Pathways for protein disulphide bond formation

Alison R. Frand, John W. Cuozzo and Chris A. Kaiser

The folding of many secretory proteins depends upon the formation of disulphide bonds. Recent advances in genetics and cell biology have outlined a core pathway for disulphide bond formation in the endoplasmic reticulum (ER) of eukaryotic cells. In this pathway, oxidizing equivalents flow from the recently identified ER membrane protein Ero1p to secretory proteins via protein disulphide isomerase (PDI). Contrary to prior expectations, oxidation of glutathione in the ER competes with oxidation of protein thiols. Contributions of PDI homologues to the catalysis of oxidative folding will be discussed, as will similarities between eukaryotic and prokaryotic disulphide-bond-forming systems.

The formation of disulphide bonds between the correct pairs of cysteine residues is essential for the folding and stability of many proteins that are secreted or localized to organelles of the secretory pathway. Nearly 40 years ago, the seminal work of Anfinsen and colleagues on the *in vitro* refolding of reduced, denatured ribonuclease A demonstrated that native disulphide bond formation can occur spontaneously[1]. The early observation that disulphide bond formation proceeded much more slowly in air than in living cells implied the existence of catalysts for oxidative protein folding.

The minimal requirements for efficient oxidative refolding *in vitro* have since been defined as a redox buffer containing both oxidizing and reducing equivalents as well as an enzymatic catalyst for thiol–disulphide exchange. Standard assays for oxidative refolding employ glutathione redox buffers in which oxidized glutathione (GSSG) provides the oxidizing equivalents necessary for protein disulphide bond formation. Under these conditions, the redox potential of the assay buffer, defined by the ratio $[GSH]^2/[GSSG]$, determines the overall rate of oxidative refolding[2,3].

The search for enzymatic catalysts of oxidative refolding led to the isolation of protein disulphide isomerase (PDI)[4]. Intensive investigation of the activities of PDI *in vitro* has since shown that the enzyme can catalyse the formation, reduction or isomerization of disulphide bonds depending upon the redox conditions of the assay and the nature of the substrate protein[5]. The activity of PDI depends on two pairs of cysteines, each of which are found in the motif Cys-x_a-x_b-Cys within a domain homologous to thioredoxin[6]. When the active-site cysteines of PDI are present in disulphide (oxidized) form, the enzyme can transfer disulphide bonds directly to substrate proteins, suggesting a physiological role for PDI as a protein dithiol oxidase[7]. However, when the active-site cysteines of PDI are present in dithiol (reduced) form, the enzyme is suited for catalysis of disulphide reshuffling. PDI is reduced in those redox buffers adjusted to give optimal refolding rates *in vitro*, and this correlation has drawn attention to the isomerase activity of the enzyme[8]. A role for PDI in the catalysis of native disulphide bond formation in the endoplasmic reticulum (ER) was first established by mutational analysis in yeast, where the *PDI1* gene was shown to be essential for cell viability and for oxidative protein folding[9,10].

In eukaryotic cells, protein disulphide bond formation proceeds within the lumen of the ER, where protein oxidation initiates upon the translocation of nascent peptide chains into the ER lumen[11]. The redox state of the ER is more oxidizing than that of the cytosol, a difference that favours the formation of structural protein disulphide bonds, and that is reflected in the relatively high intralumenal concentration of GSSG[12,13]. A net influx of oxidizing equivalents into the ER lumen is needed to support the rapid transit of secretory proteins through the ER, and to maintain a high concentration of GSSG within this organelle[12]. However, until very recently, the source of oxidizing equivalents utilized for disulphide bond formation in the ER was unclear. Genetic analysis in *Saccharomyces cerevisiae* has now defined the core pathway for protein disulphide bond formation in the eukaryotic ER.

A pathway for protein disulphide bond formation in the ER

A genetic dissection of oxidative protein folding in yeast began with the isolation of an essential and conserved gene, *ERO1* (ER oxidation), encoding a novel ER membrane protein required for protein oxidation in the ER[14,15] (Table 1). A temperature-sensitive allele of *ERO1* was identified in a screen for mutants defective in the export from the ER of secretory proteins containing disulphide bonds[14]. Mutations in *ERO1* were also isolated in a screen for *S. cerevisiae* strains with diminished oxidative capacity, a property reflected by increased sensitivity to the reductant dithiothreitol (DTT)[15]. Secretory proteins that would normally acquire intramolecular disulphide bonds remain completely reduced in the conditional *ero1-1* mutant[14]. Ero1p appears to introduce oxidizing equivalents necessary for protein disulphide bond formation into the ER lumen, a conclusion supported by the observation that a membrane-permeable thiol oxidant can substitute for *ERO1* function[14]. Moreover, overexpression of *ERO1* increases the oxidative capacity of the cell[14,15] (Table 1).

