Native disulfide bond formation in proteins

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Native disulfide bond formation is critical for the proper folding of many proteins. Recent studies using newly identified protein oxidants, folding catalysts, and mutant cells provide insight into the mechanism of oxidative protein folding *in vivo*. This insight promises new strategies for more efficient protein production.

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Current Opinion in Chemical Biology 2000, **4**:533–539

1367-5931/00/$ – see front matter

Abbreviations

BMC	(±)-*trans*-1,2-bis(2-mercaptoacetamido)cyclohexane
CPY	carboxypeptidase Y
DTT	dithiothreitol
ER	endoplasmic reticulum
Ero1p	endoplasmic reticulum oxidoreductin 1 protein
GSH	reduced glutathione
GSSG	oxidized glutathione
IGF-I	insulin-like growth factor-I
PDI	protein disulfide isomerase
RNase	ribonuclease
tPA	tissue plasminogen activator
vtPA	truncated tPA

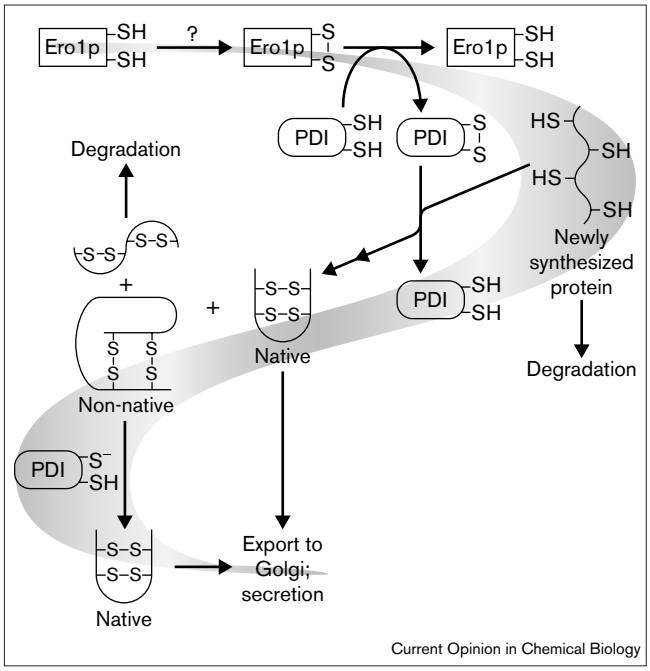
Introduction

The formation of disulfide bonds in proteins requires a sufficiently oxidizing environment [1]. Eukaryotic cells contain compartments of widely varying reduction potential ($E^{\circ\prime}$) [2]. Those proteins destined for secretion are co-translationally translocated into the oxidizing environment of the endoplasmic reticulum (ER), $E^{\circ\prime} = -0.18$ V, where they can fold and acquire their native disulfide bonds. Oxidative folding in the ER of the yeast *Saccharomyces cerevisiae* requires two proteins: endoplasmic reticulum oxidoreductin 1 protein (Ero1p) and protein disulfide isomerase (PDI).

Ero1p is a 65 kDa, membrane-associated resident of the ER lumen that is essential for *S. cerevisiae* viability. *In vivo*, Ero1p oxidizes disulfide-containing proteins. Ero1p was identified using genetic screens for proteins that either, when overproduced, confer resistance to dithiothreitol (DTT; a small-molecule reductant) or, when mutated, cause sensitivity to DTT. Addition of the thiol oxidant diamide to the growth medium can complement an Ero1p deficiency, presumably by performing the oxidative function of the missing gene product. These results indicate that the essential function of Ero1p is to oxidize newly synthesized proteins [3,4].

PDI is a 57 kDa resident of the ER that is essential for *S. cerevisiae* viability. *In vitro*, PDI catalyzes three reactions: the oxidation of thiols and the reduction and isomerization of disulfides. The two PDI active sites consist of a CXXC motif with the sequence: Cys–Gly–His–Cys. Experiments using active-site variants of PDI indicate that its essential function is the isomerization of non-native disulfide bonds (Figures 1 and 2) [5,6].

