Protein folding: **A missing redox link in the endoplasmic reticulum**

Robert B. Freedman, Angela D. Dunn and Lloyd W. Ruddock

Native disulphide-bond formation during protein folding in the endoplasmic reticulum requires oxidative machinery, the components and mechanism of which are not yet fully understood. Two recent papers have identified a novel protein component that appears to play a key role in this important redox pathway.

Address: Research School of Biosciences, University of Kent, Canterbury CT2 7NJ, UK.

Current Biology 1998, 8:R468–R470
http://biomednet.com/elecref/09609822008R0468

The lumen of the endoplasmic reticulum (ER) is a compartment specialized for protein folding; proteins destined for secretion, or for compartments accessed via the secretory pathway, enter the ER lumen unfolded, but leave it only when they are correctly folded and assembled. Recent work has focused on the nature of the quality control system at this point, but there is still much to be learnt about the cellular machinery responsible for folding and assembly in the ER. What is clear is that protein folding in this context is generally associated with the formation of native disulphide bonds, and that this is facilitated by an enzyme, protein disulphide isomerase (PDI), and by its more recently discovered homologues [1]. PDI is an excellent catalyst of disulphide-bond formation and isomerization, but any model for the way it acts on nascent proteins requires the additional participation of an oxidant, either to oxidize the substrate randomly (allowing PDI to act as an isomerase) or to reoxidize PDI (after it has acted as a direct oxidant towards reduced protein substrates). The identity of this partner oxidant remains obscure, but two recent parallel papers provide clues and a route for further investigation [2,3].

Various proposals have been put forward concerning the source of oxidizing equivalents for protein disulphide-bond formation in the ER lumen. Over 20 years ago, it was suggested (but never subsequently substantiated) that the immediate oxidant was cystamine, generated within the ER from cysteamine by a specific oxidase [4], implying that molecular oxygen was the ultimate oxidant. More recently, work on the vitamin-K-dependent system for carboxylation of nascent proteins suggested a link between this system and the system for protein disulphide-bond formation; if this were so, it would imply that vitamin K and its epoxide were the immediate source of oxidizing equivalents for protein disulphide-bond formation [5].

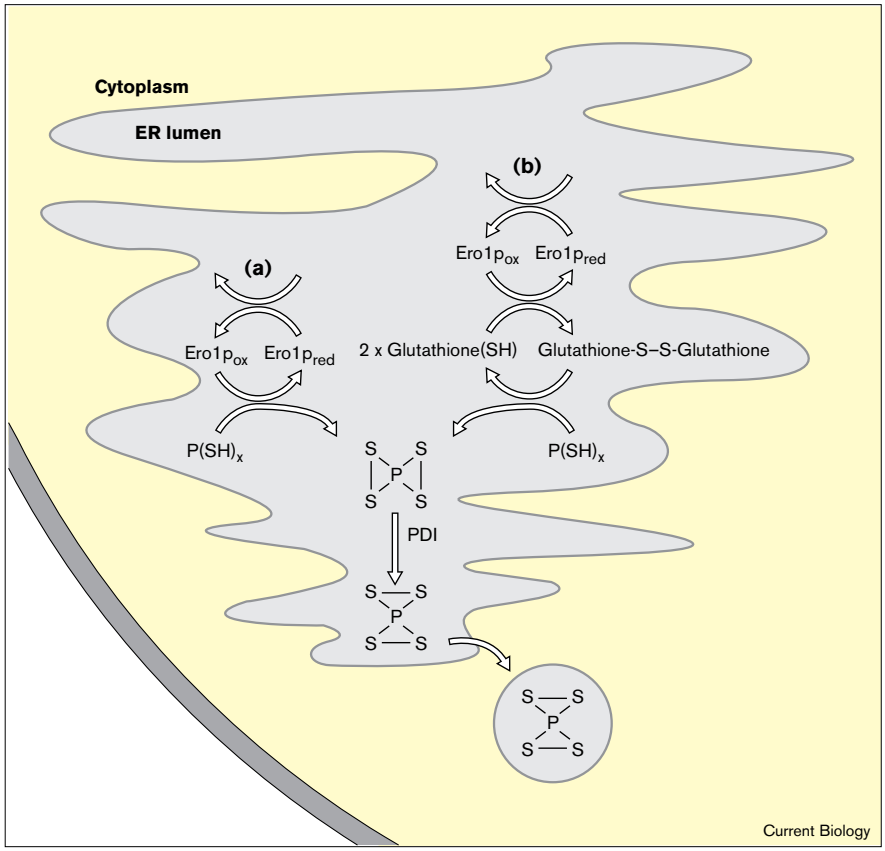
On the other hand, for many years it has been a strong presumption that the oxidant immediately involved is oxidized glutathione, the oxidized form of the eukaryotic cell's major low molecular weight thiol compound. This presumption has been strengthened by a direct study of the redox state of glutathione in the ER [6], which showed that glutathione is present in the compartment at an overall concentration similar to that in the cytosol, but in much more oxidizing proportions. There is also substantial biochemical evidence in favour of a role for glutathione. Studies on the post-translational oxidation of nascent proteins in the luminal volume of isolated microsomes show that oxidized glutathione added externally is a very effective and rapid oxidant [7,8]. In addition, studies of the secretion by yeast of recombinant mutant lysozymes have indicated that unpaired cysteine residues, lacking their normal disulphide-bonding partner, are instead secreted while disulphide-bonded to glutathione [9], suggesting a direct role for glutathione in protein oxidation. Glutathione cannot, however, be the whole story. How are the oxidizing equivalents transferred into the ER? Is it via a transport system actively concentrating oxidized glutathione, or is there an electron transfer pathway in the ER membrane that is involved in the delivery of oxidizing equivalents to nascent proteins? The two recent papers [2,3] strongly suggest the latter scenario.