Recent findings indicate that Ero1p transfers disulphide bonds directly to Pdi1p. Although the

The authors are in the Dept of Biology, Massachusetts Institute of Technology, Cambridge, MA, USA.
E-mail: ckaiser@mit.edu

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TABLE 1 – MAJOR EUKARYOTIC AND PROKARYOTIC DISULPHIDE-BOND-FORMING PROTEINS[a]

Protein	Localization	Redox state of active sites	Gene characteristics			Refs
			Properties of mutants	Suppressors	Consequences of overproduction	
Ero1p	ER membrane	Disulphide	Defective protein oxidation Defective Pdi1p oxidation Sensitivity to DTT Inviability	Oxidant (diamide) *gsh1* mutations	Resistance to DTT	14–16, 25
Pdi1p	ER lumen	Disulphide	Defective protein oxidation Sensitivity to DTT Inviability	High-copy *MPD1*, *MPD2*, *EUG1* or *EPS1*		9, 16, 17, 20–22, 40
DsbB	Bacterial cytoplasmic membrane	Disulphide	Defective protein oxidation Defective DsbA oxidation Sensitivity to DTT	Oxidant (GSSG, cystine) *dsbD* mutations	Resistance to DTT	29, 30, 36
DsbA	Bacterial periplasm	Disulphide	Defective protein oxidation Sensitivity to DTT	High-copy *dsbC* *dsbD* mutations		29–31, 36, 47
DsbC	Bacterial periplasm	Dithiol	Defective folding of proteins with multiple disulphide bonds Sensitivity to DTT	Reductant (low levels of DTT)	Suppression of *dsbA* mutations	29, 30, 37, 38
DsbD	Bacterial cytoplasmic membrane	ND	Defective folding of proteins with multiple disulphide bonds Defective DsbC reduction Sensitivity to high levels of DTT	Reductant (low levels of DTT or GSH)		29, 30, 37, 38 47

Abbreviations: DTT, dithiothreitol; ER, endoplasmic reticulum; GSH, glutathione; GSSG, glutathione disulphide; ND, not determined.
[a]Only a subset of the enzymes implicated in oxidative protein folding are shown. See Table 2 for additional eukaryotic enzymes.

active-site cysteines of yeast Pdi1p are found predominantly in disulphide form *in vivo*, those cysteines appear in dithiol form in the conditional *ero1-1* mutant (Table 1)[16]. Disulphide-linked complexes between Ero1p and Pdi1p have been captured from yeast cells, and these complexes are likely to represent intermediates in the transfer of disulphide bonds from Ero1p to Pdi1p[16] (Fig. 1).

The isolation of Ero1p–Pdi1p mixed disulphides implicated Pdi1p as a vital intermediate in the flow of disulphide bonds from Ero1p to secretory proteins. Consistent with this model, the defect in protein folding observed in cells lacking Pdi1p was traced to a defect in protein oxidation, indicating that Pdi1p is necessary for the efficient formation of protein disulphide bonds in the ER[16,17] (Table 1). The identification of active-site mutants of Pdi1p that diminish the overall oxidative capacity of the cell further highlights the significance of the oxidase activity of Pdi1p[17]. Evidence that Pdi1p engages directly in thiol–disulphide exchange with ER proteins came from the detection of disulphide-linked complexes between Pdi1p and a newly synthesized secretory protein[16]. Mixed disulphides have also been captured between mammalian PDI and viral glycoproteins folding in the ER[18].

These results provide a unified mechanism for protein oxidation in eukaryotic cells. Oxidizing equivalents flow from Ero1p to secretory proteins via Pdi1p[16] (Fig. 2). Because this pathway requires only thiol–disulphide exchange reactions between proteins in order to transmit oxidizing equivalents, mixed disulphides with small molecules such as glutathione should not be necessary as intermediates in disulphide bond formation.

Mutational analysis in yeast has suggested an essential role for Pdi1p in the isomerization of non-native disulphide bonds, based on the behaviour of a Cys-x_a-x_b-Ser active-site mutant of PDI. This form of PDI retains isomerase but lacks detectable oxidase or reductase activity *in vitro*[19]. Nevertheless, overexpression of Cys-x_a-x_b-Ser PDI can restore viability to some cells lacking *PDI1*, suggesting that only the isomerase activity of Pdi1p is essential for yeast viability[9,19]. How can these studies be reconciled with observations placing Pdi1p in a pathway for protein oxidation? It is possible that, in the absence of Pdi1p, other relatively inefficient sources of oxidase activity promote protein disulphide bond formation in the ER. This seems quite likely since several oxidoreductases homologous to Pdi1p are present in the ER lumen[20–22] (Table 2). A second possibility is that the oxidase activity of Pdi1p is indeed essential, but that Cys-x_a-x_b-Ser Pdi1p retains some oxidase activity *in vivo* associated with the formation of mixed disulphides with Ero1p or glutathione.

The role of glutathione in oxidative protein folding in the ER

The thiol–disulphide redox status of intralumenal glutathione has long been the focus of considerations of how relatively oxidizing conditions are established

within the ER[12]. Glutathione is the major small-molecule redox buffer in the ER, and the ratio of the concentration of GSH to GSSG in the ER (1:1 to 3:1) is similar to that found in redox buffers affording optimal rates for oxidative refolding *in vitro*[12]. From these observations, it was natural to suppose that GSSG serves as the primary source of oxidizing equivalents driving disulphide bond formation *in vivo*, as it does *in vitro*[12]. Accordingly, disulphide bonds in newly synthesized proteins could be thought to form through thiol–disulphide exchange reactions with GSSG or with oxidases dependent upon GSSG as a source of oxidizing equivalents[23].

