The Reactive and Destabilizing Disulfide Bond of DsbA, a Protein Required for Protein Disulfide Bond Formation *in Vivo*†

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ABSTRACT: The protein DsbA facilitates disulfide bond formation in the periplasm of *Escherichia coli*. It has only two cysteine residues that are separated in the sequence by two other residues and are shown to form a disulfide bond reversibly. Chemical modification studies demonstrate that only one of the cysteine residues has an accessible thiol group in the reduced protein. Equilibrium and kinetic characterization of thiol-disulfide exchange between DsbA and glutathione showed that the DsbA disulfide bond was 10^3-fold more reactive than a normal protein disulfide. Similarly, the mixed disulfide between the accessible cysteine residue and glutathione was 10^4-fold more reactive than normal. The overall equilibrium constant for DsbA disulfide bond formation from GSSG was only 8×10^{-5} M. These properties indicate that disulfide-bonded DsbA is a potent oxidant and ideally suited for generating protein disulfide bonds. Disulfide bonds generally increase the stabilities of folded proteins, when the folded conformation reciprocally stabilizes the disulfide bonds. In contrast, the disulfide bond of DsbA was so unstable in the folded state that its stability increased by 4.5 ± 0.1 kcal·mol^{-1} when the protein unfolded. This implies that the disulfide bond destabilizes the folded conformation of DsbA. This was confirmed by demonstrating that the reduced protein was 3.6 ± 1.4 kcal·mol^{-1} more stable than that with the disulfide bond.

Protein folding in the cell is now being studied intensively. It is known to be assisted by a variety of other proteins, which are usually classified in two groups: the chaperones, thought to prevent misfolding and aggregation by binding to polypeptide chains that are not fully folded, and the true catalysts, which accelerate protein folding by increasing the rate of slow steps (Freedman, 1992; Gething & Sambrook, 1992; Lorimer, 1992). The known catalysts consist of peptidyl prolyl isomerase, which catalyzes the cis–trans isomerization of the peptide bonds preceding proline residues, and protein disulfide isomerase (PDI),[1] which is involved in the formation of the correct disulfide bridges (Freedman, 1992).

A major question about protein folding coupled to disulfide bond formation is the identity of the oxidant that converts pairs of cysteine thiol groups to disulfide bonds. Co- and posttranslational formation of disulfide bonds occurs rapidly in the endoplasmic reticulum (ER) of eukaryotic cells, which is therefore likely to possess an efficient oxidation system. The relative levels in the ER of GSH and GSSG, the reduced and disulfide forms of glutathione, respectively, are compatible with formation of native protein disulfide bonds. The ratio of [GSH] to [GSSG] has recently been estimated to be in the range of 3:1 to 1:1 (Hwang et al., 1992), while the cytosol is more reducing, with a [GSH] to [GSSG] ratio of approximately 100:1. Although most of the glutathione may be reduced, such conditions are not incompatible with formation of protein disulfide bonds. Native protein disulfide bonds are generally stabilized by the folded protein conformation (although an exception is reported here), so they can be stable under conditions where the glutathione is predominantly reduced (Creighton, 1983). For example, the overall equilibrium constant for generation of the three disulfide bonds of native BPTI (bovine pancreatic trypsin inhibitor) using glutathione has been measured to be 8.8×10^3 M[3] (Creighton & Goldenberg, 1984). This value indicates that the native conformation of this protein, with all three disulfide bonds, is 5.4 kcal·mol^{-1} more stable than the reduced protein under cytosol-like redox conditions of [GSH] = 10 mM and [GSSG] = 0.1 mM, and intermediate forms with only some of the disulfide bonds are even more unstable than the reduced protein (Creighton, 1983). As this protein can fold and form disulfides even under cytosol-like thiol–disulfide redox conditions, the more oxidizing conditions reported for the ER by Hwang et al. (1992) are undoubtedly sufficient to facilitate disulfide bond formation in other, less stable proteins also. In addition, the ER contains large quantities of PDI, which has been shown *in vitro* to increase the rates of formation, rearrangement, and reduction of disulfide bonds in various systems (Creighton et al., 1980; Freedman et al., 1989; Gilbert, 1989a; Lyles & Gilbert, 1991; Zapun et al., 1992) and which may also be subject to net oxidation and reduction (Lundström & Holmgren, 1990).

In Gram-negative bacteria such as *Escherichia coli*, disulfide bonds are formed in proteins in the periplasm. The periplasm is topologically equivalent to the eukaryotic ER, although separated from the extracellular environment only by the outer membrane. Mutants deficient in the formation of disulfide bonds in secreted proteins were isolated and found to be defective in gene *dsbA* (Bardwell et al., 1991; Kamitani et al., 1992). The product of that gene, DsbA, is a 21-kDa soluble periplasmic protein that contains only two cysteine residues, in the sequence Cys-Pro-His-Cys at residues 30–33. This sequence is reminiscent of the active sites of the thiol–disulfide oxidoreductase (TDOR) family, which includes PDI, thioredoxin, and glutaredoxin (Holmgren, 1968; Eklund et al., 1991),

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[1] Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; CD, circular dichroism; DTT, dithiothreitol; ER, endoplasmic reticulum; GdmCl, guanidinium chloride; GSH and GSSG, the reduced and oxidized forms of glutathione, respectively; PAGE, polyacrylamide gel electrophoresis; PDI, protein disulfide isomerase; TDOR, thiol–disulfide oxidoreductase.

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in which the two cysteine residues interconvert between the dithiol and the disulfide forms. Moreover, DsbA is active in the reduction of insulin, demonstrating that it has at least some TDOR activity (Bardwell et al., 1991). There is little sequence identity between DsbA and members of the TDOR family, but it has been suggested on the basis of limited sequence similarities and similar secondary structure predictions that DsbA contains a structural domain that is common to all the TDOR family (Ellis et al., 1992).

Preliminary experiments showed that substoichiometric quantities of DsbA had little or no effect on the rates of formation, rearrangement or reduction of the three disulfide bonds of BPTI (A. Zapun and T. E. Creighton, unpublished observations) under conditions where PDI exhibits clear catalytic activity (Creighton et al., 1980; Zapun et al., 1992). This suggests a major difference in function of DsbA and PDI. When used in stoichiometric amounts and in the absence of any other disulfide reagent, however, the disulfide form of DsbA was an extremely efficient oxidizing agent for reduced BPTI (A. Zapun and T. E. Creighton, unpublished observations). Akiyama et al. (1992) observed DsbA in large excess to allow the oxidative refolding and disulfide bond rearrangements of alkaline phosphatase and ribonuclease A.

We report here an investigation of the thiol–disulfide properties of DsbA, as a first step in characterizing its biochemical function. The results demonstrate that the single disulfide bond formed between the two cysteine residues of this protein is ideally suited to be the oxidant that generates disulfide bonds in proteins in the bacterial periplasm.

