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Identification of a Protein Required for Disulfide Bond Formation In Vivo

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Summary

We describe a mutation (*dsbA*) that renders Escherichia coli severely defective in disulfide bond formation. In *dsbA* mutant cells, pulse-labeled β-lactamase, alkaline phosphatase, and OmpA are secreted but largely lack disulfide bonds. These disulfideless proteins may represent in vivo folding intermediates, since they are protease sensitive and chase slowly into stable oxidized forms. The *dsbA* gene codes for a 21,000 M_r periplasmic protein containing the sequence cys-pro-his-cys, which resembles the active sites of certain disulfide oxidoreductases. The purified DsbA protein is capable of reducing the disulfide bonds of insulin, an activity that it shares with these disulfide oxidoreductases. Our results suggest that disulfide bond formation is facilitated by DsbA in vivo.

Introduction

Many denatured proteins can spontaneously regain their correct three-dimensional structure in vitro. However, the recent discoveries of proteins that facilitate folding suggest that, in vivo, the process may be assisted by other proteins. Two classes of proteins have been implicated in folding. One class includes some factors that modify the structure of amino acids within polypeptide chains. Examples of this type are protein disulfide isomerase, which can shuffle disulfide bonds between cysteines (Freedman, 1989; 1991), and cyclophilin and FK506, which catalyze cis-trans proline isomerization in vitro (Harding et al., 1989; Takahashi et al., 1989). A second class comprises molecular chaperones that play roles in several aspects of folding, including the assembly of multimeric complexes, and in maintenance of certain proteins in translocation-competent, partially folded states (Rothman, 1989). The group of chaperones includes members of the heat shock families hsp70, GroEL, and GroES and components of the protein secretion machinery (Landry and Gierasch, 1991).

Disulfide bonds are crucial to the folding and stability of many proteins (Doig and Williams, 1991; Taniyama et al., 1991), but little is known about the in vivo mechanism of disulfide bond formation. In vitro reformation of disulfide bonds will occur, but the process is slow ($t_{1/2}$ > 15 min) and the bonds formed are not always the correct ones (Freedman, 1991). In contrast, in vivo disulfide bond formation is rapid and accurate. For example, the 17 disulfide bonds in rat albumin are formed within 30 s after chain completion in vivo, while in vitro the process takes a few hours (Peters and Davidson, 1982). This difference in rates has led to a search for factors that catalyze disulfide bond formation in vivo. Disulfide oxidoreductases such as thioredoxin and protein disulfide isomerase (PDI) can facilitate disulfide exchange reactions in vitro (Hawkins et al., 1991). Disulfide bond formation is partially defective in PDI-depleted microsomes (Bulleid and Freedman, 1988), but the in vivo role of PDI has not yet been definitively established. We describe in this paper a mutant deficient in disulfide bond formation that may help illuminate the in vivo mechanism of disulfide bond formation.

Results

The Genetic Selection for *dsbA* Mutants

A mutation affecting disulfide bond formation (*dsbA*) was isolated fortuitously using a selection designed to detect factors involved in membrane protein insertion. The selection utilizes a fusion in which β-galactosidase (β-gal), a cytoplasmic protein, is fused to the MalF protein, an inner membrane protein of Escherichia coli. In the fusion MalF–β-gal 102, β-gal is fused to the large periplasmic domain of MalF (Froshauer et al., 1988). The translocation of β-gal across the membrane is initiated but not completed; a portion of the β-gal protrudes into the periplasm. The β-gal is therefore partly embedded in the membrane, and as a result, the MalF–β-gal 102 fusion lacks β-gal activity (0.2 U). In a similar fusion, MalF–β-gal 11-1, where β-gal is fused to a cytoplasmic domain of MalF, β-gal can fold properly and 87 U of β-gal is expressed (Froshauer et al., 1988). Selection for Lac$^+$ derivatives of the strain containing the MalF–β-gal 102 fusion could yield mutations unlinked to the fusion that slowed the insertion of the MalF protein and thus allowed β-gal to fold prior to export or could yield mutations that directly affected the folding of β-gal. Among the latter class might be mutants altered in protein folding in either the periplasm or the cytoplasm.

Lac$^+$ derivatives of KM1086, a strain carrying the *malF–lacZ* 102 fusion on a lysogenic λ phage were isolated on M63 lactose minimal medium. Four independent mutations, *dsbA*1–*dsbA*4, that were unlinked to the fusion were isolated. These mutations expressed 44 ± 5 U β-gal. Each of the mutations mapped to the 87′ region of the E. coli chromosome and were linked to known genetic markers in the region (data not shown). The *dsbA*1 mutation was complemented for the Lac phenotype by the F′ 133, which carries this region, showing that it is recessive.

Cloning the *dsbA* Gene

During the complementation studies, we found that *dsbA*1 strains carrying an F factor were resistant to infection with male-specific phages, including M13. Electron microscopy revealed that these strains lacked F pili (D. Furlong, personal communication). The M13 resistance conferred by the *dsbA* mutations provided a convenient means of screening an ordered E. coli clone bank (Kohara et al., 1987) for a clone that complements the *dsbA* mutation (as described in Experimental Procedures). Lysogenization

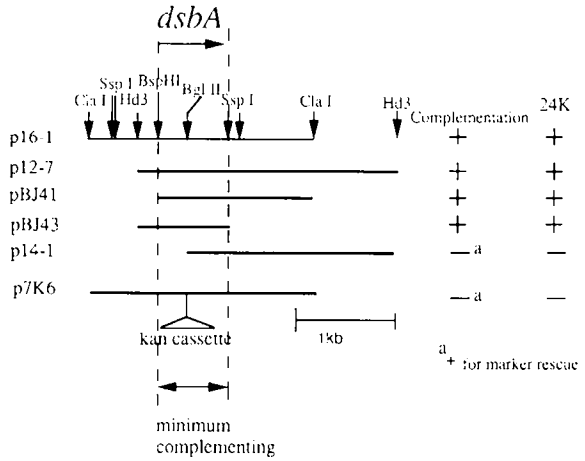

Figure 1. Physical Map of the *dsbA* Region

Horizontal lines indicate the inserts of plasmids containing portions of the *dsbA* region. Complementation was for M13 sensitivity (as described in Experimental Procedures). The presence or absence of the 24K protein was detected by maxicell analysis (Silhavy et al., 1984).