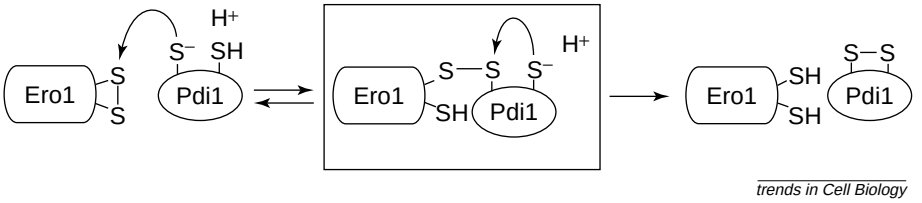

FIGURE 1

The proposed mechanism for thiol–disulphide exchange between Ero1p and Pdi1p. A disulphide bond in Ero1p undergoes nucleophilic attack by a thiolate anion derived from the N-terminal active-site cysteine of Pdi1p. The resulting mixed-disulphide intermediate can be resolved by intramolecular attack of the mixed-disulphide bond by a thiolate anion derived from the C-terminal cysteine of Pdi1p. This mixed disulphide intermediate (box) has been detected following treatment of cells with acid, a reagent that inhibits further thiol–disulphide exchange by driving thiol protonation.

Recent studies in yeast have disproved this hypothesis. These studies relied in part upon a yeast mutant that is devoid of intracellular glutathione owing to disruption of GSH1, the gene encoding the cytosolic enzyme catalysing the first and rate-limiting step in glutathione biosynthesis[24]. GSSG was found to be entirely dispensable for protein oxidation *in vivo* because oxidative protein folding proceeded with normal kinetics in cells lacking glutathione[14]. Surprisingly, disruption of *GSH1* actually restored protein oxidation in the conditional *ero1-1* mutant, indicating that GSH normally produces a load on the protein oxidation pathway[25]. Removing glutathione from the ER thus restores function to the compromised protein oxidation system of the *ero1-1* mutant[25]. Oxidation of glutathione in the ER was further shown to rely upon oxidizing equivalents ultimately derived from Ero1p as production of GSSG *in vivo* was coupled to Ero1p activity[25]. Together, these results show that glutathione oxidation occurs at the expense of protein oxidation and therefore indicate that glutathione serves as a net reductant in the ER[25] (Fig. 3). Oxidation of intralumenal GSH could result from the reduction of disulphide bonds in secretory proteins PDI or even in Ero1p (Fig. 3).

What role does glutathione normally play during oxidative protein folding *in vivo*? Studies of oxidative protein refolding *in vitro* have shown that both oxidizing and reducing equivalents are necessary for efficient refolding[2]. GSH might provide those reducing equivalents necessary to generate optimal folding conditions in the ER. Reducing equivalents from GSH might also counteract conditions of oxidative stress, a view consistent with the observation that oxidative protein folding is more readily compromised by an exogenous oxidant in cells deficient in glutathione[25]. Consistent with a role for glutathione in the ER as a net source of reducing equivalents, recent work suggests that GSH, rather than GSSG, is selectively imported into ER microsomes[26].

The functional analysis of Ero1p, Pdi1p and glutathione offers a new perspective on how protein disulphide bond formation occurs in the ER. The emerging view is that disulphide bond formation in the ER proceeds by the sequential transfer of oxidizing equivalents between proteins rather than by transfer from GSSG. An important implication of this view is that the flow of oxidizing equivalents might be controlled more by the kinetics of protein–protein interactions than by equilibration of protein dithiols and disulphides with the glutathione redox buffer. Thus, the actual redox status of a protein in the ER might be determined primarily by the relative reactivity with other redox-active proteins and might differ significantly from that predicted from equilibrium measurements of redox potential relative to glutathione. In the case of PDI itself, the redox potential as measured by equilibrium with glutathione is nearly equivalent to the thiol–disulphide redox