EXPERIMENTAL PROCEDURES

Protein and Chemicals. DsbA was isolated from an overproducing strain of *E. coli* JCB607 (Bardwell et al., 1991). Cells were submitted to a cold osmotic shock (Manoil & Beckwith, 1986) to release the periplasmic proteins. DsbA was further purified by anion-exchange chromatography on a DEAE-Sephacel (Pharmacia) column equilibrated with 10 mM MOPS (pH 7) and 1 mM EDTA and eluted with a linear gradient of 0–80 mM NaCl. The concentration of DsbA was determined from its absorbance at 280 nm, using an extinction coefficient, ϵ_{280} = 21 740 M^{-1} cm^{-1}, calculated from its amino acid sequence (Gill & von Hippel, 1989), as there was no significant difference of the absorbance at 280 nm of the reduced and oxidized forms of the protein in the presence or absence of 6 M GdmCl.

GSH and GSSG from Sigma were used without further purification. Ultrapure urea was from BRL; only freshly prepared solutions were used. All other chemicals were of reagent grade or better.

Size-Exclusion Chromatography. To determine the size of the native protein, ≈1.5 mg of purified DsbA was analyzed by gel filtration on a Sephacryl S-200 HR (Pharmacia) column equilibrated with 10 mM MOPS (pH 7) and 1 mM EDTA. Blue dextran and protein standards of cytochrome *c*, carbonic anhydrase, serum albumin, alcohol dehydrogenase, and β-amylase were used to calibrate the column.

Covalent Modification of Protein Thiols and Nondenaturing Polyacrylamide Gel Electrophoresis (Native PAGE). Solutions of DsbA, with or without 10 mM DTT, were reacted by addition of one-fourth volume of 0.5 M iodoacetamide or potassium iodoacetate in 1.5 M Tris-HCl (pH 8.7). After 2 min at room temperature, the samples were kept on ice prior to analysis by electrophoresis. Alternatively, the mixture was further incubated for 3 min after the addition of three volumes of 8 M urea and 1.5 M Tris-HCl (pH 8.7). Samples were analyzed using the high-pH nondenaturing gel electrophoresis system of Davis (1964). Gels were stained with 0.1% (w/v) Coomassie Blue in 10% (w/v) trichloroacetic acid plus 10% (w/v) 5-sulfosalicylic acid and destained in 5% (v/v) methanol plus 7.5% (v/v) acetic acid.

Circular Dichroism Spectra. CD spectra were recorded at 25 °C with a Jobin-Yvon CDVI spectrometer. The same protein solutions (≈17 μM DsbA) were used for the far- and near-ultraviolet measurements in cells with path lengths of 0.1 and 1 cm, respectively. The buffer was 0.1 M sodium phosphate (pH 7) and 1 mM EDTA and also contained 8 M urea for the spectra of the unfolded state. The unfolded and reduced sample also contained 1 mM DTT.

The thiol-blocked species were prepared as described in the preceding section but were isolated by gel filtration on a Sephadex G-25 column equilibrated with the appropriate buffer immediately after the blocking reaction. A shorter incubation with iodoacetamide (1 min) was used to prepare the DsbA reacted at a single cysteine residue (see Results); as a result, the reaction was incomplete, and this sample subsequently contained a small amount (≤10%) of protein that had been reoxidized. The reduced sample without denaturant was also purified by gel filtration because the presence of DTT prevented measurements at low wavelengths (<210 nm). The reduced sample was blocked using iodoacetic acid after the 4 h required for recording of the CD spectra and was examined by native PAGE; less than 5% had been reoxidized. The integrities of all other samples used for spectral analysis were also subsequently confirmed by native PAGE.

Fluorescence Spectra. Fluorescence emission spectra were recorded on an Aminco-Bowman Series 2 luminescence spectrometer at 25 °C with the same samples used to measure the CD spectra.

Unfolding Equilibria. The reversible unfolding of DsbA was measured by the CD ellipticity at 222 nm with a 0.1-cm path length cell at 25 °C in 0.1 M Tris-HCl (pH 7.5), 0.2 M KCl and 1 mM EDTA, containing varying concentrations of urea. The pH was adjusted after the addition of urea. Solutions of reduced DsbA also contained 1 mM reduced DTT. Spectra were accumulated between 210 and 240 nm, and the buffer spectrum was subtracted for each urea concentration.

Redox Titration. The equilibrium constant for disulfide bond formation in both folded and unfolded DsbA, with glutathione as thiol–disulfide reagent, was determined by incubating DsbA (≈34 μM) 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. The same conditions were used for measurements in 8 M urea, except that the concentration of DsbA was about 17 μM. The thiol–disulfide exchange reaction was quenched by the addition of HCl (to pH ≈2) and analyzed by HPLC on a reverse-phase Vydac C-18 column in 0.1% (v/v) trifluoroacetic acid. Samples were immediately loaded onto the reverse-phase column after the acidification, but a 15-min delay did not result in any significant change in the results obtained. The various species were separated with a gradient of acetonitrile [40–48% (v/v) in 24 min]. They were detected by their absorbance at 215 nm, and the peaks were quantified by integration. Loading the same amount of reduced and oxidized DsbA resulted in peaks of identical areas, showing that correction for relative extinction coefficients or recoveries was not necessary. The extinction coefficients and recoveries of the mixed-disulfide species were assumed to be the same as those of the reduced and oxidized proteins. The same results were obtained after

20 and 35 min of incubation, indicating that the reactions had reached equilibrium.

To correct for air oxidation, the free thiol content of the reaction mixture, $[SH]_{Ellman}$, was determined at the time of the acid quench by Ellman's assay in 6 M GdmCl with the extinction coefficient $\epsilon_{412} = 13\,700\ M^{-1}\ cm^{-1}$ (Ellman, 1959; Riddles et al., 1983). In the absence of glutathione, the oxidation of DsbA by air was negligible over the time of incubation. The concentration of GSH was calculated as $[GSH] = [SH]_{Ellman} - 2R[DsbA]$, where R is the fraction of total DsbA, [DsbA], that was reduced, as determined by HPLC. The GSSG concentration was taken as $[GSSG] = [GSSG]_0 + ([GSH]_0 - [GSH])/2$, where $[GSSG]_0$ and $[GSH]_0$ were the initial concentrations added.

Kinetic Measurements. The rates of the reaction between DsbA and GSH or GSSG were measured with a SFM-3 stopped-flow instrument from Bio-Logic, equipped with a PMS-400 photomultiplier mounted at a 90° angle to monitor fluorescence. Excitation was at 280 nm, and emission was recorded at wavelengths greater than 305 nm using a cut-off filter. The conditions were the same as those used for the redox titration, except that the final DsbA concentration was between 2 and 4 μM; GSH or GSSG was always in at least a 10-fold excess. Reduced DsbA was prepared by incubation with 10 mM reduced DTT and then removing the DTT on a Sephadex G-25 gel-filtration column equilibrated with the appropriate buffer. All solutions were degassed and filtered before use.

In the case of reduction experiments, the small amount of oxidation of GSH that occurred during storage in the syringe of the instrument was taken into account by measuring the free thiol content of the collected stopped-flow reaction mixture, using the Ellman's procedure without GdmCl and $\epsilon_{412} = 14\,150\ M^{-1}\ cm^{-1}$ (Riddles et al., 1983).

Each curve was an average of two to six measurements and could be fitted with a nonlinear least-squares procedure to a single exponential ($A = A_0 e^{-k_{obs}t}$), as expected for a pseudo-first-order reaction.

