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Forum Review

Genomics Perspective on Disulfide Bond Formation

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ABSTRACT

Disulfide bond formation, reduction, and isomerization in substrate proteins are catalyzed by designated pathways composed of thiol-dependent enzymes. Disulfides are generated in oxidizing environments, such as bacterial periplasm and eukaryotic endoplasmic reticulum (ER), but could also be formed in the cytosol. Major contributors to the formation of intramolecular disulfides in proteins are thiol/disulfide oxidoreductases containing a conserved CxxC motif (two cysteines separated by two other residues), which in turn transfer reducing equivalents to adapter or membrane-bound oxidoreductases. Disulfide bond formation is accompanied by disulfide bond reduction and isomerization processes, allowing disulfide repair and quality control. Higher eukaryotes evolved a complex network of thiol/disulfide oxidoreductases that are involved in disulfide bond formation and isomerization and thiol-dependent protein retention. Emerging evidence suggests that these ER functions might be assisted by mammalian selenocysteine-containing oxidoreductases Sep15 and SelM. *Antioxid. Redox Signal.* 5, 397–402.

INTRODUCTION

DISULFIDE BOND FORMATION, reduction, and isomerization are important posttranslational modifications in proteins that occur in most, if not all, living organisms. These redox functions are catalyzed by a large group of proteins, collectively called thiol/disulfide oxidoreductases, and are linked to various cellular processes, such as defense against oxidative stress, redox regulation of signaling proteins, protein folding, and central metabolic pathways. The best studied thiol/disulfide oxidoreductase systems are NADPH-dependent thioredoxin and glutaredoxin systems (8, 22, 46). Thioredoxins, glutaredoxins, and their homologues contain a conserved CxxC motif (two cysteines separated by two other residues), which is directly involved in redox reactions. In some proteins, one of the cysteines in the CxxC motif may be replaced with serine (14). Reaction mechanisms of thiol/disulfide oxidoreductases involve formation of a disulfide bond between the two CxxC cysteines (reductase function), reduction of the intramolecular CxxC disulfide bond (oxidase function), or formation of an intermolecular disulfide bond (isomerase function) (10, 14, 45).

In eukaryotes, disulfide bonds in substrate proteins are primarily formed in the endoplasmic reticulum (ER), which contains numerous chaperones and proteins responsible for quality control of folding. Substrate proteins undergo formation of native structure and necessary posttranslational modifications, including disulfide bond formation, isomerization, and reduction (11). Properly folded proteins are secreted or transported to Golgi or other compartments. Specific transport and proteolytic degradation systems are utilized for irreversibly misfolded proteins to prevent their accumulation in the ER (13).

DISULFIDE BOND FORMATION, REDUCTION, AND ISOMERIZATION IN *E. COLI* PERIPLASM

Escherichia coli periplasm was characterized in great detail with regard to disulfide bond formation, reduction, and isomerization, and it is often used as a model system to study redox processes in more complex eukaryotic systems.

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Disulfide bond formation in secreted *E. coli* proteins is controlled by a set of Dsb oxidoreductases (DsbA, DsbB, DsbC, DsbD, DsbE, and DsbG) (19, 45, 46). DsbA is a 21-kDa protein involved in the formation of disulfide bonds in periplasmic proteins (7) (Fig. 1). It contains two domains, one of which has a thioredoxin fold (7). High oxidizing activity of DsbA was linked to instability of its oxidized form (52). Integral membrane protein DsbB regenerates the oxidized form of DsbA. Although it has a thiol/disulfide oxidoreductase activity and contains two pairs of cysteines, including one CxxC motif, DsbB is not a thioredoxin-fold protein.

The proposed DsbB reaction mechanism involves formation of an intermediate mixed disulfide between Cys104 of DsbB and Cys33 of DsbA, followed by generation of an oxidized DsbA and reduced Cys104 and Cys130 in DsbB. Subsequently, this pair of DsbB cysteines is reoxidized with a Cys41/Cys44 redox motif (20, 24, 27, 28). Reduction of the Cys41/Cys44 disulfide is difficult to achieve *in vitro* in the presence of oxygen, but the disulfide can be reduced in the presence of membrane-bound quinones (29). This finding suggested the involvement of a respiratory chain in reoxidation of the CxxC motif in DsbB, as well as a direct link between disulfide bond formation and electron transport. To shuttle electrons to the electron transport chain, DsbB uses quinone/cytochrome oxidase/oxygen under aerobic conditions and menaquinone/fumarate reductase or nitrate reductase under anaerobic conditions (3). Inactivation of DsbA or DsbB leads to accumulation of unfolded proteins. It was suggested that DsbA/DsbB is the major disulfide bond formation system in the *E. coli* periplasm.