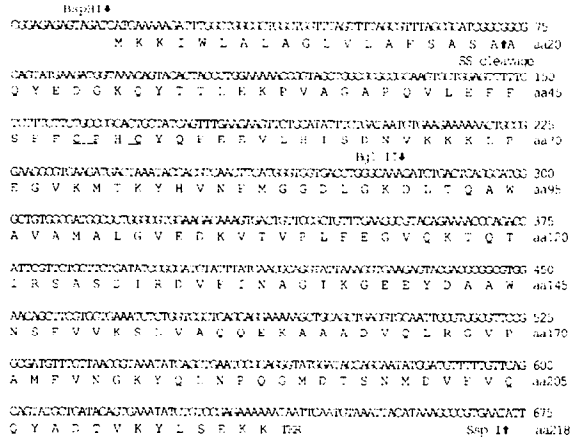

Figure 2. DsbA Sequence

Nucleotide sequence containing the *dsbA* structural gene and its amino acid sequence. The putative signal peptidase processing site is indicated with an arrow. The cysteine residues shown in Figure 3 are underlined. Restriction sites shown in Figure 1 are indicated. This sequence was obtained as part of the E. coli genome project directed by F. Blattner (see Experimental Procedures).

with λ10H11 from the clone bank restored susceptibility to M13 infection, suggesting that λ10H11 carries the wild-type *dsbA* gene. Subcloning narrowed the complementing DNA to a 658 bp region (see Figure 1 and Experimental Procedures)

The DNA sequence of this region was kindly provided by the E. coli genome project directed by F. Blattner (see Experimental Procedures). The complementing DNA contained an open reading frame that could code for a protein of 23,089 M_r (Figure 2). Maxicell analysis showed that all complementing subclones expressed a ~24,000 M_r protein (Figure 1). At the amino terminus of this open reading frame (Figure 2), there is a sequence characteristic of a cleavable signal sequence, suggesting that the *dsbA* gene product is localized in the periplasm or outer membrane. There are no long hydrophobic stretches that might indicate a membrane location for the protein. A good potential cleavage site for leader peptidase lies between two alanines at position 19 and 20. Cleavage at this position would yield a protein of 21,118 M_r. With both osmotic and chloroform shock methods of fractionation, the DsbA protein is found in the periplasmic fraction in both overexpressing cells and wild-type strains, which is consistent with the finding of an apparent signal sequence at the amino terminus of this protein (Figure 6; and data not shown).

dsbA Gene Is Nonessential

To see whether the *dsbA* gene is essential for bacterial growth, we first constructed a null allele of the *dsbA* gene (*dsbA*::kan1). Then we attempted linear transformation of a plasmid carrying the null mutation into a pair of strains: one haploid for *dsbA* and a second that contains an additional copy of *dsbA*. If the *dsb* gene were essential, then replacement of the *dsbA* gene by the *dsbA*::kan1 insertion would only be possible in the diploid strain. However, the *dsbA*::kan1 mutation could be introduced into both the haploid and diploid strains at the same frequency (see Experimental Procedures), indicating that it is not essential.

Phenotypes of *dsbA* Mutants

The phenotypes of strains carrying the *dsbA*1 and *dsbA*::kan1 mutations are similar. Both increased the β-gal activity of the MalF–β-gal 102 fusion to the same extent (data not shown). Both also caused a defect in F pilus assembly, as measured by resistance to the male-specific phage M13 in F^+ cells. Although the mutants grew normally on various rich media at all temperatures tested (23°C–43°C), they exhibited strain-dependent defects in growth on minimal medium. In the strain background JCB474 the *dsbA* mutants JCB472 and JCB566 failed to form colonies on minimal media; in the background MC4100, *dsbA* mutant colonies (JCB555) were mucoid on minimal M63 glucose media. The *dsbA*::kan1 mutants also formed mucoid colonies on rich media in the presence of sublethal levels of tetracycline and sodium arsenite and the dyes acridine orange and pyronin Y. Hypersensitivity to antibiotics and dyes is consistent with a defect in the cell envelope (Sampson et al., 1989). The enzymatic activity of alkaline phosphatase (AP) activity in JCB571 ($phoR^-$, *dsbA*::kan1), was diminished relative to the isogenic $dsbA^+$ control, JCB570, from 5- to 58-fold depending upon growth conditions.

The *dsbA* Gene Product Contains a Sequence Similar to the Active Site of Disulfide Oxidoreductases

The 21,118 M_r amino acid sequence predicted from the *dsbA* DNA sequence was compared with the GenBank release 68 using the program Fasta, which searches for regions of local similarity. Although no sequences with overall similarity to DsbA were found, a short region of local similarity between DsbA and the active sites of a

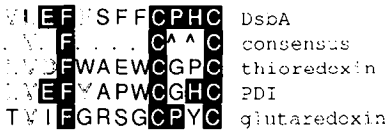

Figure 3. Comparison of the Active Sites of Disulfide Oxidoreductases with DsbA

E. coli thioredoxin, the first active site of rat PDI, E. coli glutaredoxin, and the DsbA sequence are shown. Identities are shown in black; highly similar amino acids are outlined. Sequences are from Hartman et al. (1990) and GenBank. The symbol ^ indicates a bend.

number of disulfide oxidoreductases was detected (see Figure 3) (Hawkins et al., 1991).

