Structural and Functional Characterization of DsbC, a Protein Involved in Disulfide Bond Formation in *Escherichia coli*†

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ABSTRACT: DsbC is a soluble protein of the bacterial periplasm that was identified genetically as being involved in protein disulfide formation. The gene sequence was corrected to include an additional proline residue and was then consistent with the molecular weight of the purified protein. Gel filtration and subunit hybridization indicate that DsbC is a stable dimer of identical subunits. Each subunit has a -Cys-Gly-Tyr-Cys- segment that forms an unstable and reactive disulfide bond; only the first cysteine residue is accessible, similar to thioredoxin and DsbA. The other two cysteine residues of DsbC form a buried, structural disulfide bond. The reactivities and stabilities of the active site disulfide bond of DsbC have been characterized and compared to that of DsbA. Both are very unstable and can be transferred rapidly to reduced proteins and peptides, although they differ somewhat in their kinetic reactivities. The two active sites of the DsbC dimer appear to function independently. DsbC is much more active than DsbA in catalyzing protein disulfide rearrangements, and this may be its main function *in vivo*.

Disulfide bonds are found in many secreted proteins. They are often required for folding to the biologically active conformation and for its stability. Protein disulfide formation can occur *in vitro* but is often slow and inefficient. Reactions with molecular oxygen or small disulfide molecules, such as oxidized glutathione (GSSG[1]), are required for the chemical oxidation of thiol groups to disulfide bonds. Incompletely folded protein molecules can be insoluble and subject to aggregation. Quasi-native species, with folded conformations but incomplete disulfide bonds, often with thiol groups that are inaccessible, can be stable and predominate (Creighton & Goldenberg, 1984). Intramolecular disulfide rearrangements by thiol−disulfide exchange, which are slowed by stable protein conformations, are frequently rate-limiting in protein folding (Creighton, 1986).

Cells have several classes of proteins that assist folding [reviewed by Gething and Sambrook (1992); Lorimer, 1992; Jaenicke, 1994; Wülfing & Plückthun, 1994]. Not surprisingly, catalysts assist formation of the correct disulfide bonds (Freedman, 1992). In eukaryotes, secretory proteins are translocated cotranslationally into the lumen of the endoplasmic reticulum (ER), where they rapidly fold and acquire their disulfide bonds, before proceeding further along the secretory pathway. The ER has been shown to contain glutathione at millimolar concentrations in a reduced to oxidized ratio of between 3:1 and 1:1 (Hwang et al., 1992). This environment is sufficiently oxidizing to permit thermodynamically the formation of disulfide bonds that are stabilized by the protein conformation, but the rapid rate at which disulfide formation occurs is due to the presence of large amounts of the catalyst protein disulfide isomerase (PDI). This enzyme was initially recognized for its ability *in vitro* to catalyze the formation and rearrangement of protein disulfide bonds (Givol et al., 1964; Venetianer & Straub, 1964), and its role *in vivo* has now been established in yeast (LaMantia & Lennarz, 1993). PDI is a large, complex protein of 57 kDa consisting of five domains (Edman et al., 1985), two of which are homologous in sequence to thioredoxin; each of the latter contains one active site sequence, -Cys-Gly-His-Cys-, similar to that of thioredoxin, -Cys-Gly-Pro-Cys-.

In Gram-negative bacteria such as *Escherichia coli*, extracytoplasmic proteins are translocated into the periplasmic space, where disulfide bonds form rapidly. The isolation of mutants deficient in disulfide bond formation has led to the characterization of several proteins involved in this process. The first to be recognized was DsbA (Bardwell et al., 1991; Kamitani et al., 1992), which is a soluble, monomeric periplasmic protein of 21 kDa. Although it has no apparent sequence homology to thioredoxin, its crystal structure revealed that it has a domain with a very similar fold, plus an additional helical domain; it is therefore a member of the thiol-disulfide oxidoreductase family, which includes thioredoxin, PDI, and glutaredoxin (Martin et al., 1993). Like thioredoxin and the homologous domains of PDI, it has an active site pair of cysteine residues, -Cys-Pro-His-Cys-, which can reversibly form a disulfide bond. *In vitro* experiments with hirudin and bovine pancreatic trypsin

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[1] Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; CD, circular dichroism; Da, dalton; $DsbC_{4SH}$, DsbC with its four cysteine residues in a thiol form; $DsbC_S^S$, $DsbC_{SH}^{SH}$, and $DsbC_{SH}^{SSG}$, respectively DsbC with its two active site cysteine residues linked by a disulfide bond, in the free thiol form, and with the accessible Cys98 thiol group in a mixed disulfide with glutathione; comparable designations are used for DsbA; $DsbC_{SH}^{Thr}$, $DsbC_{Val}^{SH}$, and $DsbC_{Ala}^{SH}$, DsbC variants with either Cys98 replaced by a threonine residue or Cys101 replaced by a valine or alanine residue, respectively; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; GdmCl, guanidinium chloride; GSH and GSSG, reduced and oxidized glutathione, respectively; HPLC, high-pressure liquid chromatography; PDI, protein disulfide isomerase; TFA, trifluoroacetic acid.

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inhibitor (BPTI) have shown that DsbA is very effective at introducing disulfide bonds into reduced proteins (Wunderlich et al., 1993; Zapun & Creighton, 1994). In contrast, and contrary to PDI, it has little or no disulfide isomerase activity, depending upon the particular case considered (see Discussion) (Zapun & Creighton, 1994).

The thiol−disulfide chemistry of the active site of DsbA has been thoroughly characterized (Zapun et al., 1993, 1994; Wunderlich & Glockshuber, 1993). Its disulfide bond is very unstable and reactive. The equilibrium constant for forming this disulfide bond by thiol−disulfide exchange with GSSG, a reaction commonly used to compare disulfide bond stability, was measured to be 80 μM, which is one of the lowest values found thus far. For comparison, the active site disulfide bond of thioredoxin is formed with an equilibrium constant of 10 M (Holmgren, 1981) and is therefore 6.9 kcal mol^{-1} more stable than that of DsbA, even though they are structurally indistinguishable (Martin et al., 1993). As in thioredoxin, only the more N-terminal of the two cysteine residues of DsbA has its thiol group accessible. It forms a mixed disulfide with glutathione (Zapun et al., 1993, 1994) and other substrate protein cysteine residues (Darby & Creighton, 1995). This mixed disulfide, which is probably an obligatory intermediate in all thiol−disulfide exchange reactions involving DsbA, was also shown to be extremely unstable and reactive; that with glutathione reacts with an external thiol 10^3-fold faster than does a normal mixed disulfide (Zapun et al., 1993, 1994). These properties can explain the efficiency of DsbA as a protein disulfide oxidant (Darby & Creighton, 1995).

To fulfill the role of oxidant *in vivo*, DsbA has to be recycled, reoxidized once it has transferred its disulfide bond to a substrate protein. DsbB, a predicted membrane-embedded protein with a putative active site pair of cysteine residues in its periplasmic domain, seems to serve this purpose (Bardwell et al., 1993; Missiakas et al., 1993). Mutants in the gene *dsbB* have the same phenotype as *dsbA* mutants, and much less of the oxidized form of DsbA is detected in *dsbB*$^-$ cells than in wild-type strains. How DsbB is itself oxidized and what is the ultimate source of oxidizing power remain to be elucidated.

Recently, another soluble periplasmic protein involved in this process has been identified in both *Erwinia chrisanthemi* and *E. coli* and designated DsbC (Shevchik et al., 1994; Missiakas et al., 1994). Like *dsbA*$^-$ and *dsbB*$^-$, strains, *dsbC*$^-$ cells are sensitive to high concentrations of DTT, do not grow in a minimal medium not supplemented with small disulfide molecules, have decreased alkaline phosphatase activity and motility, and are hypersensitive to benzylpenicillin; the three last properties result from extra-cytoplasmic proteins that require disulfide bonds. Alkaline phosphatase and β-lactamase were demonstrated directly not to contain all their native disulfide bonds early after translocation in each of the *dsbA*$^-$, *dsbB*$^-$, or *dsbC*$^-$ strains, in contrast to normal strains. Overexpression of DsbC can restore a wild-type phenotype in cells lacking DsbA and/or DsbB, and reciprocally, overexpression of DsbA can complement the lack of DsbC. The absence or presence of DsbC was observed not to affect the redox state of DsbA (Missiakas et al., 1994). The gene for DsbC indicated that the mature protein has a mass of 23 kDa and, like DsbA, PDI, and thioredoxin, contains a pair of cysteine residues, with the sequence -Cys-Gly-Tyr-Cys- at positions 98−101. In addition to the putative active site, DsbC has two other cysteine residues at positions 141 and 163.

We report here the structural and functional characterization of the DsbC protein. Its activity on the well-characterized pathway of disulfide bond formation in BPTI was investigated and compared to that exhibited by DsbA and PDI; DsbC appears to be more like PDI than is DsbA, having a greater disulfide isomerase activity.

EXPERIMENTAL PROCEDURES

Proteins and Chemicals. GSH and GSSG from Sigma were used without further purification. The concentration of GSH was determined by Ellman's assay (Riddles et al., 1983) and that of GSSG by its absorbance at 248 nm with the extinction coefficient ϵ_{248} = 386 M^{-1}cm^{-1}. All other chemicals were of reagent grade or better. Solutions of glutathione, DTT, urea, GdmCl, iodoacetamide, and iodoacetic acid were freshly prepared.

$DsbC^S_S$ was prepared with a method derived from that of Missiakas et al. (1994). The overproducing cells (BL21 harboring the plasmid pDM801) were grown overnight to saturation in LB medium and further incubated for 5 h with 5 mM IPTG to induce expression. The harvested cells were submitted to a cold osmotic shock to release the periplasmic proteins (Manoil & Beckwith, 1986). The shockate was incubated overnight at 4 °C with 20 mM Tris-HCl (pH 7) and 100 μM GSSG in order to convert all the DsbC into its oxidized form. If this step was omitted, about 60% of the protein was isolated in the reduced form and remained partially reduced even after several days of storage at 4 °C. The protein was purified by anion-exchange chromatography on a Q-Sepharose column (1.6 × 15 cm, Pharmacia) equilibrated with 20 mM Tris-HCl, pH 6.4, and eluted with a linear gradient of 0−0.4 M KCl in the same buffer. The concentration of monomers of DsbC and its variants was determined from its absorbance at 280 nm, using an extinction coefficient, ϵ_{280} = 16170 M^{-1} cm^{-1}, calculated from its amino acid composition (Gill & von Hippel, 1989), as there was no significant difference of the absorbance at 280 nm of the reduced or oxidized forms of the protein, in the presence or absence of 6 M GdmCl. All DsbC concentrations reported are of the monomer.

The variants of DsbC with either Cys98 replaced by a threonine or Cys101 replaced by a valine (Missiakas et al., 1994) or alanine residue, designated $DsbC^{Thr}_{SH}$, $DsbC^{SH}_{Val}$, and $DsbC^{SH}_{Ala}$, respectively, were prepared as for the wild-type protein, but the incubation in the presence of GSSG was omitted. The new replacement of Cys101 by an alanine residue to prepare $DsbC^{SH}_{Ala}$ was performed by oligonucleotide-directed mutagenesis mainly as described previously (Missiakas et al., 1994). Synthesis of the mutated DNA strand was achieved using the mutagenic primer 5′-ACCT-GTGGTTATGCGCACAAACTG-3′. A *Fsp*I restriction site was introduced simultaneously by the mutagenic primer and used to select plasmids for sequencing.

