Oxidative protein folding in the mammalian endoplasmic reticulum

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Abstract
Native disulphide bonds are essential for the structure and function of many membrane and secretory proteins. Disulphide bonds are formed, reduced and isomerized in the endoplasmic reticulum of mammalian cells by a family of oxidoreductases, which includes protein disulphide isomerase (PDI), ERp57, ERp72, P5 and PDIR. This review will discuss how these enzymes are maintained in either an oxidized redox state that allows them to form disulphide bonds in substrate proteins or a reduced form that allows them to perform isomerization and reduction reactions, how these opposing pathways may co-exist within the same compartment and why so many oxidoreductases exist when PDI alone can perform all three of these functions.

Introduction
The ER (endoplasmic reticulum) is compartmentalized away from the cytosol and maintains redox conditions that enable a distinct set of folding catalysts to facilitate the formation and isomerization of disulphide bonds [1]. During this folding process a protein may be oxidized to form disulphide bonds, reduced to allow isomerization of non-native disulphide bonds or reduced to allow unfolding and subsequent degradation. A growing family of ER oxidoreductases is supposed to be responsible for catalysing these reactions in mammalian cells, including PDI (protein disulphide isomerase), ERp57, ERp72, PDIR, PDIp and P5. The oxidoreductases are characterized by active sites homologous with the active site found in the cytosolic reductase thioredoxin, which contains a pair of cysteine residues (CXXC) that shuttle between the oxidized disulphide and reduced dithiol form [2]. The reactions that these enzymes catalyse require the individual active sites to be maintained in either the oxidized or reduced form, for catalysis of oxidation or reduction respectively [3].

The pathway for disulphide bond formation
PDI is a multifunctional protein capable of catalysing the formation, isomerization and reduction of disulphide bonds in vitro as well as being an essential subunit for the enzymes prolyl 4-hydroxylase and microsomal triacylglycerol transfer protein [2]. During disulphide bond formation an intra-chain disulphide bond between the cysteine residues within the active site is capable of accepting two electrons from the polypeptide chain substrate. This electron transfer results in the oxidation of the substrate and the reduction of the PDI active site.

The rate-limiting step in this reaction is the re-oxidation of PDI to regenerate the active-site intra-chain disulphide [4]. For a number of years, it was believed that the low-molecular mass thiol glutathione was responsible for oxidizing the PDI active sites. However, evidence suggests that this is not the case at least in yeast [5], in which depletion of glutathione does not lead to a lack of disulphide bond formation. Rather, lowering the intracellular pool of glutathione leads to suppression of a temperature-sensitive mutation in a gene called ERO1 (for ER oxidase). Ero1p has been shown to be responsible for the oxidation of PDI [6,7]; a defect in Ero1p leads to a lack of disulphide bond formation demonstrating the crucial role played by Ero1p in the oxidative folding pathway. The mechanism of oxidation is supposed to be by disulphide exchange (Figure 1) whereby Ero1p accepts two electrons from PDI and in the process forms a mixed disulphide with PDI. There is growing evidence to suggest that Ero1p is an FAD-dependent oxidase that is capable of passing electrons from PDI to the ultimate electron-acceptor oxygen [8]. However, Ero1p can also catalyse the oxidation of PDI under anaerobic [9] conditions, suggesting the possibility that alternative electron acceptors could substitute for oxygen under these conditions.

In mammalian cells, there are two members of the Ero1 family, termed ERO1L-α [10] and ERO1L-β [11],
Figure 1 | Disulphide bond exchange

(a) A thiolate anion (-S-) is formed by the deprotonation of a free thiol. It displaces one sulphur of the disulphide bond in the oxidized species, resulting in a transient mixed disulphide bond between the two proteins. A second exchange reaction then follows, where the remaining thiolate anion attacks the mixed disulphide bond and resolves it. The net result of thiol disulphide exchange is that the originally reduced protein is oxidized. This is due to the gain of electrons from the originally oxidized species, which itself is reduced. (b) For an oxidoreductase to form a disulphide bond or oxidize a substrate, it must be oxidized itself. Conversely, for an oxidoreductase to reduce a substrate it must be in a reduced form so that it is able to donate electrons. The isomerization of a disulphide bond requires that the bond is broken and then spontaneously formed. As the bond must first be broken the oxidoreductase must be in a reduced form. It is then oxidized by the destruction of the disulphide bond, leaving it in the oxidized state necessary to promote the formation of the alternative disulphide bond.

which differ in their tissue distribution and transcriptional regulation. Only ERO1L-β is induced by the unfolded protein response [11], whereas ERO1L-α is induced during hypoxia [12]. Both isoforms are capable of complementing yeast expressing a temperature-sensitive Ero1p mutant and accelerating the folding of disulphide-bonded proteins when overexpressed in mammalian cells [13], demonstrating their functional equivalence and crucial role in the oxidative folding pathway. The mammalian isoforms differ from their yeast counterpart in that they do not contain a large C-terminal domain, which is supposed to localize the yeast protein to the ER. Instead they form a complex with a protein called ERp44, which retains mammalian Ero1 in the ER through a thiol-mediated mechanism [14]. ERp44 contains a thioredoxin-like domain, which could potentially fulfill an alternative function to merely the ER retention of Ero1.

