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Forum Editorial

Oxidative Protein Folding: Many Different Ways to Introduce Disulfide Bonds

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A DISULFIDE BOND IS A COVALENT LINKAGE between two cysteines. Formation of disulfide bonds contributes to the stability, activity, and folding of many proteins. In the 1960s, Anfinsen and his coworkers showed that the formation of disulfide bonds can occur spontaneously *in vitro* in the presence of O_2 (1), which led to the long-held conviction that disulfide bond formation was a spontaneous process *in vivo*. It was 30 years later that discovery of mutations in the gene *dsbA* of *Escherichia coli* revealed that disulfide bond formation is a catalyzed process *in vivo* (2, 6). Later, increasingly, enzymes are being identified that promote the oxidative folding of proteins. Half of the articles in this forum issue highlight the mechanisms employed by various organisms to introduce disulfide bonds into proteins. A surprising variety of mechanisms and protein structures are evolved to catalyze oxidative protein folding which takes place at unexpectedly diverse cellular locations.

The most common places in which disulfide bond formation occurs are the periplasm of gram-negative bacteria and the endoplasmic reticulum (ER) of eukaryotes. The process is best studied in *E. coli* (13).

When a newly translocated protein appears in the periplasm of *E. coli*, a periplasmic protein DsbA introduces disulfide bonds directly into the protein by transferring a disulfide bond formed in its CXXC motif embedded in its thioredoxin-like fold to a pair of cysteines on the substrate (2, 5, 6). DsbA is maintained in its active oxidized state by a membrane protein DsbB, which passes electrons to quinones in the respiratory chain (13, 15). To repair a misoxidized protein, DsbC, another thioredoxin-like protein in the periplasm, reduces the disulfide bond formed between wrongly paired cysteines (13). The active site CXXC motif of DsbC is kept in the active reduced state by a membrane protein DsbD that transfers electrons directly from cytoplasmic thioredoxin to DsbC (13). Despite the knowledge that has accumulated, many interesting questions remain. For example, it is not established how DsbB uses its two pair of essential cysteines to oxidize DsbA nor how quinone oxidizes DsbB.

In this issue, Takahashi *et al.* examine the role of the cytoplasmic loop of DsbB that connects two domains of DsbB each containing a pair of redox-active essential cysteines (15). For this purpose, they introduced in-frame insertions or point mutations into the region. Their intensive biochemical analysis of properties of the mutant proteins led to the proposal that the cytoplasmic loop is important for the coordination of the two pairs of essential cysteines so that they can cooperatively facilitate the electron flow from DsbB to quinones.

A system analogous to the DsbA–DsbB pathway also exists in the yeast ER (13). Yeast uses an essential thioredoxin-like protein disulfide isomerase (Pdi1p), as a primary catalyst of oxidative folding in the ER. Pdi1p, in turn, is reoxidized by Ero1p, an essential membrane-associated protein with four essential cysteines. This organism possesses another ER membrane-associated protein, Erv2p, that, when overexpressed, can replace the function of Ero1p in oxidizing Pdi1p. In contrast to DsbB that passes electrons to quinones, both Ero1p and Erv2p have a bound FAD cofactor and can use O_2 as a direct recipient of electrons. Sevier and Kaiser discuss the mechanisms and structures of the components involved in the disulfide bond formation and compare them with the corresponding system found in the periplasm of *E. coli* (13).

Whether the ER has a reductive pathway like the bacterial DsbC–DsbD system is not clear. One unique feature of the ER is that this compartment holds a high concentration of glutathione. As Sevier and Kaiser describe (13), inactivation of the glutathione synthetic pathway suppressed the phenotypes of yeast *ero1–1* mutant, which indicates that glutathione acts as a net reductant in the ER, counteracting the oxidizing activity of the Ero1p pathway. This finding led to the proposal that the reducing equivalents from glutathione are used to reduce the wrongly paired cysteine residues of substrates or to reduce Pdi1p to act as an isomerase. Sevier and Kaiser speculate that *E. coli* needed to develop a protein-based thiol reduction pathway (the DsbC–DsbD system) instead of using the small molecule glutathione because of the

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presence of the outer-membrane pores that allow the diffusion of small molecules out of the cell (13).

For years it had been assumed that catalysis of structural disulfide bond formation takes place in the bacterial periplasm and eukaryotic ER. However, studies carried out in recent years revealed that many other compartments also have catalytic systems for disulfide bond formation.

In the gram-positive bacterium, *Bacillus subtilis*, only a very few extracytoplasmic proteins with disulfide bonds have been identified (11). Nonetheless, recent works established that this organism also has evolved catalytic systems for disulfide bond formation. Möller and Hederstedt summarize the information on the thiol–disulfide oxidoreductase found on the cell surface of this bacterium. Interestingly, in *B. subtilis*, all of the enzymes are tethered to the extracytoplasmic side of the plasma membrane, in contrast to *E. coli* DsbA which is water soluble (11). This may reflect the fact that *B. subtilis* lacks an outer membrane which, in *E. coli*, prevents the diffusion of proteins from the cell envelope.

