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Title

"Electron avenue: pathways of disulfide bond formation and isomerization"

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An important step in the folding of many secreted proteins is the formation of covalent links between pairs of cysteines, resulting in the formation of disulfide bonds. The classic studies of Anfinsen and his coworkers on the *in vitro* folding of ribonuclease showed that reduced and denatured ribonuclease could fold spontaneously under oxidative conditions, reforming the appropriate disulfide bonds (Anfinsen et al., 1961). This work was often taken to indicate that no enzyme catalyst was required for this process *in vivo*. While the slowness of this oxidation step *in vitro* might have hinted at the need for an oxidative enzyme *in vivo* to make this process more efficient, it was not until 30 years later that studies in bacteria revealed the existence of such an enzyme, DsbA (Bardwell et al., 1991).

The *dsbA* (disulfide bond) gene and its product were discovered as a result of two types of genetic studies: 1) screening or selection for mutant strains defective in the folding of bacterial cell envelope proteins and 2) screening for mutants or genes changing the cellular oxidative capacity resulting in altered sensitivity to the reductant dithiothreitol. Extensive exploitation of these approaches revealed the crucial role of 3 additional cell envelope proteins- DsbB, DsbC and DsbD- involved in two distinct pathways: the formation of disulfide bonds and the isomerization of disulfide bonds (Raina and Missiakas, 1997; Rietsch and Beckwith, 1998).

Both in eukaryotic or prokaryotic cells, the oxidation and isomerization steps are catalyzed exclusively in extracytoplasmic environments (e.g. the lumen of the eukaryotic endoplasmic reticulum and the gram-negative bacterial periplasmic space). In contrast, the cytoplasm displays a network of enzymes and molecules dedicated to the reduction of disulfide bonds (Åslund and Beckwith, 1999).

Electron movement in disulfide bond formation

DsbA is a small periplasmic protein which is a member of the thioredoxin superfamily. This family is characterized by a conserved "thioredoxin fold" and a common active site motif: cys-x-x-cys (Martin, 1995). The active form of DsbA has the two cysteines joined in a disulfide bond. The process of disulfide bond formation begins with a disulfide exchange between this oxidized form of DsbA and reduced cysteine residues of substrate proteins. This exchange results in the passage of 2 electrons to DsbA which is now in the reduced form and must be reoxidized in order to restore its activity. The reoxidation step is performed by DsbB; in *dsbB* mutants, DsbA accumulates in the reduced form (Figure 1). DsbB is a cytoplasmic membrane protein with 4 transmembrane segments and 2 periplasmic loops each containing a pair of essential cysteine residues. A likely reoxidation intermediate between DsbA and DsbB has been identified as a heterodimer containing a disulfide bond between cysteine 30 of DsbA and cysteine 104 of the second periplasmic domain of DsbB (Raina and Missiakas, 1997; Rietsch and Beckwith, 1998).

For the pathway we have described to be functional, at least one more oxidative step is required: the oxidized form of DsbB must be regenerated in order for continuous reactivation of DsbA. At the time of the discovery of DsbB, quinones and other components of the membrane electron transport systems of *E. coli* were suggested as likely recipients of electrons from DsbB, restoring the latter protein to the oxidized state (Bardwell et al., 1993). Subsequently, Ito's group showed that depleting cells of quinones or of cytochromes resulted in defects in reoxidation of DsbB (and, therefore, DsbA) (Kobayashi and Ito, 1999).

In a recent Cell, Bader et al. (Bader et al., 1999) present an impressive combination of biochemical, genetic and physiological experiments which provide compelling evidence for the role

of membrane electron transport components in the reoxidation of DsbB. Furthermore, they are able to specify which cytochromes and quinones can function in this pathway. Their report describes the reconstitution of a highly purified *in vitro* system that replicates the *in vivo* phenomena. In particular, the reoxidation of DsbB is shown to be dependent on the presence of either cytochrome *bd* or *bo* and of either a menaquinone or ubiquinone electron acceptor. These findings provide a satisfying explanation for yet another unresolved question about disulfide bond formation. It is known that the Dsb system still functions efficiently to promote disulfide bond formation under anaerobic growth conditions (Belin et al., 1994). What is the source of oxidation potential when oxygen is not present? Now, with the identification of menaquinone as an effective recipient of electrons from DsbB, a pathway via menaquinone to final electron acceptors other than oxygen appears likely and is supported by their data. Thus, DsbB would switch its use of primary electron acceptors depending on the degree of aerobiosis.