$DsbA^S_S$ was obtained as described previously (Zapun et al., 1993). PDI purified from bovine liver was kindly provided by R. B. Freedman, University of Kent. $DsbC^{SH}_{SH}$, $DsbA^{SH}_{SH}$, and reduced PDI were prepared by incubation of the appropriate isolated protein with 10 mM DTT in 0.1 M Tris-HCl (pH 8) for 10 min prior to desalting on a small Sephadex G-25 column (NAP-5, Pharmacia)

equilibrated with 1 mM HCl. BPTI (Trasylol) was a generous gift of Bayer AG.

Peptide Mapping. To localize the discrepancy in the mass of DsbC detected by mass spectrometry from that expected from its gene sequence (Lovett & Kolodner, 1991), it was cleaved by CNBr. After reduction in 100 μL of 8 M GdmCl, 17 mM DTT, and 170 mM Tris-HCl (pH 8) for 20 min, desalting on a G-25 (NAP-5) column in 1 mM HCl, and lyophilization, about 20 mg of DsbC was redissolved in 50 μL of 70% (v/v) TFA containing 1 mM DTT and 1 M CNBr. The mixture was kept in the dark for 20 h before lyophilization and redissolution in 100 μL of 1% TFA. A portion was analyzed by matrix-assisted laser desorption mass spectrometry. Of the remaining material, 60 μL was added to 1 mL of 10 mM DTT and 0.1 M Tris-HCl (pH 8) to ensure that all its disulfide bonds were reduced; the fragments were separated at 38 °C on a reverse phase HPLC column (C-18, Vydac 218TP54), with a linear gradient of acetonitrile (10−40% in 39 min) in 0.1% TFA. The fractions containing the major peaks were dried and redissolved in 50 mM $(NH_4)_2CO_3$ for further mass spectrometry analysis. The fraction containing the peptide of interest was incubated with 0.1 M iodoacetamide before separation by reverse phase HPLC, in the same way as described above, and analysis of the main peak fractions by mass spectrometry. All mass spectrometry measurements were performed by the EMBL Protein Sequencing Service.

Size-Exclusion Chromatography. To determine the size of the native proteins, 0.02−0.15 mg of DsbC or 0.06 mg of DsbA was analyzed by gel filtration on a 1 × 30 cm Superose 12 column (Pharmacia) equilibrated with either 50 mM MOPS (pH 7.5), 0.1 M NaCl, or 0.1 M sodium phosphate (pH 7), 1 mM EDTA. The proteins were also analyzed on a 1 × 30 cm Sephacryl HR-200 (Pharmacia) column, equilibrated with 50 mM MOPS (pH 7.5) containing 0.1 M NaCl, with or without 1% (w/v) SDS, or 0.6 M NaCl. Blue dextran, β-amylase, alcohol dehydrogenase, bovine serum albumin, carbonic anhydrase, α-lactalbumin, and cytochrome *c* were used to calibrate the columns.

Covalent Modification of Protein Thiols and Denaturing Polyacrylamide Gel Electrophoresis. The redox state and the accessibility of the cysteine residues under various conditions were determined by their reaction with iodoacetamide or iodoacetate. $DsbC^S_S$ as isolated was incubated at a concentration of about 200 μM for 15 min in 125 mM Tris-HCl (pH 8) and 0.75 mM EDTA, with or without 12.5 mM DTT, with or without 6 M GdmCl. Samples were reacted by addition of 1/4 volume of 0.5 M iodoacetamide in 1.5 M Tris-HCl (pH 8.7). After 3 min at room temperature, the samples were desalted on a Sephadex G-25 (NAP-5) column equilibrated with 0.1% TFA. One of the mixtures that had been incubated with DTT was further incubated for 5 min after addition of 3 volumes of 8 M GdmCl, 170 mM Tris-HCl (pH 8), and 1 mM EDTA prior to desalting. Their degrees of modification were analyzed by electrospray mass spectrometry. Other samples prepared in the same way, but reacted with iodoacetate, were analyzed electrophoretically by a modification of the cysteine-counting method (Creighton, 1980). After desalting by gel filtration in 1 mM HCl and lyophilization, the iodoacetate-reacted samples were redissolved and incubated for 10 min in 8 M GdmCl, 17 mM DTT, 170 mM Tris-HCl (pH 8), and 1 mM EDTA to reduce any remaining disulfide bonds. All free cysteine thiol groups were then reacted by addition of 1/4 volume of 0.5 M iodoacetamide in 1.5 M Tris-HCl (pH 8.7). After 3 min the samples were desalted and lyophilized again as described above. For cysteine counting, a "ladder", with different numbers of cysteine residues modified by reaction with iodoacetate, was prepared by reaction of DsbC, fully reduced in the presence of 6 M GdmCl, 12.5 mM DTT, 125 mM Tris-HCl (pH 8), and 0.75 mM EDTA, with 1/4 volume of different mixtures of 0.5 M iodoacetamide and 0.5 M iodoacetate in the ratios of 1:0, 1:1, 1:3, 1:9, and 0:1 in 1.5 M Tris-HCl (pH 8.7). After 3 min at room temperature, the five samples were mixed and immediately desalted and lyophilized as described above. The samples were analyzed using the high-pH denaturing gel electrophoresis system of Davis (1964) with 8 M urea in the separating gel (10% acrylamide) and stained with Coomassie Blue (Ewbank & Creighton, 1993).

Hybridization and Nondenaturing Polyacrylamide Electrophoresis. To determine the oligomeric state of DsbC, advantage was taken of the possibility of preparing species with different net charge, but retaining the same overall structure, by reaction of the active site cysteine residues with iodoacetate. $DsbC^S_S$ at about 150 μM was incubated for 5 min at room temperature with 1 mM DTT in 0.1 M Tris-HCl (pH 8), and then 1/4 volume of 0.5 M iodoacetate in 1.5 M Tris-HCl (pH 8.7) was added. Three min later, 3 volumes of 8 M GdmCl was added, and denaturation was left to proceed 1 min before desalting the samples on a Sephadex G-25 (NAP-5) column equilibrated in water. The same procedure was performed without DTT and iodoacetate to generate a protein without charge modification. Portions of the two samples were mixed prior to desalting into water to generate hybrid forms of the oligomer. The samples were analyzed by nondenaturing polyacrylamide gel electrophoresis at pH 7.4 on a 10% acrylamide gel with the continuous buffer system described by McLellan (1982) containing 43 mM imidazole and 35 mM Hepes. Staining was with Coomassie blue (Ewbank & Creighton, 1993).

Cysteine Reactivity of the Variants of DsbC. Ellman's assay for thiol groups (Riddles et al., 1983) was performed with the $DsbC^{Thr}_{SH}$, $DsbC^{SH}_{Val}$, and $DsbC^{SH}_{Ala}$ mutants. The time course of the reaction between the free cysteine thiols present and DTNB was followed by monitoring the absorbance at 412 nm. To ensure that the active site thiol groups were reduced, the proteins had been previously incubated with 10 mM DTT in 0.1 M Tris-HCl (pH 8.0) prior to desalting on a Sephadex G-25 (NAP-5) column in the buffer used for the Ellman's assay.

Circular Dichroism and Fluorescence Spectra. CD spectra of 14 μM DsbC were recorded at 25 °C on a Jobin-Yvon CDVI spectrometer, as described previously for DsbA (Zapun et al., 1993). The fully reduced sample ($DsbC_{4SH}$) was prepared by incubation of DsbC with 6 M GdmCl, 12.5 mM DTT, 125 mM Tris-HCl (pH 8), and 0.75 mM EDTA prior to desalting on a Sephadex G-25 (NAP-5) column equilibrated with the appropriate buffer containing 1 mM DTT. Reaction of the $DsbC_{4SH}$ protein with iodoacetate after the measurements, followed by electrophoresis as in Figure 1, confirmed that the protein remained reduced. Fluorescence emission spectra were recorded on an Aminco-Bowman Series 2 luminescence spectrometer at 25 °C with

a 5 mm square cuvette. The samples were prepared as for the CD measurements.

Reaction with Glutathione. The equilibrium constant for disulfide bond formation in the active site of DsbC, with glutathione as thiol−disulfide reagent, was determined essentially as described previously for DsbA (Zapun et al., 1993). DsbC (about 1 μM) was incubated in different redox mixtures of excess GSH and GSSG, in 0.1 M Tris-HCl (pH 7.5), 0.2 M KCl, and 1 mM EDTA at 25 °C. After 10 or 20 min, the reaction was quenched with 0.5−0.25 M HCl and the mixture subsequently diluted by adding $^1/_5$ volume of water. Samples were stored at 1 °C prior to analysis by reverse phase HPLC on a C-18 Vydac column (218TP54) at 38 °C with a gradient of acetonitrile (30−42% (v/v) in 26 min) in 0.1% TFA. The low concentrations of DsbC used in these experiments did not allow accurate quantification of the small amounts of its mixed disulfide with glutathione. This was accomplished using 45 μM DsbC and appropriate concentrations of GSH and GSSG.

Loading the same amounts of $DsbC_S^S$ or $DsbC_{SH}^{SH}$ onto the reverse phase HPLC column resulted in peaks detected at 220 nm of identical area, showing that correction for different extinction coefficients or recoveries was not necessary; the same was assumed to be the case for the mixed disulfide species. To correct for air oxidation of thiol groups, the free thiol content of the mixture was determined at the time of the acid quench by Ellman's assay (Riddles et al., 1983) as described previously (Zapun et al., 1994). The equilibrium constant for the formation of $DsbC_{Val}^{SSG}$ or $DsbC_{Ala}^{SSG}$ was measured following essentially the same procedure, but the reaction was quenched by addition of 1.5 volumes of 0.67 M HCl.

The rates of reduction of $DsbC_S^S$ and oxidation of $DsbC_{SH}^{SH}$ by excess GSH or GSSG, respectively, were determined by reverse phase HPLC analysis of the two forms of the protein in reaction mixtures that were acid quenched after various time intervals. The rate of formation of $DsbC_{Ala}^{SSG}$ by reaction of $DsbC_{Ala}^{SH}$ with GSSG was determined in the same way.

BPTI Refolding and Preparation of the Folding Intermediates. The preparation of fully reduced BPTI and the various folding intermediates, as well as the disulfide-coupled folding reactions, was performed as described previously (Zapun & Creighton, 1994), with the following exception. When measuring the direct oxidation of $(5-55,14-38)_N$ by $DsbC_S^S$, or its rearrangement by $DsbC_{SH}^{SH}$, the reaction was acid quenched after various time intervals. Aliquots of 100 μL were withdraw from the reaction mixture and mixed with 30 μL of 1 M HCl. Of each acid-quenched sample, 30 μL was added to 220 μL of 10 mM HCl to be analyzed by reverse phase HPLC to determine the redox state of DsbC. The remaining 100 μL was mixed with 100 μL of water for further HPLC analysis of the different BPTI forms.

RESULTS

Purified DsbC. About 28 mg of purified DsbC could be isolated from 1 L of cell culture. The protein was more than 98% pure as judged by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate or urea and by reverse phase HPLC. Its absorbance, fluorescence excitation and emission, and ^{1}H NMR spectra (J. Kemmink, A.Z., and T.E.C., unpublished observation) did not reveal any tightly bound cofactor or prosthetic group. Moreover, electrospray mass spectrometry of the protein as isolated, or after incubation under denaturing and reducing conditions and further purification by reverse phase HPLC, gave essentially the same molecular weight of 23458 ± 4 Da.