Disulfide Bond Formation Is Defective in the *dsbA*::kan1 Mutant

The sequence similarities between the *dsbA* gene product and disulfide oxidoreductases and the effect of *dsbA* mutations on AP activity are both consistent with *dsbA* coding for a protein involved in disulfide bond formation. Alkaline phosphatase requires disulfide bonds for its activity (DuBose and Hartl, 1991). These disulfide bonds do not form in the cytoplasm (A. Derman and J. B., submitted). Therefore, we assayed disulfide bond formation directly in *dsbA* mutants. Many disulfide-containing proteins migrate faster in sodium dodecyl sufate–polyacrylamide gel electrophoresis (SDS–PAGE) than do their reduced counterparts, presumably because the form containing disulfide is more compact. Thus, migration of proteins on SDS–PAGE under nonreducing conditions can be used as a sensitive measure of the extent of native disulfide bond formation (Scheele and Jacoby, 1982). Identical migration rates following disulfide reduction with β-mercaptoethanol demonstrate that disulfide bonds, not other posttranslational modifications, such as signal sequence cleavage, cause the differences in band position. The proteins are first exposed to iodoacetamide, which alkylates free cysteines and irreversibly blocks disulfide formation, so spontaneous formation of disulfide bonds by air oxidation does not occur (Gurd, 1972; Pollitt and Zalkin, 1983). Iodoacetamide is inactive against previously formed disulfide bonds.

The *dsbA*::kan1 mutant and its wild-type parent were pulse labeled with [^{35}S]methionine for 40 s, and free cysteines were trapped with iodoacetamide. The cells were lysed and immunoprecipitated with antisera to AP, OmpA, and β-lactamase, proteins that contain disulfide bonds. AP has two disulfide bonds; OmpA and β-lactamase each have one (Kim and Wyckoff, 1991; Pollitt and Zalkin, 1983; Tani et al., 1990). In wild-type strains, the disulfide bonds of AP, OmpA, and β-lactamase form very rapidly, with the vast majority being formed within the 40 s pulse period (see Figures 4 and 5). Therefore, disulfide bond formation is very rapid in *dsbA*$^+$ strains (A. Derman and J. B., unpublished data; Pollitt and Zalkin, 1983). Strikingly, in the *dsbA*::kan1 mutant, β-lactamase, AP, and OmpA lack disulfide bonds after a 40 s pulse. Disulfide bonds in the *dsbA*::kan1 strain are slowly acquired upon additional growth (see Figures 4 and 5). That all three proteins examined had a defect in disulfide bond formation suggests that the *dsbA* strain may be generally defective in disulfide bond formation.

The fate of the reduced (nondisulfide-bonded) proteins varied. In the *dsbA*::kan1 strain, a portion of the reduced β-lactamase becomes oxidized, but an equal portion fails to acquire disulfide bonds even during a 60 min chase period (Figure 5A). Reduced AP is very unstable; most is lost over a 5 min chase period (Figure 5B). The small amount of AP that remains has acquired disulfide bonds and is not further degraded. In contrast, reduced OmpA appears stable in vivo and slowly becomes oxidized during further growth. In wild-type strains all three proteins are stable during the chase.

Protein Folding Is Defective in the *dsbA*::kan1 Strain

Many periplasmic and outer membrane proteins fold into protease-resistant states; acquisition of protease resistance is commonly used as an indication that a protein is properly folded (Randall and Hardy, 1986). When pulse-

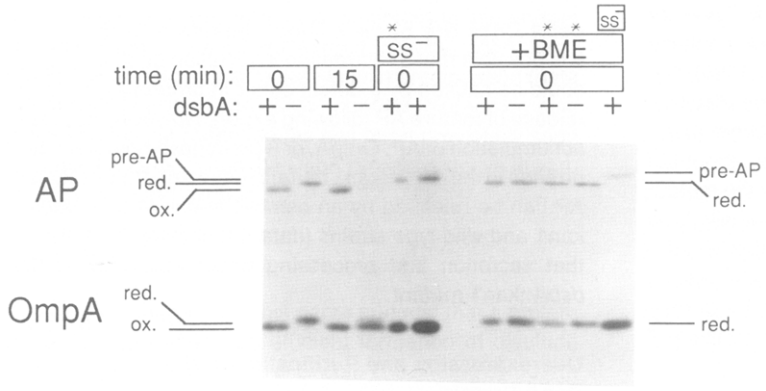

Figure 4. Effect of the *dsbA*::kan1 Mutation on Disulfide Bond Formation

dsbA$^+$ (WT) and *dsbA*::kan1 (*dsbA*−) strains were labeled with [^{35}S] methionine for either 40 s (0) or for 40 s followed by a 15 min chase period with excess cold methionine. Cells were lysed and immunoprecipitated with antisera to AP and OmpA. Autoradiogram after polyacrylamide gel electrophoresis under either nonreducing conditions (left portion) or under reducing conditions (+BME). The positions of the disulfide bonded oxidized (ox.), nondisulfide bonded reduced (red.), and precursor (pre-AP) forms of the proteins are indicated. The asterisks indicate samples that were not exposed to iodoacetamide. The strains used were JCB571 (*dsbA*::kan1), JCB570 (*dsbA*$^+$), and SM593 (*dsbA*$^+$, phoA61 [SS$^-$]). Pho A61 is a mutation in the signal sequence of phoA that prevents export.