RESULTS

Purified DsbA. About 28 mg of purified DsbA could be isolated from 1 L of cell culture. The protein was more than 98% pure as judged by SDS–PAGE, native PAGE, and reverse-phase HPLC and exhibited the expected activity in the insulin reduction assay (Bardwell et al., 1991). No protein thiols were detected by the Ellman's assay, with or without 6 M GdmCl, indicating that the purified protein was in the disulfide form. To confirm that the signal sequence was correctly processed, five rounds of N-terminal amino acid sequence analysis were performed, which confirmed the expected sequence Ala-Gln-Tyr-Glu-Asp-. Electrospray mass spectrometry gave 21 128 ± 1.3 for the molecular weight, which compares well with the 21 130 expected from the gene sequence, with the two cysteines forming a disulfide bond. Size-exclusion chromatography indicated an apparent molecular weight of 26 900, indicating that the protein was at least largely monomeric.

The purified protein appeared from its molecular weight and its absorbance, fluorescence, and nuclear magnetic resonance spectra (Kemmink, Zapun, and Creighton, unpublished observations) not to contain any tightly bound cofactors or prosthetic groups.

Cysteine Thiol Groups of DsbA. The purified DsbA protein was essentially homogeneous by native PAGE and had the approximate mobility expected from its predicted size and net charge. Incubation with iodoacetic acid or iodoacetamide in the presence of 6 M urea did not result in any alteration of the electrophoretic mobility (Figure 1, lanes a and d), as expected if the two cysteine residues were paired in a disulfide bond in the isolated protein. After treatment of the protein with excess DTT, reaction with iodoacetate in the presence of 6 M urea produced a protein with a greater mobility than the oxidized protein (Figure 1, lane c), like that expected in this electrophoretic system for the addition of two negatively charged carboxymethyl groups. Reaction of the reduced protein in the absence of denaturant resulted in an intermediate mobility (Figure 1, lane b). The alkylation reactions were performed at pH 8.7 to maximize the reactivity of the thiols, and the difference in reactivity probably reflects differences in steric accessibility: one of the cysteine residues is readily accessible to an alkylating agent, while the second one is protected and unreactive unless the protein is at least partially unfolded.

	a	b	c	d	e	f	g
DTT	-	+	+	-	+	+	-
IAAc	+	+	+	-	-	-	-
IAAm	-	-	-	+	+	+	-
Urea	+	-	+	+	-	+	-

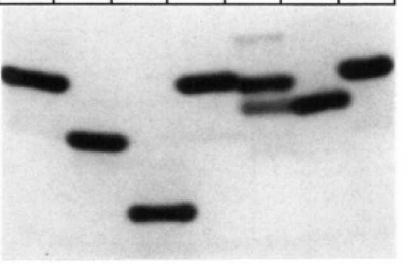


FIGURE 1: Native PAGE of purified DsbA that had been incubated with and without 10 mM DTT, followed by addition of iodoacetate (IAAc) or iodoacetamide (IAAm) to 0.1 M, with or without urea, subsequently added to 6 M.

With neutral iodoacetamide as the alkylating reagent, reacting both thiols of reduced DsbA unexpectedly produced a somewhat greater electrophoretic mobility than that of the disulfide-bonded protein (Figure 1, lane f). Reacting only the accessible cysteine residue in the absence of denaturant caused only a very small change in mobility, but the buried cysteine residue also reacted partially with iodoacetamide (Figure 1, lane e). An increase in electrophoretic mobility upon reacting both thiols is somewhat surprising, for it indicates that the protein has become more compact or more negatively charged.

CD Spectra. The secondary and tertiary structures of the DsbA protein in its various forms were compared by CD and fluorescence spectroscopy. In the far-ultraviolet, where the CD signal arises primarily from the geometry of the peptide bonds and reflects the secondary structure, oxidized DsbA and the various reduced species (with both thiols free or fully blocked with either reagent or with only the accessible thiol blocked) gave identical CD spectra (Figure 2). The near-ultraviolet spectra were also very similar for all species, except for that doubly blocked with iodoacetamide, which gave an altered spectrum. This species also gave an anomalous electrophoretic mobility (Figure 1).

The far-UV CD spectrum of DsbA did not give a unique estimate of the secondary structure content of the protein but is indicative of the presence of both β-structure and α-helix. In 8 M urea, spectra typical of denatured proteins were observed for both the reduced and oxidized forms, with no significant ellipticity at wavelengths greater than 215 nm.

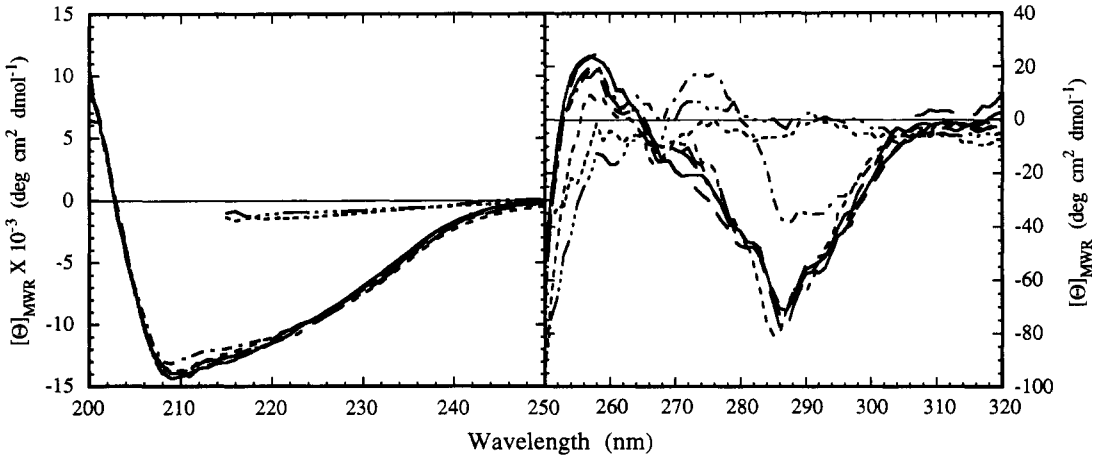


FIGURE 2: Circular dichroism (CD) spectra of DsbA in its different forms: (·····) oxidized; (——) reduced; with one cysteine residue blocked by reaction with (— —) iodoacetic acid, or (— · —) iodoacetamide; with both cysteine residues blocked with (– – –) iodoacetic acid, or (· · · · ·) iodoacetamide; (—·—) oxidized and (– – – –) reduced in 8 M urea. The buffer was 0.1 M sodium phosphate, pH 7, and 1 mM EDTA, at 25 °C.

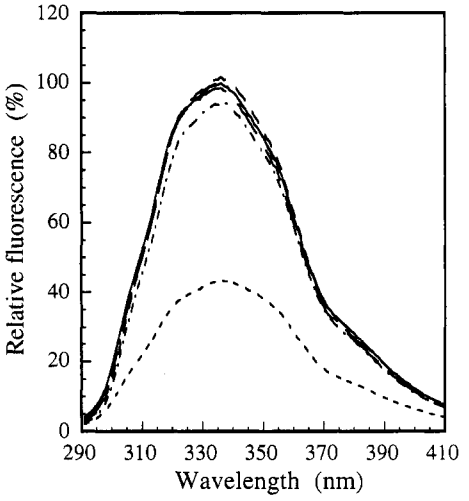


FIGURE 3: Fluorescence emission spectra, with excitation at 280 nm. The sample designation and conditions are as given in the legend to Figure 2.

