The Nonconsecutive Disulfide Bond of Escherichia coli Phytase (AppA) Renders It Dependent on the Protein-disulfide Isomerase, DsbC*

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The formation of protein disulfide bonds in the Escherichia coli periplasm by the enzyme DsbA is an inaccurate process. Many eukaryotic proteins with nonconsecutive disulfide bonds expressed in E. coli require an additional protein for proper folding, the disulfide bond isomerase DsbC. Here we report studies on a native E. coli periplasmic acid phosphatase, phytase (AppA), which contains three consecutive and one nonconsecutive disulfide bonds. We show that AppA requires DsbC for its folding. However, the activity of an AppA mutant lacking its nonconsecutive disulfide bond is DsbC-independent. An AppA homolog, AppC, a periplasmic acid phosphatase with similar structure, lacks the nonconsecutive disulfide bond but has the three consecutive disulfide bonds found in AppA. The consecutively disulfide-bonded AppC is not dependent on DsbC but is rendered dependent by engineering into it the conserved nonconsecutive disulfide bond of AppA. Taken together, these results provide support for the proposal that proteins with nonconsecutive disulfide bonds require DsbC for full activity and that disulfide bonds are formed predominantly during translocation across the cytoplasmic membrane.

Disulfide bonds between cysteine residues make an important contribution to the folding pathway and stability of many proteins. Early in vitro studies on protein folding showed that the protein RNase when denatured and its disulfide bonds reduced could refold into an active enzyme with the correct disulfide bonds formed (1). Although these results indicated that the information necessary for the proper folding of proteins was intrinsic to the amino acid sequence, the rate of disulfide bond formation in these experiments was much slower than the rates observed in living cells, and the yield of active enzyme was low. The low yields of properly folded enzyme were attributed to the formation of incorrect disulfide bonds in many of the assembled protein molecules. Thus, the in vitro studies, although remarkably successful, were unable to come close to replicating the rapid kinetics and the accuracy of in vivo disulfide bond formation. To explain this inaccuracy observed in biochemical experiments, Anfinsen and co-workers (1) postulated the existence in cells of an enzyme, protein-disulfide isomerase (PDI), which would increase the yields of active enzyme by correcting incorrectly formed disulfide bonds. Such an enzymatic activity was discovered in beef liver cells (2). The explanation for the slow in vitro rates was resolved much later with the discovery that in vivo an enzyme is required to catalyze the efficient formation of protein disulfide bonds (3–5).

Although protein-disulfide isomerase (PDI) was originally detected in the endoplasmic reticulum (ER) of eukaryotes as an enzyme that catalyzes disulfide bond isomerization in vitro, its in vivo role is still unclear. PDI is required for the formation of disulfide bonds in the endoplasmic reticulum (ER) (6, 7). However, it is not known whether PDI carries out both oxidation and isomerization reactions in the ER or whether the latter reaction might be performed by other ER proteins.

In Gram-negative bacteria, the enzyme catalyst of disulfide bond formation is the protein, DsbA, which is located in the periplasmic space of bacteria (3–5). A separate protein, DsbC, carries out protein disulfide bond isomerization (8, 9). DsbC is a “Y”-shaped homodimer with two disulfide bonds in each 20-kDa monomer (10). The C-terminal disulfide bond (Cys140–Cys163) is required for folding and stability of DsbC, whereas the N-terminal redox active cysteines (Cys98–Cys101) are required for the isomerase activity and are maintained in a reduced form by the inner membrane protein DsbD (11). It appears that cysteine 98 attacks incorrect disulfide bonds in misfolded substrates, and the resultant mixed disulfide between enzyme and substrate is resolved by cysteine 101 (11). The pairs of N-terminal cysteines (Cys98–Cys101) of the dimeric DsbC face each other in a hydrophobic cleft formed by the two arms of the Y shape. Because misoxidized substrates of DsbC are likely to be misfolded with core hydrophobic residues surface-exposed, the hydrophobic cleft of DsbC might mediate the interaction between the misfolded substrate protein and cysteines 98–101. Furthermore, DsbC possesses chaperone activity that is independent of cysteines 98 and 101 (12).