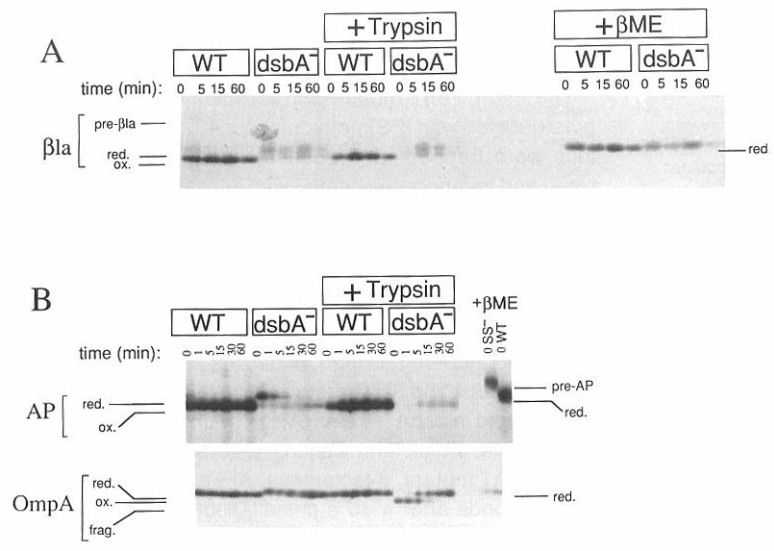

Figure 5. Kinetics of Disulfide Bond Formation and Acquisition of Protease Resistance in *dsbA*::kan1 Strains

Samples were treated as in Figure 4 except that where indicated the samples were treated with 10 mg/ml trypsin (+ Trypsin) prior to immunoprecipitation. The strains used were the same as in Figure 4, except that in (A) the pBR322 containing strains JCB 555 (*dsbA*::kan1) and JCB558 (*dsbA*⁻) were used.

labeled *dsbA*::kan1 cells were lysed and treated with 10 mg/ml trypsin prior to immunoprecipitation, the reduced (nondisulfide-bonded) AP was completely sensitive to trypsin, suggesting that disulfide bond formation is an essential step in the folding pathway. We believe that the reduced AP represents an in vivo folding intermediate, since it is sensitive to proteases and thus not properly folded, and since it chases to a stable oxidized form. A second AP folding intermediate, a form of AP that is oxidized but still trypsin sensitive and thus not yet fully folded, can be detected during the pulse (Figure 5B; K. Johnson and J. B., unpublished data).

Kinetics of OmpA Folding

Measuring the rate of acquisition of protease resistance gives an indication of the rate with which disulfide bond formation and any subsequent folding steps occur. In *dsbA*::kan1, OmpA was protease sensitive after the 40 s pulse and acquired trypsin resistance with a $t_{1/2}$ of ~5 min. In a *dsbA*⁺ strain, OmpA became almost entirely resistant to trypsin during the 40 s pulse. A discrete tryptic fragment of OmpA synthesized in the *dsbA*::kan1 strain was observed (Figure 5b). OmpA is thought to have two domains, a portion that is tightly folded and embedded in the outer membrane and a periplasmic loop that normally contains a disulfide bond (Chen et al., 1980; Tani et al., 1990). We surmise that if this periplasmic loop lacks the disulfide bond, it is sensitive to trypsin. We note from longer exposure of Figure 5B that approximately the same amount of OmpA is trypsin sensitive after a 1 hr chase for the mutant as is trypsin sensitive after a 40 s pulse for the wild-type strain. Thus, OmpA folds considerably more slowly in the *dsbA*::kan1 mutant, compared with wild type. Our results suggest that the folding of both AP and OmpA into their native protease-resistant form requires the formation of disulfide bonds.

The Disulfide Bond in β-Lactamase Is Not Essential for Protease Resistance

Interestingly, although reduced β-lactamase was protease sensitive after the pulse, it acquired protease resistance during further growth even though it is still reduced (Figure 5A). This suggests that the reduced β-lactamase can become at least partially folded without disulfide bond formation. This is consistent with the nonessential nature of the cysteines in β-lactamase (Schultz et al., 1987).

Does DsbA Act at a Step Prior to Disulfide Bond Formation?

While our data are most consistent with the DsbA protein being directly involved in disulfide bond formation, we have not ruled out the possibility that the primary role of this protein is in some other aspect of protein folding. Then, the failure to form disulfide bonds would be a secondary consequence of a more general defect in folding. If this were the case, we would expect that other periplasmic proteins not containing disulfide bonds would be defective in folding. However, we find that both ribose-binding protein and maltose-binding protein, periplasmic proteins that do not contain disulfide bonds, acquire protease resistance as rapidly in the *dsbA* mutant as they do in wild-type strains (data not shown).

dsbA Does Not Show a Major Secretion Defect

It is possible that a retardation in disulfide bond formation could be caused by a defect either in protein export or in release of mature AP following export. However, no major accumulation of AP, OmpA, or β-lactamase precursor can be seen in the *dsbA*::kan1 mutant (Figure 4). Pulse-labeled AP can be released by an osmotic shock in both *dsbA*::kan1 and wild-type strains (data not shown). This shows that secretion and processing occur efficiently in the *dsbA*::kan1 mutant.

Overexpression and Purification of the DsbA Protein

To allow biochemical characterization of the *dsbA* gene product, the DsbA protein was overexpressed and purified. In plasmid pBJ41 the *dsbA* gene was placed under

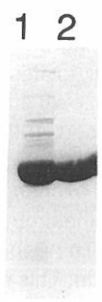

Figure 6. Overproduction and Purification of the *dsbA* Gene Product

Strain JCB607 (pBJ41) was grown to an optical density of 0.2, and IPTG was added to 5 mM. After 5 hr of growth cells were harvested. A periplasmic fraction was isolated by an osmotic shock (lane 1), loaded onto a DE52 cellulose column, and eluted with various NaCl concentrations; lane 2 shows the fraction that eluted with 50 mM NaCl. Samples were subjected to SDS–PAGE (16% acrylamide) and stained with Coomassie blue.