Figure 1



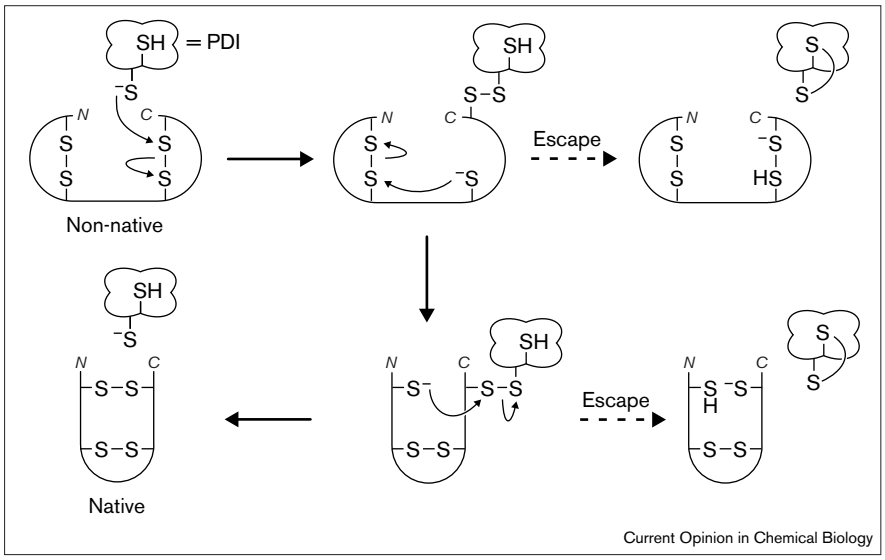
Primary pathway of native disulfide bond formation in the ER. PDI transfers oxidizing equivalents from Ero1p to reduced, unfolded proteins [9••]. Cells lacking Ero1p cannot oxidize newly synthesized proteins in the ER and are inviable. Non-native disulfide bonds must isomerize to the native state. Those proteins that do not attain the native state are degraded rather than secreted, and cells lacking a disulfide isomerase are inviable [5]. For simplicity, only one of the two active sites of PDI is shown.

The CXXC active-site motif of PDI is also found in homologous thiol–disulfide oxidoreductases [7]. In bacteria, the pathway for native disulfide bond formation involves the CXXC-containing Dsb family of proteins (DsbA–D) and may be analogous to the eukaryotic pathway [8]. This review will focus on intriguing new insights into the mechanism of oxidative folding for eukaryotic secretory proteins and how these developments are being harnessed to guide the efficient production of these proteins.

Role of protein oxidants

Oxidative protein folding involves both the oxidation of thiols and the isomerization of non-native disulfide bonds. Recently, Frand and Kaiser [9••] delineated how Ero1p

Figure 2



Putative mechanism of disulfide bond isomerization [15••]. Isomerization begins with nucleophilic attack of a thiolate provided by the catalyst (such as PDI) on a non-native disulfide bond. The resulting, covalent substrate–catalyst complex contains a substrate thiolate and can perform intramolecular thiol–disulfide exchange reactions to form native disulfide bonds and, eventually, release of the catalyst (redox-inactive mechanism). The second thiol in the active site of the catalyst can act as a clock to ensure timely rearrangement of the substrate [16]. Those substrates that are slow to rearrange can be partially reduced (and subsequently reoxidized) by a dithiol catalyst (reduction–reoxidation mechanism). Those catalysts lacking a second thiol can become trapped in mixed disulfides with the substrate.

and PDI accomplish the first step of this process (Figure 1). Ero1p and PDI form a disulfide-linked complex *in vivo*. Likewise, PDI and carboxypeptidase Y (CPY), a protein that contains five disulfide bonds, form mixed disulfides. Reduced forms of both PDI and CPY accumulate in cells lacking functional Ero1p. Reduced CPY also accumulates in the ER of PDI-depleted cells. Thus, oxidizing equivalents flow from Ero1p to PDI to substrate proteins (Figure 1).