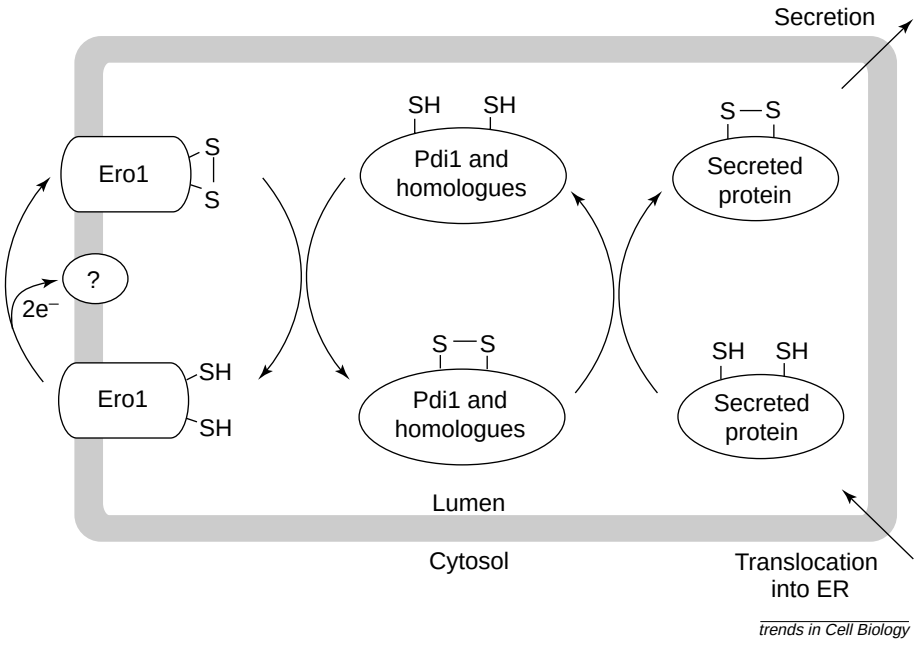

FIGURE 2

A pathway for protein disulphide bond formation in the endoplasmic reticulum (ER). Oxidizing equivalents flow from Ero1p to Pdi1p, and then from Pdi1p to secretory proteins, through a series of direct thiol–disulphide exchange reactions. Some subset of the ER oxidoreductases homologous to protein disulphide isomerase (PDI), including yeast Mpd2p, might function in parallel with Pdi1p. The mechanism for re-oxidation of Ero1p is unknown. Only one thioredoxin-like domain of Pdi1p is shown.

TABLE 2 – EUKARYOTIC HOMOLOGUES OF PDI

Protein	Size (kDa)[a]	Thioredoxin-like domains		Acidic regions	Distinctive features	Shared features	Refs
		with CXXC (a type)	without CXXC (b type)[b]				
S. cerevisiae homologues							
Pdi1p	58	2	2	1	Essential for viability	SS, HDEL, CHO	9, 10
Mpd1p	36	1	1			SS, HDEL, CHO	20
Mpd2p	32	1	–			SS, HDEL, CHO	21
Eug1p	58	2	2		Cys-x-x-Ser active sites	SS, HDEL, CHO	40
Eps1p	81	1	1		ER membrane protein	SS, KKKXXX, CHO	22
Mammalian homologues							
PDI	55	2	2	1	General peptide-binding site mapped to b′ domain	SS, KDEL	39, 48, 49
ERp72	71	3	2	1	Calcium binding	SS, KEEL	39, 50–52
ERp57	54	2	2		Interaction with nascent monoglycosylated glycoproteins	SS, QEDL	18, 39, 41, 42, 53
P5	46	2	1	1		SS, KDEL	39, 52, 54
PDIR	57	3	1			SS, KEEL	39, 55
PDIp	55	2	2		Pancreas-specific expression	SS, KEEL, CHO	39, 56

[a]Sizes for the yeast PDI proteins refer to the predicted molecular weights of each gene product based on the full DNA sequence and will not reflect signal-sequence cleavage or glycosylation of the proteins *in vivo*.
[b]The presence of b-type domains in mammalian homologues of PDI is inferred by sequence comparisons to known thioredoxin-fold domains of PDI proteins[39]. b-type domains in the yeast homologues of PDI are inferred by sequence conservation with Pdi1p in regions corresponding to the b-type domains of mammalian PDI. Any structural similarities between these regions and thioredoxin remain to be determined.
Abbreviations: CHO, potential glycosylation acceptor sites; SS, signal sequence. HDEL, KKKXXX, KDEL, KEEL and QEDL refer to the amino acid sequence of potential endoplasmic reticulum retention signals.

status of glutathione in the ER[12,27], leading to the expectation that, if PDI within the ER were in equilibrium with the glutathione redox buffer, it should partition equally between the dithiol and disulphide forms[6]. However, direct measurement of the redox status of Pdi1p *in vivo* reveals that the enzyme is almost entirely in the disulphide form[16]. This disparity can be explained if Pdi1p is not in equilibrium with the glutathione redox buffer but instead the redox status of Pdi1p represents a steady-state condition set by efficient oxidation of Pdi1p by Ero1p and relatively inefficient reduction of Pdi1p by glutathione and protein thiols. Thus the behaviour of PDI in standard *in vitro* assay conditions for oxidative protein refolding might differ fundamentally from that in the physiological environment of the ER. *In vitro*, the inefficiency of re-oxidation of PDI by GSSG might severely restrict the capacity of the enzyme to act as an oxidase[28]. *In vivo*, however, Ero1p activity appears to keep PDI in an oxidized state poised for the transfer of disulphide bonds to substrate proteins.

