Catalysis of disulphide bond formation in the endoplasmic reticulum

L. Ellgaard
Institute of Biochemistry, Swiss Federal Institute of Technology (ETH), ETH Hönggerberg, CH-8093 Zurich, Switzerland

Abstract
Disulphide bonds are critical for the maturation and stability of secretory and cell-surface proteins. In eukaryotic cells, disulphide bonds are introduced in the ER (endoplasmic reticulum), where the redox conditions are optimal to support their formation. Yet, the correct pairing of cysteine residues is not simple and often requires the assistance of redox-active proteins. The enzymes of the thiol-disulphide oxidoreductase family catalyse oxidation, reduction and isomerization, and thereby play important roles for the folding of many proteins. To allow all three redox reactions to take place concurrently in the same compartment, specific protein–protein interactions regulate the function of individual enzymes, while a careful balance of the ER redox environment is maintained. At the same time, the system must be capable of responding to changes in the cellular conditions, caused, for instance, by oxidative stress and protein misfolding. This review presents recent progress in understanding how ER redox conditions are regulated and how protein disulphides are formed in the ER of mammalian cells.

Introduction
By establishing covalent intra- and intermolecular cross-links, disulphide bonds promote efficient folding and oligomerization of many polypeptides during synthesis and maturation in the ER (endoplasmic reticulum). In contrast, incorrect formation of disulphide bonds most often leads to misfolding. Non-native conformers are prone to aggregation and will be detected by the ER quality-control system, which retains misfolded proteins and eventually targets them for ERAD (ER-associated degradation) if they fail to fold to their native conformation [1]. By the ERAD pathway, misfolded proteins are retrotranslocated to the cytosol, where they are degraded by the proteasome.

In biochemical terms, the formation of a disulphide bond from two thiol groups proceeds through the transfer of two electrons. This redox reaction requires an electron acceptor, and to catalyse this process the mammalian ER contains a number of thiol-disulphide oxidoreductases. These enzymes not only catalyse dithiol oxidation, but are also capable of reducing and rearranging (isomerizing) incorrectly formed disulphides (Figure 1A).

The classical ER oxidoreductase is PDI (protein disulphide isomerase). In Saccharomyces cerevisiae, PDI is essential for viability, and its function is relatively well characterized in this organism. Pathways for electron transfer by PDI have been delineated, and molecular mechanisms and structural features underlying disulphide bond formation in yeast have been determined in recent years. These studies have provided important general insight into protein oxidation in the ER. However, as the two systems differ in several respects, many findings concerning the yeast system cannot be extended to the mammalian system. In the present review, we will focus mainly on the latter (see [2,3] for recent reviews of the yeast system).

Thiol-disulphide oxidoreductases of the ER
Thiol-disulphide oxidoreductases catalyse thiol-disulphide-exchange reactions. During the course of an exchange reaction, transient mixed disulphides are formed between one of the two cysteine residues present in a characteristic CXXC active-site sequence motif of the enzyme, and a cysteine of the substrate. The equilibrium between the dithiol and disulphide form of the active-site cysteine residues determines the redox activity of a given thiol-disulphide oxidoreductase. For the enzyme to function as an oxidase, the two active-site cysteine residues must form a reactive disulphide. The reduced dithiol state can catalyse reduction of mispaired cysteine residues and thereby allow the protein to function as a reductase or an isomerase.

Most oxidoreductases contain one or more domains similar to thioredoxin, a disulphide reductase of the cytosol. The three-dimensional structures of thioredoxin-related domains show a typical α/β-fold with a central β-sheet surrounded by α-helices, where the residues of the CXXC motif are found at the N-terminus of an α-helix (Figure 1B).

Among the mammalian enzymes, PDI and its closest known homologue ERp57 are the best characterized. PDI is an abundant and versatile redox enzyme, which has oxidase, reductase and isomerase activity, and it interacts with a variety of substrates (reviewed in [4]). While the function of PDI as an oxidase in vivo has been highlighted in recent years.
Thiol-disulphide oxidoreductases, catalysis and structure

1


molecules, a finding that potentially indicates a role for reducing incompletely folded MHC class I heavy-chain [14]. Further-
class I complex with antigenic peptide, ERp57 has been characterized [13]. During folding and loading of the MHC complex, the interaction with ERp57 is particularly well characterized [13]. During folding and loading of the MHC class I complex with antigenic peptide, ERp57 has been implicated in disulphide bond rearrangement [14]. Furthermore, in vitro studies have shown that ERp57 is capable of reducing incompletely folded MHC class I heavy-chain molecules, a finding that potentially indicates a role for

ERp57 in preparing ERAD substrates for retrotranslocation [15].