Joly and Swartz (38) reported that dependence on DsbC for proper folding shows an interesting pattern among eukaryotic proteins expressed in Escherichia coli. The proteins that did not require DsbC contained disulfide bonds that were formed between cysteines found in consecutive positions in the proteins. In contrast, the DsbC-dependent proteins contained nonconsecutive disulfide bonds, i.e., the latter class of proteins contain a disulfide bond between two cysteines that have between them the amino acid sequence another cysteine or cysteines that is involved in a disulfide bond (see e.g. the disulfide bond between cysteines 155 and 430 in Fig. 1). Until recently, all the substrates used to study the isomerase activity

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1 The abbreviations used are: PDI, protein disulfide isomerase; ER, endoplasmic reticulum; ORF, open reading frame.
of DsbC have been eukaryotic in origin. The reason that only eukaryotic proteins expressed in E. coli were employed in studies on DsbC function is that very few of the known proteins of the bacteria contained multiple disulfide bonds. Recently, Hinner and Bardwell (13) have identified the E. coli periplasmic proteins RNase I and MepA as proteins whose stability is limited nonconsecutively in the amino acid sequence. By using four disulfide bonds, one of which involves a pair of cysteines phytase (AppA). This protein was chosen because it contains a potential substrate of DsbC the proteins RNase I and MepA as proteins whose stability is.

In this work, we used a bio-informatics approach to identify as a potential substrate of DsbC the E. coli periplasmic protein phytase (AppA). This protein was chosen because it contains four disulfide bonds, one of which involves a pair of cysteines located nonconsecutively in the amino acid sequence. By using mutant analysis of AppA and genetic engineering of an AppA homolog, App, we present evidence supporting the proposal that disulfide bond connectivity plays a critical role in making a protein DsbC-dependent. We discuss the implications of such a finding for the timing of disulfide bond formation during protein translocation.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions—Bacterial strains and plasmids used in this study are listed in Table I. Strains or plasmids were constructed using standard molecular and genetic techniques (14). Bacteria were grown in TB medium (10 g of tryptone, 5 g of yeast extract, 2 g of NaCl in 1 liter of deionized water) with 0.2% arabinose. Bacteria were grown in NZ medium (15) at 37 °C with the appropriate antibiotics. Plasmid-encoded variants of AppA and Agp were induced with 0.2% arabinose.

To generate a plasmid encoding appA or agp gene under a tightly controlled inducible promoter, we utilized the pBAD18 expression vector (16). The appA ORF was amplified by PCR from a colony of DHB4 using the primer pair 5′-GGCTTAGTCTAGACCTACCATG-3′ and 3′-Xbaa-appA (5′-CTAGTCTAGATTCACGAAGCCTGTTA-3′), whereas the appA ORF was amplified by using the primer pair 5′-GGCTTAGTCTAGACCTACCATG-3′ and 3′-Xbaa-appA (5′-CTAGTCTAGATTCACGAAGCCTGTTA-3′). The resulting fragments were digested with EcoRI and XbaI and cloned into EcoRI- and XbaI-digested pBAD18, and named pAgp155. This construct replaced the C-terminal Val412–Lys413 amino acids from AppA by the now standard technique of gene disruption (17).