This molecular weight was, however, 95 ± 4 Da greater than expected for the mature protein, with its signal sequence correctly processed, from the amino acid sequence derived from the published nucleotide sequence designated *xprA* by Lovett and Kolodner (1991). To locate the discrepancy, an aliquot of the protein was cleaved with CNBr and the entire mixture of reduced peptide fragments was analyzed by matrix-assisted laser desorption mass spectrometry. One fragment was observed to have a mass about 95 Da greater than expected, with a measured mass of 4829 Da, corresponding to residues 154−201 of the published sequence of the mature protein. This peptide was isolated by separating the entire mixture of CNBr-generated fragments by HPLC. The fraction containing this peptide was incubated with iodoacetamide under reducing and alkaline conditions in order to block all free thiol groups. After further HPLC purification, one fraction contained a peptide with a mass of 4892 Da, which is 98 Da greater than that expected for residues 154−201 with Cys163 reacted with iodoacetamide.

The nucleotide sequence of the *dsbC* gene used in this study was determined in the region corresponding to the unexpected peptide fragment. It revealed the presence of three additional bases, CCG, in addition to those at positions 1679−1681 of the sequence published by Lovett and Kolodner (1991); the same sequence was found when the *dsbC* gene was cloned from *E. coli* chromosomal DNA. The revised gene sequence encodes a DsbC protein with one additional proline residue at position 198 of the expected mature sequence. A proline residue has a mass of 97 Da, and the location of the additional residue is consistent with the peptide mapping. The revised predicted molecular weight of 23 460 Da is in good agreement with the measured value.

Size-exclusion chromatography of $DsbC_S^S$ at concentrations varying between 15 and 30 μM under various buffer conditions on two different types of matrix yielded an apparent molecular weight of about 67 kDa, indicating that it was oligomeric under the experimental conditions; hybridization experiments to be described showed it to be a dimer (see Figure 4). Incubation of DsbC with 10 mM DTT, 8 M GdmCl, both, or 1% Triton X-100 prior to loading onto the column did not alter its elution properties, showing that either the oligomerization interaction was not disrupted by those agents or the oligomer was regenerated rapidly after their removal. Similar experiments with $DsbA_S^S$ confirmed that it is a monomer under all these conditions. In the presence of 1% SDS, DsbC had the same elution properties as DsbA, showing it was monomeric under these denaturing conditions. When native DsbA and DsbC were incubated together at a concentration of about 100 μM each for 1 h at 4 °C prior to analysis, their elution properties were not affected, indicating that they did not form a stable complex under the conditions used.

Cysteine Thiol Groups of DsbC. To probe the redox state and the accessibilities of the cysteine residues of DsbC, the electrophoretic cysteine-counting method (Creighton, 1980) was used. DsbC was incubated under various conditions and

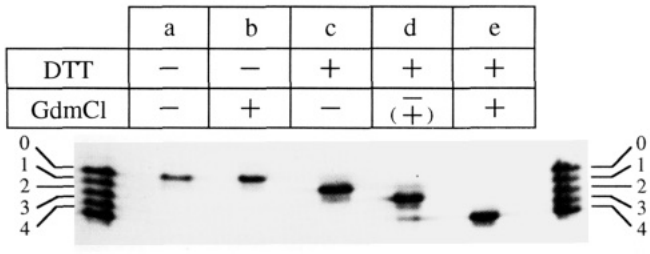


FIGURE 1: Reactivity of the cysteine thiol groups of DsbC. Purified DsbC was incubated with and without 17 mM DTT, in the presence or absence of 6 M GdmCl, followed by addition of excess iodoacetate. In the sample analyzed in lane d, GdmCl was added after the addition of the acidic reagent iodoacetate. After gel filtration, the protein samples were incubated under denaturing and reducing conditions prior to blocking all free cysteine thiols with neutral iodoacetamide. The samples were analyzed by polyacrylamide gel electrophoresis at pH 9.5 in 8 M urea, where the mobility depends upon the number of groups that had reacted with iodoacetate rather than iodoacetamide. The ladders on both sides were generated by reacting reduced, unfolded DsbC with mixtures of iodoacetamide and iodoacetate. The numbers indicate the number of cysteine residues that have reacted with iodoacetate.

then reacted with iodoacetic acid. Any free and accessible thiol group at this stage would have reacted and subsequently carry one negative charge. Thiol groups that were inaccessible or involved in disulfide bonds were then made accessible under denaturing or reducing conditions and reacted with iodoacetamide; they subsequently carry no net charge. The net charge on the protein was determined by polyacrylamide gel electrophoresis in 8 M urea (Figure 1), by comparison with fully unfolded and reduced DsbC reacted with either iodoacetate or iodoacetamide; the former has four more negative charges. Reacting the reduced protein with mixtures of iodoacetate and iodoacetamide generated species with the expected intermediate numbers of negative charges, to produce an electrophoretic "ladder of charge" (Figure 1, side lanes).

$DsbC_S^S$ as isolated did not react with iodoacetate in the absence or presence of 6 M GdmCl, showing that its four cysteine residues were involved in disulfide bonds (Figure 1, lanes a and b, respectively). Incubation in 6 M GdmCl with 13 mM DTT resulted in a fully reduced species with four accessible thiol groups that reacted with iodoacetate (Figure 1, lane e). Incubation with DTT in the absence of denaturant resulted in a species of DsbC with only one cysteine thiol that reacted with iodoacetate. (Figure 1, lane c). The subsequent addition of 6 M GdmCl allowed a second thiol to react (Figure 1, lane d). This observation is analogous to that made with thioredoxin and DsbA, where the active site disulfide bond is accessible to reducing agent and only one of the resulting free thiol groups is exposed and reactive while the other is buried (Kallis & Holmgren, 1980; Zapun et al., 1993). This species is designated $DsbC_{SH}^{SH}$. The other two cysteine residues of $DsbC_{SH}^{SH}$ were paired in a second disulfide bond that is buried and could not be reduced (even after a 30 min incubation with 10 mM DTT; data not shown) unless the protein was unfolded.

Samples incubated under the same conditions, but reacted with iodoacetamide instead of iodoacetate and without further blocking of the remaining cysteine residues, were analyzed by electrospray mass spectrometry. The masses measured were consistent with the above results (data not shown).

To identify which of the thiol groups of $DsbC_{SH}^{SH}$ was accessible and reactive, the kinetics were measured of the reaction between DTNB and variants of DsbC lacking either of the putative active site cysteine residues Cys98 and Cys101 (Riddles et al., 1983). With $DsbC_{Val}^{SH}$ and $DsbC_{Ala}^{SH}$, about 1 equiv of thiol reacted with 150 μM DTNB within 2 s (data not shown); the second-order rate constant for the reaction of the thiol in these mutants with DTNB is therefore $\gg 2000\ s^{-1}\ M^{-1}$, as expected for an accessible thiol group. With $DsbC_{SH}^{Thr}$, 1 equiv of free thiol reacted with DTNB much more slowly, with a second-order rate constant of about $100\ s^{-1}\ M^{-1}$. These results indicate that the sequence -Cys98-Gly-Tyr-Cys101- forms the active site of DsbC and that Cys98 has its thiol group accessible and reactive in the native conformation, whereas that of Cys101 is buried and much less reactive.

When DsbC was fully reduced under denaturing conditions and the denaturant was removed by gel filtration, electrophoretic analysis demonstrated that all four cysteine residues of $DsbC_{4SH}$ reacted with iodoacetate only after readdition of denaturant. After 10 min of incubation with 0.5 M iodoacetate under native conditions, fewer than 10% of the molecules had their four thiols reacted, about 20% had three thiols reacted, and the remainder had only two cysteine residues blocked (data not shown). This indicates that $DsbC_{4SH}$ can refold without any disulfide bonds and that the thiols that form the buried disulfide bond under normal conditions are then also buried and poorly reactive toward iodoacetate. Nevertheless, the absence of the buried disulfide bond makes both of the active site cysteine residues reactive to iodoacetate, probably by lowering the stability of the folded conformation or increasing its flexibility. Fully oxidized DsbC, partly oxidized DsbC with only the accessible disulfide bond reduced, and fully reduced DsbC are designated here as $DsbC_S^S$, $DsbC_{SH}^{SH}$, and $DsbC_{4SH}$, respectively.

In contrast to the alkylation experiments, Ellman's assay measured 4.0 thiol groups in $DsbC_{4SH}$ under both native and denaturing conditions. DTNB is a disulfide reagent, so its reaction with only a single cysteine thiol can result in the formation of a protein disulfide bond and the generation of two molecules of the chromophore TNB. Therefore two cysteine residues will be measured even if only one actually reacted with DTNB. With $DsbC_{SH}^{SH}$, Ellman's assay measured 1.8 free thiols, under both native and denaturing conditions, close to the 2.0 expected.

CD and Fluorescence Spectra. The secondary and tertiary structures of DsbC in its various forms were compared by CD and fluorescence spectroscopy. $DsbC_S^S$, $DsbC_{SH}^{SH}$, and $DsbC_{4SH}$ gave identical CD spectra in both the far- and near-UV (Figure 2), confirming that all three are very similarly folded.

$DsbC_S^S$ and $DsbC_{SH}^{SH}$ gave very similar fluorescence emission spectra with $\lambda_{max} = 312$ nm (Figure 3), but fully reduced $DsbC_{4SH}$ exhibited about 2-fold greater fluorescence, with $\lambda_{max} = 314$ nm. The short wavelength of λ_{max} indicates that the single tryptophan residue, Trp140, is essentially buried in all three forms of DsbC; not surprisingly, its fluorescence is quenched when the adjacent Cys141 is disulfide-bonded.

Oligomerization State. The gel filtration results yielded an ambiguous oligomerization state of DsbC; the apparent molecular weight of 67 kDa is 2.8-fold greater than that of a single polypeptide chain. Polyacrylamide gel electrophoresis in the presence of 1% SDS gave a molecular weight

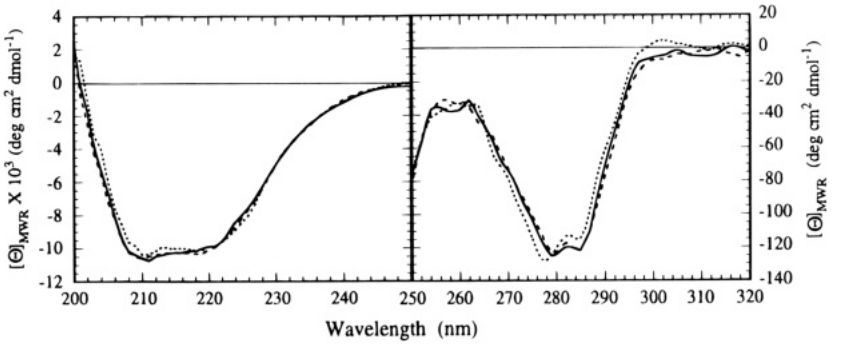


FIGURE 2: Circular dichroism spectra of (− − −) $DsbC_S^S$, (—) $DsbC_{SH}^{SH}$, and (•••) $DsbC_{4SH}$. The buffer was 0.1 M sodium phosphate (pH 7) and 1 mM EDTA at 25 °C. The two reduced samples also contained 1 mM DTT.

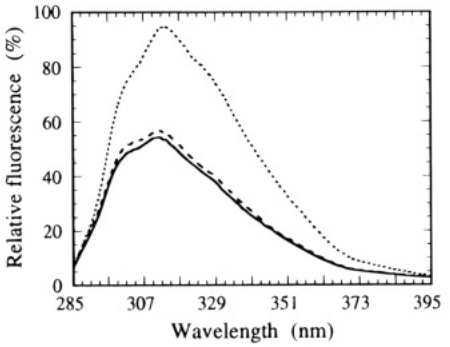


FIGURE 3: Fluorescence emission spectra of (− − −) $DsbC_S^S$, (—) $DsbC_{SH}^{SH}$, and (•••) $DsbC_{4SH}$ with excitation at 280 nm. The conditions were as in Figure 2.