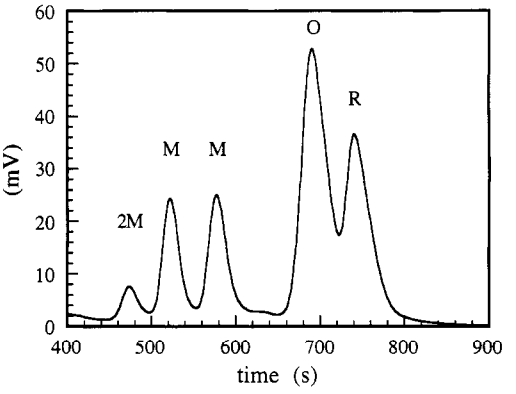


FIGURE 4: HPLC elution profile of acid trapped DsbA that had been incubated at 25 °C with 10 mM GSSG and 40 mM GSH in 8 M urea containing 0.1 M Tris-HCl (pH 7.5), 0.2 M KCl, and 1mM EDTA. The mixture was quenched by addition of HCl to pH ≈ 2, and about 35 μg of protein was loaded onto the reverse-phase column. O stands for oxidized DsbA, R for reduced, M for the two species with a single mixed disulfide with glutathione, and 2M for the double mixed disulfide.

Fluorescence Spectra. The various reduced forms of the protein with zero, one, or both thiols blocked gave very similar tryptophan fluorescence emission spectra with λ_{max} = 336 nm (Figure 3). By contrast, the disulfide form of DsbA exhibited approximately 40% of the quantum yield of the various reduced forms, but no shift of λ_{max}, suggesting that the fluorescence was simply quenched by the disulfide bond.

Thiol-Disulfide Redox Equilibrium. To investigate the oxidation–reduction properties of the two cysteine residues of DsbA in the folded and unfolded conformations, the protein was equilibrated in various mixtures of GSH and GSSG in the absence or presence of 8 M urea, and the state of the cysteine residues was determined in samples trapped with iodoacetamide or acid. When iodoacetamide-trapped samples were examined by native PAGE, various amounts of the reduced and disulfide forms of the protein were observed, as expected, plus a faint band with a mobility intermediate to that of the species singly and doubly blocked with iodoacetic acid; the faint band had the electrophoretic mobility expected of DsbA containing a mixed-disulfide with glutathione ($DsbA_{SH}^{SSG}$). GSH possesses two carboxyl groups and one amino group; the amino group has a pK of approximately 9.5 (Jung et al., 1972) and is expected to be only partially ionized at the pH of the separating gel (≈9.5). Consequently, the addition of one glutathione moiety in this system should result in the addition of somewhat more than one net negative charge, as was observed. When the redox incubation was performed in 8 M urea, an additional band with a mobility slightly greater than that of the species doubly-blocked with iodoacetic acid was present and interpreted as the double mixed disulfide ($DsbA_{SSG}^{SSG}$) (data not shown).

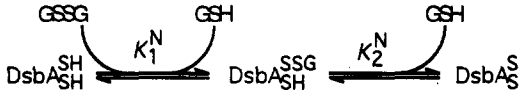
The same species were observed in acid-trapped samples separated by reverse phase HPLC (Figure 4). The five peaks were collected, any free thiols were blocked with iodoacetamide in the presence of 6 M urea, and the species present were identified by native PAGE. Their electrophoretic mobilities were consistent with them being, before trapping, the reduced, dithiol form (R), the oxidized, disulfide form (O), the two single mixed disulfides with glutathione (M, which had identical electrophoretic mobilities), and the double mixed disulfide (2M).

In the absence of urea, with the folded protein, only one single mixed disulfide species was observed and no double. This is in agreement with the finding that only one of the cysteine residues is accessible in the folded protein.

The relative concentrations of the various forms of the protein that had been equilibrated in various GSH/GSSG mixtures, in the absence and presence of denaturant, are shown

in Figures 5 and 6. The data were consistent with the expected models (Creighton, 1986; Gilbert, 1989b). In the absence of denaturant,

Scheme I



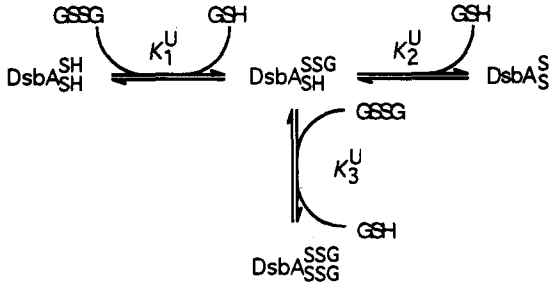
with

$$K_{SS}^N = K_1^N K_2^N = \frac{[DsbA_S^S][GSH]^2}{[DsbA_{SH}^{SH}][GSSG]} \quad (1)$$

where $DsbA_{SH}^{SH}$ and $DsbA_S^S$ are the reduced and disulfide forms of the protein, respectively, and $DsbA_{SH}^{SSG}$ is the single mixed disulfide species.

In 8 M urea, the double mixed disulfide species, $DsbA_{SSG}^{SSG}$, accumulates, and $DsbA_{SH}^{SSG}$ includes both single mixed disulfide species:

Scheme II



with, as in eq 1,

$$K_{SS}^U = K_1^U K_2^U \quad (2)$$

In 8 M urea, the two cysteine residues are probably equivalent, as identical amounts of the two one mixed disulfide species were measured (Figure 4), so they were treated as a single species. The overall and partial equilibrium constants are summarized in Table I.

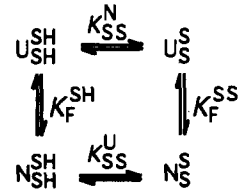
The remarkable observation is the low stability of the disulfide bond of DsbA, which is $<10^{-6}$ as stable as the weakest disulfide bond of folded BPTI (Creighton & Goldenberg, 1984). Moreover, the disulfide bond of DsbA is considerably *less* stable in the folded state than in the unfolded protein. Usually, the opposite is observed, for the native conformation generally substantially stabilizes protein disulfide bonds (Creighton, 1986). In the case of DsbA, the native conformation appears to destabilize the disulfide bond. The difference in stabilities of the disulfide bonds of the native and unfolded forms, defined as

$$\Delta\Delta G_{SS} = -RT \ln\left(\frac{K_{SS}^N}{K_{SS}^U}\right), \quad (3)$$

is 4.5 ± 0.1 kcal·mol^{-1}.

