Replacement of the Active-Site Cysteine Residues of DsbA, a Protein Required for Disulfide Bond Formation *in Vivo*

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ABSTRACT: DsbA is a periplasmic protein of *Escherichia coli* that was identified genetically as being involved in the formation of disulfide bonds in secreted proteins. Its active site contains one accessible and one buried cysteine residue, separated in the primary structure by only two other residues. These cysteine residues can form a very unstable disulfide bond that is 10^3-fold more reactive toward thiols than normal. Moreover, the mixed disulfide between the accessible cysteine residue and glutathione is 10^4-fold more reactive than normal. Site-directed mutagenesis was carried out to replace either one or both cysteine residues by serine. Cys30 is shown to be the accessible thiol, while Cys33 is shielded from the solvent. Even though the thiol group of Cys30 is exposed and reactive, it formed a very unstable mixed disulfide with glutathione. This disulfide bond was 2.17 ± 0.02 kcal mol^{-1} less stable in the native conformation than when DsbA was unfolded. If the native conformation destabilizes the mixed disulfide, the mixed disulfide must destabilize the folded conformation to the same extent. This was confirmed by demonstrating that the folded conformation of the mixed disulfide form of the mutant DsbA was 2.7 ± 0.9 kcal mol^{-1} less stable than that of the reduced form; these stability effects originated almost exclusively in the folded conformation. Replacing the cysteine residues by serine destabilized the folded conformation of the reduced protein to varying extents. This suggests that the thiol groups are involved in interactions that stabilize the folded conformation, which would cause any disulfide bonds, either inter- or intramolecular, that involve these groups to be unstable.

Many secreted proteins require disulfide bonds between some of their cysteine residues for stability of their folded conformations. Disulfide bond formation occurs co- and posttranslationally in the endoplasmic reticulum (ER)[1] of eukaryotic cells (Freedman, 1992) and posttranslationally in the periplasm of Gram-negative bacteria such as *Escherichia coli* (Bardwell & Beckwith, 1993). The formation of protein disulfide bonds *in vitro*, using air or small-molecule disulfides as oxidizing reagents, is often slower than during biosynthesis, and catalysts of this process *in vivo* are now firmly established both in eukaryotes and prokaryotes (Freedman, 1992; Bardwell & Beckwith, 1993).

Protein disulfide isomerase (PDI) is well-established to play a role in the formation of the disulfide bonds in the ER, where it is present at high concentrations (Bulleid & Freedman, 1988; Freedman, 1992). Under the redox conditions prevailing there, a [GSH]:[GSSG] ratio of between 3:1 and 1:1 (Hwang et al., 1992), disulfide bond formation in proteins is favorable (Creighton, 1983), and PDI has been shown to be an efficient catalyst for the folding of BPTI *in vitro* under such conditions and in microsomes (Creighton et al., 1980, 1993; Zapun et al., 1992).

Mutants of *E. coli* deficient in the formation of disulfides in secreted proteins were isolated and found to be defective in the gene *dsbA* (Bardwell et al., 1991; Kamitani et al., 1992). In addition to this genetic evidence, the protein product of that gene, DsbA, has been shown *in vitro* to catalyze the reduction of insulin by DTT (Bardwell et al., 1991), the regeneration of active RNase from the scrambled form in the presence of GSH (Akiyama et al., 1992) or DTT (Yu et al., 1993), and the oxidative refolding of RNase and alkaline phosphatase in the presence of GSSG (Akiyama et al., 1992; Akiyama & Ito, 1993). Co-overexpression in *E. coli* of DsbA resulted in increased yields of native, correctly folded Ragi bifunctional inhibitor (a plant protein with five disulfide bonds), in a manner dependent on the redox state of the growth medium (Wunderlich & Glockshuber, 1993b). DsbA can also enhance the rate of formation of a single disulfide bond in a model peptide (N. J. Darby and T. E. Creighton, unpublished observations) and acts as a very efficient oxidizing agent for reduced BPTI (A. Zapun and T. E. Creighton, unpublished observations).