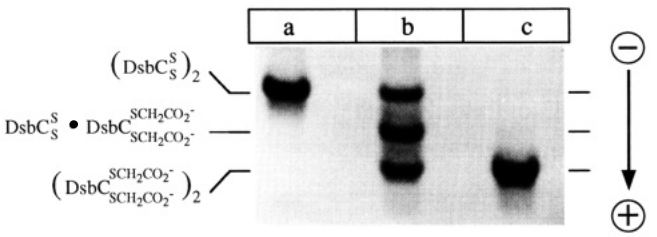


FIGURE 4: Electrophoretic demonstration by hybridization that DsbC is a dimer. $DsbC_S^S$ (lane a), DsbC with its active site thiol groups reacted with iodoacetate (lane c), and the two incubated together in 6 M GdmCl prior to desalting by gel filtration (lane b) were analyzed by nondenaturing polyacrylamide gel electrophoresis at pH 7.4. The intermediate hybrid band generated in lane b is assumed to be the dimer containing one polypeptide chain of each of the two forms of the protein in lanes a and c.

of <30 kDa, both under reducing and nonreducing conditions, indicating that the association of the subunits was not through disulfide bonds (data not shown).

The possibility of preparing a species of DsbC with its active site cysteine residues reacted with iodoacetate, and therefore carrying two negative net charges relative to $DsbC_S^S$, was exploited to prepare homo- and heterooligomers by mixing the two species under denaturing conditions and subsequently removing the denaturant by gel filtration. Hybrids should form (Eisenstein & Schachman, 1989), as no conformational changes were observed by various types of spectroscopy upon reduction of $DsbC_S^S$. The various oligomeric species could be separated on the basis of their charge at pH 7.4 by nondenaturing polyacrylamide gel electrophoresis (Figure 4). One hybrid species with an electrophoretic mobility intermediate to that of the two homooligomers was observed, indicating that DsbC was present as a stable dimer.

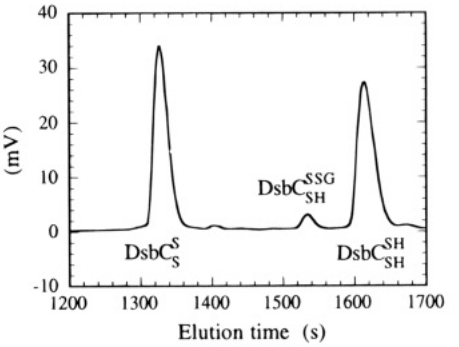


FIGURE 5: HPLC separation of acid-trapped forms of DsbC. The protein had been equilibrated at 25 °C with 40.2 mM GSSG and 2.88 mM GSH in 0.1 M Tris-HCl (pH 7.5), 0.2 M KCl, and 1 mM EDTA, prior to trapping by addition of HCl. About 150 μg of protein was loaded onto the reverse phase column.

Thiol−Disulfide Redox Equilibrium. To investigate the stability of the accessible disulfide bond of DsbC, the equilibrium constant for its formation was measured with glutathione as reagent. The protein was equilibrated in various mixtures of GSH and GSSG, the thiol−disulfide exchange reaction was quenched by acid, and the samples were analyzed by reverse phase HPLC.

$DsbC_S^S$ and $DsbC_{SH}^{SH}$ could be separated surprisingly easily (Figure 5). $DsbC_{4SH}$ was found to have the same elution properties as $DsbC_{SH}^{SH}$ under a variety of elution conditions, but it was not produced by the procedure used here (as shown electrophoretically after trapping with iodoacetate; Figure 1), as GSH was able to reduce only the active site disulfide bond of DsbC under nondenaturing conditions (data not shown). Small amounts of the mixed disulfide with glutathione $DsbC_{SH}^{SSG}$ were detected under the appropriate conditions, and its identity was confirmed by electrospray mass spectrometry, which found the mass of the protein to be increased by the expected 305 Da. As only the species $DsbC_S^S$, $DsbC_{SH}^{SSG}$, and $DsbC_{SH}^{SH}$ were present, and no hybrid forms, DsbC appeared to be a monomer under the acidic conditions used for the separation. With a given concentration of glutathione, the amount of the mixed disulfide species trapped did not depend on the conditions of the acid quench, with final HCl concentrations varying between 50 mM and 1 M; 250 mM was used routinely. The length of incubation with glutathione (10 or 20 min) prior to acid quenching and the duration of storage prior to analysis (up to 5 h) did not affect the results, showing that equilibrium had been reached and the trapping was stable.

The relative amounts of $DsbC_S^S$ and $DsbC_{SH}^{SH}$ trapped after the protein had been incubated at low concentrations (about

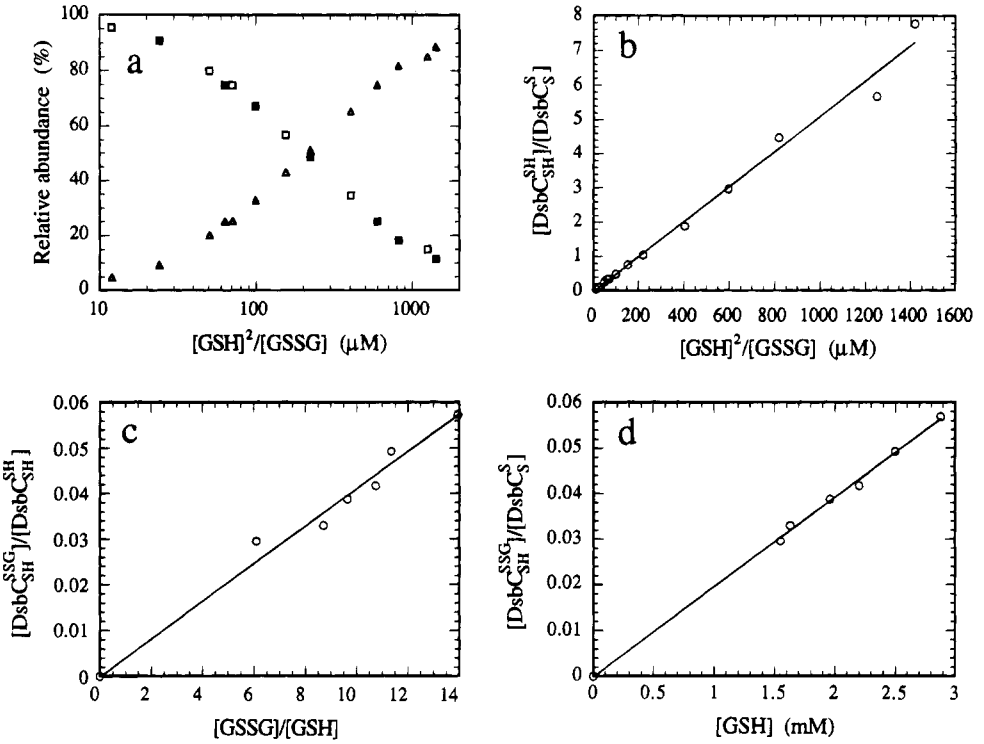
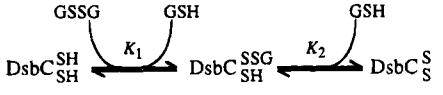


FIGURE 6: Measurement of the thiol−disulfide equilibrium of the active site disulfide bond of DsbC with glutathione. (a and b) DsbC at a monomer concentration of about 1 μM was incubated with various mixtures containing either of two concentrations of GSSG and varying amounts of GSH, in 0.1 M Tris-HCl (pH 7.5), 0.2 M KCl, and 1 mM EDTA at 25 °C. The equilibrium reaction mixtures were acid quenched and analyzed by reverse phase HPLC as in Figure 5. (a) Relative proportions of $DsbC_S^S$ (□, ■) and $DsbC_{SH}^{SH}$ (△, ▲) at varying concentrations of GSSG and GSH. Open and filled symbols were for mixtures that contained 20 and 10 mM GSSG, respectively. (b) The plot of $[DsbC_{SH}^{SH}]/[DsbC_S^S]$ versus $[GSH]^2/[GSSG]$ should be linear and gives the value of K_{SS}. The relative amounts of the mixed disulfide intermediates were measured in panels c and d using a higher concentration of DsbC, about 45 μM monomer. It was incubated with various mixtures containing between 14 and 40 mM GSSG and the amount of GSH required to keep the value of $[GSH]^2/[GSSG]$ close to 200 μM. (c) The plot of $[DsbC_{SH}^{SSG}]/[DsbC_{SH}^{SH}]$ versus [GSSG]/[GSH] should be linear and gives the value of K_1. (d) The plot of $[DsbC_{SH}^{SSG}]/[DsbC_S^S]$ versus [GSH] should be linear, and the reciprocal of the slope gives K_2. The equilibrium constants K_{SS}, K_1, and K_2 refer to eq 1, and the values are given in Table 1. The solid lines are the results of linear fits to the data.

1 μM) in various GSH/GSSG mixtures are shown in Figure 6a,b. The relative amounts of $DsbC_{SH}^{SSG}$ measured after incubation of the protein at the higher concentration of about 45 μM in GSH/GSSG mixtures chosen to optimize its accumulation are shown in Figure 6c,d. The data were consistent with the expected model of disulfide formation in a monomeric unit where only the single accessible cysteine residue of the protein can form an intermediate mixed disulfide with glutathione:



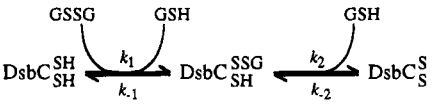
(1)

with

$$K_{SS} = K_1K_2 = \frac{[DsbC_S^S][GSH]^2}{[DsbC_{SH}^{SH}][GSSG]} \qquad (2)$$

There were no indications of cooperativity, and the two monomers of the DsbC dimer appeared to be independent. The relative concentrations of the species defined unambiguously the values $K_{SS} = (1.95 \pm 0.06) \times 10^{-4}$ M, $K_1 = (4.1 \pm 0.1) \times 10^{-3}$, and $K_2 = (5.1 \pm 0.1) \times 10^{-2}$ M. The accessible disulfide bond of $DsbC_S^S$ is nearly as unstable as that of $DsbA_S^S$; its K_{SS} value of 200 μM is only somewhat greater than the 80 μM of DsbA under similar conditions (Wunderlich & Glockshuber, 1993; Zapun et al., 1993). The mixed disulfide with glutathione is also remarkably unstable.

Kinetics of Reaction of DsbC with Glutathione. No spectroscopic difference was found between $DsbC_S^S$ and $DsbC_{SH}^{SH}$, so the rates of their interconversion by reaction with glutathione were monitored by acid quenching of the reactions after various time intervals followed by separation and quantification of the different forms of the protein by reverse phase HPLC, as in Figure 5. In each case, a large excess of glutathione was used and all the experimental kinetic data could be fitted to single-exponential functions, as expected for pseudo-first-order reactions. The kinetics in both directions were fully consistent with the expected reaction involving the transient formation of a mixed disulfide intermediate (Creighton, 1986).



(3)

There were no indications of interactions between the two monomers of the DsbC dimer.

The kinetics of oxidation of $DsbC_{SH}^{SH}$ with excess GSSG in the absence of GSH exhibited a linear dependence of the pseudo-first-order rate constant on the concentration of GSSG (Figure 7b), and no mixed disulfide intermediate accumulated, indicating that the initial step was rate-limiting, with a value for k_1 of 34 ± 1 s^{-1} M^{-1}.