Although Ero1p has been shown to be responsible for the oxidation of PDI, it is not clear whether Ero1 also catalyses the oxidation of other ER oxidoreductases. Within the yeast ER, the family of oxidoreductases includes Pdip, Mpd1p, Mpd2p, Eug1p and Eps1p, of which only Pdip and Mpd2p have been shown to be substrates of Ero1p [15]. In the mammalian ER, a growing family of oxidoreductases are potential substrates for ERO1L-α and -β, including ERp57, ERp72, P5, PDIR, PDIp, ERp18 and ERdj5 [16–18]. The exact function of these proteins is not known nor is it clear why there are so many in the lumen of the ER.

Ero1p has been shown to be an essential gene in yeast; however, overexpression of a second oxidase, Erv2p, can restore viability to an ero1-1 mutant strain [9]. Erv2p is an oxygen-dependent flavoprotein with a tightly associated cofactor that is capable of introducing disulphide bonds in protein substrates. When overexpressed in yeast it can form mixed disulphides with PDI, demonstrating that Erv2 can oxidize PDI. A homologue of Erv2 called sulphydryl oxidase has been identified in mammals, raising the possibility that alternative oxidative pathways exist also in mammalian cells [19]. This protein has three thioredoxin domains, is targeted to the secretory pathway and is an oxygen-dependent FAD-linked oxidase that can directly introduce disulphide bonds into PDI [20]. Clearly, before any exact function during oxidative protein folding can be assigned to sulphhydril oxidase or other potential oxidases, we should be able to demonstrate not only the potential for these proteins to act as oxidases
in vitro but also the requirement of these proteins to catalyse this reaction in vivo.

The pathway for disulphide bond reduction

We and others have recently investigated the redox state of several ER oxidases within mammalian cells and shown that PDI, ERp57, P5, PDIR and ERp72 are all in a predominantly reduced form at steady state [13]. After purification, these proteins are oxidized to form disulphide bonds within their active sites. This would suggest that a pathway exists to maintain these proteins in a reduced state within the cell. That PDI is predominantly reduced in mammalian cells contrasts with the situation in yeast, where PDI is clearly predominantly oxidized [15]. The yeast ER possibly is more oxidizing than the mammalian ER, explaining this discrepancy; however, despite the differences it is clear that PDI in mammalian cells can be oxidized by Ero1 and catalyses disulphide bond formation [13]. ERp57, on the other hand, does not appear to be a substrate for Ero1 as judged by a lack of oxidation of ERp57 in cells overexpressing Ero1 [13]. ERp57 has also been shown to act as a reductase, at least in vitro, to allow the breaking of non-native disulphide bonds within MHC-class I heavy chain [21]. Hence, there is a requirement for both an oxidative pathway for disulphide bond formation and a reductive pathway to allow reduction and isomerization of non-native disulphides and for these pathways to co-exist in the same intracellular compartment.

The presence of alternative pathways for maintaining ER oxidoreductases in a reduced or oxidized state would mimic the situation within the Escherichia coli periplasm where the main enzyme catalysing disulphide isomerization, DsbC, is maintained in a reduced state by DsbD. DsbD is a membrane protein and shuttles reducing equivalents to DsbC, is maintained in a reduced state by DsbD. DsbD in Escherichia coli mimic the situation within the eukaryotic ER.

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The pathways for maintaining DsbA or DsbC in either an oxidized or reduced form are kinetically segregated in the periplasm, allowing both oxidation and isomerization reactions to occur within the same compartment. It remains to be determined whether a similar system for maintaining mammalian oxidoreductases in the reduced form exists within the eukaryotic ER.

One major difference between the ER and the bacterial periplasm is the absence of a glutathione buffer within the periplasm. Hence, the presence of a glutathione buffer itself may maintain the mammalian ER oxidoreductases in a reduced state, eliminating the requirement for a separate pathway for their reduction. As yet, no pathway has been identified that could maintain glutathione (GSH) in a reduced state within the ER; however, there are oxidases such as Ero1 and sulphhydryl oxidase that may oxidize GSH to GSSG (glutathione disulphide) [5,20]. Maintaining a pool of GSH within the ER could be brought about by continuous transport from the cytosol where it is reduced by glutathione reductase. Some evidence exists to suggest the presence of a transport system that allows the selective passage of GSH rather than GSSG [24]. Removal of GSSG from the ER could be by an as yet unidentified glutathione reductase, by secretion of GSSG or by GSSG directly oxidizing protein thiols.

Interestingly, when GSH has been eliminated either from yeast or mammalian cells by removal of the enzyme involved in the first step of glutathione synthesis, glutamate–cysteine ligase, both yeast and mammalian cells are viable, but do require reducing agent to be included in their growth medium [5,25]. As the isomerization and therefore the reduction of substrates by PDI is an essential function, it follows that GSH may not be essential for the reductive pathway to function.

Conclusion

The formation of native disulphide bonds in proteins relies not only on their ability to catalyse the oxidation of disulphide bonds, but also to catalyse the isomerization of non-native disulphides. Hence, the reductive pathway as well as the oxidative pathway is essential to ensure correct folding of secretory proteins. Our understanding of the oxidative pathway has been boosted due to the discovery of Ero1; however, our understanding of the reductive pathway in eukaryotes is limited. One of the main questions still to be resolved is how the two seemingly opposing pathways can exist within the same intracellular compartment.

References


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