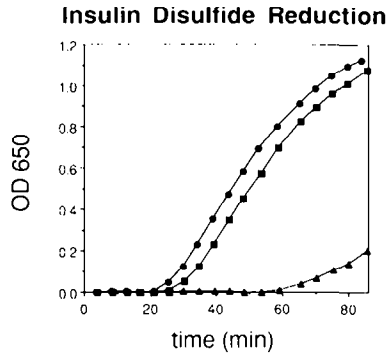

Figure 7. *dsbA* Protein Facilitated Reduction of Insulin by Dithiothreitol

The incubation mixtures contained 0.1 M potassium phosphate (pH 7.0), 2 mM EDTA, 0.13 mM insulin, and 0.33 mM dithiothreitol. Purified DsbA was added to ~5 μM (squares). Thioredoxin from E. coli 0.7 μM served as a positive control (circles). Dithiothreitol alone without added protein served as a negative control (triangles). The reduction of insulin and its resulting precipitation were monitored by following optical density at 650 nm.

the transcription and translational control of the *trc* promoter in pKK233-2. In strain JCB607 (pBJ41), the *dsbA* gene product was massively overproduced and present almost exclusively in the periplasmic fraction. The periplasmic shock fluid (>70% DsbA) was adjusted to pH 7.0 and loaded onto a DE52 cellulose column that had been equilibrated with 10 mM MOPS (pH 7.0). The fraction that eluted with 50 mM NaCl is >90% *dsbA* gene product (Figure 6). Forty milligrams of DsbA protein could be recovered from 1 liter of induced culture.

DsbA Protein Can Act to Reduce Insulin Disulfide Bonds In Vitro

The reduction of insulin by a disulfide oxidoreductase can be assayed by a rapid spectrophotometric assay. Insulin contains two polypeptide chains, A and B, that are linked by two interchain disulfide bonds. When these bonds are broken, the free B chain is insoluble and precipitates. Both PDI and thioredoxin are known to catalyze this disulfide exchange reaction in vitro. Dithiothreitol reduces PDI and thioredoxin, and these proteins in turn reduce the insulin substrate in a disulfide exchange reaction. The purified DsbA protein was incubated with insulin and found to stimulate insulin precipitation in a dithiothreitol-dependent manner (Figure 7). A control extract from the *dsbA*::kan1 strain JCB609 did not stimulate insulin precipitation (data not shown). In the same assay, purified thioredoxin also promoted insulin reduction, as has been previously seen (Holmgren, 1979). Thus the DsbA protein is active in vitro in disulfide bond exchange.

Discussion

The results presented in this paper suggest that a protein factor is required for the efficient formation of disulfide bonds in exported proteins of E. coli. The finding of mutant bacteria that are defective in the process demonstrates the importance of such an activity in vivo.

We have isolated a mutation in a gene, *dsbA*, that, by several criteria, is involved in disulfide bond formation. First, *dsbA* mutants are severely defective in the formation of disulfide bonds in vivo. Second, the *dsbA* gene product, a 21,000 M_r protein, contains a sequence motif found in disulfide oxidoreductases. Third, the purified protein exhibits an activity, the reduction of insulin disulfide bonds, common to this class of enzymes. The *dsbA* gene product may be acting directly to facilitate disulfide bond formation. Alternatively, it may alter the redox potential of the periplasmic space in a way that favors disulfide bond formation. The possibility that DsbA is involved at a step in protein folding prior to disulfide bond formation seems unlikely based on the in vitro activity of the protein disulfide bond exchange and the finding that proteins that do not contain disulfide bonds apparently fold normally in *dsbA* mutants.

Why Is It Required That Disulfide Bond Formation Be Facilitated?

The difference in the thiol/disulfide redox state between the cytoplasm and the periplasm may be sufficient to prevent disulfides in the cytoplasm and to drive disulfide bond formation in the periplasm. However, spontaneous oxidation may not proceed at a physiologically relevant rate. Although at least some proteins can attain the correct disulfide bonds during refolding in vitro, this process is often considerably slower than might be expected for effective assembly in vivo (Freedman, 1991; Peters and Davidson, 1982). Disulfide bonds contribute significantly to the stability of proteins in many cases. In E. coli, as in eukaryotes, disulfide bonds are commonly found in exported proteins but are rare in cytoplasmic proteins (Gilbert, 1990). Slow assembly in a protease-rich environment such as the bacterial periplasm could lead to rapid degradation of an incompletely folded protein, as is clearly the case with AP.

Disulfide bond formation is not totally eliminated in strains carrying the *dsbA*::kan1 mutation. The remaining disulfide bonds may form by spontaneous air oxidation or they may be catalyzed by another enzymatic system. Therefore, the viability of the *dsbA*::kan1 mutant does not

establish whether disulfide bonds are essential for E. coli growth. Besides the deficiency in disulfide bond formation, the *dsbA*::kan1 mutation has severe effects on growth, including preventing colony formation on minimal media in some strain backgrounds. This defect may be due to the effect of the mutation on the assembly of sugar transport proteins in the cell envelope. Several of these have cysteine residues involved in thiol/disulfide exchange thought to be essential for sugar transport (Konings and Robillard, 1982; Robillard and Konings, 1981). The defect in assembly of F pili in *dsbA* mutants may reflect the existence of pilin proteins that contain essential disulfide bonds.