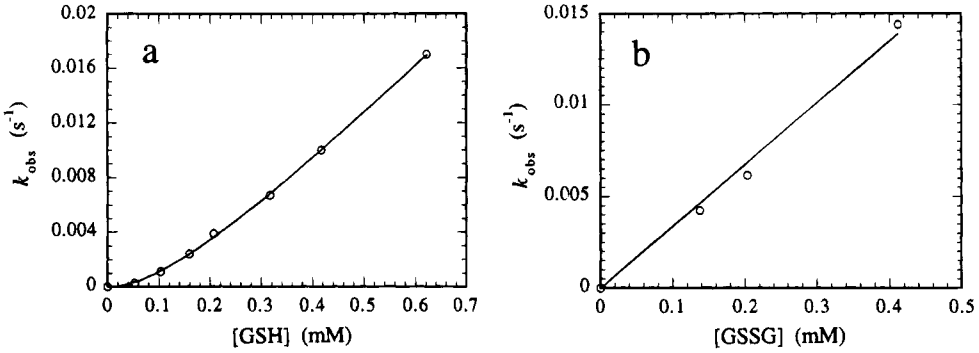


FIGURE 7: Dependence of the observed pseudo-first-order rate constants for the reaction of $DsbC_S^S$ and $DsbC_{SH}^{SH}$ on the concentrations of GSH and GSSG, respectively. Conditions were as in Figure 6. (a) Rate of reaction of $DsbC_S^S$ with GSH. The solid curve was generated using eq 4 and the rate constants given in the text. (b) Rate of reaction of $DsbC_{SH}^{SH}$ with GSSG. The solid curve was generated using the equation $k_{obs} = k_1[GSSG]$ with $k_1 = 34\ s^{-1}\ M^{-1}$.

Table 1: Equilibrium and Kinetic Constants for the Thiol−Disulfide Exchange Reactions between DsbC or DsbA and Glutathione

		DsbC	DsbA[b]	P[c]
equilibria[a]	K_{SS} (M)	$(1.95 \pm 0.06) \times 10^{-4}$	$(8.3 \pm 0.1) \times 10^{-5}$	$k_{intra}/(1\ s^{-1}\ M^{-1})$
	K_1	$(4.1 \pm 0.1) \times 10^{-3}$	$(6.1 \pm 0.1) \times 10^{-3}$	4
	K_2 (M)	$(5.1 \pm 0.1) \times 10^{-2}$	$(1.28 \pm 0.02) \times 10^{-2}$	$k_{intra}/(4\ s^{-1}\ M^{-1})$
kinetics[a]	k_1 ($s^{-1}\ M^{-1}$)	34 ± 1	41 ± 1	8
	k_{-1} ($s^{-1}\ M^{-1}$)	$(8.3 \pm 0.5) \times 10^3$	$(6.7 \pm 0.3) \times 10^3$	2
	k_2 (s^{-1})	1.9 ± 0.1	60 ± 16	k_{intra}
	k_{-2} ($s^{-1}\ M^{-1}$)	38 ± 1	$(4.6 \pm 0.9) \times 10^3$	4

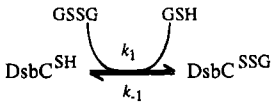
[a] The equilibrium and rate constants are defined in eqs 1 and 3, respectively. [b] Revised values of Zapun et al. (1993). Subsequent work revealed that the initial quenching procedure might not have been adequate to trap the mixed disulfide (Nelson & Creighton, 1994). After optimization to give maximum amounts of mixed disulfide, the reaction was quenched with a final concentration of 0.5 M HCl. The slight revisions in the rate and equilibrium constants do not affect the conclusions draw previously. [c] Values expected for a normal protein, with its thiol groups normally accessible and reactive. k_{intra} is the rate constant for the intramolecular step in forming the disulfide bond, which differs in different peptides and proteins; it is usually in the range 10^{-3}−$10^5\ s^{-1}$ depending upon the conformational change to make the disulfide bond. The conditions were 25 °C in 0.1 M Tris-HCl (pH 7.5), 0.2 M KCl, and 1 mM EDTA.

During the reduction of $DsbC_S^S$ by excess GSH in the absence of GSSG, the mixed disulfide intermediate was not populated substantially, so the observed pseudo-first-order rate constant, k_{obs}, is expected to be given by

$$k_{obs} = \frac{k_{-1}k_{-2}[GSH]^2}{k_2 + k_{-1}[GSH]} \quad (4)$$

At low GSH concentrations, when $k_2 \gg k_{-1}[GSH]$, the reaction is second-order with respect to the concentration of GSH, and the observed rate constant is given by $k_{obs} \approx k_{-1}k_{-2}[GSH]^2/k_2$. At high GSH concentrations, when $k_{-1}[GSH] \gg k_2$, the rate determining step is the initial attack of GSH on $DsbC_S^S$ and can be approximated as $k_{obs} \approx k_{-2}[GSH]$. The observed kinetics showed both expected types of behavior (Figure 7a) and defined unambiguously the values $k_{-1}k_{-2}/k_2 = (1.59 \pm 0.13) \times 10^5\ s^{-1}\ M^{-2}$ and $k_{-2} = 38 \pm 1\ s^{-1}\ M^{-1}$. The individual rate constants can be obtained from these data and the equilibrium constants and are given in Table 1. The overall equilibrium constant calculated from the rate constants, $K_{SS} = k_1k_2/k_{-1}k_{-2}$, is 212 ± 24 μM, which is very close to the value measured directly.

Reaction of DsbC Variants with Glutathione. Accurate kinetic and equilibrium measurements of the reaction of DsbC with glutathione depend upon efficient trapping of the mixed disulfide intermediate by acid. The quenching seemed to be adequate, as the amount of mixed disulfide detected was the same with different trapping procedures. However, some of the reactions are rapid and intramolecular, and the thiol groups of DsbC and GSH probably differ markedly in their pK_a values, as with DsbA, where difficulties in acid trapping have been encountered (Nelson & Creighton, 1994). Most of these uncertainties can be alleviated by genetically replacing the second cysteine residue of DsbC, Cys101. In this case, only the mixed disulfide with glutathione can be formed.



(5)

with

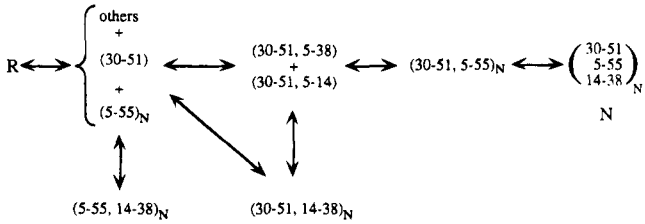
$$K_1 = \frac{k_1}{k_{-1}} \quad (6)$$

This mixed disulfide would be expected to be as unstable as the one formed by the wild-type protein, for which a K_1 value of 4×10^{-3} was measured. Cys101, which is buried in DsbC, was replaced by an alanine residue to disturb the structure as little as possible. The equilibrium constant K_1 was measured to be $(2.24 \pm 0.01) \times 10^{-2}$ (data not shown), which is 5 times larger than the value measured with the wild-type protein. A very similar discrepancy of 6-fold was found with DsbA when the corresponding buried cysteine residue was replaced by Ser (Zapun et al., 1994). These discrepancies appear to be due to perturbations of the reactivity of the accessible cysteine residue by the replacement of the adjacent Cys, probably by local perturbation of the protein structure. This was also indicated by a much

greater discrepancy measured when Cys101 in DsbC was replaced by the more bulky Val. The measured value of K_1 of 0.56 ± 0.02 (data not shown) is in this case 140-fold greater than that measured with the wild-type protein and close to the value of unity expected for a normal mixed disulfide bond. Whatever the reason for the discrepancy, the low value of K_1 measured with $DsbC^{SH}_{Ala}$ confirms that the mixed disulfide of DsbC with glutathione is much less stable than a typical mixed disulfide.

The kinetics of reaction of $DsbC^{SH}_{Ala}$ with GSSG were measured, but the reverse reaction with the small amounts of GSH generated by the forward reaction was significant. The data obtained with different concentrations of GSSG were therefore modeled using the program of numerical simulation REDKINS written by J. Ewbank and N. Darby (Ewbank, 1992). The initial rates gave the value of $k_1 = 66 \pm 4\ s^{-1}\ M^{-1}$, and the equilibria reached were consistent with the above value of K_1, which gave the value of $k_{-1} = (2.9 \pm 0.2) \times 10^3\ s^{-1}\ M^{-1}$. These values are very close to those measured with the wild-type protein, differing only by factors of 2 and 3, respectively. These measurements confirm the general accuracy of the rate and equilibrium constant values measured with the wild-type protein.

Effects of DsbC on the Disulfide Folding of BPTI. BPTI is a small protein of 58 residues with three disulfide bonds. Its disulfide-coupled folding pathway is unusually well-characterized and understood. It was established by rigorous kinetic analysis and replacing the various cysteine residues (Creighton, 1978; Creighton & Goldenberg, 1984; van Mierlo et al., 1993, 1994).



(7)

R is the fully reduced and unfolded protein with six free thiols. The intermediates are depicted by numbering the cysteine residues paired in disulfide bonds according to their position in the primary sequence. Those that tend to adopt the native-like conformation are denoted by the subscript N.

The first disulfide bond is formed randomly in a statistical manner (Darby & Creighton, 1993), but the resulting species are in rapid equilibrium, and (30−51) predominates because it is stabilized by some elements of native structure (van Mierlo et al., 1993). In (30−51), Cys5, Cys14, and Cys38 are in flexible and unstructured parts of the molecule, and they can readily form any of the three possible disulfide bonds to generate the intermediates (30−51, 5−14), (30−51, 5−38), and $(30-51, 14-38)_N$. This latter species accumulates to large quantities during refolding, especially at low pH, because it has a stable quasi-native conformation and does not readily form the 5−55 disulfide bond. This is not due to inaccessibility of the Cys5 and Cys55 thiol groups but to a kinetic block in actually forming the disulfide bond. This species is in rapid equilibrium by intramolecular disulfide rearrangements with the (30−51, 5−14) and (30−51, 5−38) intermediates, which in turn rearrange intramolecularly to form $(30-51, 5-55)_N$. The missing disulfide bond 14−38 is readily made in this last intermediate.

The one-disulfide intermediate $(5-55)_N$ also tends to adopt a quasi-native conformation (van Mierlo et al., 1991) and is relatively stable at neutral pH. Because of its quasi-native conformation, $(5-55)_N$ can very rapidly form the 14−38 disulfide bond to generate the most stable quasi-native species $(5-55, 14-38)_N$, which has the thiol groups of Cys30 and Cys51 buried in the hydrophobic core and unreactive (Creighton & Goldenberg, 1984). It can accumulate even more than $(30-51, 14-38)_N$ during refolding of BPTI because its disulfide rearrangements are extremely slow.

The effects of catalytic amounts of DsbC on the disulfide refolding of BPTI in the presence of 0.5 mM GSH and 2 mM GSSG were examined at pH 7.5 and 25 °C. Although not physiological, because DsbC probably does not function in the presence of glutathione, these conditions are the same as used previously with DsbA and mammalian PDI (Zapun & Creighton, 1994; Creighton et al., 1993) and permit comparison of the various catalysts. The concentrations of reduced BPTI and the Cys-X-Y-Cys active sites of the catalysts were almost identical in each case. Figure 8B compares the kinetics of disappearance of the fully reduced unfolded BPTI (R) and the appearance of the native three-disulfide-bonded protein (N) in the absence and presence of DsbC, DsbA, or PDI.

