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# Building bridges: disulphide bond formation in the cell

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## Summary

**Disulphides are often vital for the folding and stability of proteins. Dedicated enzymatic systems have been discovered that catalyse the formation of disulphides in the periplasm of prokaryotes. These discoveries provide compelling evidence for the actual catalysis of protein folding *in vivo*. Disulphide bond formation in *Escherichia coli* is catalysed by at least three 'Dsb' proteins; DsbA, -B and -C. The DsbA protein has an extremely reactive, oxidizing disulphide which it simply donates directly to other proteins. DsbB is required for the reoxidation of DsbA. DsbC is active in disulphide rearrangements and appears to work synergistically with DsbA. The relative rarity of disulphides in cytoplasmic proteins appears to be dependent upon a disulphide-destruction machine. One pivotal cog in this machine is thioredoxin reductase.**

## The need for a catalyst *in vivo*

Disulphide bridges are so important for protein stability that simple reduction of these bonds will, in many cases, cause the protein to unfold. Although vital, disulphides are often very slow to form *in vitro*. In contrast to disulphide-free proteins, which may fold in the millisecond time range, *in vitro* folding of proteins that contain disulphides can take hours or even days (Gilbert, 1994; Sosnick *et al.*, 1994). This seems unrealistically slow from the perspective of the growing cell. There is an obvious need for a protein to be able to reach the native state much more rapidly. Indeed, disulphides *in vivo* form within seconds. This discrepancy in rates led to the discovery of protein disulphide isomerase (PDI), a eukaryotic catalyst for disulphide bond formation (Goldberger *et al.*, 1963). However, investigating the role of PDI in eukaryotes has been hampered by its multi-domain-multisubunit structure, its multifunctional nature, lack of three-dimensional structure, and technically demanding assay techniques. The Dsb catalytic system in *Escherichia coli* appears to be experimentally more amenable to analysis (Creighton and Freedman, 1993). In the short time since the discovery of *dsbA* as a gene required for disulphide bond formation in *E. coli*, a detailed picture is starting to emerge of how disulphide bond catalysis occurs in prokaryotes.

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Dsb mutants are generally defective in disulphide bond formation. DsbA and DsbB are required for the formation of disulphides in compartments external to the inner membrane. Defects in disulphide bond formation have been demonstrated in several periplasmic proteins, the periplasmic domain of an inner membrane protein, in the periplasmic domain of OmpA (an outer membrane protein) and in proteins located on the outer surface of the outer membrane and in proteins which are secreted into the media (Bardwell *et al.*, 1991; Kamitani *et al.*, 1992; Bardwell *et al.*, 1993; Belin and Boquet, 1993; Whitley and von Heijne, 1993; Dailey and Berg, 1993; Bardwell *et al.*, 1994; Belin *et al.*, 1994; Pugsley, 1992; R. Taylor, personal communication). In most cases, this defect in disulphide bond formation results in protein instability and a resulting decrease in the protein's steady-state level. This decrease can be dramatic: snake toxin steady-state level is 40 000-fold reduced in DsbA$^-$ strains (Belin *et al.*, 1994). Although the rate of disulphide bond formation is severely reduced in *dsbA* and *dsbB* strains, it is still detectable. The residual levels of disulphide bond formation found in *dsbA* and *dsbB* null mutants may be due to the presence of DsbC, another enzyme involved in disulphide bond formation (Shevchik *et al.*, 1994; Missiakas *et al.*, 1994).