Unfolding Equilibrium. The effect of the protein conformation on the stability of the disulfide must reflect the inverse effect of the disulfide bond on the stability of the protein conformation (Creighton, 1986). This linkage relationship between the stabilities of the disulfide bond and of the conformation is expressed by the simple thermodynamic cycle:

Scheme III



where N_{SH}^{SH} and N_S^S represent the protein in the folded conformation with and without the disulfide bond, respectively, and U_{SH}^{SH} and U_S^S are the corresponding forms of the unfolded protein. The horizontal equilibrium constants K_{SS}^N and K_{SS}^U are for disulfide formation, with a disulfide reagent like GSSG that is not depicted:

$$K_{SS}^N = \frac{[N_S^S][GSH]^2}{[N_{SH}^{SH}][GSSG]} \qquad K_{SS}^U = \frac{[U_S^S][GSH]^2}{[U_{SH}^{SH}][GSSG]} \quad (4)$$

The vertical equilibrium constants K_F^{SH} and K_F^{SS} are of protein conformation stability in the dithiol and disulfide forms, respectively:

$$K_F^{SH} = \frac{[N_{SH}^{SH}]}{[U_{SH}^{SH}]} \qquad K_F^{SS} = \frac{[N_S^S]}{[U_S^S]} \quad (5)$$

The difference in free energy of folding between the two forms is given by

$$\Delta\Delta G_{fold} = -RT \ln\left(\frac{K_F^{SS}}{K_F^{SH}}\right) \quad (6)$$

This cycle predicts that

$$\frac{K_F^{SS}}{K_F^{SH}} = \frac{K_{SS}^N}{K_{SS}^U} \quad (7)$$

Equations 3 and 6 produce from this

$$\Delta\Delta G_{fold} = \Delta\Delta G_{SS} \quad (8)$$

In other words, whatever effect the folded conformation has on the stability of the disulfide bond, the disulfide bond must have the same effect on the stability of the folded conformation. As the disulfide of native DsbA was less stable than that of the unfolded form, $K_{SS}^N < K_{SS}^U$, the disulfide-bonded protein should be less stable to unfolding than the reduced one, $K_F^{SS} < K_F^{SH}$.

To confirm this relationship, the urea-induced unfolding of reduced and oxidized DsbA was monitored by CD. In both cases, the data were consistent with the unfolding transition being cooperative and two-state and were analyzed accordingly. As expected, and shown in Figure 7a, oxidized DsbA was found to be less stable than the reduced form, with midpoints for unfolding at 4.2 ± 0.6 M urea and 5.6 ± 0.5 M urea, respectively. Linear extrapolation of the folding free energies (Pace, 1986) was used to estimate the free energy of folding, ΔG_{fold}, of both forms in the absence of urea, as shown in Figure 7b. The results gave ΔG_{fold} of −8.7 ± 0.8 and −12.3 ± 0.6 kcal·mol^{-1} for the oxidized and reduced species, respectively. The difference in stability, $\Delta\Delta G_{fold}$, is 3.6 ± 1.4 kcal·mol^{-1}, in reasonable agreement with the difference in stability of the disulfides in the folded and unfolded states, $\Delta\Delta G_{SS}$, of 4.5 ± 0.1 kcal·mol^{-1}.

Assuming that the unfolding curves for both forms of the protein had equal slopes gave $\Delta\Delta G_{fold}$ of 3.1 ± 0.1 kcal·mol^{-1}. The greater discrepancy with $\Delta\Delta G_{SS}$ suggests that the difference in slope might be significant.

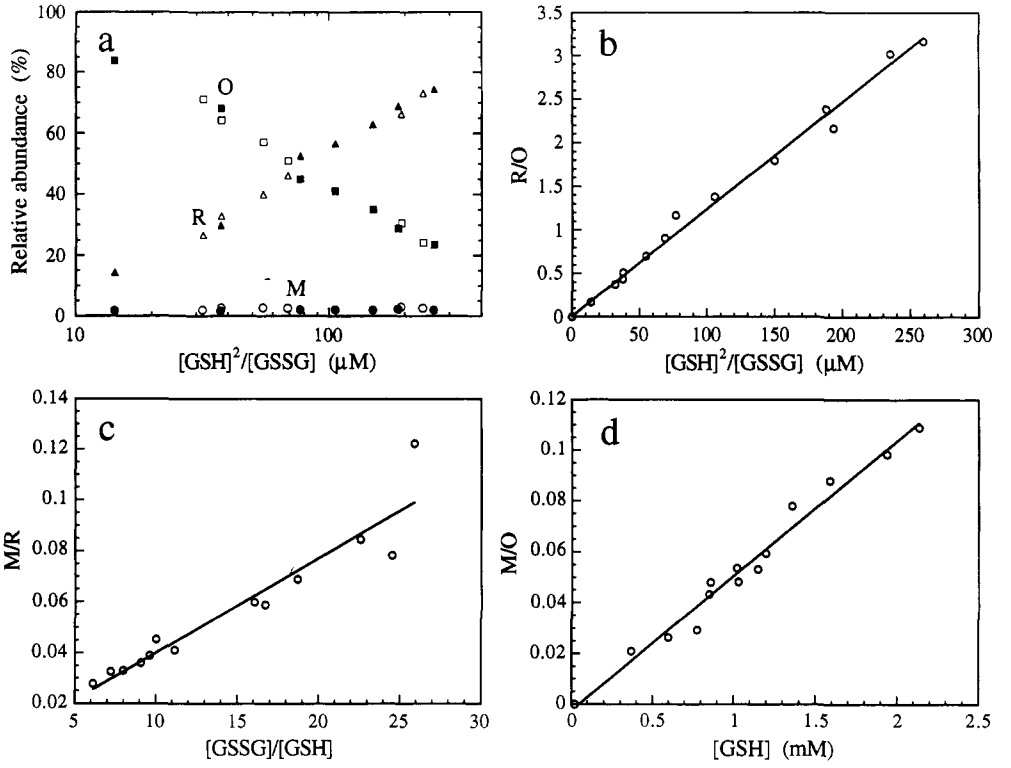
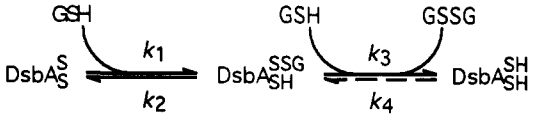


FIGURE 5: Measurement of the thiol–disulfide equilibrium of DsbA with glutathione. DsbA (≈34 μM) was incubated in various mixtures containing either of two different concentration of GSSG and varying amounts of GSH in 0.1 M Tris-HCl (pH 7.5), 0.2 M KCl, and 1 mM EDTA at 25 °C. The reaction mixtures were acid quenched, and the relative amounts of the different species were quantified by reverse-phase HPLC. (a) (□, ■), O = oxidized, (△, ▲), R = reduced, and (○, ●) M = single mixed disulfide. Open and filled symbols are for mixtures that contained 20 mM and 10 mM GSSG, respectively. (b) The plot of R/O vs $[GSH]^2/[GSSG]$ should be linear and gives the value of K_{SS}^N. (c) The plot of M/R vs [GSSG]/[GSH] should be linear and gives the value of K_1^N. (d) The plot of M/O vs [GSH] should be linear, and the reciprocal of the slope gives K_2^N. The equilibrium constants K_{SS}^N, K_1^N, and K_2^N refer to Scheme I, and the values obtained are given in Table I. Solid lines are results of linear fits to the data.

Kinetics of Reduction and Oxidation of DsbA with Glutathione. The rates of reduction and oxidation of DsbA were followed fluorometrically, using the large difference in emission intensities of the oxidized and reduced forms (Figure 3). The kinetics of reduction by excess GSH, in the absence of added GSSG, were fully consistent with a model assuming the transient formation of a mixed-disulfide intermediate (Creighton, 1986; Gilbert, 1989b; Zhang & Snyder, 1988).