Mature DsbA is a soluble, monomeric 21-kDa periplasmic protein of 189 amino acid residues (Bardwell et al., 1991; Zapun et al., 1993; Wunderlich et al., 1993). It contains only two cysteine residues, which are separated in the primary structure by only two other residues and which reversibly form a disulfide bond. This disulfide bond is extremely unstable and therefore very oxidizing. The equilibrium constant for thiol–disulfide exchange with glutathione was measured by various means to be 80 μM at pH 7.5 (Wunderlich & Glockshuber, 1993a; Zapun et al., 1993), which is one of the lowest values known. Interestingly, similar values of 42–60 μM have been reported for at least one of the two corresponding disulfide bonds of PDI (Hawkins et al., 1991; Lyles & Gilbert, 1991), although a somewhat greater value of 3 mM has been

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[1] Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; Cam, carboxamidomethyl group; CD, circular dichroism; Cm, carboxymethyl group; $DsbA_{SH}^{SH}$ and $DsbA_{S}^{S}$, the protein DsbA with free Cys30 and Cys33 thiol groups and with a disulfide bond between them, respectively, while the variants of DsbA with Cys30, Cys33, or both replaced by serine residues are designated as $DsbA_{33SH}^{30OH}$, $DsbA_{33OH}^{30SH}$, and $DsbA_{33OH}^{30OH}$, respectively, with the SH representing a cysteine residue and the OH a serine residue; DTT, dithiothreitol; ER, endoplasmic reticulum; GSH and GSSG, the reduced and oxidized forms of glutathione, respectively; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PDI, protein disulfide isomerase; MOPS, 3-morpholinopropanesulfonic acid.

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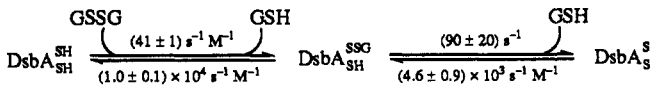
Scheme 1



measured in a different way by Lundström and Holmgren (1993).

The kinetics of the reaction of DsbA with glutathione are presented in Scheme 1. Cysteine thiol groups are indicated for the sake of simplicity here and throughout the paper as the protonated forms, even though the thiolate anion is the actual reactive species.

The rate constants of Scheme 1 show that DsbA is well suited to introduce disulfide bonds directly into other proteins (Zapun et al., 1993), which is probably its main role *in vivo*. The disulfide bond of DsbA$_S^S$ is 10^3-fold more reactive toward GSH than a normal protein disulfide, which makes it a potent oxidant. The mixed disulfide DsbA$_{SH}^{SSG}$ is 10^4 times more reactive, which is even more important for fulfilling this oxidative function (see Discussion).

Our previous studies had shown that the two cysteine residues of DsbA are not equivalent, one of them being readily accessible to alkylating reagents, while the other is unreactive and presumably buried and inaccessible (Zapun et al., 1993). In the present paper, we report the identification of the two cysteine residues, using site-directed mutagenesis to replace one or both by serine residues. The striking feature of the instability of the mixed disulfide with glutathione, and its expected effect on the stability of the folded conformation, are confirmed directly. Moreover, the effects of these replacements on the stability of the protein provide valuable clues as to why the disulfide bonds of DsbA are so unstable.

EXPERIMENTAL PROCEDURES

Most of the experimental procedures were those used previously in the study of wild-type DsbA (Zapun et al., 1993).