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Antibody Production—Anti-AppA rabbit polyclonal antiserum was generated by using the overexpression plasmid pPhy190 (18). E. coli BL21 strains harboring pPhy190 were grown overnight in 2 liters of TB medium (12 g of casein peptone, 24 g of yeast extract, 4 g of glycerol, and 20 g of NaCl in 1 liter of deionized water) with 1 mM isopropyl-β-D-galactopyranoside and 40 μg/ml kanamycin at 37 °C in an Erlenmeyer flask. Because of the Kill cassette in pPhy190, AppA is found predominantly in the supernatant. Bacteria were therefore removed by centrifugation at 10,000 rpm for 15 min. The supernatant was subjected to another round of centrifugation to remove any residual cell debris. The proteins in the supernatant fraction were precipitated by slowly adding NH₄SO₄ to a final concentration of 80% w/v and subjected to centrifugation at 10,000 rpm for 30 min. The resulting pellet was resuspended in dialysis buffer (50 mM Na₂HPO₄ at pH 8, 300 mM NaCl, and 5% glycerol) and dialyzed in 2 liters of the same buffer for 2 h at 4 °C. The dialyses buffer was changed three times. The dialyses resulted in the formation of protein precipitants that were removed by centrifugation at 10,000 rpm for 10 min. The final protein concentrate was subjected to another round of centrifugation to remove any residual cell debris. The proteins in the supernatant fraction were precipitated by slowly adding NH₄SO₄ to a final concentration of 80% w/v and subjected to centrifugation at 10,000 rpm for 30 min. The resulting pellet was resuspended in dialysis buffer (50 mM Na₂HPO₄ at pH 8, 300 mM NaCl, and 5% glycerol) and dialyzed in 2 liters of the same buffer for 2 h at 4 °C. The dialyses buffer was changed three times. The dialyses resolved in the formation of protein precipitants that were removed by centrifugation at 10,000 rpm for 10 min. The final protein concentrate was applied to a nickel column, and the fusion protein AppA-6his was eluted into fractions with an imidazole linear gradient (0–200 mM). Fractions were checked for activity and purity in an SDS-polyacrylamide gel. Those fractions that showed high activity and relative purity
were pooled together and used to raise antibodies in rabbit (Covance, Denver, PA).

**Assay for Acid Phosphatase Activity**—Quantitative measurements of acid phosphatase activity for AppA and Agp were made with the substrate p-nitrophenyl phosphate as described earlier (19) with slight modifications. Assays were performed in 100 mM sodium acetate at pH 4.5 for AppA (20) or 250 mM glycine at pH 2.5 for AppA (21) because of their varying pH optima. DHB4 cells expressing appA or app were grown in the presence of 0.2% arabinose until mid-log in NZ media at 37 °C. 100 µl of appropriately diluted cells were incubated with 900 µl of 25 mM p-nitrophenyl phosphate at 37 °C, and the reaction was stopped with the addition of 200 µl of 10 mM NaOH. The acid phosphatase activity was determined by measuring the absorbance of the formed p-nitrophenol at 410 nm. One unit was defined as 1,000 × A410 per min/ml in analogy with β-galactosidase activity (22). All experiments were conducted in duplicate and replicated at least twice.

**Thiol Redox State Analyses**—To determine the in vivo redox state of the proteins, free thiol groups were trapped with 15 mM 0.5-kDa redox reagent 4-acetamido-4-maleimidylstibene-2,2’-disulfonic acid (Molecular Probes, Eugene, OR). When indicated the proteins were reduced with the reductant dithiothreitol (15).

**Identification of Disulfide-bonded Proteins in E. coli**—All Swiss-Prot records with names ending in “_ECOLI” were downloaded January 13, 2004. The records were classified according to subcellular localization as determined from the “KW” and “CC” lines. Our subcellular localizations “Cytoplasmic,” “Periplasmic,” “Inner membrane,” and “Outer membrane” are generally derived from the KW lines, but in some cases other information in the record has been used. The subcellular localizations classified as Cytoplasmic are derived from the CC lines. “Signal” proteins are those listed with signal sequences but without being specific to one of the compartments.