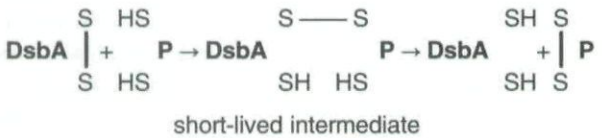
The Dsb mutants exhibit pleiotropic phenotypes. This is not unexpected, since the activity of many disulphide-bonded proteins is altered in Dsb mutants and these proteins play various roles in growth. Processes such as motility, assembly of pili, resistance to reducing agents and benzylpenicillin, and transformation with DNA are adversely affected (Dailey and Berg, 1993; Bardwell *et al.*, 1993; Peek and Taylor, 1992; Tomb, 1992; Missiakas *et al.*, 1993). Many virulence determinants normally contain disulphide bonds and so virulence is severely affected in Dsb mutants (R. Taylor, personal communication). *dsbA* mutants also show secondary effects on such processes as transcription (Pugsley, 1993). Many of these defects are severe enough to allow their use as selections or

screens for Dsb mutants. Defects in activity of alkaline phosphatase, acid phosphatase, and enterotoxin, flagellar assembly or DTT sensitivity have all been used as selectors or screens for isolating Dsb mutants.

The Dsb system appears to be widespread in prokaryotes. Genes homologous and functionally equivalent to *dsbA* have been isolated from *Vibrio cholerae, Haemophilus influenzae, Erwinia chrysanthemi,* and *Legionella* (Peek and Taylor, 1992; Tomb, 1992; Yu *et al.*, 1992; V. E. Shevchik, G. Condemine and J. Robert-Baudouy, submitted; L. Sadosky, personal communication). A gene able to complement the motility defect of the *dsbA* null mutant but showing no overall homology to DsbA has recently been isolated from the Gram-positive organism *Bacillus brevis* (S. Udaka, personal communication). The motility selection was also used to isolate *dsb* genes from *Legionella and Erwinia* and may prove to be generally useful. A *dsbB* homologue is present in *Pseudomonas aeruginosa* as orf12, an open reading frame of unknown function (Kato *et al.*, 1989).

### DsbA: the key catalyst

DsbA plays a pivotal role in disulphide bond formation so it has been the subject of intense scrutiny. DsbA$^-$ bacteria are severely defective in the formation of disulphide bonds (Bardwell *et al.*, 1991; Kamitani *et al.*, 1992; Bardwell *et al.*, 1993; Missiakas *et al.*, 1993). Mature DsbA is a soluble, monomeric 21 kDa periplasmic protein of 189 amino acids (Bardwell *et al.*, 1991; Wunderlich *et al.*, 1993a). The DsbA protein is capable of rapidly oxidizing hirudin, RNase A, Bovine Pancreatic Trypsin Inhibitor (BPTI), and a mutant lysozyme *in vitro* (Wunderlich *et al.*, 1993b; Kanaya *et al.*, 1994; Zapun and Creighton, 1994). The active site in DsbA contains two cysteine residues that can react to form a disulphide bond. This disulphide within DsbA is extremely oxidizing and tends to react very rapidly with free thiols. This reaction results in the rapid and virtually unidirectional transfer of a disulphide from DsbA to its substrate proteins.




short-lived intermediate

The transfer of the disulphide from DsbA to a folding polypeptide is a two-step process in which oxidized DsbA first forms a mixed disulphide with the free thiol of the protein and then a second thiol on the protein reacts with the mixed disulphide, resulting in the transfer of the disulphide bond to the protein. This transfer is very rapid because of the extreme reactivity of the DsbA disulphide and because of the instability of the mixed disulphide. DsbA reacts very rapidly with thiols, 1000-fold faster than normal protein disulphides do (Wunderlich *et al.*, 1993b; Zapun *et al.*, 1993). DsbA is several orders of magnitude faster in disulphide exchange reactions than small-molecule disulphides and other known disulphide oxidoreductase proteins (Wunderlich *et al.*, 1993b). The catalytic efficiency of DsbA is based on these extremely fast disulphide exchange rates. Why is the DsbA disulphide so reactive? Although many factors can influence the kinetics and thermodynamics of the reaction in Fig. 1, we will consider only three: pKa, peptide binding, and redox potential (Gilbert, 1990; 1994).