Scheme IV



At very low concentrations of GSSG, the reverse reaction from $DsbA_{SH}^{SH}$, with rate constant k_4, can be neglected, whereas the other reverse step is unimolecular. All the experimental time-course curves could be fitted to single exponential functions, so the steady-state accumulation of the mixed-disulfide intermediate was rapid and to only a low level, which was confirmed by simulations of the reaction using the final rate constants (see Scheme V). In this case, the pseudo-first-order rate constant for reduction by excess GSH is expected to be given by

$$k_{obs} = \frac{k_1 k_3 [GSH]^2}{k_2 + k_3 [GSH]} \quad (9)$$

The observed kinetics were consistent with this model. At low GSH concentrations, when $k_2 \gg k_3[GSH]$, the reaction was second-order with respect to [GSH], and the observed rate constant is $k_{obs} \approx k_1 k_3 [GSH]^2 / k_2$. At high GSH concentrations, $k_3[GSH] \gg k_2$, and the rate-determining step is the initial attack of GSH on $DsbA_S^S$, with $k_{obs} \approx k_1[GSH]$. The observed kinetics showed both types of behavior, and Figure 8a,a′ shows a fit to equation 9 of k_{obs} versus [GSH] with the rate constants of Table II.

The kinetics of oxidation of DsbA with excess GSSG, in the absence of added GSH, were described by the reverse process. Both reverse steps involve GSH and can be ignored in its absence. A linear dependance of the pseudo-first-order rate constant on [GSSG] was observed over a wide range (Figure 8b,b′), so the first step, with rate constant k_4, must have been rate limiting under the experimental conditions (Table II).

The equilibrium constant calculated from the overall rate constants, $K_{SS}^N = k_2 k_4 / k_1 k_3$, is $(8.1 \pm 0.4) \times 10^{-5}$ M, which is the same as the value measured directly by equilibrium titration, $(8.1 \pm 0.2) \times 10^{-5}$ M.

Similar kinetics of reduction of DsbA were measured using mercaptoethanol rather than GSH, with a rate constant lower by the expected 2-fold (Creighton, 1975). The thiol and disulfide forms of mercaptoethanol and glutathione are comparable, so the unusual kinetics observed with glutathione were not specific for this reagent.

DISCUSSION

The protein DsbA seems ideally suited to be the oxidizing agent that introduces disulfide bonds into proteins secreted into the periplasm of *E. coli*. The disulfide bond between its two cysteine residues is very unstable. The redox equilibrium constant with glutathione was found to be 81 μM. With

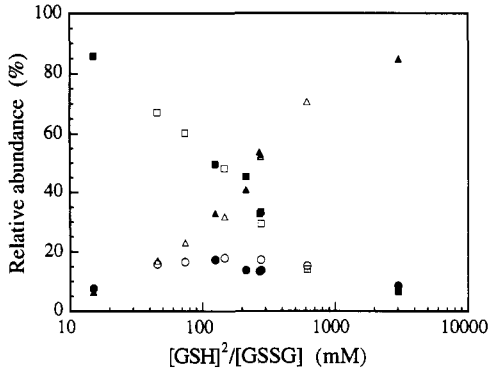


FIGURE 6: Measurement of thiol–disulfide equilibrium of DsbA with glutathione in 8 M urea. The species designation and the conditons were as given in the legend to Figure 5, except for the presence of denaturant and lower concentrations of DsbA (≈17 μM) and GSSG (10 or 5 mM). The concentration of double mixed disulfide was negligible under these conditions (*cf.* Figure 4).

Table I: Equilibrium Constants for the Thiol–Disulfide Exchange Reactions between DsbA and Glutathione[a]

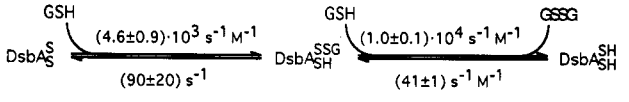
no denaturant	8 M urea
$K_{SS}^{N} = (8.1 \pm 0.2) \times 10^{-5}$ M	$K_{SS}^{U} = (1.7 \pm 0.2) \times 10^{-1}$ M
$K_1^{N} = (3.7 \pm 0.4) \times 10^{-3}$	$K_1^{U} = (2.1 \pm 0.1)$
$K_2^{N} = (1.9 \pm 0.1) \times 10^{-2}$ M	$K_2^{U} = (8.8 \pm 0.2) \times 10^{-2}$ M
	$K_3^{U} = 0.5$[b]

[a] The parameters are defined in Schemes I and II. The conditions were 25 °C in 0.1 M Tris-HCl (pH 7.5), 0.2 M KCl, and 1 mM EDTA. [b] The value for K_3^{U} was calculated from the single experiment shown in Figure 4, as the double mixed disulfide species did not accumulate to substantial quantities under the conditions used in the other experiments.

natural disulfide bonds in folded proteins, the values are generally much greater than 1 M; for example, the least stable disulfide of native BPTI has an equilibrium constant of 187 M (Creighton & Goldenberg, 1984). The value of 81 μM for DsbA is one of the lowest values published so far, although in the same range as the values of 42 and 60 μM reported for PDI (Hawkins et al., 1991a; Lyles & Gilbert, 1991), which suggests that the corresponding cysteine residues of PDI may have properties similar to those observed here for DsbA.

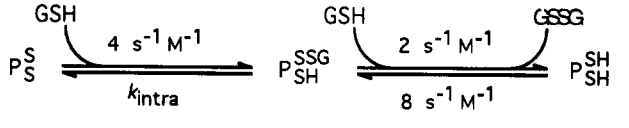
The rate constants at pH 7.5 for the overall kinetic scheme for the reaction between DsbA and glutathione can be obtained using the partial equilibrium constants measured by direct titration (Table I) and the kinetic data:

Scheme V



This can be contrasted with the behavior under the same conditions of a typical exposed protein disulfide bond, with normal reactivities of the protein thiols and disulfides (Creighton, 1980, 1986; Creighton & Goldenberg, 1984) and where the intramolecular step for forming the protein disulfide is k_{intra}:

Scheme VI



Comparison of the two reactions indicates that the reactivity of the DsbA disulfide with the thiol of GSH is about 10^3 times greater than expected for a normal disulfide bond. Moreover, the mixed-disulfide between DsbA and glutathione is also very susceptible to attack from GSH, reacting nearly 10^4 times faster than is normal. The reaction between GSSG and the accessible thiol of reduced DsbA to form the mixed-disulfide is 10 times faster than normal. By contrast, the disulfide of DsbA is formed at more standard rates, similar to those observed in cases where the protein thiols are kept in proximity by the folded conformation (Creighton & Goldenberg, 1984).

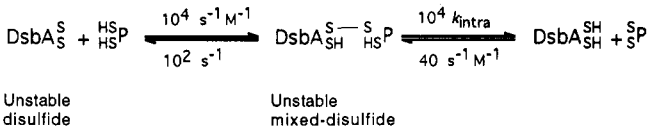
Table II: Experimental Rate Constants for the Reduction or Oxidation of DsbA by Glutathione at 25 °C, pH 7.5[a]

reduction	$k_1k_3/k_2 = (5.04 \pm 0.15) \times 10^5$ s^{-1} M^{-2}
	$k_1 = (4.6 \pm 0.9) \times 10^3$ s^{-1} M^{-1}
	$k_3/k_2 = (1.10 \pm 0.25) \times 10^2$ M^{-1}
oxidation	$k_4 = (41 \pm 1)$ s^{-1} M^{-1}

[a] The rate constants are defined in Scheme IV. The experimental conditions were as described in the footnote to Table I.