A considerable number of ER thiol-disulphide oxidoreductases are found in mammalian cells in addition to PDI and ERp57 (Figure 2, ). Several new enzymes, such as ERp18/19 [16,17], ERp46/EndoPDI [17,18], TMX [19,20] and ERdj5 [21], have recently been described in the literature. Moreover, we and others have identified a number of additional, potential ER thiol-disulphide oxidoreductases by database searching (unpublished work), so that the total number of the human enzymes of this family is approaching 20. Many of these proteins remain poorly characterized at the experimental level.

The mammalian ER oxidoreductases differ significantly with respect to a number of features, such as size, active-
site sequences, and by being membrane-bound or soluble. Therefore functional differences can also be anticipated. Whereas PDI and ERp57 show broad specificity and interact with numerous substrates, other family members could work as specialized oxidoreductases and interact with separate and specific substrates. It is also probable that certain cellular conditions, such as hypoxia or ER stress resulting from protein misfolding, regulate expression and perhaps even substrate interactions. Finally, some of these enzymes could perform distinct redox functions, as is the case in the peri-
plasmic space of Escherichia coli where separate pathways for oxidation and reduction exist. Candidate targets for the enzymes in a separate pathway for reduction would be ERAD substrates that need to be reduced before retrotranslocation [22]. However, dedicated disulphide reductases have not been detected in eukaryotes to date (Figure 2, ).

Generation of disulphides by Ero1

Whereas the function of PDI as a dithiol oxidase has been investigated for many years, the exact nature of the electron acceptor for oxidation by PDI only became clear with the identification of the Ero1p protein in S. cerevisiae [23,24]. The subsequent biochemical and structural characterization has shown that Ero1p is a FAD-binding protein that can transfer electrons from PDI to molecular oxygen [25–28]. In this pathway, molecular oxygen is the source of the oxidizing equivalents that are transferred directly from Ero1p by disulphide exchange to PDI, which then in turn oxidizes substrate proteins (Figure 2, ).

In human cells, two proteins closely related to Ero1p, Ero1-Lα and Ero1-Lβ, have been identified [29,30]. Both proteins can complement the thermosensitive ero1-1 yeast strain, both form mixed disulphides with PDI, and central functional characteristics are conserved between the orthologues [29–31]. For instance, all Ero1 proteins rely on two pairs of conserved cysteine residues for catalytic activity. The recently solved structure of an Ero1p fragment [28] and a number of mutational and functional studies [6,26,30– 33] have provided insight into the catalytic mechanism of the enzyme. Most importantly, an active-site CXXC motif abuts the FAD cofactor, whereas the cysteine residues of a
Figure 2 | Pathways for disulphide-bond formation in the ER of mammalian cells

Approx. 20 proteins of the thiol-disulphide oxidoreductase family are found in the ER of mammalian cells (1). With the exception of PDI, the type(s) of redox reactions performed by these enzymes in vivo is most often not clear, but overall they catalyse substrate protein oxidation, reduction and isomerization. Reoxidation of active-site cysteine residues in PDI after substrate oxidation is achieved by Ero1 (2). Other, as yet unknown, thiol oxidases might exist. The involvement of disulphide reductases in a separate pathway for reduction/isomerization (3) is currently speculative (box of broken lines). The ER glutathione redox buffer probably modulates the redox state of thiol-disulphide oxidoreductases and/or substrate proteins (4). Broken arrows indicate the flow of electrons.

CXXXXC motif that probably engage in disulphide exchange with PDI are placed on a flexible loop that can approach the CXXC motif. Disulphide exchange between active-site cysteine residues of PDI and those of the CXXXXC motif, followed by intrachain disulphide exchange between cysteine residues of the two cysteine pair motifs in Ero1, would allow electron transfer to O₂ through the FAD moiety. Observation of the implied transient intrachain disulphide would therefore provide direct support for this mechanism.