It is clearly important for the cell to control both the location and timing of protein folding. Chaperonins are thought to keep proteins in a partially unfolded, secretion-competent state while they are in the cytoplasm. Other factors may assist in the folding of proteins following secretion. DsbA may be one such factor. Another factor is PDI (discussed below) and BiP, which is thought to assist in protein folding within the endoplasmic reticulum (Rothman, 1989). The mechanism of BiP action however, is not yet well defined. Restriction of the cellular compartment in which disulfide bond formation occurs may be one way to regulate protein folding.

Our results reveal the importance of disulfide bond formation in the protein folding pathway for AP, OmpA, and β-lactamase in vivo. In the *dsbA*::kan1 cells, assembly proceeds normally up to disulfide bond formation. Alkaline phosphatase, which lacks its disulfide bonds, even though it has been properly secreted and processed, is not folded into its final conformation since it is sensitive to protease in vivo and to added protease in vitro. The small fraction of AP that survives proteolysis chases into an apparently native and active protease-resistant form that contains disulfide bonds. Thus, the formation of disulfide bonds appears to be an essential step in AP's folding pathway. However, even after disulfide bond formation has taken place, it is possible to detect a protease sensitive, incompletely folded AP. Thus it appears that disulfide bond formation is not the last step on the folding pathway. Reduced OmpA is also not fully folded since it is sensitive to added protease. However, it is not as unstable in vivo as AP and eventually folds into a protease-resistant form. β-lactamase, in contrast, folds into a protease resistant form in the absence of disulfide bond formation. This appears to be a case where disulfides are not essential for function. Thus, the *dsbA*::kan1 mutation allows one to examine easily the in vivo importance of disulfides in proteins.

The Nature of the DsbA Activity

Protein disulfide oxidoreductases catalyze the exchange of disulfide bonds between a source of a disulfide and free sulfhydryl groups. The disulfides may be on small molecules such as oxidized glutathione or may be present within proteins (Gilbert, 1990). Thus, protein disulfide oxidoreductases can act to make or break protein disulfides or shuffle disulfide bonds within proteins.

DsbA, like thioredoxin and PDI, is active in an assay for protein disulfide oxidoreductases that detects disulfide reduction in insulin. Thioredoxin is a well characterized ubiquitous 12K protein that appears to act in the cytoplasm as a dithiol reductant (Holmgren, 1989). PDI is a 57K protein biochemically similar to thioredoxin; however, it is present in the endoplasmic reticulum where it may function as a redox buffer (Gilbert, 1990; Hawkins et al., 1991; Krause et al., 1991). The specific activity of these three proteins in disulfide bond exchange is low (See Figure 6; Hawkins et al., 1991; Lundstrom and Holmgren, 1990; Lyles and Gilbert, 1991). The DsbA protein appears to be less active than thioredoxin. This may be because we are pushing the reaction in the reverse of the physiological direction; the assay is for disulfide reduction, while in vivo, DsbA appears to act to facilitate the formation of disulfide bonds.

Is DsbA a PDI or a Member of a New Family of Disulfide Oxidoreductases?

Disulfide bond formation is defective in PDI-depleted microsomes, leading to the implication that DsbA may be a prokaryotic PDI. However, there exist several differences between PDI and DsbA. Protein disulfide isomerase is named for its role in disulfide bond isomerization. The proteins examined in the *dsbA* mutant did not have incorrect disulfide bonds, they lacked disulfide bonds. PDI has been implicated in glycosylation, an activity that is not known to exist in E. coli. (LeMantia et al. 1991). PDI mutants have been isolated in yeast. Unlike the *dsbA*::kan1 insertion, null mutants in PDI are not viable, perhaps reflecting PDI's multiple roles (Freedman, 1991; LeMantia et al. 1991). It will be of great interest to determine whether disulfide bond formation is affected in PDI mutants. PDIs share considerable sequence homology with each other; rat and yeast PDI are 31% identical. PDI is also homologous to thioredoxins; rat PDI, for example, contains an 81 amino acid region 36% identical to E. coli thioredoxin (Edman et al., 1985). Although the *dsbA* sequence has 3 to 5 amino acids in common with the active site of these and other disulfide oxidoreductases (Figure 3), it is not closely related to the PDI-thioredoxin family and may represent a member of a new protein family of oxidoreductases. A *dsbA* homolog has recently been described by Peek and Taylor, who have characterized a gene from Vibrio cholera (*tcpG*) whose predicted amino acid sequence shares 40% amino acid identity with amino acids 19 to 150 of DsbA. Interestingly, *tcpG*, like *dsbA*, encodes a periplasmic protein essential for pilus biogenesis that, when isolated, is active in insulin reduction. Based on its sequence, the disulfide exchange activity, and the deleterious effect of mutants on pili and cholera toxin, it is proposed that TcpG may be involved in disulfide bond formation and also may act as a chaperone (Peek and Taylor, personal communication).