Apparently, only a small fraction of the PDI active sites are reduced in the ER [9••]. Yet, the dithiol form of PDI is required for catalysis of disulfide isomerization, its essential function (Figure 2). An $E^{\circ\prime}$ of –0.18 V for the ER was found by measuring the concentrations of reduced glutathione (GSH) and oxidized glutathione (GSSG) [2]. This value is in gratifying agreement with the *in vitro* optimum for the oxidative folding activity of PDI [7]. In a solution of $E^{\circ\prime}$ = –0.18 V, fully 50% of the PDI active sites should be reduced [6]. Either the ER is much more oxidizing than was believed previously or, *in vivo*, PDI is maintained out of equilibrium with its environment and away from its *in vitro* optimum. Determining the relative contributions of oxidase and isomerase activities during catalysis by PDI remains an interesting challenge.

Although the *in vivo* oxidase role of PDI has been underappreciated, this role is not essential. Even though oxidation of newly synthesized proteins is significantly impaired in the absence of PDI [9••], a variant of PDI with CGHS active sites (using single-letter amino acid code) can complement a wild-type PDI deficiency. This variant cannot catalyze reduction or oxidation, but it is an efficient catalyst of disulfide isomerization (a redox-inactive process) [5,6]. Perhaps Ero1p can oxidize proteins directly and PDI is required for the proper folding of Ero1p, which contains 14 cysteine residues. Alternatively, Ero1p-independent oxidation pathways may exist in the ER.

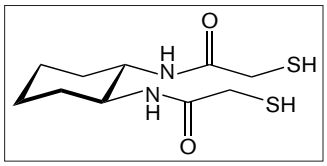
A role for GSSG in the oxidation of proteins has been ruled out [10•]. GSSG has long been thought to be the primary source of protein oxidation, with the accumulation of GSSG being a result of its selective import into the ER [2]. Yet, GSSG can be formed in the ER from GSH by the action of Ero1p. Mutant strains of yeast unable to synthesize glutathione show no defect in protein disulfide formation. Indeed, knocking out glutathione synthesis restores viability to cells lacking functional Ero1p. Disulfide bonds form efficiently in the ER of these cells. Taken together, these results indicate that glutathione contributes net reducing equivalents to the ER and actually competes with protein thiols for oxidation (see also Update). What is the role of glutathione in the ER? Most likely, it acts as a buffer against transient changes in oxidative stress [10•]. Perhaps it also helps to maintain the essential pool of reduced PDI.

If cells lacking Ero1p and glutathione can exhibit normal oxidation kinetics, then the ER must possess other oxidants. One candidate is sulfhydryl oxidase, an enzyme that catalyzes the oxidation of thiols by O_2 [11]. Another putative electron acceptor is flavin-containing monooxygenase, an O_2- and NADPH-dependent catalyst of thiol oxidation that is localized to the cytosolic face of the ER [12]. The appeal of both sulfhydryl oxidase and flavin-containing monooxygenase as putative oxidants is the direct link they provide to the ultimate electron acceptor, O_2.

Ero1p is a primary source of protein oxidation in the ER. How Ero1p becomes oxidized remains unknown (Figure 1). Ero1p performs a function analogous to that of the bacterial protein DsbB [8]. In *Escherichia coli*, the respiratory chain

Table 1

Properties of BMC.

Structure (see right)	
Molecular mass (Da)	262
$E^{\circ\prime}$ (V)	–0.24
pK_a	8.3; 9.9
% Thiolate*	0.05

*Calculated for the conditions: $E^{\circ\prime}$ solution = –0.18 V; pH 7.0; 30°C. Data from [15••].

can oxidize DsbB and perhaps provide the ultimate source of protein oxidation [13,14]. Ero1p may possess an Fe–S cluster that accepts electrons from its active-site cysteine residues and transfers them to the respiratory chain, enabling further rounds of oxidation [3,4] (see also Update).

Importance of covalent catalysis

Although efficient oxidation is undoubtedly important, the isomerization of non-native disulfide bonds may often limit the folding rate of many proteins. Indeed, the essential *in vivo* function of PDI is to catalyze the unscrambling of disulfide bonds in other proteins [5,6]. The simplest mechanism for PDI-catalyzed disulfide isomerization is shown in Figure 2. A striking feature of this mechanism is that it requires only the provision of a reactive thiolate on the part of the catalyst.