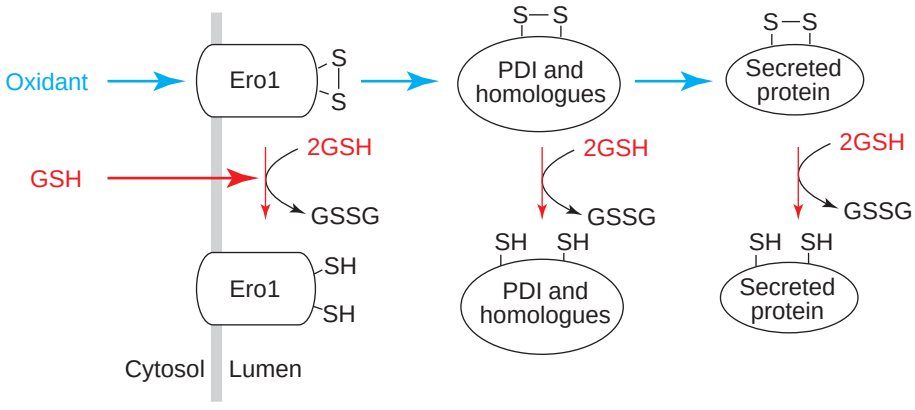

FIGURE 3

Redox balance in the endoplasmic reticulum (ER). Oxidation of glutathione competes with protein oxidation in the ER. Reduced glutathione (GSH) imported into the ER lumen engages in thiol–disulphide exchange reactions, leading to the net reduction of secretory proteins and the net oxidation of GSH. GSH could interact with ER oxidoreductases and proteins folding in the ER. Reducing equivalents contributed by GSH (red) might be important for maintaining optimal redox conditions for protein folding, by counterbalancing the flow of oxidizing equivalents through Ero1p (blue arrows). GSSG, oxidized glutathione; PDI, protein disulphide isomerase.

Similarities in eukaryotic and prokaryotic disulphide-bond-forming pathways

The pathway for protein disulphide bond formation in the bacterial periplasm provides a useful analogy for the

protein oxidation system in eukaryotes. Two enzymes drive disulphide bond formation in periplasmic proteins: the thioredoxin-like thiol–disulphide oxidoreductase DsbA and the cytoplasmic membrane protein DsbB[29,30] (Table 1). The active-site cysteines of DsbA form a disulphide bond that is transferred directly to periplasmic proteins[31], after which DsbA is efficiently re-oxidized by DsbB[32] (Fig. 4). This oxidation pathway proceeds entirely through the protein-to-protein transfer of oxidizing equivalents via thiol–disulphide exchange, as demonstrated by the capture of disulphide-linked heterodimers between DsbA and DsbB[33,34]. A small-molecule intermediate such as glutathione is unlikely to serve as an intermediate in this pathway, in part because the permeability of the outer membrane of Gram-negative bacteria does not allow for the concentration of low-molecular-weight thiol compounds in the periplasm.

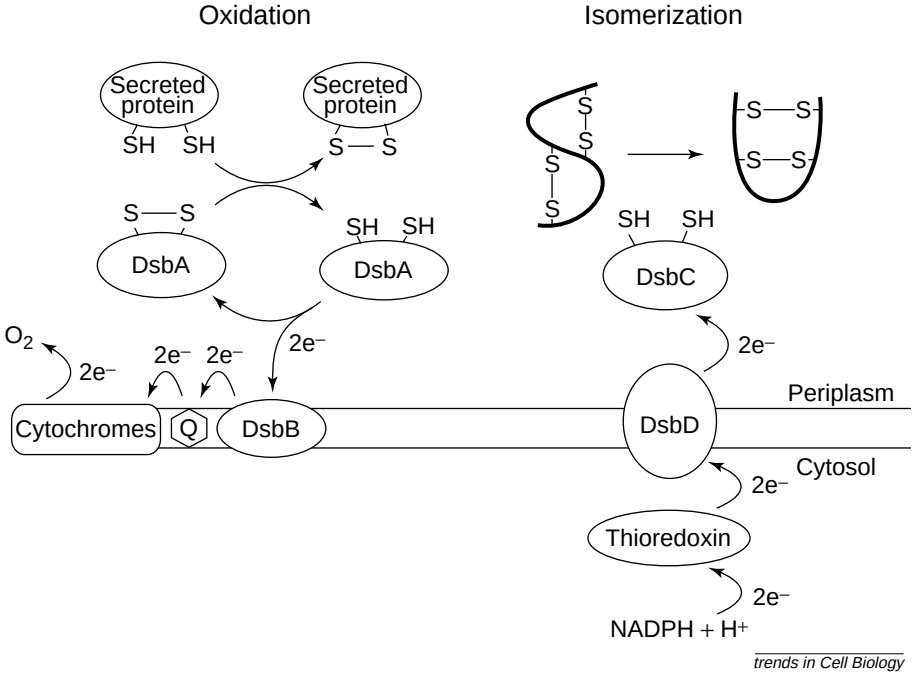

FIGURE 4

Pathways for protein disulphide bond formation and isomerization in the *Escherichia coli* periplasm. Secreted proteins are oxidized through thiol–disulphide exchange reactions with the soluble oxidoreductase DsbA, which is then re-oxidized by the cytoplasmic membrane protein DsbB. Under aerobic growth conditions, electrons flow from DsbB to molecular oxygen via ubiquinone (Q) and cytochrome *bo* or *bd* oxidase. The oxidoreductase DsbC catalyses disulphide bond rearrangements. Electrons flow from cytoplasmic thioredoxin to DsbC via the cytoplasmic membrane protein DsbD.