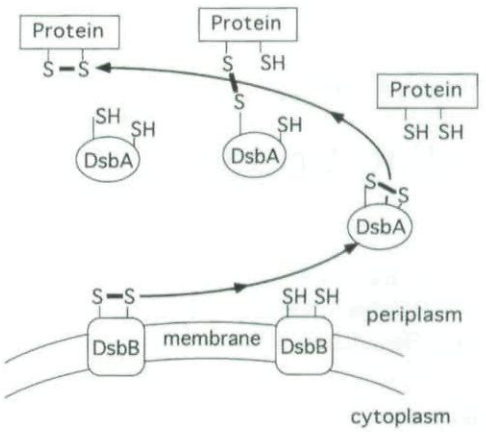



**Fig. 1.** A pathway for disulphide bond formation in bacterial periplasmic proteins. The direction of disulphide transfer is shown by the curved arrow. Proteins are secreted in a reduced state. The DsbA protein binds the unfolded chain, and then rapidly transfers its disulphide to the folding protein. Reduced DsbA is then reoxidized by DsbB, an integral membrane protein. DsbC (not shown) may be be involved in disulphide isomerization.

### pKa

Thiols can only react in disulphide exchange processes when they are deprotonated to the S$^-$ thiolate ion form. The measure of how easily a thiol becomes deprotonated is called its pKa. This determines not only the extent of ionization at any given pH, but also the intrinsic chemical reactivity of the sulphur atom, even when it is part of a disulphide bond. Thus the pKa values of the thiols are important in determining the speed of thiol disulphide exchange reactions. The rate of disulphide exchange can be predicted if the pKa values of all participating sulphur groups are known (Houk *et al.*, 1987).

The pKa of the first cysteine of DsbA Cys-30 has the extremely low value of $\approx$3.5 (Nelson and Creighton, 1994). This means that it will be half ionized at pH 3.5 and fully ionized and reactive at neutral pH. The low pKa value explains the unusual ability of DsbA to function at pH 4. Since the pKa of protein thiols is usually around 8.6, and the proportion ionized drops 10-fold for every pH unit below the pKa, only a tiny proportion of the average

protein's thiols will be reactive at pH 4, but DsbA is still ionized and reactive (Wunderlich *et al.*, 1993b; Nelson and Creighton, 1994; Zapun and Creighton, 1994).

The low pKa of Cys-30 also helps explain why mixed disulphides between DsbA and its substrates are so unstable. The low pKa allows Cys-30 to stabilize the negative charge that develops during disulphide transfer, the negative charge ends up on Cys-30, and it leaves as a thiolate ion. Thus the very low pKa of the first thiol in DsbA helps explain not only the high rate of disulphide interchange but also the direction of the disulphide transfer. However, the observed reaction rates are all about 100-fold greater than can be predicted solely by considering the pKa values. Thus the extremely fast reaction rates of DsbA with the free thiol glutathione can only be partly explained by the low pKa of Cys-30 (Nelson and Creighton, 1994).

### Peptide binding

DsbA greatly speeds the acquisition of disulphide bonds in proteins when present in catalytic quantities and thus is an actual catalyst of protein folding *in vitro* (Wunderlich *et al.*, 1993b). The ability of DsbA to bind peptide can also help explain the very rapid rates of reaction between DsbA and reduced proteins (Wunderlich *et al.*, 1993b). The three-dimensional structure of DsbA shows a deep groove running alongside the accessible cysteine of DsbA, suggesting a possible site for peptide binding (Martin *et al.*, 1993a).