Like DsbA, periplasmic proteins DsbC, DsbG, and DsbE are thioredoxin-fold thiol/disulfide oxidoreductases, but these three enzymes are responsible for rearrangement of incorrectly formed disulfides in substrate proteins (7, 19). DsbC is a dimer of two 23-kDa subunits. Each subunit contains a CxxC redox motif and two additional distant cysteines (53). The N-terminal cysteine in the CxxC motif directly attacks disulfides to form mixed intermolecular disulfides that could be either resolved by formation of correct disulfide bonds in substrate proteins or completely reduced by DsbC. DsbC is thought to be a major protein disulfide isomerase in the *E. coli* periplasm (7), whereas DsbG and DsbE are additional disulfide isomerase enzymes that recognize different, yet overlapping sets of substrates (7, 19). The latter two oxidoreductases appear to have a chaperone activity (7, 50). To function as disulfide isomerases, DsbC, DsbG, and DsbE must be in the reduced state even though they are located in the strongly oxidizing periplasm. A membrane-bound 59-kDa protein DsbD functions as reductase for these proteins (6, 18, 19, 25) (Fig. 1). One of three DsbD domains possesses a thioredoxin fold and has a CxxC redox motif. The reduced state of DsbD is maintained by an NADPH-dependent cytosolic thioredoxin system, whereby electrons are transferred directly from thioredoxin to a C-terminal domain of DsbD (7). The occurrence of two distinct systems for oxidation and isomerization/reduction of disulfide bonds in the *E. coli* periplasm (DsbA,B and DsbC,D,G,E, respectively) suggests specificity of each system and the lack of direct cross-interaction (3). The function of oxidoreductases in the periplasm is also dealt with in the accompanying article by Ortenberg and Beckwith (39).

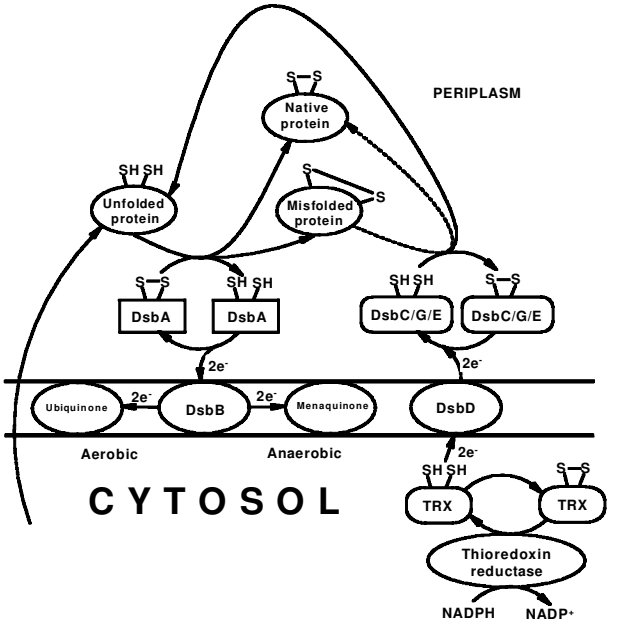

FIG. 1. A model for disulfide bond formation and isomerization pathways in the periplasm of *E. coli*. Thiol/disulfide oxidoreductases DsbA and DsbB comprise a major disulfide bond formation system, which is integrated into the respiration chain. DsbC, DsbG, and DsbE are involved in isomerization/reduction of incorrectly formed disulfide bonds in proteins and could also function as chaperones. The redox state of the DsbC,G,E system is controlled by a thioredoxin-dependent membrane reductase DsbD. Arrows indicate the direction of electron transfer, except in the case of disulfide bond isomerization that does not involve net change in redox state (shown by dashed arrow). TRX, thioredoxin.