The half-time for the disappearance of R, which is indicative of the rate of disulfide formation in the protein, was about 80 s in the absence of any catalyst. This was found previously to be decreased by PDI to less than 25 s (Creighton et al., 1993) and less so by DsbA to 65 s (Zapun & Creighton, 1994), but DsbC had no influence on the rate of disappearance of R. In contrast, DsbC exhibited a marked effect on the rate of appearance of native BPTI. About 56% of the BPTI molecules were fully refolded after 16 min in the presence of DsbC, whereas in the same time only 13% N appeared in the absence of any accessory protein. DsbA produces only a marginal rate enhancement, with about 20% of the BPTI in its native form after the same time. The effect of DsbC is, however, less than that of PDI, which caused 50% of the BPTI molecules to be fully refolded after only 3 min, a 27-fold increase in rate. The rate enhancement for the appearance of N produced by DsbC in this experiment was about 5-fold, compared to a 1.5-fold increase produced by DsbA.

In the case of PDI, the overall rate enhancement resulted both from faster protein disulfide bond formation and from faster intramolecular disulfide rearrangements in the quasi-native intermediates $(30-51, 14-38)_N$ and, to a lesser extent, $(5-55, 14-38)_N$. The faster disappearance of R indicated that DsbA was also catalyzing disulfide bond formation, and this activity has been confirmed and explained in detail with a model peptide (Darby & Creighton, 1995). However, DsbA had no significant effect on the quasi-native species $(5-55, 14-38)_N$, and $(30-51, 14-38)_N$ disappeared only slightly faster. This latter effect was shown to be due to the direct introduction of the 5−55 disulfide bond into $(30-51, 14-38)_N$ by $DsbA^S_S$ to form native BPTI, bypassing the rearrangement steps rather than catalyzing them (Zapun & Creighton, 1994).

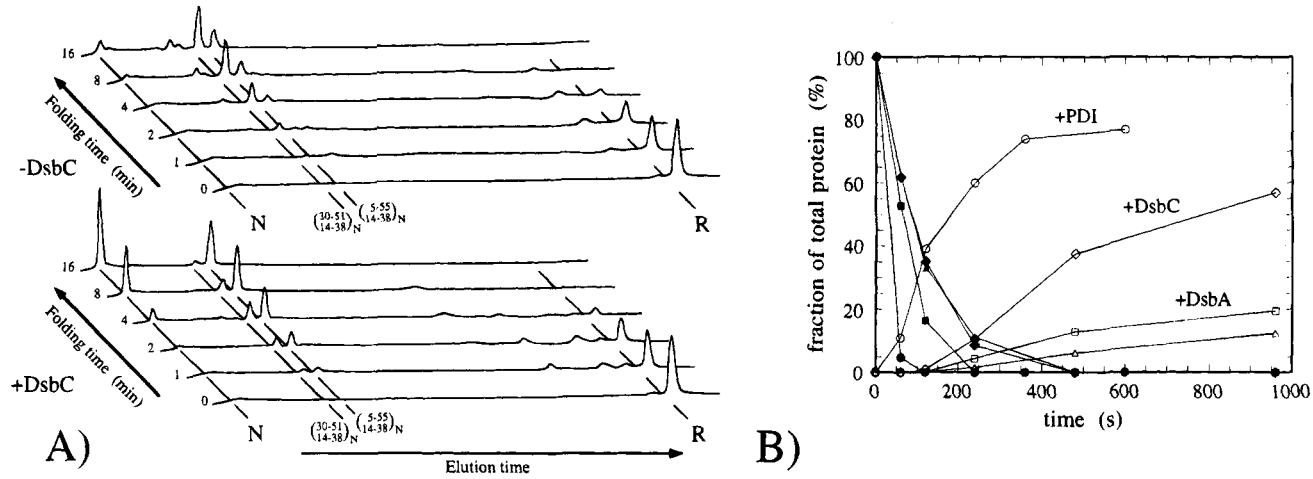


FIGURE 8: Effects of small quantities of DsbC, DsbA, and PDI on the refolding of BPTI in the presence of 0.5 mM GSSG and 2 mM GSH. The conditions were as in Figure 6. At time zero, fully reduced BPTI (R) was added to the mixture of glutathione with and without the catalysts. Portions were removed at the indicated times and acidified; the BPTI species trapped were separated and quantified by reverse phase HPLC. The concentration of BPTI was 9.6 μM in the experiment with PDI and 12 μM in the others. (A) Reverse phase HPLC analysis of the species trapped in the absence and presence of 8 μM DsbC monomer. The reduced (R) and native (N) forms of BPTI are indicated, as well as two quasi-native two-disulfide species. (B) Quantification of the native, N (open symbols), and reduced, R (solid symbols), forms of BPTI: ($\triangle$, $\blacktriangle$) in the absence of any catalyst, ($\diamond$, $\blacklozenge$) in the presence of 8 μM DsbC monomer, ($\circ$, $\bullet$)in the presence of 8 μM DsbA, and ($\square$, $\blacksquare$) in the presence of 1.6 μM PDI dimer. In each case, the ratio of cysteine residues of BPTI to those of the Cys-X-Y-Cys motif of the catalyst was 4.5:1. The data for refolding in the presence DsbA are from Zapun et al. (1994), and those in the presence of PDI are from Creighton et al. (1993).

In contrast to both PDI and DsbA, DsbC did not appear to catalyze disulfide bond formation under those conditions, as indicated by the unchanged rate of disappearance of R (Figure 8B). Rather, the enhanced rate of appearance of N caused by DsbC seemed to result from faster intramolecular rearrangement of $(30-51, 14-38)_N$, as illustrated in Figure 8A. Both the very stable intermediate $(5-55, 14-38)_N$ and N were formed more rapidly. This can be the result of efficient catalysis by DsbC of the rearrangements of $(30-51, 14-38)_N$, which partitions almost equally between $(30-51,5-55)_N$ and $(5-55, 14-38)_N$ (Creighton & Goldenberg, 1984). The amount of $(5-55, 14-38)_N$ also seemed to decrease at later times, suggesting a slight catalytic effect of DsbC on its rearrangements to the more stable $(30-51, 5-55)_N$ and then to form N.

Effects of $DsbC^{SH}_{SH}$ on the Disulfide Rearrangements of the Stable Quasi-Native Intermediates of BPTI. To measure the activity of DsbC on the intramolecular rearrangements of $(30-51, 14-38)_N$, this isolated intermediate at a concentration of 10 μM was incubated with 3 μM $DsbC^{SH}_{SH}$ at pH 7.5 and 25 °C in the absence of small thiol or disulfide molecules. Whereas $(30-51, 14-38)_N$ undergoes very slow spontaneous rearrangement and air oxidation with a half-time of about 2 h (Figure 9A), the presence of the catalytic amount of DsbC reduced this half-life to less than 10 min (Figure 9B). An equivalent amount of reduced PDI, in terms of Cys-X-Y-Cys active sites, also exhibited a dramatic effect, reducing the half-life of $(30-51, 14-38)_N$ to about 1 min, about 10-fold faster than with DsbC (Figure 9C). DsbA has no effect on this reaction (Zapun & Creighton, 1994).

In the presence of DsbC, $(30-51, 14-38)_N$ was converted to $(5-55, 14-38)_N$ in about 35% of the total BPTI while the rest of the molecules were converted to $(30-51, 5-55)_N$ and N; the latter presumably arose primarily from air oxidation. The relative persistence of $(5-55, 14-38)_N$ indicates that DsbC was ineffective in rearranging it to the more stable $(30-51,5-55)_N$. Some catalysis of rearrangement of $(5-55, 14-38)_N$ could be detected using stoichiometric amounts of $DsbC^{SH}_{SH}$. Adding 10 μM $DsbC^{SH}_{SH}$ to 10 μM $(5-55, 14-38)_N$ caused about 31% of it to be rearranged to $(30-51, 5-55)_N$ and 6% to N after 64 min at pH 7.5 and 25 °C (data not shown). In the absence of DsbC, $(5-55, 14-38)_N$ did not rearrange detectably, while catalytic amounts of PDI catalyzed its rearrangement substantially. In summary, DsbC was able to catalyze disulfide rearrangements of $(30-51,14-38)_N$ and $(5-55, 14-38)_N$ much more so than DsbA but less than PDI.

$DsbC^S_S$ as an Oxidant of Reduced BPTI. The disulfide bond of $DsbC^S_S$ is nearly as unstable and reactive to thiol groups as that of $DsbA^S_S$ (Zapun et al., 1993; Wunderlich & Glockshuber, 1993), suggesting that it also should be readily transferred by thiol−disulfide exchange (as to glutathione in eq 3) to any protein that could readily form disulfide bonds. This was confirmed using a small model unfolded peptide of 28 residues, with two cysteine residues at positions 2 and 27 that has been studied previously (Darby et al., 1994; Darby & Creighton, 1995). The disulfide bond of $DsbC^S_S$ was transferred to it stoichiometrically, as expected from their relative stabilities, and too rapidly to be measured, with an apparent second-order rate constant value greater than 10^5 s^{-1} M^{-1} (data not shown).

Disulfide transfer from $DsbC^S_S$ was further demonstrated with reduced BPTI. Figure 10 shows a time course of the refolding of reduced BPTI using stoichiometric amounts of $DsbC^S_S$, relative to the disulfide bonds to be formed in BPTI, as the only oxidant. With 30 μM $DsbC^S_S$, 10 μM reduced BPTI completely disappeared after 15 s. This rapid reaction was also observed when $DsbA^S_S$ was used as oxidant, but the spectrum of species generated was markedly different in the two cases. With $DsbA^S_S$, a mixture of many species with disulfide bonds presumably formed at random ("scrambled") was formed rapidly, and conversion by disulfide rearrangements to the normal intermediates occurred only slowly. In contrast, $DsbC^S_S$ caused the appearance of the normal intermediates $(30-51, 14-38)_N$, $(5-55, 14-38)_N$,

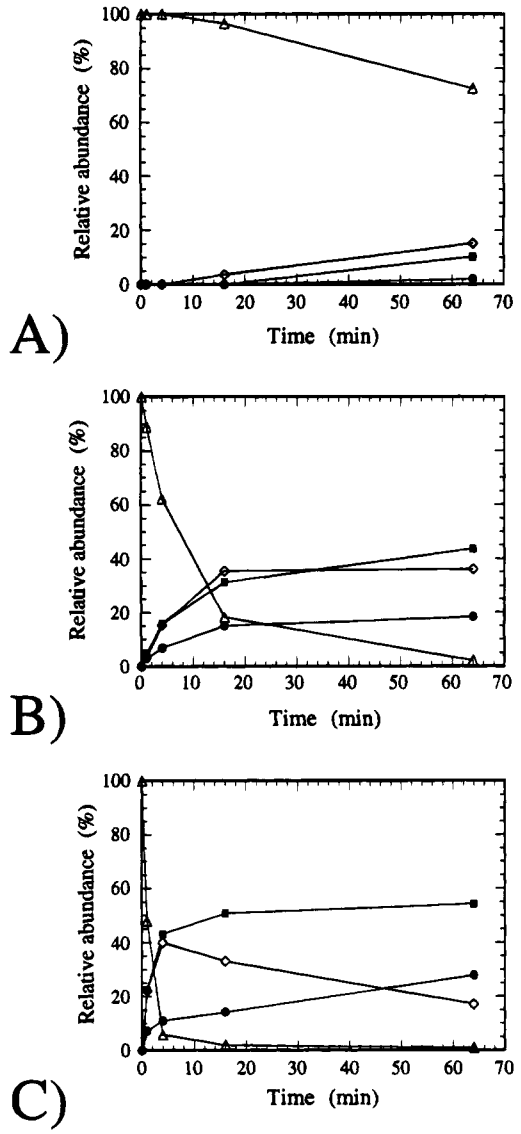


FIGURE 9: Effects of DsbC and PDI on the isolated intermediate $(30-51, 14-38)_N$. $(30-51, 14-38)_N$ (10 μM) was incubated without any catalyst (A), with 3 μM $DsbC_{SH}^{SH}$ monomer (B), or with 1.5 μM reduced PDI monomer (C), in the absence of glutathione. The conditions were as in Figure 6. The BPTI species that were acid trapped at various times were separated and quantified by reverse phase HPLC: △, $(30-51, 14-38)_N$; ◇, $(5-55, 14-38)_N$; ■, $(30-51, 5-55)_N$; and ●, N.

and $(30-51, 5-55)_N$ at even the earliest time point (15 s) of the measurements. However, the total amount of protein detected seemed to be lower initially and recovered later; therefore, many scrambled forms were also present initially, but they rapidly rearranged to the predominant intermediates. Of these species, only the quasi-native form $(5-55, 14-38)_N$ persisted after 16 min, whereas both $(30-51, 14-38)_N$ and $(30-51, 5-55)_N$ had disappeared. The reduced BPTI was almost fully refolded after 16 min, with only a minor fraction present as $(5-55, 14-38)_N$. This is faster than with $DsbA_S^S$ (Figure 11A), for which 2.5-fold greater concentrations were required to produce comparable kinetics of appearance of N (Zapun & Creighton, 1994). Even the accumulation of $(5-55, 14-38)_N$ was transient, as its relative amount was decreasing at the late times.