### Redox potential

Apart from the speed at which DsbA reacts, it is also important to consider its oxidizing power, that is whether it prefers to retain its disulphide or transfer it to other thiols. A scale of relative oxidation potentials based on glutathione has been developed for comparing disulphides thermodynamically. This scale compares the ability of a protein (P) to react with oxidized glutathione (GSSG). The measure is simply the equilibrium constant for the following reaction:

$$\begin{matrix} \text{SH} & & & & \text{S} & & \\ \text{P} & + & \text{GSSG} & \leftrightarrow & \text{P} & + & 2\text{GSH}. \\ \text{SH} & & & & \text{S} & & \end{matrix}$$

The smaller the equilibrium constant the more the equation lies on the left and the more the oxidation of glutathione to GSSG is favoured. Therefore, strongly oxidizing disulphides such as that of DsbA have small equilibrium constants. The equilibrium constant of DsbA with glutathione is $10^{-4}$ M, one of the smallest known, and thus it will tend to oxidize glutathione strongly (Wunderlich and Glockshuber, 1993a; Zapun *et al.*, 1993). As the oxidation potential of folding protein is also generally much higher than that of DsbA, oxidation of protein thiols by DsbA is very favourable thermodynamically.

### Why is DsbA so oxidizing?

The reduced form of DsbA is considerably more refractory to urea denaturation that the oxidized form (Wunderlich *et al.*, 1993a; Zapun *et al.*, 1993). This is very unusual, as disulphides normally stabilize proteins (Wunderlich *et al.*, 1993a; Zapun *et al.*, 1993). What is the source of this instability? There is no indication from the high-resolution X-ray structure of oxidized DsbA that the disulphide bond is strained — all bond lengths and angles are close to the optima and all alpha carbons can be perfectly superimposed over the much more stable active site of thioredoxin (Martin *et al.*, 1993a). Rather, mutational studies suggest that both thiol groups are involved in interactions that stabilize the folded conformation of the reduced form (Zapun *et al.*, 1994). Eliminating a thiol either by mutation or by forming a disulphide bond removes these stabilizing interactions and thus destabilizes DsbA. The energy derived from forming these stabilizing interactions could help to drive disulphide transfer from DsbA to its substrates. The oxidizing power of DsbA may be due to these interactions, to the low pKa of the first thiol, and probably to other factors as well.

### The structure of DsbA

The three-dimensional structure of DsbA was recently determined by X-ray crystallography (Martin *et al.*, 1993a,b). This structure indicates that DsbA is a member of the thioredoxin superfamily, despite low sequence identity with thioredoxin. The C alpha atoms of 71 residues in the DsbA thioredoxin domain can be lined up with the corresponding residues in thioredoxin, with a root mean square difference in position of only 1.8 Å. In addition to its thioredoxin-like domain, DsbA also has a second, helical domain which is inserted in the middle of the thioredoxin domain. As in many enzymes, the active site of DsbA is situated at a domain interface, between the helical domain and the thioredoxin domain. Surrounding the active site of DsbA are deep grooves and hydrophobic patches that could be substrate binding surfaces.

Thioredoxin, glutaredoxin and PDI are capable of very rapid disulphide exchange reactions like DsbA, and all appear to operate using a similar catalytic mechanism via an exposed reactive *N*-terminal cysteine (Gilbert, 1994; Hawkins and Freedman, 1991; Holmgren, 1989). However, thioredoxin and glutaredoxin are thought to act as disulphide reductants and thus have very different redox potentials and *in vivo* roles from DsbA and PDI. Detailed comparison of DsbA with other disulphide oxidoreductases may reveal reasons why DsbA and PDI are so oxidizing.