**RESULTS**

**Search for Potential Substrates of DsbC**—Based on previous studies indicating that the consecutiveness of disulfide bonds in a protein was a critical factor in determining whether it would be a substrate for DsbC, we performed a search for proteins known to contain nonconsecutive disulfide bonds in the native organism for DsbC, *E. coli*. By using the SwissProt data bank, we searched for all *E. coli* periplasmic proteins containing nonconsecutive disulfide bonds based on their crystal structure (Table I). Only a single protein, AppA, fulfilled this criterion. AppA was first identified as a histidine acid phosphatase (21) that hydrolyzes phosphate moieties from sev-

**Table I**

| Compartment | No. | Cys<sup>a</sup> | S-S<sup>b</sup> | Real S-S | Crossing S-S<sup>c</sup> |
|-------------|-----|---------------|----------------|----------|------------------------|
| Cytoplasm   | 476 | 433           | 7              | 6        | 0                      |
| I.M.        | 557 | 476           | 5              | 3        | 0                      |
| Periplasm   | 176 | 131           | 27             | 18       | 1<sup>d</sup>          |
| O.M.        | 111 | 88            | 17             | 3        | 0                      |
| Unclassified| 3203| 2724          | 15             | 10       | 2<sup>e</sup>          |
| Total       | 4836| 4100          | 115            | 50       | 5                      |

<sup>a</sup> Determined from the sequence and the signal annotation.

<sup>b</sup> Determined from the “FT DISULFID” lines.

<sup>c</sup> If the first residue number on an “FT DISULFID” line is lower than the second residue number of the preceding line, the bonds must cross because the lines are ordered by residue number of the first cysteine.

<sup>d</sup> AppA.

<sup>e</sup> Heat-stable enterotoxins.

<sup>f</sup> OxyR and heat-stable enterotoxin.

**Fig. 1. Structure and disulfide bond pattern of AppA**

**A.** Crystal structure of AppA (25) and Agp (20). The disulfide bond forming cysteine pair residues numbers is indicated. **B.** Schematic diagram representing disulfide bond connectivity of AppA and Agp. The residue number of individual cysteines is indicated.

and we asked whether this protein, missing its isomerase but not its chaperone activity (11), was sufficient to restore full AppA activity. The AppA activity in the strain expressing this mutant *dsbC* remained at a low level, indicating that the defect seen in the *dsbC* deletion strain was because of the missing disulfide isomerase activity (data not shown).

Western blots were carried out on the same samples to examine the level and redox state of AppA (Fig. 2B). Similar amounts of AppA protein were observed in wild-type cells and cells deleted of their copies of *dsbA* or *dsbC*, indicating that the reduced protein or misoxidized protein is not degraded by cell envelope proteases. Alkylation of free thiul groups by 4-acet-
amido-4′maleimidylstibene-2,2′-disulfonic acid showed that all of the cysteines in AppA are reduced in the absence of the periplasmic oxidant DsbA. In cells devoid of the isomerase DsbC, the redox state of AppA was similar to that of wild-type cells. These results indicate that correct disulfide bond formation in AppA is essential for its folding into an active conformation rather than its stability. We repeated the experiments with several different constructs expressing different levels of AppA (from a single chromosomal copy to a plasmid with a strong trc promoter), and we observed the same dependence on DsbA and DsbC (data not shown). Similar results were obtained when the experiments were repeated in a ΔappA strain (data not shown), confirming previous data that the chromosomal copy of the wild-type appA is not expressed in rich media at log phase (26).

We also asked whether a second periplasmic disulfide bond isomerase, DsbG (27), might play a role in isomerization of AppA. However, deletion of the dsbG gene had no effect on AppA activity (data not shown). Furthermore, a double mutant, containing deletions of both dsbC and dsbG, showed no greater reduction of activity than the dsbC mutant itself, indicating that the residual activity of AppA in the dsbC mutant was not due to DsbG activity (data not shown).

**Properties of AppA Mutants Missing Cysteine Residues**—The observation that the absence of DsbC resulted in decreased activity of AppA indicates that at least some of the disulfide bonds of the enzyme are required for its proper assembly or that a misoxidized disulfide bond can interfere with that assembly. To begin to understand which disulfide bonds are essential for AppA activity, we generated mutants in which each of the eight cysteine residues was changed to serine separately.