Each CGHC active site of PDI possesses a high disulfide reduction potential ($E^{\circ\prime}$ = –0.18 V) and an often unprotonated thiol (pK_a = 6.7). By combining these properties, it can be calculated that during the conditions of efficient *in vitro* oxidative folding ($E^{\circ\prime}$ = –0.18 V, pH 7.0, and 30°C), 33% of PDI active sites contain a thiolate [6]. To effect the mechanism shown in Figure 2, PDI can be pared down to a thiolate.

Support for this mechanism (Figure 2) comes from the activity of the dithiol: (±)-*trans*-1,2-bis(2-mercaptoacetamido)cyclohexane (BMC) [15••]. The physical properties of BMC are listed in Table 1 and are similar to those of PDI. Unlike PDI, BMC cannot bind to a protein substrate. Nevertheless, using only covalent interactions, BMC is able to catalyze native disulfide bond formation, both *in vitro* and *in vivo*.

In vitro, BMC catalyzes the reactivation of scrambled ribonuclease (RNase) A, a substrate with four non-native disulfide bonds [15••]. In this assay, BMC and PDI produce similar yields of folded RNase A. GSH and a monothiol analogue of BMC each give lower yields than do the dithiols. In effect, the second thiol provides an intramolecular clock for substrate-induced thiol–disulfide exchange [16]. Those substrates that are slow to rearrange can be released through the formation of a disulfide bond within the catalyst. These partially reduced intermediates now have a second thiolate to induce additional disulfide rearrangements and can eventually be reoxidized by the catalyst. The contribution of this reduction–reoxidation mechanism is determined in part by the effective concentration of the active-site thiols [1,17]. The redox-inactive mechanism requires at least two productive thiol–disulfide exchanges within the substrate before catalyst release (Figure 2). A higher effective concentration within the catalyst will decrease the contribution of the redox-inactive mechanism.

Table 2

Production of folded acid phosphatase in yeast*.

Condition	Relative yield
PDI (basal)	1
PDI (basal) plus BMC (0.2 mg/ml)	3
PDI (15-fold overproduction)	3

*Data from [15••,18].

BMC added to the growth medium of *S. cerevisiae* cells producing *Schizosaccharomyces pombe* acid phosphatase, which has eight disulfides, causes more efficient protein folding *in vivo* and results in a threefold increase of secreted, active enzyme [15••]. This increase is equivalent to that achieved with co-overproduction of PDI (Table 2) [18]. The accordance of achievable yield between exogenously added BMC and endogenously produced PDI suggests a shared mechanism of action. Like PDI, BMC may function *in vivo* as a direct catalyst of disulfide isomerization. Still, BMC could act either to reduce a greater fraction of the basal PDI present in the ER, increasing its efficiency as an isomerase, or to oxidize acid phosphatase directly (after having been oxidized itself). Regardless, at optimum concentrations of BMC, a step other than native disulfide bond formation probably limits the secretion of acid phosphatase.

BMC may provide better methods for the production of disulfide-bonded proteins. The action of BMC *in vivo* may allow for increased yields of active protein simply through its addition to the growth medium. BMC-based redox buffers may also prove useful for the efficient *in vitro* folding of proteins from inclusion bodies.

Support for the biological relevance of the mechanism shown in Figure 2 comes from the observation by Molinari and Helenius [19•] of transient mixed disulfides between PDI and a newly synthesized viral glycoprotein *in vivo*. These complexes provide the first demonstration of a covalent interaction between PDI and a substrate in mammalian cells. Together with the observation of disulfide-linked complexes between PDI and CPY or Ero1p in yeast cells [9••], these studies support an *in vivo* role for the mechanism of PDI catalysis derived from *in vitro* studies (Figure 2). It is not clear whether these complexes involve substrates undergoing oxidation or isomerization (or both).