### DsbB: the recycler

It is clear that DsbA can act catalytically to speed the rate of protein folding *in vitro*; it also must act catalytically in the cell since the levels of oxidized substrates can exceed the level of DsbA by more than 500-fold (Akiyama *et al.*, 1992; Belin *et al.*, 1994). The ability of DsbA to actually accelerate the rate of protein folding sets it apart from molecular chaperones, which generally act by slowing unproductive steps in protein folding. So far only the transfer of the disulphide from DsbA to substrate proteins has been considered. How is oxidized DsbA regenerated? The protein that appears to be responsible for the reoxidization of DsbA is an inner membrane protein called DsbB (Bardwell *et al.*, 1993; Missiakas *et al.*, 1993; Belin and Boquet, 1993) (see Fig. 1). DsbB$^-$ mutants are severely defective in disulphide bond formation. In *dsbB* mutants no oxidized DsbA is found, whereas in wild-type cells DsbA is found in a mixture of oxidized and reduced forms. DsbB contains four cysteine residues, facing the periplasmic side of the membrane, that are essential for its function (G. Jander, N. Martin, J. Beckwith, D. Missiakas and S. Raina, personal communication). These cysteines may play a role in the oxidation of DsbA. DsbB may act by transferring disulphide bonds directly to DsbA. The source of these oxidizing equivalents is unknown, but an intriguing possibility is that the electrons generated by disulphide bond formation are coupled through DsbB to the electron-transport chain. The terminal electron acceptor in the disulphide bond formation process is also unknown, but it is clear from the ability of DsbA to function anaerobically *in vivo* that it need not be oxygen (Belin *et al.*, 1994).

DsbB as a stationary redox reagent provides a possible way of coupling disulphide bond formation to energy input; such a couple could provide an explanation for the apparent ability of *E. coli* to maintain an oxidizing environment in the presence of substantial levels of strong reductants. GSH and cystine can influence disulphide bond formation if present in the media in millimolar concentrations (Bardwell *et al.*, 1993; Wunderlich and Glockshuber, 1993b). However, the oxidizing status of the periplasm is not dependent upon the presence of these small-molecule redox components. *E. coli* can grow and form disulphides normally either in the total absence of exogenous thiols or can override the presence of up to 7 mM of the strong reductant dithiolthreitol (DTT) (Bardwell *et al.*, 1993; Missiakas *et al.*, 1993).

### DsbC and others

Additional genes involved in disulphide bond formation have been isolated by two approaches. In one, genes that rescue the severe motility defect of DsbA null strains have been cloned by complementation (V. E. Shevchik, G. Condemine, and J. Robert-Baudouy, submitted). In the second approach, DTT-sensitive mutants were sought (Missiakas *et al.*, 1993; 1994). Two genes have been cloned out of *E. coli* and *Erwinia chrysanthemi* that compliment *dsbA*: one is *dsbA* itself, and the second, *dsbC*, encodes a 24 kDa periplasmic protein that shows disulphide oxidoreductase activity *in vitro*. It possesses an active-site sequence characteristic of the thioredoxin superfamily. Replacement of either cysteine residue of the predicted active site C-G-Y-C inactivates DsbC. Recent experiments performed by A. Zapun and T. E. Creighton in collaboration with D. Missiakas and S. Raina show that DsbC is much more active than DsbA in disulphide-rearrangement reactions. In addition, mixtures of DsbC and DsbA are much more active than either protein is independently. This, together with the observation that the phenotypes of *dsbA dsbC* double mutants are stronger than either *dsbA* or *dsbC* single mutants, suggests that DsbC in some way assists DsbA. DsbC may be particularly important in isomerization reactions. Another possibility is that DsbC is a component in a pathway of disulphide formation independent of the DsbA/B pathway (Shevchik *et al.*, 1994a; Missiakas *et al.*, 1994). Knockout mutations in *Erwinia dsbC* show no visible defect *in vivo*, in contrast to the severe defects seen in *dsbA* or *dsbB* nulls (Shevchik *et al.*, 1994). This might reflect the fact the periplasmic proteins assayed contained only 1–2 disulphides. Proteins that contain multiple disulphides are apparently rare in prokaryotes relative to eukaryotes so there may be a correspondingly smaller need in prokaryotes for an isomerase function.