VACCINIA VIRUS: DISULFIDE BOND FORMATION IN A REDUCING ENVIRONMENT OF CYTOSOL

A mechanism of disulfide bond formation in a highly reducing environment is provided by recent studies on vaccinia virus (48). To replicate in the cytoplasm of infected cells, vaccinia virus must produce proteins containing disulfide bonds. A whole pathway of disulfide bond formation has been identified and characterized in this system and is depicted in Fig. 2. The first protein in the pathway is E10R, an Erv1-like flavin adenine nucleotide (FAD)-dependent sulfhydryl oxidase. E10R specifically oxidizes an adapter protein A2.5L by shuttling electrons to molecular oxygen. In turn, A2.5L specifically oxidizes a thioredoxin-fold protein G4L. Unlike thioredoxin-fold thiol/disulfide oxidoreductases, A2.5L contains a CxxxC redox motif, but G4L has a typical CxxC motif. G4L works as an oxidase for membrane proteins L1R and F9L, which are essential for formation of intracellular mature virions. In addition, the reduced form of

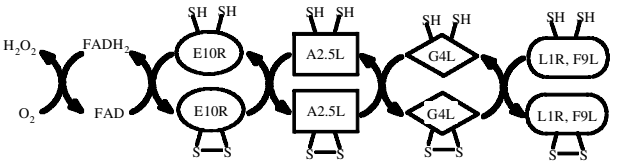

FIG. 2. A model for disulfide bond formation in vaccinia virus proteins in the cytosol of infected mammalian cells. Protein E10R, a homologue of a FAD-dependent sulfhydryl oxidase Erv, oxidizes a thioredoxin-fold protein G4L via an adapter oxidoreductase A2.5L. Oxygen is the terminal electron acceptor in the pathway. This system is a simple analogue of ER and periplasm disulfide bond formation pathways.

G4L appears to function as reductase or isomerase for misfolded L1R and F9L.

DISULFIDE BOND FORMATION IN THE ER

Formation of disulfide bonds in the ER of eukaryotic cells is illustrated in Fig. 3 and has been best characterized for *Saccharomyces cerevisiae*. In this system, the function of yeast protein Ero1 is analogous to that of bacterial DsbB. The active center of Ero1 contains a CxxCxxC redox motif that is directly involved in reoxidation of protein disulfide isomerase (PDI), which in turn oxidizes substrate proteins (5, 12). Although Ero1 proteins have no hydrophobic domains for membrane insertion, they strongly associate with membranes as peripheral proteins (41). Humans have two Ero1 homologues, Ero1-Lα and Ero1-Lβ, with the latter protein having higher activity toward oxidation of PDI. An additional difference between human Ero1s is their patterns of expression. Whereas Ero1-Lα is expressed constitutively, expression of Ero1-Lβ is induced by unfolded protein response (4, 40). It was suggested that Ero1-Lβ works as a redox compensator by increasing oxidative equivalents in the ER in face of large supplies of reduced proteins (36). Unlike bacterial DsbB that is reactivated by transferring electrons to the respiratory chain, the mechanism of Ero1 reactivation is not known. *S. cerevisiae* Ero1 employs FAD as a cofactor (51), but this finding cannot fully explain Ero1 reactivation properties as Ero1 appears to be active under anaerobic conditions and independently of the presence of FAD (13, 51). Interestingly, mutations in yeast *ero1* could be complemented by a mutation in glutathione synthetase gene (9). This observation suggested the existence of additional mechanism(s) of generation of oxidative equivalents in the ER, and the major candidate for such role appears to be a FAD-dependent, soluble sulfhydryl oxidase Erv2, a homologue of mitochondrial sulfhydryl oxidase Erv1 (49). Erv2 has a CxxC redox motif located in the C-terminal portion of the protein (16). The finding of complementation of Ero1 deficiency by a defect in glutathione synthesis (9) suggested that glutathione may possibly function as a reductant in the ER and regulate redox homeostasis by buffering the Ero1 activity.

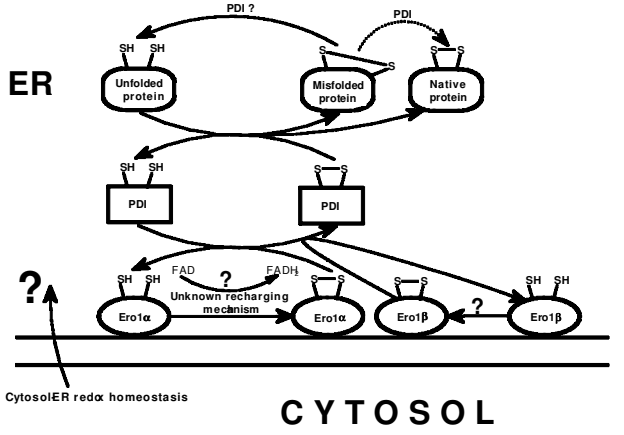

FIG. 3. A model for disulfide bond formation and control in the ER of mammalian cells. Two membrane-associated oxidases Ero1α and Ero1β are involved in the oxidation of PDIs, which in turn transfer oxidizing equivalents to substrate proteins. PDIs are involved in oxidation, rearrangement, and possibly reduction of disulfide bonds in unfolded and misfolded proteins. PDIs work in a complex with chaperones as part of the quality control system. A mechanism for regeneration of oxidative equivalents in Ero1 is not well understood. Arrows indicate the direction of electron transfer, except in the case of disulfide bond isomerization that does not involve net change in redox state (shown by dashed arrow).