Molinari and Helenius [19•] also found that PDI has substrate specificity *in vivo*. Although PDI was only observed in mixed disulfides with one of two disulfide-containing

viral proteins, its homolog ERp57 was found linked to both substrates. A pancreas-specific homolog of PDI, but not PDI itself, has been found to specifically recognize peptides containing tyrosine or tryptophan residues [20]. The biological relevance of substrate specificity during PDI catalysis is not yet clear.

The *in vitro* folding pathway of proteins with multiple disulfide bonds is complex [21]. During the folding of reduced RNase A, PDI has little effect on the distribution of single-disulfide intermediates formed in glutathione [22] or DTT [23] redox buffers. The rate-limiting step during the *in vitro* folding of RNase A [23] and lysozyme [24] involves isomerization of partially oxidized intermediates. PDI increases the rate of oxidative folding *in vitro* by isomerizing substrates that are neither fully oxidized nor fully reduced.

Structural basis for catalysis by PDI

The three-dimensional structure of PDI remains unknown. PDI consists of four domains with structural homology to the CXXC-containing oxidoreductase thioredoxin plus an acidic carboxy-terminal domain [25]. The first and fourth domains contain the two active sites. Recent studies have identified an important role for the third domain in substrate binding [26,27]. The carboxy-terminal domain is not required for catalytic activity [28].

Recent crystal structures of a PDI homolog from *Pyrococcus furiosus* composed solely of two catalytic domains [29] and of a dimeric *E. coli* thioredoxin active-site variant with enhanced isomerase activity [30] provide clues as to how the thioredoxin-like domains of PDI may interact. In both structures, the thioredoxin domains form a continuous β-sheet (though the interfaces occur at opposite edges of the β-sheet). This arrangement suggests that the thioredoxin domains of PDI may be colinear.

A crystal structure of the dimeric bacterial PDI homolog DsbC provides a slightly different model for the structure of PDI [31•]. The DsbC dimer contains two catalytic domains and two dimerization domains. This arrangement is reminiscent of the four thioredoxin-like domains in PDI. The dimer structure is V-shaped, with the two CXXC active sites pointing towards each other across a large, hydrophobic cleft. This cleft seems well-suited for the binding of unfolded proteins. The catalytic and dimerization domains in a DsbC monomer are connected by a hinged linker helix that allows for conformational changes associated with substrate recognition. Such flexibility has been implicated in catalysis by PDI [27], though it is not known if PDI possesses interdomain linkers similar to those in DsbC [32]. Knowledge of the structure of intact PDI will enable a more detailed model of the mechanism by which PDI catalyzes native disulfide bond formation.

Importance of redox environment

Stable disulfide bonds rarely form in the cytosol [33]. The bacterial cytosol ($E^{\circ\prime} = -0.27$ V) is normally even more reducing than its eukaryotic counterpart ($E^{\circ\prime} = -0.23$ V) [2] and is not a good environment for the production of properly folded, multiply disulfide-bonded proteins. Of course, bacterial expression systems can produce large quantities of heterologous proteins. Unfortunately, insoluble aggregates of these proteins tend to form when they misfold, and the *in vitro* folding of these inclusion bodies can be difficult and time-consuming [34].

One approach to this problem has been to secrete disulfide-bonded proteins along with PDI to the more oxidizing periplasm in *E. coli* [35]. This approach is hit-or-miss, as rat PDI does not improve yields of human tissue plasminogen activator (tPA), a 17-disulfide protein, but yeast PDI increases the yield by 50%. In addition to problems of specificity, PDI activity may be hindered by the redox environment of the periplasm. The yield of active tPA obtained by coproduction of yeast PDI was lower than that obtained from cells overproducing DsbC [36], whose CXXC active site is maintained in the reduced state [8]. Perhaps the periplasmic environment is too oxidizing for efficient catalysis of disulfide isomerization by PDI. The ability of PDI to function in place of the periplasmic oxidant DsbA during the oxidative folding of *E. coli* alkaline phosphatase supports this idea [35].