To test further the role of the disulfide bonds of AppA in its activity, we constructed mutant forms of AppA where each of the cysteine pairs involved in disulfide bonds was mutated to serines. As with the single cysteine mutants, all of the mutant strains exhibited reduced amounts of AppA activity. Although all (except Cys200 and Cys210) of the single cysteine mutants of AppA described earlier displayed significantly diminished activity (<10% of wild type, Fig. 3), the double cysteine mutants of AppA discussed here displayed higher activities than did the single mutants (Fig. 4). The differences in activities between the single and double mutants may be due to the presence of unpaired cysteines in the former constructs that could enhance the probability of forming incorrect disulfide bonds, as suggested above. The misoxidized complexes with the single cysteine mutants are most likely misfolded and susceptible to protease degradation as indicated by the Western blots (Fig. 3). This is not the case when both the partner cysteines are mutated (Fig. 4). Most interestingly, cysteines 200 and 210, which are normally joined in a disulfide bond, showed the least loss of activity (70% for Cys200 and 35% for Cys210 in comparison to wild type). This was not surprising as a triple mutant of AppA (C200N/D207N/S211N) has been selected in a screen for increased stability studies of AppA (28, 29).

**Mutant AppAs That Contain Only Consecutive Disulfide Bonds Are Independent of DsbC**—With the collection of mutants each lacking one of the disulfide bonds of AppA, but still expressing some level of activity, we had an opportunity to assess the role of each disulfide bond on DsbC dependence. We measured the activity of various mutated AppA constructs expressed in dsbC+ and ΔdsbC null mutant cells (Fig. 4). The results show that the mutant AppA lacking its nonconsecutive disulfide bond C155S/C430S does not require DsbC for its remaining activity. In contrast, mutants lacking the Cys199/Cys130 or Cys200/Cys210 disulfide bonds retained dependence on DsbC. The AppA Cys404→Cys413 double cysteine mutant, which
AppA/C$_{430}$ proteins were independent of DsbC for their activity and C$_{430}$ to alanine. The AppA/C$_{155}$S/C$_{430}$A, AppA/C$_{155}$A/C$_{430}$S, and C$_{430}$ to serine resulted in DsbC independence of AppA. To test whether the specific amino acid change of cysteines 155/430 is the primary reason for the dependence of AppA on DsbC. It is possible that the specific amino acid change of cysteines 155/430 to serine resulted in DsbC independence of AppA. To test this alternative explanation, we changed both cysteines 155 and 430 to alanine. The AppA$_{Cys155/430}$, AppA$_{Cys155/430}$S, and AppA$_{Cys155/430}$S proteins were independent of DsbC for their activity (data not shown). All of the samples were further analyzed by Western blot with anti-AppA antibodies. The levels of AppA protein expressed by the mutant version missing the different disulfide bonds were between 50 and 100% of that seen with the wild-type AppA (data not shown). Elimination of individual disulfide bonds in the protein may lead to misfolded protein but not to a protein that is significantly susceptible to degradation.

The AppA homolog Agp does not require DsbC for its folding into active enzyme. The existence of a close structural homolog of AppA with a slightly different pattern of disulfide bonds has provided us with a tool to examine more deeply the basis of the DsbC dependence of AppA. Agp is classified as a histidine acid phosphatase and hydrolyzes phosphate moieties from a variety of sugars, exhibiting the highest phosphatase activity against glucose 1-phosphate at an optimum pH of 5.5 (20). Agp is localized to the periplasm and shows 30% identity and 50% similarity to AppA (Fig. 6). The crystal structure of Agp has been solved recently (30), revealing three consecutive disulfide bonds placed similarly to those of AppA but lacking the fourth nonconsecutive disulfide bond seen in AppA (Fig. 1). The structures of the two proteins, AppA and Agp, are largely superimposable (Fig. 1).