DNA Manipulation and Site-Directed Mutagenesis. Isolation of plasmid DNA and DNA restriction, ligation, and analysis were carried out using standard procedures (Sambrook et al., 1989). PCR was performed using an Omnigen TR3 CM220 thermal reactor (Hybaid). The mutants with either or both Cys30 and Cys33 replaced by serine were constructed using PCR with the expression plasmid pBJ41 (Bardwell et al., 1991) as template. The oligonucleotides, which encompass a unique *Bss*HII restriction site and encode the T to A base changes necessary to change cysteine to serine and to create the DsbA$_{33SH}^{30OH}$, DsbA$_{33OH}^{30SH}$, and DsbA$_{33OH}^{30OH}$ mutants were, respectively, 5′-GTAGCTGGCGCGCCGCAAGTGCTG-GAGTTTTTCTCTTTCTTCAGCCCGCAC-3′, 5′-GTA-GCTGGCGCGCCGCAAGTGCTGGAGTTTTTCT-CTTTCTTCTGCCCGCACACAGCTATCAG-3′, and 5′-GTAGCTGGCGCGCCGCAAGTGCTGGAGTTTTT-CTCTTTCTTCAGCCCGCACACAGCTATCAG-3′. The first and second base changes (shown in bold characters) generate *Eco*R57I and *Alu*I restriction sites, respectively, which were used to screen the transformed clones for successful mutations. The complementary primer included a unique *Bgl*II restriction site.

The PCR products were digested with restriction enzymes *Bss*HII and *Bgl*II and purified after electrophoresis in agarose gels. The pBJ41 plasmid was digested with the same enzymes, and the original insert to be replaced was removed. The resulting linearized vector, purified from agarose gel, was then ligated with the PCR fragments and transformed into JCB684, a DsbA$^-$ expression strain of *E. coli* (*dsbA*::Kan; J. C. A. Bardwell, personal communication).

JCB684 and all three selected mutant clones formed mucoid colonies on minimal medium M63 unless supplemented with a disulfide reagent such as cystine, indicating that they were defective in DsbA activity (J. C. A. Bardwell, personal communication). JCB684 transformed with pBJ41 (encoding wild-type DsbA) grew normally on M63 medium.

The coding sequences of the three mutants were verified by nucleotide sequencing (Sequenase II, U.S. Biochemical Corp.).

Protein Purification. Cells were submitted to a cold osmotic shock to release the periplasmic proteins (Manoil & Beckwith, 1986). Glutathione in the osmotic shockate was determined using the G-400 kit from Bioxytech S.A. The DsbA was purified by anion exchange chromatography on a DEAE-Sephacel (Pharmacia) column equilibrated with 10 mM MOPS (pH 7), being eluted with a linear gradient of 0–80 mM NaCl.

Covalent Modification of Protein Thiols. Reduced DsbA$_{33OH}^{30SH}$ was prepared by incubation of the isolated protein for 20 min with 10 mM DTT in 10 mM MOPS (pH 7) prior to gel filtration against the appropriate buffer. DsbA$_{33Scam}^{30OH}$ was prepared by incubation of DsbA$_{33SH}^{30OH}$ for 20 min with 10 mM DTT and 6 M urea, followed by addition of one-fourth volume of 0.5 M iodoacetamide in 1.5 M Tris-HCl (pH 8.7). After 2 min at room temperature, the alkylating reagent was removed by gel filtration.

To probe the accessibility of the cysteine residues (Zapun et al., 1993), protein samples were incubated for 20 min with 10 mM DTT and then 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, only the accessible cysteine residue has normally reacted. Alternatively, the mixtures were further incubated for 3 min after addition of 3 volumes of 8 M urea and 1.5 M Tris-HCl (pH 8.7), which caused the normally buried cysteine residue to react also. The samples were kept on ice prior to analyze by electrophoresis.

Redox Titration. The equilibrium constant for reaction of DsbA$_{33OH}^{30SH}$ with glutathione (Scheme 2) was measured in 0.1 M Tris-HCl (pH 7.5), 0.2 M KCl, and 1 mM EDTA by the same procedures as used for wild-type DsbA, using reverse-phase HPLC to separate and quantify reduced DsbA$_{33OH}^{30SH}$ and DsbA$_{33OH}^{30SSG}$ (Zapun et al., 1993). Loading the same amount of the two proteins resulted in peaks of identical area, showing that correction for any differences in relative extinction coefficients and recoveries was not necessary. The same results were obtained after 20 or 30 min of incubation, indicating that equilibrium had been reached.