The Genetic Selection for *dsbA* Mutants

It is interesting to speculate as to how a selection designed to isolate mutations affecting integral membrane protein insertion gave rise to mutations affecting disulfide bond formation instead. The *dsbA* mutations confer a Lac$^-$ phenotype on strains carrying the MalF–β-gal 102 fusion. In these strains, β-gal is thought to be enzymatically inactive because it is imbedded in the cytoplasmic membrane. A

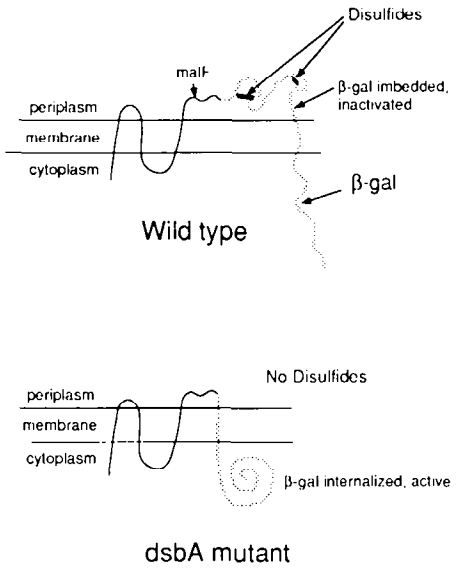

Figure 8. Topological Model of the MalF–β-gal Fusion in *dsbA*⁺ and *dsbA* Strains

portion of β-gal protrudes into the periplasm, and early in β-gal there are several cysteines. In *dsbA*⁺ strains, disulfide bonds may form between pairs of these cysteines and the bonds may fold the periplasmic portion of β-gal into a conformation that stabilizes the transmembrane structure. The *dsbA* mutations should retard the formation of disulfide bonds in the β-gal, thereby destabilizing the transmembrane structure. As a result of this destabilization, the β-gal might be pulled back into the cytoplasm by the folding of that portion that has remained behind. As a result, the protein might be able to assemble into an active enzyme (Figure 8). Consistent with this model, it has been reported that it is possible for a protein that is partially secreted to be retracted into the cytoplasm (Schiebel et al., 1991).

Experimental Procedures

Bacterial strains are described in Table 1.

Complementation for *dsbA* Using Phage and Plasmid Clones

M13 sensitivity was monitored by infecting strain JCB477 with M13mp131, looking for a complementation, which leads to β-gal activity. M13mp derivatives form blue plaques on lawns of M13 sensitive cells that contain the *lacZ* omega fragment on plates with Xgal and isopropyl β-D-thiogalactopyranoside (IPTG), but do not form any plaques at all on *dsbA* E. coli, since it is M13 resistant. An ordered λ library of E. coli DNA has been constructed by Kohara et al. (1987). Phage known to map to the *metE-glnA* region were spotted on a lawn of JCB477 (*dsbA*1) on plates spread with 10^8 M13mp131, 0.2 mg/ml Xgal, and 2 mM IPTG. Phage or plasmids that complemented *dsbA*1 allowed infection by M13mp131, resulting in blue cells. λ10H11 complemented *dsbA*1. To narrow down the location of *dsbA*, λ10H11 was digested with HindIII, and fragments were subcloned into pBR322. Six complementing subclones were found to contain the same 2.5 kb insert. Subclone p16-1, which contains an overlapping ClaI fragment cloned into pBR322, also restored M13 sensitivity to *dsbA* mutants containing an F factor (Figure 1). In addition to restoring the M13 sensitivity, these plasmids also restored the Lac phenotype of the MalF–β-gal fusion 102 carried by KM1103 (*dsbA*1), KM1102 (*dsbA*3), KM1101 (*dsbA*2), and KM1104 (*dsbA*4). These results suggest that the *dsbA*1, *dsbA*2, *dsbA*3, and *dsbA*4 mutations may lie within the same gene. The restriction map of p16-1 matches that of the region at kb3662 on the Kohara restriction map of E. coli. This region corresponds to

Table 1. E. coli Strains Used

Strain	Genotype or Phenotype
JCB472	JCB474 *dsbA*1 zih-12::Tn*10*
JCB473	JCB474 zih-12::Tn*10*
JCB474	F′(*traD*30 *proAB* $LacI^q$ *lacZ*ΔM15)Λ(*lacpro*) *thi strA sbcB hsdR*⁻
JCB477	JCB474 *dsbA*1 zih-12::Tn*10*
JCB504	MC1000 *recD*1903::miniTn10tet^s zih12::Tn*10* (λD69)
JCB505	MC1000 *recD*1903::miniTn10tet^s zih12::Tn*10* (λ*dsbA*′)
JCB513	JCB474 (λD69)
JCB514	JCB474 (λ*dsbA*′)
JCB555	MC4100 (λD69) zih12:Tn*10* pBR322 *dsbA*::kan1
JCB558	MC4100 (λD69) zih12::Tn*10* pBR322
JCB566	JCB474 zih12::Tn10 *dsbA*::kan1
JCB567	JCB474 zih12::Tn*10*
JCB570	MC1000 *phoR* zih12::Tn*10*
JCB571	MC1000 *phoR* zih12::Tn*10* *dsbA*::kan1
JCB572	JCB502 *dsbA*::kan1
JCB607	*rna met* pBJ41 pMS421
JCB609	*rna met dsbA*::kan1
KM1086	MC4100 $malT^c$ (λmalF-lacZ 102)
KM1101	KM1086 *dsbA*2
KM1102	KM1086 *dsbA*3
KM1103	KM1086 *dsbA*1
KM1104	KM1086 *dsbA*4
MC1000	*araD*139 Δ(*araABC-leu*)7679 *galU galK* Δ(*lac*)X74 *rpsL thi*
MC4100	*araD*139 Δ(*argF-lac*)U169 *rpsL*150 *relA flbB*5301 *deoC*1 *ptsF*25 *rbsR*
SM593	MC1000 *phoR phoA*61

The source of all strains was this study. The exceptions are MC1000 and MC4100, from Silhavy et al. (1984) and SM593, from Michaelis et al. (1983). For gene symbols, see Bachmann (1990). Phenotypic symbols were derived from gene symbols, except as follows: tet, tetracycline; kan, kanamycin; s, sensitivity.

87.35 min on the E. coli restriction map (Bachmann, 1990) and is consistent with both the mapping data and the complementation of *dsbA*1 by λ10H11. p14-1 was derived from p12-7 by digestion with BamHI and BglII and religation. p7K6 was derived from p12-7 by digestion with BglII and ligation with the kanR cassette on a 1.75 kb BamHI fragment from λ1105 (Way et al., 1984). λdsbA' contains the 2.5 kb HindIII fragment from p12-7 cloned into λD69 (Silhavy et al., 1984).