The screen for DTT-sensitive mutants used by Missiakas *et al.* (1994) to find DsbC has allowed the isolation of mutants in genes involved in various aspects of disulphide metabolism, including both proteins involved in disulphide bond formation in the periplasm (DsbA,B,C) and proteins involved in controlling the thiol/disulphide redox status of the cytoplasm (thioredoxin and thioredoxin reductase), and in previously unidentified genes. The exciting possibility that the new genes identified using this approach are also involved in disulphide metabolism is currently being tested (D. Missiakas and S. Raina, personal communication).

### Comparison with PDI

DsbA and PDI are both similar to thioredoxin, have very similar redox potentials and reaction mechanisms and both are thought to facilitate disulphide bond formation *in vivo*. Detailed comparisons of the similarities and differences between DsbA and eukaryotic PDI have been published (Zapun *et al.*, 1993; Bardwell and Beckwith, 1993). Here I will only consider the following two questions.

### Is DsbA or DsbC a prokaryotic protein disulphide isomerase?

All protein disulphide oxido-reductases like thioredoxin, the Dsb proteins and PDI can catalyse disulphide oxidation, reduction and internal rearrangement (isomerization) reactions in the appropriate redox buffer. Deciding which reaction is physiologically relevant depends upon careful consideration of the efficiencies and conditions required for the various reactions *in vitro*, and direct observation of mutant phenotypes *in vivo*. The rate at which DsbA oxidizes other proteins is at least 1000-fold greater than the rate at which it reduces them, suggesting that it is primarily an oxidant (Zapun *et al.*, 1993; Zapun and Creighton, 1994).

Isomerization reactions require both the oxidation and the reduction of disulphide bonds. DsbC is much more efficient than DsbA in rearrangement reactions *in vitro* (A. Zapun, T. E. Creighton, D. Missiakas and S. Raina, personal communication), suggesting that the major role of DsbC *in vivo* may also be as an isomerase. Facilitation of RNase, BPTI, IGF-1 and hirudin isomerization by DsbA has been detected (Akiyama *et al.*, 1992; Zapun and Creighton, 1994; Wunderlich *et al.*, 1993b; Joly and Swartz, 1994). However, these reactions are rather slow (the turnover number with IGF-1 is an unimpressive 0.2 per minute) and require large concentrations of DsbA. In contrast, DsbA is able to oxidize proteins very rapidly. It oxidizes BPTI, for example, with a second-order rate constant of $>4000\,s^{-1}M^{-1}$, and in at least several cases directly inserts disulphides that otherwise are incorporated only by rearrangements. The primary *in vivo* role of DsbA is suggested to be in the direct introduction of disulphides into proteins. It's role in the intramolecular isomerization of incorrect disulphides, if any, is likely to be secondary (Zapun and Creighton, 1994). Thus the designation of DsbA as a prokaryotic protein disulphide isomerase is dubious. DsbC is a much better candidate.

### Does the essential role of PDI lie in its oxidoreductase activity?

In both DsbA and PDI, the first cysteines of their active sites are exposed and reactive. A eukaryotic PDI mutant where the second cysteines are replaced by serine is viable and shows a defect in disulphide bond formation. This has led to the suggestion that the essential activity of PDI may not lie in its isomerase activity (LaMantia and Lennarz, 1993). However, experiments in prokaryotes suggest that these variants may retain partial oxidoreductase activity. DsbA mutants in which the second cysteine is replaced by serine or alanine retain most of the unusual reactivity and instability of normal DsbA. These mutants catalyse insulin and GSSG reduction, and oxidative folding of hirudin, and they retain partial activity *in vivo* (Zapun *et al.*, 1994; M. Wunderlich and R. Glockshuber, personal communication; author's unpublished observations). At first glance, one would think that destroying either cysteine would result in a DsbA molecule unable to carry out disulphide exchange reactions, However, DsbA that has its second cysteine converted to serine is still reactive with glutathione, and forms an unstable mixed disulphide like wild-type DsbA. Since high levels of glutathione are available to PDI *in vivo*, the eukaryotic PDI mutants may also be able to work in oxidative folding through mixed disulphides with glutathione.