As Koehler *et al.* discuss, the intermembrane space of mitochondria (IMS) harbors a number of proteins that have disulfide bonds (7). Recent findings by Mesecke *et al.* showed that there is a disulfide relay system in the IMS comprised of Mia40p and Erv1p (7, 9). Like DsbA and PDI do for newly translocated reduced protein (5, 10), oxidized Mia40p binds newly imported reduced proteins via the formation of a mixed disulfide complex. The subsequent resolution of the complex allows the imported protein to be folded in the IMS. The reduced Mia40p is then recycled back to its oxidized active form by Erv1p that can pass electrons to O_2.

Regardless of the parallels seen among the disulfide relay systems, differences also exist. For example, depletion of Erv1p or conditions reducing Mia40p blocked the import of the substrates, leading to the model that the Mia40p-Erv1p system couples the oxidative folding of the substrates to their import into the IMS (9). On the other hand, the involvement of the DsbA–DsbB or Pdi1p–Ero1p system in the protein translocation process is not known. In addition, Mia40p lacks a CXXC active site motif, a hallmark of the disulfide bond-forming enzymes DsbA and Pdi1p. Moreover, the substrates of Mia40p identified are so called small Tim proteins and Cox17p that have a conserved pattern of cysteines (a twin CX3C motif or twin CX9C motif), into which Mia40p is thought to introduce disulfide bonds (7, 9). Such specific arrangement of cysteine residues is not observed for the substrates of DsbA or Pdi1p. It will be interesting to see whether Mia40p can introduce disulfide bonds into proteins without these motifs.

It should be mentioned that, as Koehler *et al.* and Mesecke *et al.* noted (7, 9), the existence in the IMS of many disulfide bonded proteins and the machinery for disulfide bond formation itself may not be so surprising given the evolutionary relationship of the mitochondrial IMS to the bacterial periplasm.

In contrast to the IMS, ER or bacterial periplasm, the cytoplasm harbors many pathways to keep the cysteines of proteins in the reduced state and few proteins are known there to maintain persistent disulfide bonds (4, 8). One exception is the eukaryotic antioxidant enzyme Cu,Zn-superoxide dismutase (SOD1), which catalyzes the removal of superoxide anion radical. Furukawa and O'Halloran summarize post-translational modifications in SOD1 (3).

This dimeric protein has a highly conserved pair of cysteines, which are found in the disulfide state in the isolated form of the protein. Given the mostly cytosolic localization of the protein, it remained unclear how disulfide bond is formed in this reducing environment (3). In addition to disulfide bond formation, maturation of SOD1 requires three other modifications: copper and zinc acquisition and dimerization. Earlier studies established that the essential copper cofactor is acquired by SOD1 in yeast with the assistance of the copper chaperone for SOD1, CCS. Surprisingly, the role of CCS was not restricted to the insertion of the copper (3). Elegant work by O'Halloran, Furukawa and their colleagues showed that CCS promotes the formation of the essential disulfide in SOD1 *in vivo* and *in vitro* in an O_2-dependent manner. Importantly, oxidation of SOD1 by CCS can occur even in the presence of excess reductant. Once formed, this disulfide bond is kinetically stable, which allows this protein to retain the disulfide bond persistently in the cytoplasm (3).

It has long been considered that the generally reducing environment of the cytoplasm prevents oxidative folding in this compartment. However, as already discussed, this is not always the case. Increasingly, systems are being identified that promote formation of structural disulfide bonds in cytoplasm. In addition to CCS, which catalyzes disulfide bond formation in SOD1 in the yeast cytosol (3), the vaccinia virus protein G4L, together with E10R and A2.5L, is reported to introduce disulfide bonds into viral proteins in the cytoplasm (12). Moreover, studies of redox systems in the bacterial cytoplasm show that, with only one or two mutations, the cytoplasm can be converted to an environment in which efficient disulfide bond formation can take place without changing the overall reductive nature of this compartment (14). Thus, each of the oxidative pathways withstands the coexistence of reductive pathways within the same compartment. These observations indicate that direction of electron flow in each pathway is strictly regulated by substrate specificity.

It is often observed that a single compartment houses several proteins that may carry out similar functions. For example, as Sevier and Kaiser discuss, the yeast ER holds two enzymes, Ero1p and Erv2p, that can oxidize Pdi1p and a total of five PDI homologues (13). Similar redundancy is observed for the thioredoxin and DsbB homologues found on the cell surface of *B. subtilis* as described by Möller and Hederstedt (11). Defining specific redox partners of each component of the pathways and the protein structures that underline their substrate specificity would make key contributions to the understanding of the mechanism and the physiological role of each enzyme.

As we saw above, a huge variety of mechanisms, protein structures and small molecules are being identified to be involved in oxidative folding that takes place at diverse cellular locations. However, I feel that we are just at the starting point in a sense that very little is known about the actual molecular mechanisms by which each component accomplishes its task. For example, the bacterial DsbA introduces disulfide bonds into proteins in the periplasm. The proteins are supposed to take unfolded states during translocation and start to fold when a part of the polypeptide appears in the periplasm. We

still do not even know the answer to such a fundamental question as at what point of translocation and protein folding, disulfide bonds are introduced into the protein by DsbA (5). Further studies are needed to unravel the molecular mechanisms of oxidative protein folding in the cells.

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ABBREVIATIONS

CCS, copper chaperone for SOD1; ER, endoplasmic reticulum; IMS, intermembrane space of mitochondria; PDI, protein disulfide isomerase; SOD1, Cu,Zn-superoxide dismutase.

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