Electron movement in protein disulfide bond isomerization

The early studies of Anfinsen's group focussed attention on the need for a protein disulfide bond isomerase (PDI). Based on his suggestion that an enzyme might be necessary to compensate for incorrect disulfide bonds arising during the spontaneous oxidative process, Anfinsen and his coworkers proceeded to identify such an activity in cell extracts (Goldberger et al., 1963). In this case, the discovery of PDI preceded the detection of a protein with a similar activity in bacteria, DsbC, by over three decades (Shevchik et al., 1994; Missiakas et al., 1994).

Studies over the last several years in both prokaryotes and eukaryotes have also illuminated aspects of the pathway leading to disulfide bond isomerization (Figure 2). Like DsbA, protein disulfide bond isomerases, including DsbC, contain thioredoxin domains. Via the reduced form of

their cys-x-x-cys active site, they are able to attack disulfide bonds of misoxidized proteins and promote shuffling of non-native disulfide bonds to obtain the properly oxidized protein (Figure 2). The mixed disulfide bond intermediate formed during this process might be resolved in two different ways: 1) transfer of the mixed disulfide bond to form a new disulfide bond in the substrate protein and release of DsbC in the reduced state or 2) transfer of the disulfide bond to DsbC and restoration of the reduced form of the substrate protein (Walker and Gilbert, 1997). In the latter case, the substrate protein would be reoxidized by DsbA giving the system another chance to form the correct disulfide bond.

Either because isomerization occurs by mechanism 2 or because of the oxidative environment of the periplasm, the isomerization pathway of *E. coli* requires additional proteins to maintain DsbC in the reduced state. In a striking parallel to the DsbB-DsbA system, DsbC is dependent on the cytoplasmic membrane protein DsbD for the regeneration of its reduced cys-x-x-cys active site; in *dsbD* mutants, oxidized DsbC accumulates (Raina and Missiakas, 1997; Rietsch and Beckwith, 1998). However, in contrast to the DsbB-DsbA pathway which uses intramembranous electron transfer components, DsbD is involved in electron transfer with cytoplasmic proteins. The cytoplasmic thioredoxin pathway passes electrons to DsbD to maintain the latter's DsbC-reducing activity. The mechanism for this electron transfer pathway has yet to be worked out, as DsbD is a complex protein containing 9 potential transmembrane segments, a thioredoxin-like domain at its carboxy terminus and a number of cysteine residues.

What about eukaryotes?

The findings in bacteria have led to increased interest in the mechanisms for disulfide bond formation and isomerization in the ER of eukaryotic cells. The most extensively studied catalyst,

PDI, contains two thioredoxin domains and can perform both oxidation and isomerization reactions *in vitro*. There is also *in vivo* evidence supporting a role for PDI in both processes. Recently an ER membrane-associated protein, Ero1, has been identified that provides oxidizing potential to the lumen of the ER (Frand and Kaiser, 1998; Pollard et al., 1998). Ero1 is essential for the formation of disulfide bonds in the ER. In this issue of Cell, Frand and Kaiser, present evidence that Ero1 is likely responsible for the direct oxidation of PDI. First, they show that PDI is ordinarily found with its active cys-x-x-cys sites largely in the oxidized state. However, in mutants lacking Ero1, these same cysteines are reduced. Second, they identified a disulfide-bonded heterodimer between Ero1 and PDI as a likely intermediate in the oxidation pathway.