$DsbA_S^S$ has been shown to be able to introduce the absent 5–55 disulfide bond directly into the $(30-51, 14-38)_N$ species, thereby bypassing the normal rearrangement pathway of folding (Zapun & Creighton, 1994). $DsbC_S^S$ was found to have the same activity, but disulfide rearrangements to $(30-51, 5-55)_N$ and $(5-55, 14-38)_N$ also occurred, complicating kinetic analysis. The disulfide rearrangements are likely to have been catalyzed by the $DsbC_{SH}^{SH}$ generated in the reaction; whether $DsbC_S^S$ is also active in the process could not be determined.

The Cys30 and Cys51 thiol groups of the more stable $(5-55,14-38)_N$ quasi-native species are totally buried and unreactive (Creighton & Goldenberg, 1984), and $DsbA_S^S$ was unable to introduce a disulfide bond between them (Zapun & Creighton, 1994). Nevertheless, $DsbC_S^S$ was able to do this, with an apparent bimolecular rate constant of 57 $\pm$ 5 s^{-1} M^{-1} (data not shown). An excellent correlation was observed between the disappearance of $DsbC_S^S$ and $(5-55,-14-38)_N$ and the appearance of $DsbC_{SH}^{SH}$ and native BPTI in the initial stages of the reaction, but the concentration of $DsbC_{SH}^{SH}$ did not increase as much as expected at later times. The second disulfide bond of $DsbC_S^S$ was shown to not be involved in the reaction, as no $DsbC_{4SH}$ was generated, and the discrepancy was variable, so it is tentatively attributed to air oxidation. The rate at which the final disulfide bond was introduced into $(5-55,14-38)_N$ by $DsbC_S^S$ was slow compared to the rate at which $DsbC_S^S$ reacts with unfolded proteins and peptides ($>10^5$ s^{-1} M^{-1}) but more rapid than expected with totally buried thiol groups.

Refolding of BPTI Using DsbA as the Oxidant and DsbC as a Catalyst. The ability of DsbC to rearrange disulfide bonds in the folding intermediates of BPTI, and the lack of such an activity in DsbA, suggested that *in vivo* DsbA might be the oxidant of protein disulfides and DsbC the catalyst of disulfide rearrangements. To test this possibility, stoichiometric amounts of $DsbA_S^S$ were used as the oxidant of reduced BPTI, and smaller quantities of $DsbC_{SH}^{SH}$ were also added. The time course under those circumstances is shown in Figure 11. In the presence of just $DsbA_S^S$, a mixture of many unidentified species was rapidly generated and slowly rearranged to N and $(5-55, 14-38)_N$ as observed previously (Zapun & Creighton, 1994). When a small amount of $DsbC_{SH}^{SH}$ was included, the half-time for appearance of N was decreased from 16 to 5 min. After 4 min, most of the BPTI molecules were in the various native-like forms N, $(30-51, 14-38)_N$, and $(5-55, 14-38)_N$ in the presence of DsbC, whereas in its absence a large spectrum of species remained after 16 min.

When $DsbC_S^S$ was added instead of the reduced form, similar kinetics of refolding were observed, presumably because the $DsbC_S^S$ participated in the oxidation of BPTI and was consequently rapidly reduced. The effect was proportional to the amount of DsbC added (Figure 12).

Oxidation of $DsbC_{SH}^{SH}$ by $DsbA_S^S$. To consider the thiol–disulfide status of DsbC *in vivo*, it is necessary to know the rate at which it is oxidized by $DsbA_S^S$. The rate of this reaction was measured by incubating $DsbC_{SH}^{SH}$ with an excess of $DsbA_S^S$; the reaction was quenched by acid after various time intervals, and the different DsbC species were separated and quantified by reverse phase HPLC. No mixed disulfide between the two proteins was apparent, and the data could be fitted to a single-exponential function, as expected for a pseudo-first-order reaction (data not shown), which corresponded to a second-order rate constant of 110

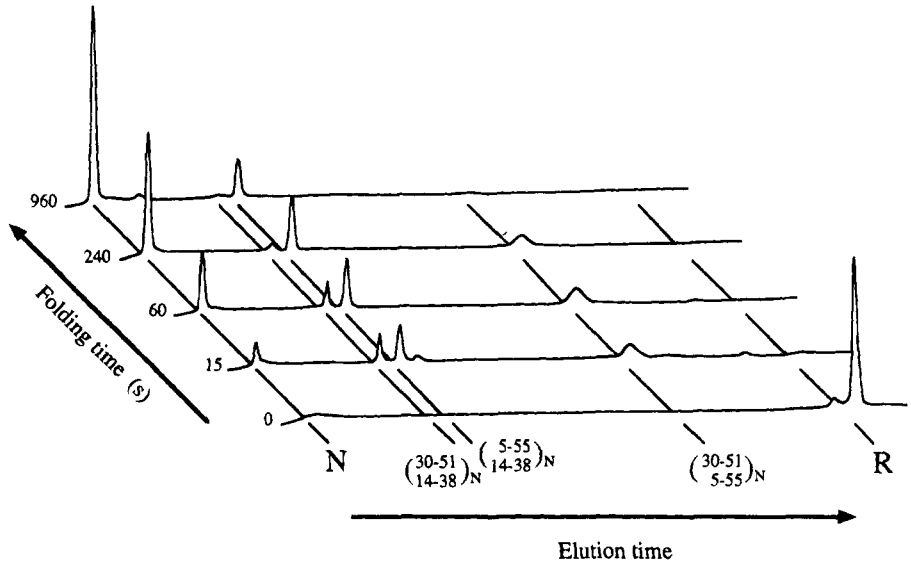


FIGURE 10: Refolding of BPTI with $DsbC_S^S$ as oxidant. Reduced BPTI (10 μM) was mixed with 30 μM $DsbC_S^S$ monomer under the conditions of Figure 6. Aliquots of the reaction mixture were acid quenched at the various times and the forms of BPTI present analyzed by reverse phase HPLC.

$\pm$ 10 s^{-1} M^{-1}. From their respective equilibrium constants with glutathione, the value of the equilibrium constant for their direct thiol–disulfide exchange can be calculated to be 2.3 $\pm$ 0.1, which gives the value of the rate of the reverse reaction:

$$\begin{array}{ccc} DsbA_S^S & \xrightarrow{110\pm10\ s^{-1}M^{-1}} & DsbA_{SH}^{SH} \\ + & & + \\ DsbC_{SH}^{SH} & \xleftarrow{48\pm6\ s^{-1}M^{-1}} & DsbC_S^S \end{array} \quad (8)$$

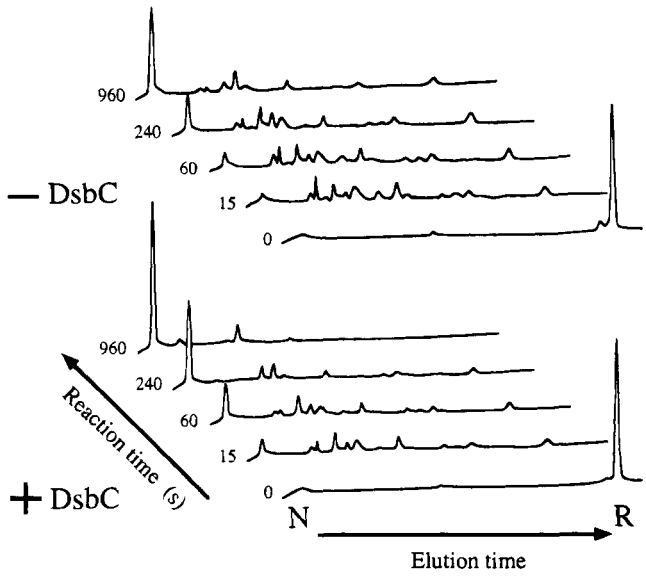
DISCUSSION

The structural and functional properties of DsbC reported here are fully consistent with it having a role in protein disulfide formation in the bacterial periplasm, as indicated by the phenotypic consequences of mutational inactivation of its gene (Missiakas et al., 1994; Shevchik et al., 1994). It is a soluble protein that can form an unstable and reactive disulfide bond between a pair of cysteine residues at its active site, which can be rapidly transferred to a reduced protein. These thiol and disulfide groups react with those of glutathione more rapidly than do those of normal proteins (Table 1), indicating that the active site of DsbC stabilizes the transition state for thiol–disulfide exchange. These properties are similar in some ways to those of DsbA, except that DsbC is a dimer and has a second, unreactive disulfide bond; also, its reactive disulfide bond has somewhat different reactivities, and it has a much greater ability to catalyze rearrangements of protein disulfide bonds.

DsbC is likely to be a member of the thioredoxin family, which includes glutaredoxin, PDI, and DsbA. Its revised 216-residue primary structure has no detectable homology to any of these proteins, except to a very small extent with a DsbA homologue from *Vibrio cholerae* (Missiakas et al., 1994), but it does have a -Cys-Gly-Tyr-Cys- segment in which only the first cysteine thiol group is accessible and reactive and where the two cysteine residues reversibly form a reactive disulfide bond without major conformational change, as in the other thioredoxin-like proteins (Kallis & Holmgren, 1980; Hawkins & Freedman, 1991; Bushweller et al., 1992; Brandes et al., 1993; Zapun et al., 1993). The sequence of DsbA appeared to be no more homologous to the other known members of this family, but it was found to have a three-dimensional structure very similar to that of thioredoxin but with an insertion of 76 additional residues to generate a bilobed structure (Martin et al., 1993). DsbC is likely to be similarly related structurally to thioredoxin, but the positions of the active site cysteine residues in its primary structure indicate that there are probably large insertions of residues both before and after the active site cysteine segment. The far-UV CD spectrum of DsbC (Figure 2) is at least compatible with it containing a thioredoxin-like domain. In addition, DsbC has a second pair of cysteine residues, at positions 141 and 163, that are normally paired in a buried disulfide bond that seems to have primarily a structural role.

The reactive disulfide bond of DsbC is nearly as unstable as that of DsbA. The equilibrium constant for its formation by interchange with glutathione is only 200 μM, slightly greater than the 80 μM measured for DsbA (Zapun et al., 1993; Wunderlich & Glockshuber, 1993). These values contrast with those of $10-10^5$ M that are measured for normal stabilizing disulfide bonds in folded proteins (Creighton & Goldenberg, 1984). The low stability of the DsbC reactive disulfide bond implies that it destabilizes the folded conformation of DsbC, as has been demonstrated for DsbA (Zapun et al., 1993; Wunderlich et al., 1993b). The mixed disulfide between DsbC and glutathione is also particularly unstable and reactive, which may be due to the reactive thiol group of $DsbC_{SH}^{SH}$ having a very low pK_a value, similar to the value of 3.5 measured for DsbA (Nelson & Creighton, 1994).