The reactivity of DsbA with glutathione was not specific to this reagent, and no indications for binding of glutathione to DsbA were observed. Therefore, the kinetics of thiol/disulfide exchange of DsbA with glutathione can be interpreted to predict the likely reactivity of DsbA with another protein, P:

Scheme VII



These properties of DsbA predict that it should transfer its disulfide bond to a folding protein at a rate more than 10^3 times greater than does GSSG. In contrast, intact protein disulfides should be reduced by DsbA only slightly faster than by GSH.

The high reactivity of the disulfide of DsbA is compatible with its postulated biological role only because the mixed-disulfide is also very unstable. Under relatively oxidizing conditions where disulfides are favored, a reagent like GSSG with a 10^3-fold more reactive disulfide bond would simply convert all protein thiol groups rapidly into mixed disulfides, which usually have normal stabilities (Creighton, 1986). The instability of the mixed disulfide between the protein substrate and DsbA should prevent this situation from occurring. Internal attack from the free thiol left in DsbA would reverse the reaction to the initial state, or a favored external attack by another free thiol of the substrate protein would lead to formation of a new disulfide bond in the protein.

Considering DsbA as the direct oxidant of protein thiols is consistent with the genetic evidence: in the absence of active DsbA, periplasmic proteins accumulate in the reduced form, and DsbA can be replaced by high concentrations of small molecule disulfides in the medium.

The question remains as to how DsbA itself is oxidized to the disulfide form. Spontaneous air oxidation of the isolated protein was observed to be too slow to be the *in vivo* reaction. A gene, *dsbB*, that codes for a cytoplasmic membrane protein that is required for the oxidation of DsbA has recently been identified (Bardwell et al., 1993).

The physical and structural basis for the striking redox properties of DsbA should be apparent from the forthcoming study of its three-dimensional crystal structure (Martin et al., 1993), but some information about its cysteine residues is available from this study. Only one of the cysteine thiols is

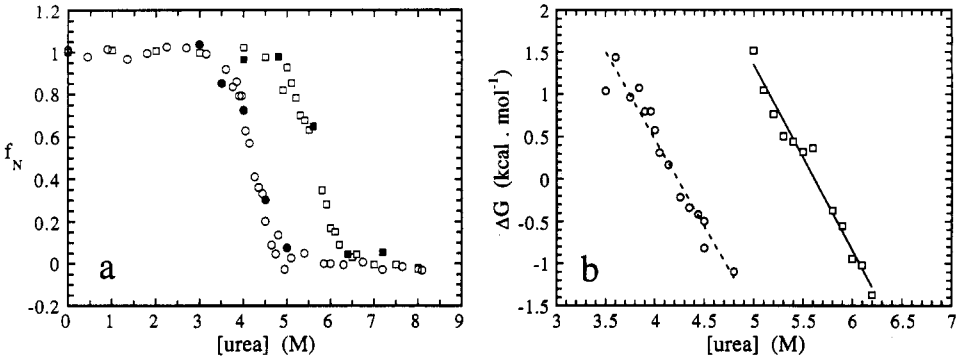


FIGURE 7: Urea-induced equilibrium unfolding transition of (○, ●) oxidized and (□, ■) reduced DsbA. (a) The fraction of molecules fully folded (f_N) as a function of the urea concentration, as measured by CD at 222 nm. Open symbols are of samples prepared by the addition of urea to the protein; filled symbols are of samples prepared by dilution from 8 M urea. Conditions were as given in the legend to Figure 5. (b) Linear extrapolation of the free energy of unfolding through the transition region. The free energy of unfolding is defined as $\Delta G_{unfold} = -RT \ln (1-f_N/f_N)$.

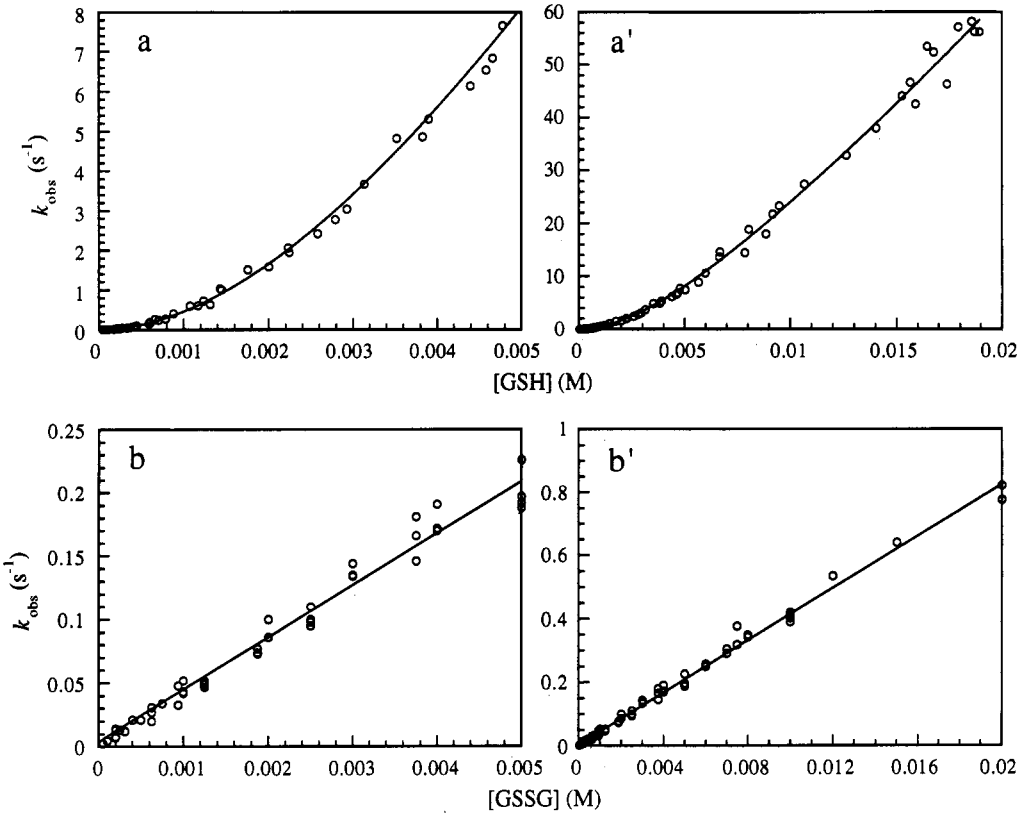


FIGURE 8: Dependence of the observed pseudo first-order rate constants for reduction and oxidation of DsbA on the concentration of reduced and oxidized glutathione, respectively. (a, a') Rate of reduction over two ranges of concentration of GSH. The solid curve was generated using eq 5 and the rate constants given in Table II. (b, b') Rate of oxidation over two ranges of concentration of GSSG. The solid curve was generated using the equation $k_{obs} = k_4[GSSG]$ with $k_4 = 41\ s^{-1}M^{-1}$.

normally reactive with alkylating reagents, and this is undoubtedly the one that participates in mixed-disulfide formation with glutathione or its substrate proteins. Preliminary experiments with mutant forms of DsbA, in which each cysteine residue has been replaced by a serine, have identified the accessible cysteine as Cys30, which is the more N-terminal (unpublished observation). As mentioned above, the reactivity of this cysteine is about 10-fold greater than expected for a normal protein thiol at pH 7.5 (Creighton, 1986), which may simply be a consequence of it having an abnormally low pK. The inaccessibility of the other cysteine thiol (Cys33) presumably inhibits its ionization and reaction with any disulfide other than the mixed disulfide of Cys30. The reasonably rapid rate at which this latter reaction occurs is compatible with Cys30 and Cys33 being held in proximity by the folded conformation of DsbA. The instability of both the intramolecular disulfide of DsbA and the intermolecular mixed disulfide can probably be explained by an unfavorable environment for any disulfide involving Cys30.