In both prokaryotic and eukaryotic systems, a membrane-associated oxidoreductase is thus implicated in the direct transfer of disulphide bonds to a soluble, thioredoxin-like oxidoreductase. Aside from the fact that the ER contains glutathione and the periplasm does not, the similarities between the key components of eukaryotic and prokaryotic disulphide-bond-forming systems are striking. For example, mutations in both *PDI1* and *dsbA* disrupt the oxidation of secretory proteins, and PDI can complement mutations in *dsbA* when targeted to the bacterial periplasm[16,31,35]. The *ero1* mutant of *Saccharomyces cerevisiae* appears primarily defective in re-oxidation of Pdi1p[16], and, in similar fashion, the *Escherichia coli dsbB* mutant is primarily defective in re-oxidation of DsbA[36] (Table 1).

In the bacterial periplasm, the isomerization of non-native disulphide bonds proceeds through a separate pathway analogous to the DsbA–DsbB system. Two components of this pathway are the periplasmic oxidoreductase DsbC and the cytoplasmic membrane protein DsbD[29,30] (Fig. 4 and Table 1). DsbC is likely to serve as a catalyst for disulphide reshuffling *in vivo* because the loss of DsbC selectively disrupts the folding of those proteins with multiple disulphide bonds[37]. Consistent with activity as an isomerase, the active-site cysteines of DsbC are found in dithiol form. Maintenance of DsbC in dithiol form requires a net influx of reducing equivalents from DsbD (Fig. 4), possibly to counteract a tendency for DsbC to be oxidized by DsbB[30,38]. Alternatively, if catalysis by DsbC involves the reductive cleavage of inappropriate disulphide bonds with the concomitant oxidation of DsbC, then DsbD might be necessary to regenerate reduced, active DsbC[30]. In this case, complete disulphide bond formation in the substrate protein would require an additional oxidation step likely to be performed by DsbA[30]. Interestingly, reducing equivalents delivered to DsbC from DsbD are derived from cytoplasmic thioredoxin, indicating that DsbD transmits reducing potential across the cytoplasmic membrane[38] (Fig. 4).

The family of PDI homologues

Several oxidoreductases homologous to PDI are found in the ER of both yeast and mammalian cells (Table 2). These PDI homologues have been implicated in diverse processes including not only oxidative protein folding but also the assembly of multiprotein complexes and the recognition of misfolded proteins in the ER[39]. Erp57 and Erp72 are mammalian homologues of PDI induced under conditions of ER stress[39] (Table 2). Yeast homologues of PDI expressed in the ER lumen are Mpd1p, Mpd2p, Eug1p and Eps1p[20–22,40]. Although Pdi1p is the only oxidoreductase strictly required for yeast viability, overproduction of any one of these enzymes can at least partially substitute for the loss of *PDI1*[20–22,40]. The *EPS1* gene has recently been implicated in the ER retention of a misfolded variant of Pma1p, the plasma membrane ATPase[22].

One attractive possibility is that individual members of the PDI family might be dedicated to the catalysis of disulphide bond formation, reduction or isomerization *in vivo*, just as DsbA and DsbC work in separate pathways to catalyse disulphide bond formation and isomerization in the bacterial periplasm. For example, Mpd2p might serve as a dedicated oxidase, given that the enzyme is a substrate for Ero1p[16]. If homologues of PDI do serve as dedicated catalysts of specific redox reactions, then an intriguing question arises as to how their specificity could be established.