The unstable and reactive disulfide bond of $DsbC_S^S$ could be transferred rapidly *in vitro* to reduced BPTI and to a peptide with only two cysteine residues. As in the case of DsbA, that will occur only if the conformation of the protein permits it to form the disulfide bond readily. Whether this is the *in vivo* role of DsbC is uncertain. Studies both *in vitro* and *in vivo* indicate that this is the primary role of $DsbA_S^S$, and the resulting $DsbA_{SH}^{SH}$ is believed to be reoxi-



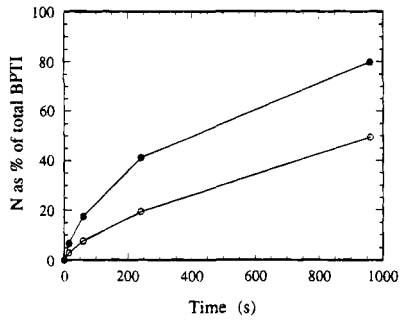


FIGURE 11: Refolding of BPTI with $DsbA_S^S$ as oxidant in the absence or presence of DsbC. Reduced BPTI (10 μM) in the absence or presence of 3.4 μM $DsbC_{SH}^{SH}$ monomer was mixed with 30 μM $DsbA_S^S$. The conditions were as in Figure 6. (A) Reverse phase HPLC elution profile of aliquots of the reaction mixture that had been acid quenched at the various times. (B) Quantification of the native BPTI, N, generated in the absence (○) or presence (●) of DsbC.

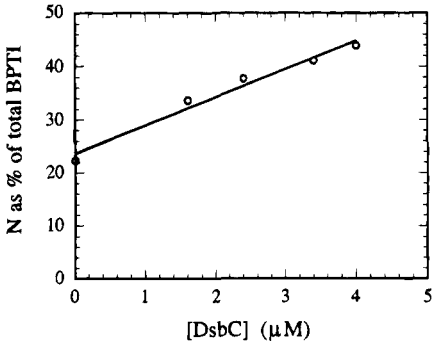


FIGURE 12: Effect of DsbC on the refolding of BPTI with $DsbA_S^S$ as oxidant. The experiment was as described in the legend to Figure 11, but the concentration of DsbC monomer was varied and the amount of native BPTI, N, was quantified after 4 min of incubation.

dized by the membrane protein DsbB (Bardwell et al., 1993; Missiakas et al., 1993; Dailey & Berg, 1993). As would be expected, mutational inactivation of either or both of the *dsbA* and *dsbB* genes gave similar phenotypes. Inactivation of the *dsbC* gene, however, produced phenotypic effects that were relatively independent of the *dsbA* and *dsbB* genes, suggesting that DsbC functions independently and that it is not reoxidized by DsbB *in vivo* (Missiakas et al., 1994). Until a mechanism for reoxidizing $DsbC_{SH}^{SH}$ is found *in vivo*, its *in vitro* activity of transferring disulfide bonds to reduced proteins is of uncertain physiological significance, other than to keep it in the reduced form, $DsbC_{SH}^{SH}$.

$DsbC_{SH}^{SH}$ was not very susceptible to air oxidation *in vitro*. It was reoxidized by $DsbA_S^S$ *in vitro* but at a rate considerably slower than that at which $DsbA_S^S$ reacts with other protein thiol groups; $DsbA_S^S$ in the periplasm would be expected to react primarily with newly translocated proteins rather than with $DsbC_{SH}^{SH}$. The observed rate constant for the reaction between $DsbC_{SH}^{SH}$ and $DsbA_S^S$, 110 s^{-1} M^{-1}, is very similar to that expected, 140 s^{-1} M^{-1}, from their respective rates of reaction with GSH and GSSG, if it is assumed that the reaction between the two to form the mixed disulfide occurs at the same rate as that between GSH and the disulfide form of each protein:

$$DsbA_S^S + DsbC_{SH}^{SH} \underset{60\ (s^{-1})}{\overset{4.6 \times 10^3\ (s^{-1}M^{-1})}{\rightleftharpoons}} DsbA_{SH}^{S}\!-\!\!-\!S\,HS\,DsbC \underset{38\ (s^{-1}M^{-1})}{\overset{1.9\ (s^{-1})}{\rightleftharpoons}} DsbA_{SH}^{SH} + DsbC_S^S \quad (9)$$

The rate constants are from Table 1. This agreement between the observed and calculated rates suggests that there are unlikely to be any specific interactions between DsbA and DsbC and that the reaction between them is not of particular physiological significance.

The greatest functional difference between DsbA and DsbC is that the latter has much more ability to catalyze the rearrangement of protein disulfide bonds. This was evident when catalytic amounts of DsbC were present during refolding of reduced BPTI with either GSSG (Figure 8) or $DsbA_S^S$ (Figure 11) as the oxidant. DsbC must have been increasing the rate of disulfide rearrangements in the stable quasi-native intermediates that accumulate in each case and in the initial intermediates in which disulfide bonds are introduced randomly by $DsbA_S^S$; the former activity was confirmed with the isolated intermediates. In contrast, DsbA has very little such isomerase activity, although it has often been considered to be the equivalent of eukaryotic PDI in the bacterial periplasm. Disulfide rearrangements normally occur by thiol−disulfide interchange between thiol and disulfide groups present simultaneously in the protein. If all the cysteine residues of a protein are paired in disulfide bonds, no intramolecular disulfide interchange is possible, even if the protein is fully unfolded. In this case, any thiol group that transiently reacts with one of the protein disulfide bonds, to form a mixed disulfide and liberate a free cysteine thiol group, will catalyze disulfide interchange; the accessible cysteine thiol group of $DsbA_{SH}^{SH}$ is very reactive, with a very low pK_a (Nelson & Creighton, 1994). Not surprisingly, $DsbA_{SH}^{SH}$ has been shown to have this disulfide interchange activity (Bardwell et al., 1991; Akiyama et al., 1992; Wunderlich et al., 1992; Joly & Swartz, 1994), but it is low and comparable to that of thioredoxin (Pigiet & Schuster, 1986), which also has a reactive thiol group with a low pK_a but is not believed to act physiologically as a protein disulfide isomerase. In the more defined systems of the well-characterized disulfide folding transitions of reduced BPTI and α-lactalbumin, DsbA was shown to have little or no

detectable activity in rearranging disulfide bonds of species with both disulfide bonds and thiol groups, whether folded or unfolded (Zapun & Creighton, 1994), whereas both PDI and DsbC have substantial activities.

Disulfide rearrangements are usually important *in vitro* when folding is coupled to protein disulfide formation (Creighton, 1978), and they are catalyzed by PDI in eukaryotes. No other catalyst of disulfide rearrangements is known in prokaryotes, so the results presented here make it tempting to speculate that this is the *in vivo* role of DsbC in the periplasm of Gram-negative bacteria. The complementary role of DsbA is to introduce disulfide bonds into proteins at random, and it is recycled by DsbB (Bardwell et al., 1993). This conclusion must be tentative until all the genes and proteins involved have been characterized, but it is consistent with the observation that inactivation of the *dsbC* gene inhibits disulfide formation most in proteins with multiple disulfide bonds (D.M., and S.R., unpublished observations), which are most likely to be dependent upon disulfide rearrangements. Also, the rate observed *in vitro* for correct disulfide formation in reduced BPTI with DsbA as oxidant and DsbC as catalyst of disulfide rearrangements (Figure 12) is comparable to the rate observed with recombinant BPTI in the periplasm of *E. coli* (Marks et al., 1986; Ostermeier & Georgiou, 1994).

Is it possible to rationalize the different disulfide isomerase activities of PDI, DsbC, and DsbA? Catalysts could act by the accessible thiol group of their reduced state transiently undergoing thiol−disulfide exchange with one of the protein disulfide bonds to form a mixed disulfide between the catalyst and the protein and to liberate a thiol group in the protein. Disulfide interchange will result in the protein if a thiol group different from the one initially released reacts with this mixed disulfide to generate a different disulfide bond in the protein. In the absence of any other effects, an increase over the spontaneous rate of thiol−disulfide interchange will be observed simply because DsbA and DsbC participate in thiol−disulfide exchange reactions much more rapidly than do normal protein thiol and disulfide groups (Table 1; Darby & Creighton, 1995). If there were no other free thiol groups in the original protein, disulfide interchange will require that the liberated thiol group undergo interchange with a second disulfide bond of the protein, which could not be catalyzed by the same active site of the enzyme. It may be significant that both DsbC and PDI have two active sites in their respective dimer and monomer and might be able to react simultaneously with two disulfide bonds of a protein substrate, whereas monomeric DsbA has only one.

An effective catalyst of disulfide rearrangements should form a mixed disulfide bond with the protein that is intrinsically reactive, and this is the case with DsbC (Table 1). The instability of the disulfide bond of $DsbC_S^S$ is reflected kinetically in a rapid rate of reaction of its mixed disulfide with GSH; in contrast, the reactivity of the disulfide bond of $DsbC_S^S$ is much more normal. The situation is quite different with $DsbA_S^S$, whose disulfide bond is only slightly more unstable. Its instability is reflected kinetically in a rapid rate of reaction of the disulfide bond of $DsbA_S^S$ itself. This is the type of kinetic behavior expected for a catalyst designed to transfer its disulfide bond to another protein (Zapun et al., 1993) rather than to catalyze disulfide rearrangements. On the other hand, the mixed disulfides with glutathione of DsbA and DsbC reacted with GSH at similar rates (Table 1), but the competing reaction of forming the disulfide bond of $DsbC_S^S$ is considerably slower than for $DsbA_S^S$ (Table 1). The mixed disulfide bond of DsbC has a 40-fold greater probability than that of DsbA of reacting with an external thiol, such as that of GSH, and presumably also with another cysteine residue of a protein mixed disulfide than with its own buried cysteine residue to generate $DsbC_S^S$. This factor helps to explain the greater isomerase activity of DsbC.

In the cases where disulfide rearrangements are inhibited by folded conformations, they will be increased in rate if the conformational restrictions are relaxed by unfolding the protein. This would be facilitated by the conformation being weakened by breaking the initial protein disulfide bond and the catalyst binding the protein in an unfolded conformation (Darby & Creighton, 1995). It is very likely that DsbC interacted to at least some extent with reduced BPTI and the peptide studied here, at least transiently, for both reacted with $DsbC_S^S$ orders of magnitude more rapidly than did GSH or $DsbA_{SH}^{SH}$. $DsbA_S^S$ also reacts rapidly with reduced proteins and peptides, but its disulfide bond is intrinsically more reactive. It probably also binds proteins (Darby & Creighton, 1995) but may do so less tightly than DsbC.

Catalysts can also bypass disulfide rearrangements in quasi-native species by incorporating the missing disulfide bonds directly. This activity with the BPTI quasi-native species has been described in PDI (Creighton et al., 1980), DsbA (Zapun & Creighton, 1994), and now with DsbC. In particular, $DsbC_S^S$ was much more effective than $DsbA_S^S$, as it was able to introduce the 30−51 disulfide bond into the very stable quasi-native $(5-55,14-38)_N$, whereas $DsbA_S^S$ was not. The Cys30 and Cys51 thiol groups are totally buried in $(5-55,14-38)_N$; whatever the exact mechanism by which it does this, $DsbC_S^S$ is likely to have been able to gain access to these buried thiol groups by at least transiently unfolding the quasi-native conformation. This is compatible with DsbC binding unfolded proteins more tightly than does DsbA.

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