond formation by maintaining thioredoxin-like reductases in their active reduced form, or by directly reducing folding substrates. When glutathione is depleted from cells, or the glutathione reduction pathway is inhibited, the human PDI family member ERp57 cannot return to its reduced form after oxidative stress, demonstrating a role for glutathione in ERp57 reduction (53). However, a large fraction of the glutathione in the ER forms mixed-disulfide bonds with proteins (9), suggesting reduced glutathione can reduce substrate proteins directly.

Notably, the cytosol appears to be the source of reduced glutathione that is necessary to modulate protein folding in the ER during redox stress (53, 82). Since a pathway to generate reduced glutathione has not been identified in the ER, a continuous flux of reduced glutathione from the cytosol may be required to maintain a pool of reduced glutathione in the ER for protein reduction. The abundance of oxidized glutathione in the ER may reflect the accumulation of trapped, spent glutathione. Preferential transport of reduced glutathione, over oxidized glutathione, into rat liver microsomes has been observed, supporting the existence of a selective facilitated transport system in the ER membrane (8).

A striking difference between the emerging eukaryotic reductive pathway and the prokaryotic DsbC–DsbD pathway is the use of the small molecule glutathione, rather than a protein, as the carrier of reducing equivalents. The permeable outer membrane separating the periplasmic space from the external medium may preclude the use of a small redox compound like glutathione. However, it is possible the small molecule cysteine plays a role in reduction reactions within the periplasm; mutants of the cysteine transporter *cydDC*, which are defective in the export of cysteine from the cytoplasm to



**FIG. 7. Pathways for protein reduction/isomerization.**

Disulfide bond isomerization in the periplasm is catalyzed by the soluble oxidoreductase DsbC, which is maintained in a reduced form by the cytoplasmic membrane protein DsbD. DsbD is kept in a reduced state by the cytoplasmic protein thioredoxin, which ultimately obtains electrons from NADPH through thioredoxin reductase. A protein reduction pathway in the ER is driven by the small molecule reduced glutathione (GSH), which is likely transported from the cytoplasm into the ER by an unidentified small molecule transporter. GSH is maintained in a reduced state in the cytoplasm by glutathione reductase, which ultimately obtains electrons from NADPH. GSH may transfer electrons to a protein reductase (like mammalian ERp57), which catalyzes substrate protein reduction, or GSH may transfer electrons directly to folding proteins. *Thin arrows* depict the flow of electrons.

the periplasm, exhibit pleiotropic phenotypes including sensitivity to DTT, loss of motility, and periplasmic cytochrome assembly defects which are similar to those observed with *dsbA* or *dsbB* mutants (87). Despite the difference in small molecule electron carriers, the eukaryotic and prokaryotic pathways for reduction/isomerization share fundamental mechanistic features. Both pathways shuttle electrons from a reductant in the cytoplasm across a membrane. In prokaryotes, electrons from cytoplasmic thioredoxin are transported across the cytoplasmic membrane through sequential thiol–disulfide exchange reactions between three cysteine pairs within the integral membrane protein DsbD, while in eukaryotes reduced glutathione is likely transported across the membrane by a polytopic membrane protein acting as a small molecule transporter. NADPH is the ultimate cytoplasmic electron source for both the eukaryotic and prokaryotic pathways, which is coupled to glutathione reductase or thioredoxin reductase activity respectively (44, 53, 89). DsbD maintains the soluble thioredoxin-like protein DsbC in a reduced form, which also parallels the potential role for glutathione in reducing thioredoxin-like ER proteins, like ERp57.

### SPECIFIC TRANSFER BETWEEN OXIDOREDUCTASES

Specificity in the disulfide transfer reactions that occur in the ER and periplasm is important not only to drive unique protein oxidation and reduction pathways, but also to prevent cross-transfer of oxidizing or reducing equivalents between the protein oxidation and reduction pathways, which could result in incapacitation of either pathway. Spurious oxidation or reduction of proteins and small molecules in the ER can also lead to unnecessary cellular damage from reactive oxygen species (ROS), a byproduct of the Ero1 and Erv2 catalytic cycles (40, 113).

Kinetic data show a large kinetic barrier separates the oxidative (DsbB–DsbA) and reductive (DsbD–DsbC) pathways in the periplasm (93). Disulfide exchange between DsbB and DsbC is prevented through the dimerization of DsbC, which appears to insulate the active site from recognition by DsbB (6). Segregation of the oxidative and reductive pathways in eukaryotes is likely achieved through a similar kinetic isolation, as glutathione has been demonstrated to be a poor substrate for both Ero1 and Erv2 (33, 97, 111).