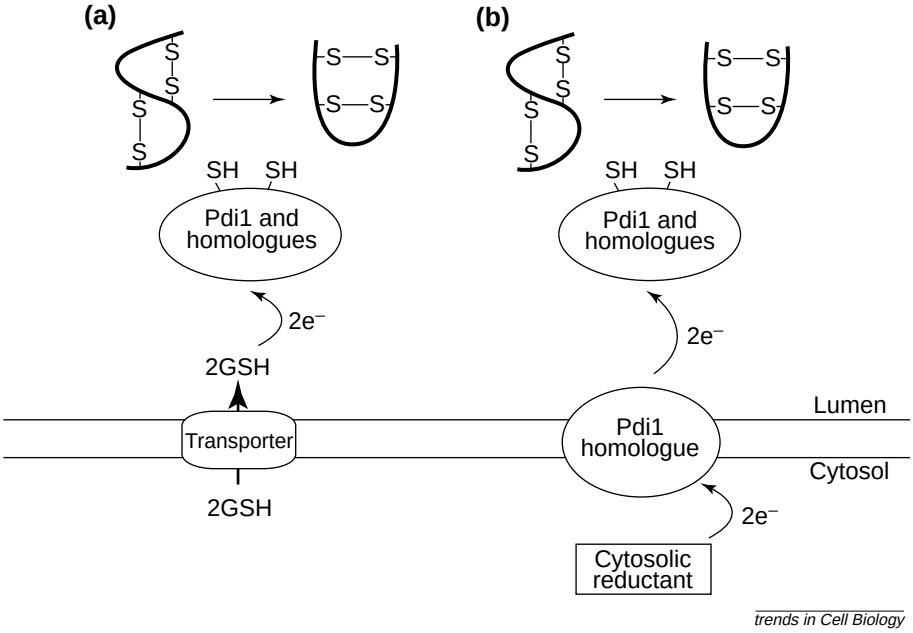

FIGURE 5

Model pathways for the isomerization of disulphide bonds in the endoplasmic reticulum (ER). Thioredoxin-like oxidoreductases localized to the ER lumen catalyse disulphide bond rearrangements. In (a), reduced glutathione (GSH) imported into the ER lumen provides reducing equivalents needed to maintain the isomerase(s) in the active, dithiol form. In (b), a membrane-localized oxidoreductase transmits electrons to isomerase(s) from cytosolic reductants.

The catalytic efficiency of Ero1p towards different homologues of PDI might vary widely and in part determine the catalytic niche of each enzyme. The redox potential of the active site of each enzyme could also be adapted to favour oxidase or reductase activity. However, the exact redox potential of an enzyme might not fully account for its functional specificity. For example, DsbA and DsbC have similar redox potentials yet appear in different oxidation states and perform discrete functions in the bacterial periplasm. In the ER, functional distinctions among the redox activities of PDI homologues must be maintained in the presence of a glutathione redox buffer[12]. It is possible that the PDI homologues might undergo thiol–disulphide exchange reactions with molecules such as Ero1p much more rapidly than they do with glutathione, kinetically isolating glutathione from certain disulphide-bond-forming pathways[28].

Individual PDI homologues have been shown to interact physically with discrete classes of secretory proteins, adding yet another layer to the potential specificity of these enzymes *in vivo*[39]. Such interactions can be independent of oxidoreductase activity and in some cases appear to involve thioredoxin-like domains that lack a Cys-x_a-x_b-Cys motif[39] (Table 2). Mammalian ERp57 provides an example of an enzyme interacting with a specific class of substrates. This enzyme engages directly in thiol–disulphide exchange reactions with nascent glycoproteins folding in the ER but interacts only with monoglycosylated substrates via a tripartite interaction with calnexin or calreticulin[18,39,41]. Association with calnexin stimulates the isomerase activity of ERp57 *in vitro*[42].

There are serious obstacles to dissecting the specific functions of individual members of the PDI family through biochemical or genetic analysis. These enzymes can, according to how an assay is configured, display activity either as oxidases, reductases or isomerases *in vitro*. The potential for functional redundancy among these enzymes complicates genetic analysis, especially because the loss of these gene products, through the accumulation of misfolded proteins, often leads to the general induction of ER oxidoreductases and chaperones with the unfolded protein response (UPR)[43]. Thus, defects in oxidative protein folding associated with the loss of any one PDI homologue might be largely obscured through a compensatory induction by the UPR of enzymes with redundant activities.

Models for disulphide bond isomerization in eukaryotes

The similarities observed thus far between eukaryotic and prokaryotic disulphide-bond-forming systems suggest that commonalities might also be found between disulphide bond isomerization pathways in eukaryotes and the DsbC–DsbD system in prokaryotes. Eukaryotic secretory proteins typically contain more disulphide bonds than their prokaryotic counterparts, indicating that there might be an even greater need for disulphide bond reducing or reshuffling functions in the ER than in the periplasm. However, genetic tests for the requirements for the oxidative folding of proteins with multiple disulphide bonds have not yet been developed in eukaryotes, so the components of isomerization pathways in the ER remain unclear. PDI and some subset of the PDI homologues are likely to serve as the immediate catalysts for disulphide reshuffling in the ER[4,19] (Fig. 5). However, one would predict that the active-site cysteines of a dedicated isomerase would be found in dithiol form *in vivo*, a criterion not met by yeast Pdi1p or Mpd2p[16]. If Pdi1p functions largely as an oxidase, then other PDI homologues might be needed in addition to Pdi1p to catalyse disulphide reshuffling in the yeast ER. Eug1p is a strong candidate for a dedicated isomerase in yeast as the active sites of this enzyme have the sequence Cys-x_a-x_b-Ser, a configuration that should preclude activity as an oxidase or reductase[40]. GSH imported into the ER lumen could serve a physiological role analogous to that of prokaryotic DsbD, by keeping isomerases in their reduced, active form (Fig. 5). Alternatively, a PDI homologue associated with the ER membrane, such as Eps1, might perform this reductive function by transmitting reducing equivalents to ER isomerase(s) from the cytosol (Fig. 5).

Where do the oxidizing equivalents come from?

Although Ero1p appears to be the key conduit for introducing oxidizing equivalents into the ER lumen, it remains unclear how Ero1p itself is re-oxidized. Because Ero1p engages in thiol–disulphide exchange with Pdi1p, the identification of one or more

redox-active cysteine pairs is anticipated in Ero1p. These active-site cysteines might be found amongst the seven conserved cysteine residues of Ero1p, three of which appear in the sequence Cys-x-x-Cys-x-x-Cys near the C-terminus of Ero1p[14,15].