These findings make it quite tempting to draw analogies between the eukaryotic and prokaryotic systems. According to this view, Ero1 and DsbB carry out similar functions, insuring that their respective disulfide bond-forming partners, PDI and DsbA, are kept oxidized. Further, the evidence so far raises the possibility that PDI combines the functions of DsbA and DsbC in one protein, being responsible for both the oxidation and isomerization steps. If this is the case, it means that some kind of balance must be maintained between the oxidized and reduced states of PDI, in order for it to carry out both classes of reactions. Alternatively, one of the other PDI homologues that have been identified may be the main contributor to isomerization.

One important difference between eukaryotes and bacteria is that significant amounts of glutathione, both oxidized and reduced, are found in the ER but none has been detected in the bacterial periplasm. Until recently, the presence of oxidized glutathione in the ER has been thought to provide the oxidizing power for disulfide bond formation. However, this proposal has been ruled out by genetic studies of Cuozzo and Kaiser (Cuozzo and Kaiser, 1999). Mutant yeast cells lacking

glutathione are still capable of efficient disulfide bond formation. Frand and Kaiser propose that the actual role of glutathione in the ER may be to function in its reduced form to maintain a fraction of PDI active as an isomerase. At any rate, the precise means by which electrons are passed back and forth through the oxidation and isomerization pathways has yet to be fully clarified.

Exciting questions remain

With many or perhaps all of the bacterial proteins identified that are required for disulfide bond formation, study has focussed on their mechanism of action. Many interesting questions remain. For example, how does DsbC recognize misfolded proteins? How can it efficiently insure that correct disulfide bond form as a result of its activity? Does DsbA act on proteins as they are being translocated into the periplasm or after they have fully emerged? How does this set of Dsb proteins, nearly all of which contain very similar thioredoxin domains, avoid deleterious interactions with each other? Recent reports show that when cytoplasmic thioredoxin is forced to be exported to the periplasm, it acts as an oxidant, and also depends on DsbB for oxidation (Debarbieux and Beckwith, 1998; Jonda et al., 1999). This apparent lack of strict specificity for substrates of DsbB poses the question of how other periplasmic proteins with thioredoxin domains that must remain reduced avoid this oxidation step.

But, perhaps equally fascinating and more mysterious is the mechanism whereby the electrons required for these oxidative and reductive processes are passed in or through membranes. For the pathway leading to disulfide bond formation, which bacterial electron transport components are employed under different environmental conditions? How does DsbB, a small protein, recognize and interact with these components? Is the step between DsbB and quinones actually occurring in the hydrophobic interior of the membrane or at the periplasmic surface? Is

there any movement of domains of the protein within or between these two locations?

In the case of the isomerization pathway in bacteria, the electron flow from the cytoplasm to the cell envelope may only require a series of transfer steps between proteins, in contrast to the DsbB reoxidation pathway. How are electrons transferred from thioredoxin on the cytoplasmic side of the membrane to DsbD and thence to the periplasmic side of the same membrane protein? Are there movements of domains of DsbD?

Finally, the recent reopening of the issue of disulfide bond formation in the ER raises analogous questions about pathways of electron transfer. How does glutathione get transferred to the ER? What is the source of oxidizing power for Ero1? Are there other proteins involved in these electron transfer processes? Does PDI carry out both oxidation and isomerization reactions *in vivo* which would require different oxidation states of its cysteines? If so, how is this balance maintained? If not, what other proteins are important for these processes?

All of these questions show that the mechanism elaborated by the cell to achieve the oxidation of cysteine residues is tremendously intricate when compared to earlier views of the problem and to the simplicity of the primary reaction: the formation of a disulfide bond that ties.

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Figure legends:

Figure 1: A complete disulfide bond formation pathway in *E. coli*? The green arrows indicate the oxidation reaction catalyzed by DsbA. The black arrows pointing leftward along the membrane starting with DsbB on the right represent the direction of the flow of electrons. The hexagon with Q in it represents quinones. Although not indicated in this figure, it has been proposed that electrons in DsbB are transferred from the pair of cysteines that are involved in the oxidation of DsbA to the pair of cysteines located in the amino-terminal periplasmic domain of DsbB and thence to the electron transport chain (Kishigami and Ito, 1996).