F' 133 was obtained from B. Bachmann. It carries the region from *ilv* at 84.5 to *argH* at 89 min. pBJ41 was produced by isolating the *dsbA* gene on a BspHI-HindIII fragment and cloning it into the Pharmacia expression vector, pKK233-2, that had been cleaved with NcoI and HindIII. pBJ43 was made by cutting p16-1 with HindIII and SspI and subcloning into pUC18. pMS421 is a *lacIQ*, *strR*, *spcR*, pSC101 *ori* plasmid obtained from Mimi Susskind.

Test for Essentiality

A method involving linear transformation was used (Bardwell and Craig, 1988). A kanamycin resistance cassette was inserted in p16-1 into a *BglII* site near the middle of the 23K open reading frame generating p7K6. The plasmid carrying this insertion mutation (*dsbA*::kan1) failed to express a 23K protein and failed to complement *dsbA*1, even though it was present in multiple copies. p7K6 was linearized and transformed into the chromosome of strains JCB504 and JCB505, as described by Russell et al. (1989). After transformation, kanamycin-resistant colonies were selected and the proportion that was ampicillin sensitive (indicating that linear transformation had occurred) was determined. The number of ampicillin-resistant colonies, (those in which the p7K6 plasmid remains intact), provides an internal control for transformation efficiency. If the *dsbA* gene were essential, we would be able to recover Kan^R amp^S transformants in the meridiploid but would be unable to interrupt the *dsbA* gene in the haploid. However, similar proportions of amp^S kan^R transformants were recovered from parallel transformations into the *dsbA* haploid and diploid; 13% (35/271) amp^S in JCB504; 16%(12/76) amp^S in JCB505.

To confirm the transformation results, the *dsbA*::kan1 mutation was moved by P1 into haploid and diploid strains using the closely linked Tn*10*::zig12. The *dsbA*::kan1 mutation was >90% linked to Tn*10*::zig12 in transduction into both the diploid and haploid strains, JCB513 and JCB514, indicating that it is nonessential. P1 transductions and selection for λ lysogens were as described (Silhavy et al., 1984).

Biochemical Tests

The turbidimetric assay of insulin disulfide reduction was as described by Holmgren (1979). DsbA protein concentration was estimated using the method of Bradford (1976).

Sequence Analysis

F. Blattner, D. Daniels, G. Plunkett III, and V. Burland are in the process of sequencing the E. coli genome, and they kindly made available their sequence data from the kb3662 region. Compilation of their data showed a small gap and several ambiguities that needed to be resolved. The sequence was completed using M13 clones of the region kindly supplied by D. Daniels and Albert Nakano.

General Methods

Procedures for AP enzyme assays and preparation of periplasmic proteins by osmotic shock and chloroform shock were essentially as described previously (Manoil and Beckwith, 1986; Michaelis et al., 1983; Ames et al., 1984). Anti-PhoA antibody was made by C. Gardel in our laboratory. Anti-OmpA antibody was kindly provided by Carol Kumamoto. Anti-β-lactamase antibody was purchased from 5 Prime → 3 Prime Inc.

Labeling, Proteolysis, Immunoprecipitation, and PAGE

E. coli were grown in M63 glucose containing 18 amino acids (minus met and cys), 1 milligram per liter thiamine, and 1 mM $MgSO_4$ (Silhavy et al., 1984). Cells were labeled with 50 μCi/ml [^{35}S]methionine for 40 s and then were either chased with cold met (0.05%), or their disulfide bond formation was blocked by adding iodoacetamide to 10mM and holding on ice for 15 min. Not adding iodoacetamide resulted in complete oxidation of proteins during processing. Cells were spun down and resuspended in 0.5 vol of cold spheroplast buffer (0.5 M sucrose, 100 mM Tris [pH 8.0], 0.5 mM EDTA). Lysozyme was added to 2 μg/ml and EDTA was added to 7 mM. After a 15 min incubation on ice, cells were lysed by adding Triton X-100 to 1%, and trypsin was added to 10 μg/ml. After 20 min of proteolysis on ice the protease inhibitor phenymethylsulfonyl fluoride was added to 1 mM, and trypsin inhibitor to 2.5 mg/ml was added to all samples. An equal volume of 2 × KISDS buffer (100 mM Tris-HCl [pH 8.0], 300 mM NaCl, 4% Triton X-100, 2 mM EDTA, 0.06% SDS) was added and the lysate was frozen in a mixture of dry ice and ethanol and then thawed. The freezing followed by thawing was repeated two more times. After spinning out the debris, antibody was added, and samples sat overnight on ice at 4°C. Samples were then immunoprecipitated as previously described (Froshauer et al., 1988). Oxidized and reduced forms of proteins were distinguished by SDS–PAGE under nonreducing conditions. Optimal separation of oxidized and reduced forms was obtained by electrophoresis on a Bio-Rad 38 cm × 50 cm Sequigen electrophoresis apparatus using 0.8 mm spacers. When required, protein samples were reduced by boiling in 0.7 M β-mercaptoethanol for 3 min.

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GenBank Accession Number

The accession number for the sequence reported in this paper is M77746.

Notes Added in Proof

We recently learned that Ito and his colleagues have identified the same gene by a very different approach.

The data referred to as A. Derman and J. B., submitted, are now in press: Derman, A., and Beckwith, J. B., Escherichia coli alkaline phosphatase fails to acquire disulfide bonds when retained in the cytoplasm. J. Bacteriol., in press.