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Review

Forming disulfides in the endoplasmic reticulum ☆

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ABSTRACT

Protein disulfide bonds are an important co- and post-translational modification for proteins entering the secretory pathway. They are covalent interactions between two cysteine residues which support structural stability and promote the assembly of multi-protein complexes. In the mammalian endoplasmic reticulum (ER), disulfide bond formation is achieved by the combined action of two types of enzyme: one capable of forming disulfides *de novo* and another able to introduce these disulfides into substrates. The initial process of introducing disulfides into substrate proteins is catalyzed by the protein disulfide isomerase (PDI) oxidoreductases which become reduced and, therefore, have to be re-oxidized to allow for further rounds of disulfide exchange. This review will discuss the various pathways operating in the ER that facilitate oxidation of the PDI oxidoreductases and ultimately catalyze disulfide bond formation in substrate proteins. This article is part of a Special Issue entitled: Functional and structural diversity of endoplasmic reticulum.

1. Introduction

Newly synthesized proteins fold into functional 3-dimensional conformations stabilized by van der Waals interactions, salt bridges, hydrogen bonds and the hydrophobic effect. In addition to these non-covalent interactions, a large number of proteins in the secretory pathway, or those destined as cell surface receptors, form disulfides; covalent bonds formed between the side chains of two cysteine residues. Protein disulfide bonds are primarily formed as a result of a thiol-disulfide exchange reaction, with PDI exchanging its active site disulfide with its substrate. Potentially any two cysteine residues in close spatial proximity can form a disulfide bond. Therefore, the formation of a native disulfide bond in a polypeptide containing several cysteine residues can become problematic. Non-native disulfides can prevent correct folding and have to be reduced or isomerized to form the correct, native disulfides [1,2]. Hence, the formation of native disulfides is often a rate-limiting step during the folding and maturation of many disulfide-containing secretory proteins [3,4].

Disulfide bonds are crucial for the biosynthesis and function of many proteins. They promote structural stability, facilitate the assembly of multi-protein complexes and can modulate redox-dependent functions in response to changes in the cell [5]. Disulfide bonds can also mediate the formation of productive folding intermediates, and have been shown in the ER to promote thiol-mediated protein retention [6,7]. These functions illustrate the importance of disulfide bonds in numerous cellular processes and highlight the need for a proper understanding of the proteins involved and the mechanisms regulating their formation.

In the last few decades, significant progress has been made toward unraveling the mechanistic details of protein disulfide bond formation in prokaryotic and eukaryotic organisms. Thus we now know that enzyme-catalyzed disulfide bonds can be formed in a number of specialized cellular locations, including the endoplasmic reticulum, the bacterial periplasm [8] and the inter-membrane space of the mitochondria [9]. While significant progress has been made in understanding the enzyme-catalyzed oxidative pathways operating in the ER, no corresponding reduction pathways operating in this organelle have been identified to date.

2. Environment of the ER and disulfide bond formation

The environment of the ER is distinct from the cytosol in two important aspects that ensure the formation of disulfide bonds in proteins translocated across the ER membrane; namely the presence of an optimized redox buffer and disulfide exchange proteins. Like the cytosol, the ER contains a redox buffer, maintained by the cysteine-containing tripeptide, glutathione (γ-L-glutamyl-L-cysteinylglycine). However, unlike the cytosol the glutathione buffer is much more oxidizing. In the cytosol, glutathione is predominantly in the reduced form (GSH) with only very low concentrations of oxidized glutathione (GSSG) [10]. A high GSH:GSSG ratio is maintained by a robust reductive pathway consisting of glutathione reductase coupled to NADPH. Such a system, in general, prevents disulfide formation. In addition, thioredoxin and thioredoxin reductase coupled to NADPH act to reduce any disulfides formed within cytosolic proteins [11]. In contrast, the ER contains no

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obvious candidates for a glutathione or a thioredoxin reductase and as a consequence the GSH:GSSG ratio is much lower and disulfides can form. In addition, most of the thioredoxin domain-containing proteins present in the ER are more likely to form than break disulfides due to the more positive reduction potential of their active sites than cytosolic thioredoxin. For example, the active site disulfide of thioredoxin has a reduction potential of approximately −270 mV [12] whereas the active site disulfide of PDI has a reduction potential of approximately −165 mV [13]. This means that the thioredoxin disulfide is more stable than the PDI disulfide so PDI is more likely to exchange its disulfide with a substrate protein rather than reduce an existing disulfide.

In addition to providing an environment for disulfide formation, the ER contains proteins that are dedicated to protein folding [14–16]. Such folding factors can form multi-protein complexes that act in concert to allow folding and disulfide formation in newly synthesized proteins. Many of these folding factors, such as BiP, calnexin and calreticulin, do not catalyze protein folding, but rather shield newly synthesized proteins from other competing 'off pathways' such as aggregation, thereby maintaining newly synthesized polypeptides in a 'folding competent' conformation [16].