The *ERO1* gene (*endoplasmic reticulum oxidoreductin 1*, YML130c) from the yeast *Saccharomyces cerevisiae* was isolated by the two groups [2,3] using independent screens for temperature-sensitive mutants, with subsequent confirmation of the identification of *ERO1* by selecting for genes from a yeast genomic library which complemented the defective phenotypes. Frand and Kaiser [2] screened temperature-sensitive mutants for defects in the export of secretory proteins from the ER, whereas Pollard *et al.* [3] exploited the fact that any mutant defective in components of the redox pathway may be sensitive to the reductant dithiothreitol (DTT), so they screened temperature-sensitive mutants for DTT sensitivity. In a separate, more direct screen, Pollard *et al.* [3] also isolated the *ERO1* gene from a genomic library by selecting for growth on normally lethal levels of DTT. Both groups reported that the *ERO1* mutants are sensitive to DTT, and conversely that overexpression of the wild-type *ERO1* gene leads to DTT resistance. The DTT sensitivity, together with genetic analysis by both groups which established that the *ERO1* gene is essential for viability in *S. cerevisiae*, indicates that the *ERO1* gene product, Ero1p, may play a crucial role in the cell.

Ero1p has a predicted molecular weight of 65 kDa, a putative amino-terminal signal sequence that directs ER

Figure 1

Potential schemes for the oxidation of nascent secretory proteins. The protein substrate (P) may be oxidized **(a)** directly by Ero1p or **(b)** by oxidized glutathione, which in turn is reoxidized by Ero1p. In this case, PDI is acting primarily as an isomerase. Alternatively PDI may be acting as the oxidant, as well as an isomerase, with the oxidizing equivalents to reoxidize PDI coming either from Ero1p, from oxidized glutathione or from another oxidant.



localization and nine potential *N*-linked glycosylation sites. Genes homologous to *S. cerevisiae ERO1* are present in a wide range of eukaryotes including humans, suggesting conservation of function. Within the most highly conserved region of Ero1p is the sequence Cys–X–X–Cys–X–X–Cys (where X is any amino acid), a motif that specifies part of the binding site for an iron–sulphur cluster in proteins related to ferredoxin. Tagging an epitope to the carboxyl terminus of Ero1p allowed both groups to elucidate that the protein is *N*-glycosylated, but the glycosyl moiety does not contain α1–6 mannose [2], suggesting that the tagged protein is restricted to the ER. In addition, Pollard *et al.* [3] indicate that epitope tagging of Ero1p may have an effect on its activity *in vivo*. More direct evidence for the localization of Ero1p comes from immunofluorescence experiments which showed that it colocalizes with the known ER-resident protein Kar2p/BiP [3]. Given that Ero1p does not contain the classic yeast ER-retention signals HDEL (His–Asp–Glu–Leu) or KKXX (Lys–Lys–any–any), it was suggested that Ero1p is retained in the ER by being tightly associated with the membrane, or with membrane components. This suggestion is indirectly supported by the requirements for extraction of tagged Ero1p from membrane-containing fractions of cell extracts [2].

Both groups have demonstrated that Ero1p is linked to the formation of disulphide bonds in the ER, by showing that the transport of invertase from the ER was independent of the presence of Ero1p while that of carboxypeptidase Y required Ero1p. The transport of invertase is known to be independent of redox status — for example, it is unaffected by the presence of DTT — while that of carboxypeptidase Y requires the formation of its five native disulphide binds and is blocked by the presence of DTT. In addition Frand and Kaiser [2] showed that the dependence on Ero1p that is seen for the transport of carboxypeptidase Y is also seen for Gas1p, a protein which may contain up to seven disulphide bonds in its extracellular domain. This effect on the secretion of disulphide-bond-containing proteins, combined with the link between DTT sensitivity and Ero1p production, is consistent with the prediction that *ERO1* is involved in the system for maintaining the redox state of the ER.