### Disulphide bridge demolition

Disulphides are rare in cytoplasmic proteins and common in periplasmic proteins. There are good reasons why disulphide bonds are not normally permitted in cytoplasmic proteins. The thiol group is one of the most reactive sidechains and is used at the active site of a number of enzymes. Oxidation of these or other thiols in a protein can disrupt function. This is reflected by the common addition of DTT to enzyme assay solutions to protect proteins from inactivation through oxidation. The scarcity of disulphides in cytoplasmic proteins appears to be not just because of the lack of catalysts that form disulphides. In addition, there appears to be a system in the cytoplasm that actively destroys cytoplasmic disulphides. Mutations selected to allow disulphide bond formation in the cytoplasm identify thioredoxin reductase as a pivotal component of the disulphide destruction machine (Derman *et al.*, 1993). In the absence of thioredoxin reductase, disulphide bond formation occurs in cytoplasmically localized alkaline phosphatase and mouse urokinase. Thioredoxin reductase probably does not directly reduce disulphide bonds in cytoplasmic proteins. It is hypothesized that in the absence of thioredoxin reductase an unidentified substrate of thioredoxin reductase accumulates in an oxidized form and this directly oxidizes proteins. It is also possible that the lack of thioredoxin reductase alters the thiol/disulphide redox status of the cytoplasm in a more indirect manner. Thioredoxin itself is expected to accumulate in an oxidized form in thioredoxin reductase mutants but this alone is not sufficient to drive disulphide bond formation in *trxB* mutants (Derman *et al.*, 1993). In wild-type strains, disulphides are presumably passed from proteins, through unknown intermediate(s), to thioredoxin reductase and then destroyed by the oxidation of NADPH to $NADP^+$. Why are these mutants that form disulphides in cytoplasmic proteins not only viable but healthy? One possible reason is that the rate of cytoplasmic disulphide bond formation is not as rapid in these mutants as it is in the periplasm. Thus cytoplasmic proteins may fold rapidly enough to separate and bury their cysteines before oxidation can occur.

Two thioredoxin-like molecules that may play a role in disulphide metabolism have recently been isolated. HelX is a periplasmic protein present in *Rhodobacter capsulatus* with ≈25% amino acid sequence identity to thioredoxin (Beckman and Kranz, 1993). TlpA is an inner membrane protein from *Bradyrhizobium japonicum* that contains a large periplasmic domain with ≈30% amino acid sequence identity to thioredoxin. TlpA has disulphide oxidoreductase activity *in vitro* (Loferer *et al.*, 1993; Loferer and Hennecke, 1994). Both HelX and TlpA play roles in cytochrome biosynthesis. In the absence of HelX, *c*-type cytochromes are not present, and TlpA mutants show strongly reduced levels of the apoproteins of subunit I of cytochrome $aa_3$. A potential function of HelX is to keep the cysteines of *c*-type apocytochromes in a reduced state — a requirement for their covalent attachment to haem. Specific interactions between HelX and *c*-type cytochromes may allow them to escape the oxidative power of the Dsb system.

## Biotechnological implications

Recent developments have added a new urgency to the desire to understand the normal process of disulphide bond formation. Many of the proteins that are useful for therapeutic purposes have disulphide bonds. The DsbA–DsbB system is clearly required for disulphide bond formation in the expressed recombinant proteins tPA, urokinase, BPTI, snake toxin and probably many, if not all, recombinant proteins expressed in the periplasm of *E. coli* (Bardwell *et al.*, 1993; Belin *et al.*, 1994; M. Ostermeier and G. Georgiou, submitted). Manipulation of the redox environment of the periplasm by DsbA overexpression in conjunction with addition of redox compounds to the medium can have a dramatic effect on recombinant protein production. Using this approach, a 14-fold increase in yield was obtained with RBI (Wunderlich and Glockshuber, 1993b). Both DsbA overexpression and addition of reduced glutathione to the media were necessary; simple overexpression of DsbA alone had little effect. RBI is a heavily disulphide bonded alpha-amylase/trypsin inhibitor. Properly folded, its five disulphide bridges cross-link this protein into a complex topology. With overexpressed alkaline phosphatase (two disulphide bonds) a fourfold increase in yield could be obtained by simple overexpression of DsbA. This suggests that DsbA only becomes limiting for disulphide bond formation when its substrates are overexpressed (R. Glockshuber, personal communication).