It will be of interest to learn how the folded conformation of DsbA incorporates the energetically unfavorable properties of its disulfide bond. Much of the strain can be relieved by unfolding the protein, and the disulfide is then about 4 kcal·mol^{-1} more stable. The strain can also be relieved by reducing the disulfide bond. The stability of folded DsbA is then greater by the same amount, as expected from the linkage relationship between the disulfide bond and conformational stability (Scheme III; Creighton, 1986).

This is a very unusual situation, as disulfides generally stabilize protein folded conformations, in part by disfavoring the unfolded state by decreasing its conformational entropy (Pace et al., 1988). A disulfide between cysteine residues

separated by only two others, however, as also occurs in thioredoxin (Dyson et al., 1990), would not be expected to have a large entropic stabilizing effect. In DsbA, this slight stabilizing contribution is more than counterbalanced by other interactions in the folded state that disfavor the disulfide bond. That the effect of the DsbA disulfide is primarily on the folded state is shown by the anomalous kinetics of reduction of the disulfide in the folded state and by the disulfide in the unfolded state being apparently normal, with a stability similar to those measured in other systems. The equilibrium constant measured for disulfide bond formation in unfolded DsbA, 170 mM (Table I), is similar to that for unfolded thioredoxin, 26 mM (Lin & Kim, 1989); both proteins have cysteine residues separated by two other residues, but the amino acids are different, -Pro-His- in DsbA and -Gly-Pro- in thioredoxin. Also, the stabilities of the mixed disulfides with both thiols of unfolded DsbA were within a factor of 2 of the values expected if they were comparable to the disulfide of GSSG; in this case, the expected values are $K_1^U = 4$ and $K_3^U = 1$ (Scheme II, Table I).

The structural reasons underlying the instability of the disulfide bond of DsbA are probably subtle, since there were no indications of any substantial conformational changes by the protein upon making or breaking the disulfide bond. No substantial difference was detected between the disulfide and dithiol forms of DsbA by far- and near-UV CD spectroscopy (Figure 2). The fluorescence emission spectra exhibited no shift of λ_{max} (Figure 3), arguing against a modification of the exposure of the two tryptophan residues to the solvent. The lower quantum yield of the oxidized form is probably due to quenching by the disulfide bond (Kishore et al., 1983).

It is instructive to compare the properties of DsbA with those of the functionally related proteins PDI, thioredoxin, and glutaredoxin, which also form reversible disulfides between cysteines separated by two residues in their sequence. PDI is involved in the formation of correct protein disulfide bonds in the ER of eukaryotic cells (Freedman et al., 1989). Thioredoxin is a small ubiquitous protein found in all living cells that takes part in a large variety of thiol–disulfide oxidoreduction processes. It is thought to act generally as a dithiol reductant (Holmgren, 1985). Accordingly, its disulfide bond is relatively stable, with an equilibrium constant with glutathione of 10 M (Lin & Kim, 1989). By contrast, the active disulfides of PDI are extremely unstable and oxidizing, with a value of 42–60 μM for the equilibrium constant with glutathione (Hawkins et al., 1991a; Lyles & Gilbert, 1991), similar to the value measured here for DsbA and suggesting that they may have similar functions. Disulfide bond formation is partially defective in PDI-depleted microsomes (Bulleid & Freedman, 1988). It may be that the primary in vivo role of PDI is not isomerization of disulfide bonds, as its name suggests, but in the oxidation of proteins. Depending on the redox conditions, PDI and thioredoxin participate in the same thiol–disulfide exchange reactions, although with very different efficiencies (Hawkins et al., 1991b; Lundström & Holmgren, 1990). PDI possess two domains highly homologous to thioredoxin, and both proteins belong to the TDOR family, which also includes glutaredoxin (Eklund et al., 1991). A sequence alignment between DsbA and thioredoxin obtained with limited sequence similarity and secondary structure assignment suggested that DsbA might also be homologous and structurally related to the TDOR family (Ellis et al., 1992).

DsbA is also similar to PDI and thioredoxin in that only the more N-terminal of the active pairs of cysteine residues is accessible and reactive. The pK of this cysteine residue in thioredoxin and PDI is abnormally low, 6.7 as compared to a more typical 8.7 (Kallis & Holmgren, 1980; Vuori et al., 1990; Hawkins & Freedman, 1991). A similar low pK value of the accessible cysteine residue of DsbA would explain its slightly high reactivity with GSSG ($k_4 = 41$ s^{-1} M^{-1}). The same rate constant is calculated for the same partial reaction between GSSG and PDI, using the rate constant for reduction by GSH, $k_1k_3/k_2 = 10^6$ s^{-1} M^{-2} (Gilbert, 1989a), and the overall equilibrium constant with glutathione, $K_{SS} = 42$ μM (Hawkins et al., 1991a). Therefore, the functional properties of DsbA support the suggestion (Ellis et al., 1992) that it is homologous to proteins of the TDOR family. Moreover, the far-UV CD spectrum of DsbA (Figure 2) is compatible with the protein containing secondary structure like that in the thioredoxin domain (Eklund et al., 1991).

It is also of interest that no structural modifications of thioredoxin or glutaredoxin have been observed upon breakage of its disulfide bond using the same probes (fluorescence and CD) that were used in this work with DsbA (Holmgren, 1972; Kelley et al., 1987; Sandberg et al., 1991). The crystal structure of oxidized thioredoxin (Katti et al., 1990) is virtually identical to the solution structure of the reduced form determined by NMR (Dyson et al., 1990). In the case of thioredoxin, the folded conformation of the oxidized form is 3.1–3.5 kcal·mol^{-1} more stable than that of the dithiol species, as expected from the stability of its disulfide bond (Lin & Kim, 1989). If thioredoxin, PDI, and DsbA share a similar structure around their active sites, as suggested by the sequence alignment (Ellis et al., 1992), and in both cases do not exhibit significant conformational change upon reduction of their disulfide bond, it is remarkable that the disulfide bond is stabilizing in thioredoxin and destabilizing in DsbA and PDI. In the absence of substantial structural differences between the reduced and oxidized proteins, these effects may lie in subtle conformational differences in the active site (Dyson et al., 1990) or possibly changes in the surface hydration properties as proposed for thioredoxin (Kaminsky & Richards, 1992a,b).

NOTE ADDED IN PROOF

Using a different method, R. Glockshuber and colleagues obtained values for the equilibrium constant between glutathione and DsbA that are comparable to ours (Wunderlich & Glockshuber, 1993). Their work also showed that the folded conformation of the disulfide form of DsbA is less stable than its dithiol form (M. Wunderlich, R. Jaenicke, and R. Glockshuber, personal communication).

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