PDI, a substrate for Ero1, contains one to four thioredoxin modules, each with CxxC or CxxS motifs (15, 45, 51). In the active form of the enzyme, CxxC groups are present in the oxidized (disulfide) state, but in a mammalian PDI homologue, functional CxxC motifs may occur in a reduced state (15). Ero1 proteins have strong specificity for PDI in the ER thiol oxidation pathway. PDI synthesis is regulated by cellular redox status and concentration of unfolded proteins via transcriptional gene activation (37, 38, 47). The general function of PDI is to oxidize cysteines, but PDI is also involved in a rearrangement of disulfide bonds and protein quality control and could function as a chaperone. A eukaryotic analogue of the bacterial disulfide bond reduction/isomerization DsbD-DsbC,G,E system is not known. Whether the reduced form of PDI can function in this capacity is also not clear (13).

Some PDI homologues, such as human ERp44, contain the CxxS motif in place of the CxxC motif (1, 14). The lack of the C-terminal CxxC cysteine in the active site precludes the role of ERp44 in generating disulfide bonds. However, the initial half-reaction—formation of intermolecular disulfides with substrate proteins—may proceed. Expression of the ERp44 gene, like that of Ero1-Lβ, is induced by accumulation of unfolded proteins in the ER. Thus, CxxS-containing PDI homologues may be involved in the isomerization of disulfide bonds or in the retention of misfolded proteins in the ER, and this idea is supported by available experimental data (1). The role of PDI in the ER is also discussed in the accompanying article by Kersteen and Raines (26).

THIOL/DISULFIDE OXIDOREDUCTION: BIOINFORMATICS APPROACHES

Research over the last 3 years on disulfide bond formation and protein folding control led to significant expansion in our

understanding of these processes, but the fundamental mechanisms of how disulfide bond formation is accomplished and regulated in higher eukaryotes are largely unexplained. One direction of significant potential is the application of bioinformatics methods for identification and functional characterization, on a genome-wide basis, of participants in the pathways of disulfide bond formation, reduction, and isomerization. In our laboratory, we identify proteins containing conserved redox motifs, such as the CxxC motif, in the context of defined secondary structure patterns. Interestingly, redox motifs involving one or two cysteines are often located immediately upstream of an α-helix, and this phenomenon can be seen in structurally distinct proteins. Moreover, residues around the active site, including those located between two cysteines of the CxxC motif, are often highly conserved. Although the CxxC motif is the best characterized redox motif, information on additional motifs is emerging. Examples are a CxC motif in the 15-kDa protein (17, 33) and the CxxxC motif in the vaccinia virus A2.5L protein (48). Redox motifs containing two cysteines can often tolerate a natural replacement of one of the cysteines with a structurally or functionally similar amino acid (*e.g.,* serine in thioredoxins and glutaredoxins, alanine or glycine in methionine sulfoxide reductases, or threonine in glutathione peroxidases and peroxiredoxins) (14; Fomenko and Gladyshev, unpublished observations). In such derivative redox motifs, reaction mechanisms differ from the classical thiol/disulfide mechanism described for thioredoxin (22, 35). It was suggested that an amino acid replacing cysteine in the redox centers is often involved in stabilization of a reactive thiolate. The presence of an α-helix dipole downstream of the redox motif might also stabilize the thiolate (14), although this idea has been disputed.

Analysis of the *S. cerevisiae* genome revealed the presence of five PDIs and single Ero1 and Erv2 proteins, all these proteins being ER-resident oxidoreductases. In higher eukaryotes, the number of proteins involved in thiol disulfide reduction, formation, and isomerization appears to be even larger. Analysis of the human proteome for homologues of known oxidoreductases revealed the presence of at least six PDIs, two Ero1s, and two previously uncharacterized ER-resident glutathione peroxidase homologues. Possible functions of glutathione peroxidases may include protection against oxidative stress, assistance in formation of disulfide bonds by using peroxides as electron acceptors, and control of the redox state in the ER. It should be noted that glutathione is one of the candidates to regulate ER redox state; the ratio of reduced to oxidized glutathione (GSH/GSSG) is ~30-fold lower in the ER compared with that in the cytosol (23).