In some cases, recombinant proteins are difficult to export into periplasm, where they can be exposed to the Dsb systems. A solution to this problem is provided by synthesizing proteins in the cytoplasm of *trxB* mutants, which allow disulphide bond formation to occur in the cytoplasm (Derman *et al.*, 1993). The rates of disulphide bond formation are slower in the cytoplasm of *trxB* mutants than the Dsb-catalysed rate in the periplasm, but substantial levels of oxidized proteins accumulate. As an example, active murine urokinase and alkaline phosphatase accumulate in the cytoplasm of *trxB*-deficient mutants but not in wild-type cells.

## Implications for *in vitro* protein folding

The rate and type of pathways followed in protein folding are dependent upon the nature of the redox reagent used (Rothwarf and Scheraga, 1993). A dithiol redox reagent such as DsbA reacts in a very different way with proteins than does the GSSG/GSH redox pair commonly used in protein folding experiments *in vitro*. DsbA forms only short-lived mixed disulphides with protein thiols because the effective concentration of the second thiol of DsbA is very high. When a linear disulphide reagent such as glutathione is used, stable mixed disulphides form between the protein and glutathione. This can generate kinetic traps in the oxidation process which slow folding and greatly complicate the folding pathway. The use of linear disulphide reagents such as glutathione increases the number of species on a refolding pathway by a factor as great as 10, favouring multiple pathways. The use of excessively high concentrations of oxidized glutathione can result in the formation of excessive mixed disulphides, reducing the rate of protein disulphide bond formation by tying up free protein thiols. Regeneration with a dithiol reagent such as DsbA results in fewer types of pathways and favours formation of intramolecular disulphide bonds. This is a simple consequence of the absence of stable mixed disulphides between DsbA and its substrates.

Is the pathway followed for oxidative folding the same *in vivo* as it is *in vitro*? This question is difficult to address directly because of the paucity of techniques available for studying protein folding *in vivo*. Early indications suggest that the two pathways may be similar but there is at least one important difference: although DsbA is able to oxidize proteins very rapidly *in vitro*, the initial disulphides formed are not the correct ones (Zapun and Creighton, 1994; Wunderlich *et al.*, 1993b). This is in contrast to the situation *in vivo* where disulphide bond formation is apparently both rapid and accurate. It is possible that this is a simple consequence of the much higher concentration of DsbA *in vivo* than can be achieved *in vitro*. However, it is also possible that additional folding factors such as DsbC must be added to *in vitro* folding reactions before we can reproduce *in vitro* the rapid, accurate and efficient folding observed *in vivo* (Akiyama and Ito, 1993; A. Zapun and T. Creighton, personal communication). The proper oxidation of proteins is very dependent upon conformation during folding. If the conformation keeps the cysteine residues together, they will form disulphides very rapidly, but if the conformation

keeps them apart, disulphides will not form. It is possible that chaperones may play a role in preventing improper interactions that lead to improper disulphide bonds.

## Conclusions

The old observation that disulphides form in periplasmic proteins but not in cytoplasmic proteins is beginning to be explained in detail at the molecular level. There appears to be a 'Dsb machine' present in the periplasm that catalyses disulphide bond formation. A key component of this machine is DsbA, which rapidly and unidirectionally transfers its disulphide to proteins. Conversely, there appears to be a 'disulphide-destruction machine' present in the cytoplasm.

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