Work in bacteria has shown that DsbB is re-oxidized predominantly through the transfer of electrons to molecular oxygen via later stages of the respiratory electron transport chain. Accordingly, depletion of intracellular pools of haem or ubiquinone and menaquinone will disrupt the flow of oxidizing equivalents into the DsbB–DsbA system[44,45]. Moreover, *in vitro*, electrons flow from DsbB directly to ubiquinone associated with either cytochrome *bd* or cytochrome *bo* oxidase, and these two haem-containing terminal oxidases then shuttle electrons to molecular oxygen[32] (Fig. 4). When *E. coli* are grown anaerobically, menaquinone might transfer electrons from DsbB to alternative acceptors[32].

In yeast, at least two cytochrome-based systems that reside in the ER membrane are responsible for sterol and unsaturated fatty acid biosynthesis[46]. Both systems transfer electrons directly to molecular oxygen and could potentially serve as electron sinks for the relatively small number of electrons released during disulphide bond formation in the ER lumen. However, all of these electron transport chains are thought to be oriented to the cytosolic face of the ER, so, if they are in fact coupled to re-oxidation of Ero1p, then a lipid-soluble small-molecule electron acceptor might be needed to shuttle between the lumenal and cytosolic leaflets of the ER membrane. As yet, a small-molecule electron acceptor for re-oxidation of Ero1p has not been identified. Because Ero1p behaves like an integral membrane protein, it is also possible that a portion of Ero1p has direct access to proteins of the ER-based electron-transport machinery.

Summary and future prospects

The work reviewed here places Ero1p and Pdi1p in an enzymatic pathway for protein disulphide bond formation in the ER[16] and demonstrates that intralumenal GSH competes with protein thiols for oxidizing equivalents derived from Ero1p[25]. These studies provide a solid framework for further genetic and biochemical analysis of oxidative protein folding in eukaryotes. It will be of great interest to see whether analogies between eukaryotic and prokaryotic systems continue to hold as more details of the mechanism of Ero1p re-oxidation emerge. The contributions of individual PDI homologues to oxidative protein folding in the ER might become clearer once genetic tests are developed to identify factors required for disulphide bond isomerization *in vivo*. Further, the reconstitution of oxidative protein folding driven by Ero1p should enable a biochemical dissection of the interactions of Ero1p, the PDI homologues and glutathione with secretory proteins in an environment more closely resembling the ER lumen than could previously be achieved under assay conditions in which oxidizing equivalents are supplied by GSSG. Analysis of oxidative protein folding under native conditions should elucidate how cells meet the specific requirements for disulphide bond formation in a wide array of secretory proteins.

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Stem cells show their potential

Sally Lowell

The past year has seen some dramatic breakthroughs in stem cell research[1]. The January 2000 Keystone conference* brought together researchers ranging from developmental biologists to clinical haematologists to discuss progress in this exciting field. Despite these diverse backgrounds, several common themes emerged during the conference.

*Keystone Symposium on Stem Cells, Asymmetric Cell Division and Cell Fate; Keystone, Colorado, USA; 17–22 January 2000. Organized by M.T. Fuller, C.Q. Doe and I.L. Weissman.

Fate decisions

Stem cells are defined by their ability to self-renew and to generate differentiated cells[2]. They produce all the tissue types that make up an adult organism during development, and are responsible for the renewal and repair of adult tissues, such as the blood, the lining of the gut, and the epidermis.

How is diversity generated in the daughters of stem cells? It is easy to see how invariant asymmetric cell divisions could always generate one stem and one nonstem daughter. However, most strategies are based on populational asymmetry, with cell fate being responsive to extrinsic factors in the local environment.

A well-studied example of an invariant strategy is the asymmetrical localization of cell fate determinants, such as numb, within a pre-mitotic precursor cell (Fig. 1). This, together with correct orientation of the mitotic spindle, generates daughters with distinct identities. The machinery that directs asymmetric cell division and spindle localization is being uncovered in genetically tractable organisms such as *Drosophila*, *Caenorhabditis elegans* and yeast[2]. It now seems that this machinery could be conserved in vertebrates. S. Temple (Albany, USA) has demonstrated, by using long-term time-lapse microscopy, that mouse cortical neuronal precursors follow stereotyped reiterative asymmetric cell divisions that correlate with asymmetric localization of the mouse homologue of numb.

Extrinsic signals that maintain *Drosophila* germline stem cells, such as the nucleoplasmic factor PIWI, are provided by a specialized somatic microenvironment within the ovary, a ‘stem cell niche’. H. Lin (Durham, USA) has now found a PIWI homologue that is associated with the male germline in vertebrates.

Feedback mechanisms also influence the balance of two different fates within a population. For example, the signalling protein Delta, expressed on differentiating neurons, suppresses differentiation of neighbouring precursors. In newly discovered variations on this theme, Delta directs neural crest stem cells to differentiate into glia

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