3. PDI thiol-disulfide oxidoreductase family

Disulfide bond formation in the ER is catalyzed by the PDI family of dithiol-disulfide oxidoreductases [17]. About 20 proteins have been assigned to this family to date; with 15 members containing the typical thioredoxin-like fold, with characteristic CXXC motifs in their active site. The number and position of the thioredoxin domains and their active site chemistries vary depending on the particular enzyme [17]. These proteins catalyze thiol-disulfide exchange reactions by acting as electron acceptors during disulfide bond formation (oxidation reaction) or as electron donors during breaking of disulfides (reduction reaction). The PDI proteins also catalyze isomerization reactions by rearranging non-native to native disulfides. The ability of a particular PDI enzyme to act as an oxidant or a reductant depends in part on the redox potential of the CXXC active site [13,18]. As mentioned previously, the majority of PDI proteins contain active sites disulfides that are relatively unstable in comparison to the CXPC motif of cytosolic thioredoxin [19]. A notable exception is ERdj5, which contains four CXXC motifs, with three of these containing the CXPC motif [20]. Consistent with the similarity to cytosolic thioredoxin, the active site disulfides in ERdj5 are quite stable and this enzyme has been shown to act as a reductant, breaking disulfides to facilitate the unfolding of proteins destined for degradation in the cytosol [21]. While it was thought the functions of some of the PDI proteins would be redundant because of the similarities in their active sites, there is now accumulating evidence for substrate specificities and defined roles [22].

PDI was the first ER oxidoreductase to be extensively characterized. It is an essential enzyme in yeast, and has been shown to function as a chaperone as well as introduce disulfide bonds into substrates [23–25]. PDI contains four thioredoxin-like domains namely a, b, b′, and a′. Only the a and a′ domains contain the canonical CXXC active site motif [18]. The low pK_a of the first cysteine of the CXXC motif allows this residue to participate in disulfide bond formation [26]. The crystal structure of both a reduced and oxidized version of the human protein has been solved [27,28]. Overall the four PDI domains form a twisted 'U' conformation, with the catalytic domains a and a′ situated at the top, facing each other; while the domains b and b′, are sandwiched between the catalytic domains, on the inside of the 'U' [28]. The interior of the U is hydrophobic, particularly at the b and b′ domains and is the principal binding site for peptides and misfolded regions of substrates [29]. However, other domains of PDI have been implicated in substrate binding [30]. The presence of such hydrophobic regions explains how in addition to facilitating disulfide formation in newly synthesized proteins, PDI may also function as a chaperone, inhibiting aggregation of misfolded proteins [31].

Conformational differences between the reduced and oxidized human PDI indicate a large inter-domain rotation around the a′b′ region resulting in a transition from an open (oxidized) to a closed (reduced) structure (Fig. 1). In the open conformation the binding cleft could accommodate larger polypeptides which may have folded but not formed their disulfides. In contrast, the binding cleft in the reduced protein is much smaller restricting binding to smaller or less folded polypeptides. Such conformational changes are intriguing and may indicate a possible mechanism to ensure the release of polypeptides from PDI once the disulfide transfer has occurred. Indeed a recent paper using a model single domain protein with one disulfide bond indicates that protein folding occurs prior to disulfide formation [2]. While this work used a single domain of PDI as the catalyst, the basic premise that folding occurs prior to disulfide formation would necessitate a large binding cleft within full-length PDI to accommodate the folded polypeptide substrate.

When PDI and other oxidoreductases introduce disulfides into newly synthesized proteins, their active sites have to be re-oxidized to allow for further rounds of disulfide formation. This function is fulfilled by specific ER-resident oxidases, which do not directly introduce disulfides into newly synthesized proteins. These enzymes catalyze the first step in disulfide formation by transferring oxidizing equivalents to the PDI proteins, which then introduce these disulfides into nascent polypeptides [32–35].

4. ERO1 oxidative pathway

The first of the ER oxidative pathways for *de novo* disulfide formation to be identified involves the ER oxidoreductin 1 protein (Ero1). This enzyme was initially characterized in yeast where it was shown to be essential for disulfide formation in PDIp [36,37]. Mammals and other vertebrates have two Ero1 paralogs, namely: Ero1α which is present in all tissues and Ero1β which shows some tissue specific expression [38,39]. The mammalian Ero1 proteins are able to complement yeast Ero1p temperature-sensitive mutants [38]. The Ero1 proteins are flavoenzymes that use FAD as a cofactor to transfer electrons from PDI to molecular oxygen, forming hydrogen peroxide in the process [40–42]. Ero1 contains a cluster of cysteine residues responsible for its catalytic cycle. A pair of cysteine residues, present on an unstructured flexible loop, and termed the shuttle cysteines, constitutes the 'outer' active site. At the C-terminus of the protein are two additional cysteines, present in a CXXC motif, and lying in close proximity to the bound cofactor FAD. These cysteines comprise the 'inner' active site. The first step in disulfide exchange between Ero1and PDI proteins involves the flexible loop shuttle cysteines which directly accept electrons from PDI. Through an internal disulfide exchange reaction, these electrons are shuttled to the 'inner' active site which pass them onto the FAD, and ultimately onto the final acceptor oxygen. In addition to disulfide exchange reactions, the flexible loop has been suggested to contain a binding site for PDI [43].

While Ero1 activity is important for disulfide bond formation, unregulated Ero1 could generate high concentration of hydrogen peroxide, which could affect cell viability. Overexpression of a deregulated yeast Ero1 mutant results in inhibition of cell growth [44] while the overexpression of human Ero1α results in an unfolded protein (stress) response [45]. Thus, the activity of the Ero1 proteins are regulated via a negative feedback mechanism that involves pairs of non-catalytic cysteine residues that restrict the movement of the 'outer' shuttle disulfide, thereby preventing disulfide exchange with the 'inner' active site [41,43,44,46]. These regulatory disulfides need to be reduced to activate the enzyme and allow electron transfer between the active sites. One current view is that PDI itself can regulate Ero1 activity by reducing the regulatory disulfides when the ER PDI pool becomes predominantly reduced and reform the regulatory disulfides once sufficient oxidized PDI is formed [46,47].