The divergent polypeptides containing the second cysteine pairs in the Ero1, Erv2, and DsbB family members may be tailored to drive the interaction with specific oxidoreductases. For Ero1 and Erv2, the unstructured nature of the loop and tail containing the second cysteine pair may mimic the characterized substrate for PDI, an unfolded polypeptide. Movement of the domains encompassing the redox-active cysteine pairs may also direct the flow of electrons along specific pathways. For Ero1 and Erv2, movement of the mobile loop or tail close to the catalytic core may promote disulfide exchange between the second cysteine pair and the active site, while movement away from the core domain may allow interaction with soluble PDI (36, 37).

The auxiliary polypeptide domain containing the secondary cysteine pair may also restrict access to the Cys-x-x-Cys active site, limiting the nonspecific oxidation of proteins and thiols within the ER or periplasm. Physical isolation of the active site cysteine pair from large substrates is supported by the Ero1 and Erv2 structures, which show the active site cysteine pair buried beneath the protein surface, underneath the flexible loop domain in Ero1 (36) or covered by the flexible tail and dimer interface in Erv2 (37). Restricted accessibility of the DsbB Cys-x-x-Cys active site to large substrates like DsbA is suggested by the observed decrease in disulfide transfer between reduced DsbA and a DsbB mutant with the secondary cysteine pair replaced with serines, relative to the transfer seen between reduced DsbA and wild-type DsbB (35, 92).

Structural changes that allow substrates direct access to the active site cysteine pair, bypassing the need for the secondary cysteine pair, have been described for both Ero1 and Erv2 (99, 114). The altered activity of these mutants toward selected substrates suggest that blocking access to the active site cysteine pair normally prevents the nonspecific oxidation of proteins or molecules in the ER (99, 114). The active site cysteine pair of DsbB has been calculated to have a redox potential as high as  $-69$  mV, forming the most oxidizing disulfide bond characterized to date (35). However, recent experiments suggest the  $-69$  mV value does not reflect the redox potential of the cysteine pair alone, which has been determined to be  $-207$  mV, but rather includes a contribution from residual bound quinone (52). Nonetheless, it seems likely exposure of a disulfide–quinone pair with such a high propensity toward oxidizing substrates could result in indiscriminate oxidation of proximal proteins with free cysteines.

### CONCLUSIONS

Important progress has been made over the past several years toward understanding how disulfide bonds are formed in cellular proteins in the ER of eukaryotes and the periplasmic space of prokaryotes. Striking similarities have been revealed between the eukaryotic and prokaryotic protein oxidation and reduction pathways at functional, mechanistic, and structural levels. Recognition that all known biosynthetic disulfide bond formation processes share the same fundamental process will provide the groundwork for future studies focusing on the multiple homologs and paralogs of the protein components of the major disulfide bond formation pathways, which appear to form unique substrate specific oxidation and reduction pathways throughout the cell.

It will be interesting to further characterize how the multiple homologs and paralogs are integrated into the characterized oxidation and reduction pathways and whether additional uncharacterized redox pathways using these homologs exist. It will also be exciting to see the identity and functions of the cellular redox pathways using the Erv family, which are now emerging. Using the structural features conserved between the Ero1 and Erv2 core domains, it may now be possible to identify new unrecognized oxidoreductases in eukaryotic or prokaryotic cells by developing motif-searching algorithms similar to those used to recognize thioredoxin-like

domains, expanding the cellular oxidoreductase family even further.

Most of the core enzymes within the disulfide bond formation pathways, and their homologs, appear to contain a redox active pair within one of two characterized structural folds, an Erv-like or a thioredoxin-like domain. Fusion of additional domains to the thioredoxin or Erv-like structural blocks, may provide diversity to the oxidoreductase family and allow for specific thiol-transfer reactions. Future domain swapping, site-directed mutagenesis, and *in vitro* biochemistry experiments can be used to assess the molecular determinants within these auxiliary domains that confer substrate specific thiol-disulfide exchange events. Finally, some of the greatest insight into the specificity of the disulfide bond formation pathways will likely come from structural studies of enzyme-substrate complexes, including complexes between Ero1-PDI, Erv2-PDI, and DsbB-DsbA.

### ABBREVIATIONS

DTT, dithiothreitol; ER, endoplasmic reticulum; GSH, reduced glutathione; PDI, protein disulfide isomerase; ROS, reactive oxygen species.

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Date of first submission to ARS Central, November 3, 2005;  
date of acceptance, November 24, 2005.