SELENOCYSTEINE AND ER FUNCTION

In some thiol/disulfide oxidoreductases, active-site cysteine may be naturally replaced with selenocysteine, the 21st amino acid in protein (21). Selenocysteine is encoded by the TGA codon, and selenocysteine-containing proteins can be found in a wide range of organisms (17). About two dozen selenoproteins occur in the human genome (Kryukov and Gladyshev, unpublished observations), at least two of them being ER-resident proteins, Sep15 (30) and SelM (31). Sep15 and SelM genes are expressed in various tissues and organs, but their expression patterns are not identical. The highest level of Sep15 is found in the prostate, whereas SelM is highly expressed in the brain. Interestingly, Sep15 was found to occur in a tight complex with UDP-glucose:glycoprotein glucosyltransferase (UGTR) (30). UGTR is a 150-kDa protein involved in quality control of protein folding. It functions as a conformation-dependent folding sensor by glucosylating N-linked glycans in misfolded glycoproteins, allowing new cycles of protein folding by a calnexin/calreticulin chaperone system (42, 43). UGTR recognizes partially misfolded proteins at the level of individual domains, but appears to be inactive with completely unfolded proteins. The calnexin/calreticulin system involves chaperones, interacts with PDIs, and assists in the formation of correct structures in substrate proteins. These processes appear also to require disulfide bond reduction, but as discussed above, reductive processes in the ER have not been thoroughly investigated. In this regard, one of the candidate reductases might be Sep15. Sep15 could also be involved in thiol-dependent retention processes, which are essential for correct folding of oligomeric proteins. Thiol retention systems generally function by monitoring the redox state of certain cysteines that are involved in formation of intermolecular (or in some cases intramolecular) disulfides (44). Sep15 and SelM are distant homologues (Fig. 4). Whether SelM also forms complexes with UGTR is not known. Interestingly, mammals possess two UGTR homologues (2), but UGTR2 has no known activity. It would be interesting to determine its possible association with SelM.

CONCLUDING REMARKS

Disulfide bonds are formed, reduced, and isomerized primarily by thiol disulfide oxidoreductases containing the CxxC motif. Additional redox proteins may be involved that contain dicysteine or CxxC-derived motifs. In each case, designated electron transfer systems evolved that specifi-

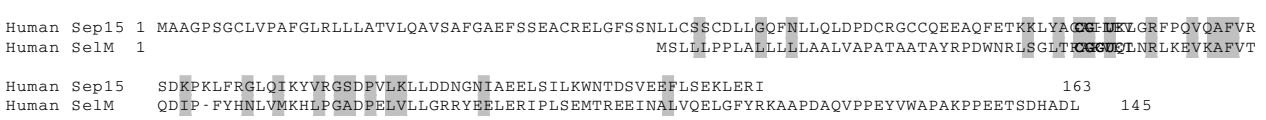

FIG. 4. Homology between human Sep15 and SelM selenoproteins. Identical amino acids are indicated in gray. The putative selenocysteine-containing redox motif is shown in bold. U is selenocysteine.

cally transfer reducing and oxidizing equivalents to a terminal oxidant (oxygen) and a reductant (NADPH), respectively. Disulfide bond formation is best characterized for bacterial periplasm, eukaryotic ER, and eukaryotic cytosol, and in each case thioredoxin-fold proteins containing CxxC motifs are the disulfide-forming enzymes. The disulfide bond reduction and isomerization pathway was characterized for bacterial periplasm, but specific ER-resident reductases are not known. Some redox proteins appeared to replace catalytic cysteines with selenocysteines. Selenocysteine is more active than cysteine in redox proteins, and it would be very interesting to determine the functions of ER-resident selenoproteins. Bioinformatics approaches should yield important information regarding disulfide bond formation, reduction, and isomerization, at the level of both identifying redox proteins and integrating available information into system-wide mechanisms that govern redox processes involving cysteines.

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ABBREVIATIONS

ER, endoplasmic reticulum; FAD, flavin adenine dinucleotide; NAD, nicotinamide adenine dinucleotide; PDI, protein disulfide isomerase; UGTR, UDP-glucose:glycoprotein glucosyltransferase.

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