The idea that *ERO1* is involved in maintaining the ER's redox state is further supported by the finding of Frand and Kaiser [2] that addition of the oxidant diamide, at the correct concentrations, reverses the effects of the loss of Ero1p. In contrast, in a mutant strain that lacks the PDI-encoding *PDI1* gene, addition of diamide does not reverse

the effects of the loss of PDI, indicating that Ero1p and PDI perform distinct functions (and both are essential). In addition, both groups also found that synthesis of Ero1p is induced, like the synthesis of other ER-resident proteins, in response to an increase in unfolded proteins in the ER, and Ero1p contains an unfolded-protein response element (UPRE) in its promoter. Pollard *et al.* [3] additionally found, by using reporter constructs under the transcriptional control of the Kar2 UPRE, that *ERO1* mutants not only show an induction of the unfolded-protein response, but that this response is induced in the mutants even in the absence of stress, indicating that an *ERO1* mutation leads to a more general defect in the folding of proteins in the secretory pathway.

The two papers make a strong case that Ero1p is involved in the pathway of secretory protein oxidation in yeast, and the presence of genes homologous to *ERO1* across a wide range of eukaryotes suggests that the pathway is conserved. The work provides little direct biochemical evidence, however, and only limited indirect information on the properties of Ero1p. This makes it difficult to reconstruct the complete pathway and to identify all the components and their interactions. The suggestion that Ero1p is a membrane-associated ferredoxin-like protein is plausible and worth further investigation, but the identity of its redox partners remains obscure. There are no clues yet about the source from which it derives its oxidizing equivalents, nor about the target to which it transfers them. Does it oxidize an intermediate carrier, such as glutathione, does it act directly on reduced proteins (which are then isomerized by PDI) or does it recycle PDI from the reduced to the oxidized state (see Figure 1)?

The evidence, as yet, is inconclusive; Pollard *et al.* [3] even suggest that Ero1p may be a sensor rather than an active component of the pathway, while Frand and Kaiser [2] suggest that there is no requirement for oxidized glutathione as an oxidant. These contradictions demonstrate that there is still a large gap in our knowledge of a key redox pathway in secretory cells. The great value of the two recent papers is that they have placed a large piece into this gap in the jigsaw puzzle, and they are therefore bound to stimulate and facilitate the search for the adjacent pieces.

References

1. Freedman RB: **The formation of protein disulphide bonds.** *Curr Opin Struct Biol* 1995, **5:**85-91.
2. Frand AR, Kaiser CA: **The *ERO1* gene of yeast is required for oxidation of protein dithiols in the endoplasmic reticulum.** *Molecular Cell* 1998, **1:**161-170.
3. Pollard MG, Travers KJ, Weissmann JS: **Ero1p: a novel and ubiquitous protein with an essential role in oxidative protein folding in the endoplasmic reticulum.** *Molecular Cell* 1998, **1:**171-182.
4. Ziegler DM, Poulsen LL: **Protein disulphide bond synthesis: a possible intracellular mechanism.** *Trends Biochem Sci* 1977, **2:**79-81.
5. Soute BAM, Groenen-van Dooren MMCL, Holmgren A, Lundstrom J, Vermeer C: **Stimulation of the dithiol-dependent reductases in the vitamin K cycle by the thioredoxin system.** *Biochem J* 1992, **281:**255-259.
6. Hwang C, Sinskey AJ, Lodish HF: **Oxidized redox state of glutathione in the endoplasmic reticulum.** *Science* 1992, **257:**1496-1502.
7. Creighton TE, Bagley CJ, Cooper L, Darby NJ, Freedman RB, Kemmink J, Sheikh A: **On the biosynthesis of bovine pancreatic trypsin inhibitor (BPTI). Structure, processing, folding and disulphide bond formation of the precursor in vitro and in microsomes.** *J Mol Biol* 1993, **232:**1176-1196.
8. Marquadt T, Hebert DN, Helenius A: **Post-translational folding of influenza hemagglutinin in isolated endoplasmic reticulum-derived microsomes.** *J Biol Chem* 1993, **268:**19618-19625.
9. Hayano T, Inaka K, Otsu M, Taniyama Y, Miki K, Matsushima M, Kikuchi M: **PDI and glutathione-mediated reduction of the glutathionylated variant of human lysozyme.** *FEBS Lett* 1993, **328:**203-208.