In this study, we used the ability of Agp to hydrolyze 4-nitrophenyl phosphate at pH 4.5 to study its activity when expressed in strains lacking DsbC. The activity of Agp was strongly dependent on DsbA, indicating that Agp requires its disulfide bonds for activity (Fig. 5). However, it showed very little if any dependence on DsbC. These assays were repeated seven times; the average was 95% ± 10% of the activity of Agp when expressed in wild-type, dsbA, or dsbC mutant strains. These assays were repeated seven times; the average was 95% ± 10%. These results are consistent with the model in which the dependence of AppA on DsbC for full activity is because of its nonconsecutive disulfide bond. Alternatively, it could be that the difference in the dependence of the two proteins on DsbC is due to the difference between having three and four disulfide bonds, irrespective of their consecutive nature. However, because MepA and other proteins that are DsbC-dependent also have three disulfide bonds, the dependence of AppA cannot simply be due to the number of disulfide bonds. Nevertheless, the differences seen between AppA and Agp could be due to a combination of factors, including both the number and the relative positioning of the cysteines involved in disulfide bonds.

The conversion of Agp to a protein containing a nonconsecutive disulfide bond in AppA dependence. The results described above show that AppA is dependent on DsbC for its proper folding and that this dependence disappears with the elimination of the nonconsecutive Cys$_{155}$—Cys$_{430}$ disulfide bond. We proceeded to ask whether the introduction of such a nonconsecutive disulfide bond into Agp would make Agp dependent on DsbC. Alignment of the AppA amino acid sequence with that of Agp reveals that there are gaps in the Agp sequences in the region surrounding the Cys$_{155}$—Cys$_{430}$ disulfide bond. We replaced C$_{155}$ and C$_{430}$ with Ala in Agp, creating a new protein. Furthermore, the x-ray crystal structures of AppA and Agp revealed that the structures are highly similar surrounding the Cys$_{155}$—Cys$_{430}$ disulfide bond. Based on the structural and amino acid sequence information, we replaced C$_{155}$ and C$_{430}$ with Ala in Agp, creating a new protein. The resulting single (Agp$_{155}$ or Agp$_{430}$) and double (Agp$_{155/430}$) peptide insertions into Agp were tested for dependence on DsbC. Wild-type and the mutant Agp constructs were expressed from an arabinose promoter in pBAD18, in wild-type, or ΔdsbC cells. The activities of all constructs were measured and standardized to wild-type expression levels (Fig. 7). Unlike wild-type Agp, both Agp$_{155}$ and Agp$_{430}$ displayed significantly decreased activity (~50%) when expressed in ΔdsbC cells. This effect is reminiscent of the...
Nonconsecutive Disulfide Bond of E. coli Phytase on PDI

We have presented evidence that supports the proposal that a protein-disulfide bond isomerase is required for the proper folding of those proteins that contain one or more nonconsecutive disulfide bonds. This proposal implies that the action of sulfhydryl oxidases (e.g. DsbA or PDI) on substrate proteins often leads to the introduction of incorrect disulfide bonds when the protein to be assembled contains multiple disulfide bonds, at least one of which is formed between cysteines located nonconsecutively in the final three-dimensional structure. The results described here in conjunction with previous work are striking enough to suggest that enzymes such as DsbA act on substrate proteins to preferentially form disulfide bonds in a sequential manner, proceeding along the polypeptide chain from one terminus to the other. The most obvious way in which this could occur is if DsbA interacts with cysteines in substrate proteins as they are being translocated along the polypeptide chain from one terminus to the other. The nature of DsbA is acting. If DsbA acts at this stage, the nature of the final folded structure of the protein would have little relevance as to which cysteines get joined into disulfide bonds. Therefore, DsbA would generally form disulfide bonds between cysteines that are located consecutively in the amino acid sequence as it appears in the periplasm. If a nonconsecutive disulfide bond is required for the properly folded form of the protein, this sequential process of disulfide bond formation would lead to an incorrect disulfide bond, hence the requirement for disulfide bond isomerases, which are found in organisms throughout the biological kingdom.