In the *E. coli* periplasm, the prior overproduction of DsbC or DsbA (the bacterial analogue of Ero1p) doubles production of insulin-like growth factor-I (IGF-I) relative to the simultaneous coproduction of the proteins [37]. The abundance of Dsb proteins in the periplasm during production of IGF-I leads, however, to increased aggregation rather than more efficient *in vivo* folding. Fortunately, IGF-I can be folded easily from inclusion bodies, and 8.5 g of IGF-I can be isolated from 1 liter of cell culture. This high yield may be due to protection of translocated IGF-I from proteolysis or assistance in its translocation. Surprisingly, most of the DsbA in cells after its transient overproduction is found in the reduced form rather than the oxidized form that normally accumulates and is responsible for catalysis of protein oxidation in the periplasm [8]. The decrease in periplasmic folding efficiency when IGF-I is produced subsequent to DsbA or DsbC overproduction argues against improved thiol–disulfide exchange as a cause of the boost in yield [37].

Manipulation of the redox environment in the bacterial cytosol may allow for more efficient oxidative protein folding *in vivo* [38••]. Such a strategy has been made possible by the development of *E. coli* strains that grow normally despite having an oxidizing cytosol. These strains were isolated as suppressors of the slow-growing phenotype displayed by cells lacking the genes for thioredoxin reductase and glutathione synthesis. The cytosolic folding of four normally secreted, multiply disulfide-bonded proteins was more efficient in these strains than in wild-type cells. Thioredoxin, which normally acts as a cytosolic reductant, acts as a protein oxidant in these strains [39],

consistent with its ability to act as an oxidant when exported to the periplasm [40,41].

The cytosolic folding of truncated tPA (vtPA), which has nine disulfides, can be improved by coproduction of active-site thioredoxin variants with higher $E^{\circ\prime}$. The optimum yield of vtPA in the cytosol is obtained with coproduction of DsbC in these mutant strains, which gives a 200-fold increase in folding efficiency relative to wild-type cells (Table 3). This result underscores the importance of maintaining a dithiol catalyst for the shuffling of non-native disulfide bonds (Figure 2). Surprisingly, the coproduction of PDI in the cytosol had little effect on the cytosolic folding of vtPA. The folding of vtPA in the cytosol of the mutant strains was more efficient than was its folding in the periplasm of wild-type cells (Table 3) [38••]. Oxidation may be slower in the cytosol than in the periplasm, decreasing the need for disulfide isomerization, and thus explaining the greater efficiency of cytosolic folding.

Modulation of the redox environment in the cytosol may be an effective strategy for the production of eukaryotic secretory proteins in bacteria. Folding efficiency in this system could be improved further by coproduction of a disulfide isomerase with active-site properties appropriate for the redox environment. The mechanism of disulfide bond isomerization (Figure 2) can guide the design and choice of this catalyst.

Table 3

Production of utPA in bacteria*.

Condition	Relative yield
Wild-type cytosol	0.1
Oxidizing cytosol	1
Wild-type periplasm plus DsbC	10
Oxidizing cytosol plus DsbC	21

*Data from [38••].

Conclusions

The recent past has provided many exciting advances in the understanding of native disulfide bond formation. Among these is a new-found appreciation of the role played by protein oxidants such as Ero1p in the ER. This protein most probably functions as the primary electron acceptor in the PDI-catalyzed oxidation of newly synthesized proteins. Further, the long-held hypothesis that glutathione is the primary oxidant in the ER has been disproved. The elaboration of the roles played by other protein oxidants in the ER and identification of the oxidant for Ero1p remain interesting challenges. The observation of covalent complexes between PDI and newly synthesized proteins supports the mechanism of PDI catalysis developed from *in vitro* studies. Catalysis of protein folding by BMC, a small-molecule mimic of PDI, reveals the most important properties ($E^{\circ\prime}$ and pK_a) of PDI. Small-molecule PDI mimics could be useful for the efficient preparation of secretory protein, both *in vivo* and *in vitro*. The structure of PDI, which would aid in the design of novel catalysts and provide a more detailed understanding of its catalytic mechanism, has remained elusive. The structure of DsbC, however, provides a hint of how the four thioredoxin-like domains of PDI might interact. Lastly, the development of *E. coli* strains with an oxidizing cytosol provides a new strategy for efficient *in vivo* native disulfide bond formation. The modulation of the redox environment in the eukaryotic cytosol or the ER, perhaps through altered levels of glutathione or Ero1p (or both), in addition to the provision of properly designed folding catalysts, may provide further improvements in efficient protein production. Such a strategy depends on a firm understanding of the mechanism by which native disulfide bonds form.