To correct for air oxidation of thiols, the free thiol content of the mixture, $[SH]_{Ellman}$, was determined at the time of the acid quench by Ellman's assay in the absence of guanidinium chloride, using the extinction coefficient ϵ_{412} = 14 150 M^{-1} cm^{-1} (Riddles et al., 1983). GSH being in all cases in large excess over protein, the contribution of the reduced protein to $[SH]_{Ellman}$ was neglected, and $[SH]_{Ellman}$ was taken as a measure of [GSH]. The concentration of GSSG was taken as $[GSSG] = [GSSG]_0 + ([GSH]_0 - [GSH])/2$, where $[GSSG]_0$ and $[GSH]_0$ were the initial concentrations added.

Kinetics of Reduction of DsbA$_{33OH}^{30SSG}$ with GSH. DsbA$_{33OH}^{30SSG}$ at a concentration of about 1 μM in the redox titration buffer with 10, 20, or 40 μM GSH was incubated for various times, when the reaction was quenched by acid. The two species DsbA$_{33OH}^{30SSG}$ and DsbA$_{33OH}^{30SH}$ were separated and quantified by HPLC as above.

Reduction of Insulin. Catalysis of the reduction of insulin by DTT was assayed according to Holmgren (1979).

RESULTS

Purification of the DsbA Variants. The variants of DsbA with Cys30, Cys33, and both replaced by serine residues, designated respectively as $DsbA^{30OH}_{33SH}$, $DsbA^{30SH}_{33OH}$, and $DsbA^{30OH}_{33OH}$, had physical properties like those of normal DsbA and were purified by very similar procedures. The protein $DsbA^{30SH}_{33OH}$ was sometimes isolated as the mixed disulfide with glutathione, $DsbA^{30SSG}_{33OH}$. This was apparent from its electrophoretic mobility, which corresponded to the addition of about 1.5 negative charges to $DsbA^{S}_{S}$ (the glutathionyl moiety contains two carboxylic groups and one amino group that is partially deprotonated at the electrophoresis pH of 9.5). The electrophoretic mobility of $DsbA^{30SSG}_{33OH}$ was not modified upon incubation with iodoacetamide or iodoacetate, unless it was previously reduced with DTT. After reduction by DTT, incubation with excess GSSG restored the initial electrophoretic mobility (data not shown). Its molecular mass was determined by electrospray mass spectrometry to be 21 422 ± 3 Da, corresponding well to that expected for the mixed disulfide with glutathione, 21 421 Da. The other purified single cysteine mutant, $DsbA^{30OH}_{33SH}$, had the expected molecular mass of 21 115 Da, without the glutathione moiety.

Formation of the mixed disulfide $DsbA^{30SSG}_{33OH}$ was found to result from air oxidation of the glutathione released along with DsbA upon osmotic shock of the cells and to occur only when there was a delay of 2–3 days in the purification process prior to the chromatography step. Glutathione was shown to be present in the osmotic shockate of cells grown on M9 minimal medium (≈5 μg of glutathione/mL of culture) and to originate from the cells, not from the growth medium. Osmotic shock is known to release from the cytoplasm up to 20% of the nucleotides, as well as some small proteins such as thioredoxin and elongation factor Tu (Lunn & Pigiet, 1982).

Formation of the mixed disulfide only with the Cys33 → Ser mutant suggested that the reactive thiol of DsbA corresponds to Cys30.