Our explanation for the formation and isomerization of disulfide bonds is based on the following assumptions. 1) Disulfide bond formation takes place during protein translocation. Although this has not been shown in bacteria, it is the case in eukaryotic cells where disulfide bond formation occurs during translocation of proteins into the ER (32–36). 2) DsbA is relatively indiscriminate in its reactions with cysteines of substrate proteins. This assumption derives from the evidence that when proteins not ordinarily translocated into the periplasm are forced to do so, unwanted disulfide bonds form that are not seen in the proteins in their native locations (3, 37). One can imagine that at least some portions of partially translocated polypeptide chains appearing in the periplasm do not have significant three-dimensional structure and that these are the chains on which DsbA is acting. If DsbA acts at this stage, the nature of the final folded structure of the protein would have little relevance as to which cysteines get joined into disulfide bonds.

Our evidence supporting the importance of nonconsecutive disulfide bonds is as follows. First, the E. coli periplasmic protein, phytase (AppA), contains three consecutive disulfide bonds and one nonconsecutive one. We have found that this protein shows a strong in vivo dependence on DsbC for its full activity. Expression of appA in cells deleted for dsbC displayed a 3–4-fold decrease in activity. Furthermore, we show through use of active site cysteine mutants of DsbC that this dependence requires the disulfide bond isomerase activity of DsbC. As nearly all previously studied substrates of DsbC have been eukaryotic proteins cloned into the bacteria, this represents the first direct evidence for the requirement of a native substrate for the isomerase activity of DsbC. We believe it likely that the RNase I and MepA proteins shown previously to require DsbC for proper folding do so because of the isomerase activity of DsbC, although the effect...
of mutants of the two redox active cysteines of DsbC in helping assemble these proteins is not known (13). Other than these three E. coli proteins, the only protein native to the bacteria with more than two cysteines tested previously for DsbC dependence, alkaline phosphatase, contained two consecutive disulfide bonds and was not dependent.

Second, the AppA homolog, Agp, provided us with a means to further examine the role of nonconsecutive disulfide bonds in determining DsbC dependence. Agp and AppA show strong similarity at the level of amino acid sequence, and their three-dimensional structures are almost completely superimposable. In addition, Agp contains three disulfide bonds at similar positions in the structure and sequence as three of the four disulfide bonds in AppA. However, Agp is missing the fourth disulfide bond found in AppA, which is the nonconsecutive one. Expression of Agp in cells deleted for dsbC showed little decrease in activity of the protein compared with a wild-type background. Thus, we believe it likely that the nonconsecutive disulfide bond of AppA is the factor making AppA DsbC-dependent. Because Agp and AppA are so similar in structure, it seems less likely that structural differences between the two proteins play a role in the differences seen in the dsbC− background.

Third, we were concerned that another explanation for the difference between App and AppA in their DsbC dependence is simply that the difference in number of disulfide bonds (three in the former and four in the latter) is responsible. However, it is already known that proteins with three disulfide bonds can be dependent on DsbC (11, 38). Furthermore, melanoctye growth stimulating activity has only two nonconsecutive disulfide bonds and is 50% dependent on DsbC (38). Nevertheless, to test this possibility, we constructed a set of mutants of AppA in which a single pair of cysteines involved in each of the four disulfide bonds was changed to serines. All of these proteins missing one of their disulfide bonds exhibited some AppA enzymatic activity. Two such mutant appA constructs, each of which lacked one of the consecutive disulfide bonds, displayed significant dependence on DsbC. Thus, proteins with only three disulfide bonds can show strong dependence on DsbC. A third mutant lacking the nonconsecutive (155–430) disulfide bond of AppA was no longer dependent on DsbC. The fourth mutant, lacking a consecutive disulfide bond, did not appear to be DsbC-dependent, but its AppA activity was so low that the significance of this result is not clear.

Finally, we were able to convert App from a protein that was DsbC-independent to one that required DsbC for assembly into active enzyme by introducing two cysteines into the protein that could generate a disulfide bond analogous to the AppA155–430 nonconsecutive disulfide bond. Because it is likely that this alteration of App resulted in very little change in structure of the protein (Fig. 1), we suspect it is the nonconsecutiveness, not any changes in structure, that leads to the DsbC dependence.