The effect of gene knockdown by RNAi (RNA interference) of the human Ero1α on oxidative folding in tissue-culture cells has not been investigated to date. However, overexpression of either Ero1 protein shifts the redox state of PDI towards the oxidized form [6]. This, in turn, can influence protein oxidation. When overexpressed in mammalian cells, Ero1-Lα and Ero1-Lβ accelerate oxidative folding of co-expressed immunoglobulin J chains, which are substrates of PDI [6,34]. While both human Ero1 proteins therefore play a role in regulating the function of PDI by promoting its oxidation, there is presently no indication that either protein promotes the oxidation of the other CXXC-containing ER thiol-disulphide oxidoreductases. Potentially, Ero1-independent pathways for oxidation of ER oxidoreductases other than PDI exist (Figure 2, 2). An interesting candidate oxidase is the quiescin/sulphydryl oxidase, a flavoprotein that oxidizes protein diethiols while reducing oxygen to H₂O₂, but its role in oxidative folding in the ER is still unclear [35].

Maintaining a redox balance

The balance that PDI has to strike between its functions as an oxidase, reductase and isomerase illustrates the point that mechanisms need to be in place to modulate the ER luminal redox environment and the redox state of individual proteins.

The role of glutathione, the primary ER redox buffer, in disulphide-bond formation and regulation of the ER redox conditions has been contemplated for years (Figure 2, 4). Whereas the cytosol maintains a ratio of GSH/GSSG of approx. 100:1, the corresponding ratio in the ER is relatively more oxidizing [36,37]. A number of roles have been proposed for GSH in the ER [2,34,38]. For instance, it could function to counteract oxidative stress by directly sweeping up surplus oxidizing equivalents. In this connection, disulphide-bond formation catalysed by Ero1 has been shown to contribute significantly to the total production of reactive oxygen species in the cell [3,39]. During ER stress that induces the UPR (unfolded protein response), signalling through the ATF4 and Nrf2 transcription factors is important to counteract oxidative stress through the up-regulation of genes that increase glutathione synthesis [39,40]. The finding that GSH production is up-regulated in cells overexpressing Ero1L-α indicates the presence of an additional signalling pathway where the altered redox state of the ER is sensed directly and transmitted to the nucleus [34]. In addition, GSH could play a role in reducing wrongly
formed disulphides and active-site cysteine residues of thiol-disulphide oxidoreductases, such as those of PDI and ERp57 that are found predominantly in the reduced form under steady-state conditions [6].

How then is protein oxidation in the ER promoted? Oxidative folding by PDI is probably maintained by the small fraction of oxidized PDI present at steady state, and modulated by up-regulation of the two human Ero1 proteins. Thus, Ero1L-α is up-regulated by hypoxia [41], and Ero1L-β is up-regulated by ER stress that induces the UPR [29,41]. In response to accumulation of misfolded proteins in the ER, the UPR leads to the transcriptional up-regulation of many proteins involved in ER protein folding and ERAD, while reducing the amount of protein translocated into the ER through a decrease in translational initiation [42]. The transcriptional regulation of the Ero1 proteins allows the cell to increase its capacity for oxidative folding when the redox state of the cell is perturbed, and under conditions where ER protein folding is compromised. Whether ER thiol-disulphide oxidoreductases in addition to PDI function as oxidases is currently not clear.

The relatively high concentration of GSSG in the ER could arise, at least in part, from the reduction of active-site disulphides in PDI and other oxidoreductases by GSH, and from the reduction of incorrectly paired disulphides in substrate proteins. Regarding its potential role in oxidative site disulphides in PDI and other oxidoreductases by GSH, oxidases is currently not clear.

of additional oxidoreductases. In parallel, more biochemical and structural studies of ER oxidoreductases are needed to obtain a better understanding of oxidative protein folding at the molecular level. Finally, the regulation of ER redox homeostasis, and the exact nature of the molecules and the signalling pathways involved will be important topics to address.

The author thanks all members of the Ellgaard laboratory for helpful discussions. J. Riemer for help with preparing figures and Dr L. Ruddock for critical reading of the paper. This work was supported by grants from the Swiss National Science Foundation and the ETH Zurich.

References


©2004 Biochemical Society

Received 21 June 2004