Reactivities of the Cysteine Thiol Groups of DsbA. The accessibilities of the various cysteine residues were probed through their reactivity to neutral and acidic alkylating reagents in the absence or presence of a denaturant. The products were examined by native PAGE at pH 9.5, where the mobility is proportional to the hydrodynamic volume and net charge of the protein. By using similar neutral and acidic reagents, reactivity of a protein thiol group will be detectable by electrophoresis, as at least one reagent will cause a change in electrophoretic mobility.

As expected, the double mutant $DsbA^{30OH}_{33OH}$, without any cysteine residues, had the same mobility as $DsbA^{S}_{S}$, and its mobility was not modified by iodoacetate or iodoacetamide, with or without added denaturant (Figure 1, bottom).

That the Cys30 thiol group is fully accessible to the solvent and to thiol reagents was confirmed with protein $DsbA^{30SH}_{33OH}$. Samples treated with acidic iodoacetate had electrophoretic mobilities greater than those treated with iodoacetamide by the amount corresponding to a single charge difference (Figure 1, middle). The same difference was observed in the presence and absence of urea, indicating that the reaction with the alkylating reagents occurred in the folded conformation.

$DsbA^{30SH}_{33OH}$ had the electrophoretic mobility expected for a DsbA molecule that carries one additional negative charge compared to $DsbA^{S}_{S}$, suggesting that the single thiol was fully ionized. Reaction with neutral iodoacetamide resulted in a decrease in electrophoretic mobility corresponding to the loss of this negative charge, whereas reaction with iodoacetic acid did not change the electrophoretic mobility, as expected.

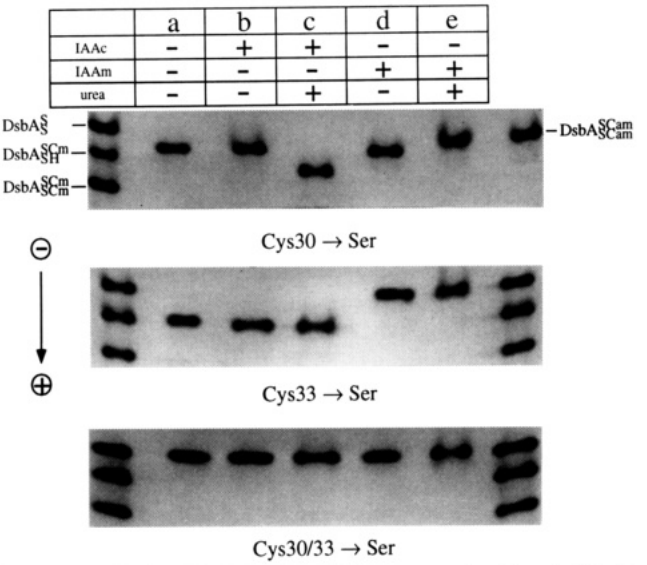


FIGURE 1: Native PAGE of the DsbA mutants incubated with 10 mM DTT, followed by the addition of buffer and either iodoacetate (IAAc) or iodoacetamide (IAAm) to 0.1 M, with or without urea subsequently added to 6 M.

The protein $DsbA^{30OH}_{33SH}$ confirmed that the Cys33 thiol group is buried and unreactive to thiol reagents in the native conformation. Incubation of $DsbA^{30OH}_{33SH}$ with either alkylating reagent in the absence of denaturant did not result in any modification of its electrophoretic mobility (Figure 1, top, lanes b and d). Under these conditions, a normal accessible thiol group reacts with a half-time of less than 1 s. Reaction with both types of reagent led to altered electrophoretic mobilities only in the presence of urea (lanes c and e). Similar results were obtained for reaction with GSSG (data not shown). These observations indicate that the thiol group of Cys33 is buried and inaccessible to alkylating reagents in the native conformation of $DsbA^{30OH}_{33SH}$.