Update

Additional proof that glutathione is not the primary thiol oxidant in the ER has been found [42]. The amount of glutathione excreted from yeast, subsequent to the overproduction of secretory proteins, does not correlate with the number of disulfide bonds in the secreted proteins. Moreover, glutathione excretion is dependent on O_2 and independent of functional vesicular transport. These results demonstrate that excreted glutathione does not result primarily from thiol oxidation in the ER and are consistent with a role for glutathione in protecting the ER from oxidative stress.

The gene for a human homolog of Ero1p, Ero1-L, has been cloned and its encoded protein has been characterized [43]. In yeast, Ero1-L can complement an Ero1p deficiency. Two cysteine residues in Ero1-L were found to be required for its function as an oxidant, consistent with a CXXC motif being conserved in the Ero1 family.

Acknowledgements

We thank EA Kersteen and TA Klink for helpful discussions and critical reading of the manuscript. KJW was supported by a WARF predoctoral fellowship and Chemistry-Biology Interface Training Grant GM08505 (National Institutes of Health). Work in the authors' laboratory was supported by grant BES-9604563 (National Science Foundation).

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9. •• Frand AR, Kaiser CA: **Ero1p oxidizes protein disulfide isomerase in a pathway for disulfide bond formation in the endoplasmic reticulum.** *Mol Cell* 1999, **4**:469-477.

This paper provides the first demonstration of a direct interaction between Ero1p and PDI. Redox-sensitive PDI–Ero1p and PDI–CPY complexes are trapped *in vivo* and identified by immunoprecipitation. With PDI production turned off, reduced CPY and oxidized Ero1p accumulate in the ER. No complexes between Ero1p and CPY are found. In wild-type cells, only a small fraction of the free PDI molecules are modified by an alkylating agent. In cells lacking functional Ero1p, almost all of the free PDI is modified, indicating that Ero1p maintains PDI in a mostly oxidized state. The authors conclude that the predominant pathway of protein oxidation in the ER is driven by Ero1p and mediated by PDI.

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This study abolishes the long-held notion that glutathione is the predominant protein oxidant in the ER. The production of GSSG *in vivo* is found to correlate with Ero1p activity. Growth of cells lacking functional Ero1p is restored by a knockout of glutathione biosynthesis, as is the oxidation of CPY. Induction of the unfolded protein response correlates with intracellular glutathione levels. The lack of glutathione causes an increase in sensitivity to exogenous addition of diamide, a thiol oxidant, indicating a role for reduced glutathione in protecting the ER against hyperoxidizing conditions.

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BMC, a small-molecule mimic of PDI, is synthesized and characterized. BMC increases the reactivation of scrambled RNase A *in vitro*. The yield of active RNase A is similar to that obtained with PDI and higher than that obtained with monothiol reagents. Further, addition of BMC to the growth medium of yeast cells producing acid phosphatase (like overproduction of PDI) causes an increase in the secretion of active enzyme. BMC is thus the first example of a small-molecule enzyme mimic that functions both *in vitro* and *in vivo*.

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This paper provides the first direct evidence that PDI is directly involved in the oxidative folding of glycoproteins. Mammalian cells were infected with a virus that causes all cellular protein synthesis to become dedicated to the production of viral proteins. These infected cells were pulse-labeled with radioactive sulfur, and the newly synthesized viral proteins were separated by two rounds of gel electrophoresis: the first under non-reducing conditions and the second in the presence of reductant. The two disulfide-containing viral proteins were found to form mixed disulfides with PDI and its homolog Erp57, but not with Erp72. The amount of these complexes present in the ER peaked 10 min after the pulse and then decreased steadily, illustrating the transient nature of these folding intermediates. Blocking proper glycosylation of the nascent proteins interfered with their oxidative folding.

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