Figure 2: Players involved in the pathway for isomerization of disulfide bonds in *E. coli* (the topology of DsbD is based on membrane protein topology prediction algorithms; E.J. Stewart and J. Beckwith, unpublished results). Black arrows indicate the direction of electron flow. The large green arrow indicates the isomerization reaction catalyzed by DsbC

Figure 1

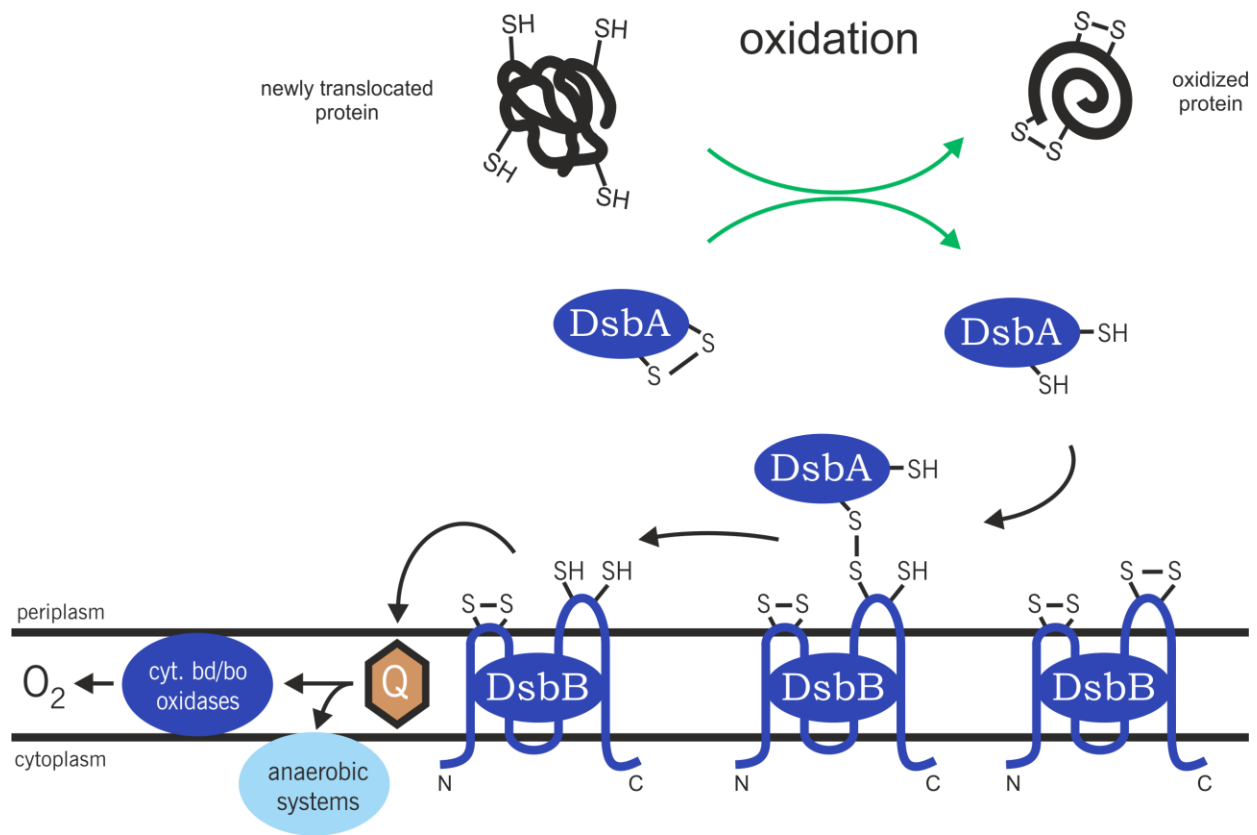


Figure 2