It is perhaps surprising that so few proteins with multiple disulfide bonds have been detected in E. coli. Eukaryotic cells appear to have large numbers of such proteins including peptide hormones and membrane receptors. There are presumably other DsbC substrates remaining to be identified. Assuming that for an E. coli protein to be a potential substrate of DsbC it requires multiple disulfide bonds, we searched the Swiss Protein Data Bank for such candidates. It was simplest for our purposes to restrict ourselves to likely periplasmic proteins, even though outer membrane and cytoplasmic membrane proteins can contain disulfide bonds. Of the 176 proteins categorized as periplasmic, 131 contain at least a single cysteine residue and 46 contain 3 or more cysteines. For most of these, we do not know whether the proteins form disulfide bonds. Nevertheless, these analyses indicate that 25% of all periplasmic proteins have the potential to be misoxidized by DsbA and require the isomerization of DsbC to be active. Of the 27 published disulfide bonded structures, only AppA has been shown to have a nonconsecutive disulfide bond.

Although the data that exist are consistent with our explanation for DsbC dependence of proteins, there are several reasons for thinking that the actual situation may sometimes be more complex. First, in the case of the metalloprotease elastase from Pseudomonas aeruginosa, DsbA catalyzes the formation of a C-terminal disulfide bond (Cys270–Cys297) prior to the formation of the N-terminal disulfide bond (Cys301–Cys397) (39). This “post-translocational” disulfide bond formation is dependent on the folding of elastase. Tommassen and co-workers (39) have demonstrated that the N-terminal disulfide bonds of elastase are not formed until the proper folding of the C terminus is achieved resulting in autoprocessing of the elastase proenzyme. Thus, some disulfide bonds may only be formed once the protein achieves a certain tertiary structure, which does not occur until the C-terminal disulfide bond-dependent autoprocessing has occurred. Second, some proteins appear to require for their folding pathway the formation of disulfide bonds between cysteines that are not found in the final active protein (40). Third, the distance between cysteines that are destined to be joined in a consecutive disulfide bond and the greater proximity of one or both of them to other cysteines may enhance the probability that DsbA will “make mistakes.” In this case, some proteins may be found that show consecutive disulfide bonds in their final structure but still exhibit at least some DsbC dependence. Finally, some degree of folding of the protein during the translocation process may bring closer cysteines that are not meant to be joined in the final structure. This is indeed the case at least in vitro, where partial folding of the protein α-lactalbumin in the presence of calcium is necessary for the formation of native disulfide bonds (41). In the absence of calcium, α-lactalbumin is less ordered, and the protein is fully oxidized following a nonnative disulfide bond pattern.

Finally, although AppA activity shows DsbC dependence, there is still a residual enzymatic activity of 25–30% that found in a wild-type DsbC− background. This could be due to one of several factors. 1) There is another low level isomerase activity in the periplasm. This cannot be DsbG, as the double mutant dsbC, dsbG shows the same activity as the single dsbC mutant. However, when DsbG is overexpressed, it does restore ~75% AppA activity. The lack of involvement of DsbG in AppA assembly in wild-type strains may be due either to low levels of expression (DsbG is expressed at ~25% the level of DsbC (42)) or low affinity for AppA. Either of these deficits may be overcome by increasing expression, or there could be a small molecule reductant in the media or periplasm that reduces misoxidized AppA, allowing DsbA to form the correct S–S bonds. 2) The frequency of formation of incorrect disulfide bonds catalyzed by DsbA when confronting a protein with a nonconsecutive disulfide bond may not be 100%. Some fraction of translocating proteins may assume more of the features of the three-dimensional structure before DsbA has acted on them. 3) The AppA activity that remains in the dsbC− mutant may not be due to protein with correct disulfide bonds but rather due to one or several species of AppA containing incorrect disulfide bonds that still exhibit some enzymatic activity, even though they are not folded entirely properly. Our results showing activity of mutant proteins missing one or another disulfide bond is consistent with this explanation.

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2 M. Berkmen, D. Boyd, and J. Beckwith, unpublished results.
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Nonconsecutive Disulfide Bond of E. coli Phytase on PDI

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