The electrophoretic mobility of the $DsbA^{30OH}_{33SH}$ species suggests that the Cys33 thiol group was partially ionized during electrophoresis at pH 9.5, for the electrophoretic mobility was decreased somewhat by reaction with neutral iodoacetamide and increased substantially by acidic iodoacetic acid. This interpretation is tentative, however, for covalent modification of Cys33 increases the mobility of the protein for reasons that are not understood (Zapun et al., 1993). Spectral and reactivity measurements confirm that Cys30 has the lower pK_a value in $DsbA^{30SH}_{33SH}$ and is fully ionized at most pH values (J. W. Nelson and T. E. Creighton, unpublished observations).

Circular Dichroism Spectra. All the forms of the mutant proteins studied here gave CD spectra indistinguishable from that of normal DsbA (Figure 2), showing that the amino acid changes and covalent modifications did not alter the structure substantially. The anomalous spectrum of $DsbA^{SCam}_{SCam}$ observed previously in the near-UV remains unexplained (Zapun et al., 1993).

Thiol–Disulfide Redox Titration of $DsbA^{30SSG}_{33OH}$. The instability of the mixed disulfide between glutathione and the normal protein, $DsbA^{SSG}_{SH}$, did not allow its isolation and characterization, for the mixed disulfide was rapidly displaced to form the DsbA intramolecular disulfide bond. The

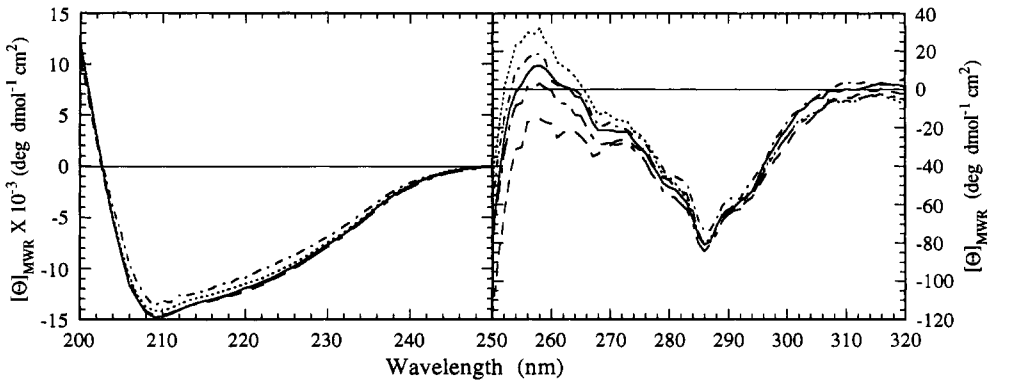


FIGURE 2: Circular dichroism (CD) spectra of mutants of DsbA: (—) $DsbA^{30OH}_{33SH}$, (– – –) $DsbA^{30OH}_{33SCam}$, (– · –) Dsb^{30SH}_{33OH}, (– · · –) $DsbA^{30SSG}_{33OH}$, and (···) $DsbA^{30OH}_{33OH}$. The protein concentration was about 14 μM in 0.1 M sodium phosphate (pH 7) and 1 mM EDTA. Spectra were recorded at 25 °C with a Jobin-Yvon CD VI spectrometer equipped with cells with path lengths of 0.1 and 1 cm, for the far-UV and near-UV measurements, respectively.

Scheme 2

$$K_{SSG} = \frac{[DsbA^{30SSG}_{33OH}][GSH]}{[DsbA^{30SH}_{33OH}][GSSG]}$$

$DsbA^{30SH}_{33OH}$ variant cannot form the protein disulfide bond, so its mixed disulfide form, $DsbA^{30SSG}_{33OH}$, could be studied directly (Scheme 2). This reaction is analogous to the first-half equilibrium of Scheme 1.

The value of K_{SSG} was measured by quantifying the relative amounts of the two different forms of the protein generated by incubation with varying concentrations of GSH and GSSG. The experimental data shown in Figure 3 indicated that the values of K_{SSG} in the absence and presence of 8 M urea were, respectively, $K^N_{SSG} = (39 \pm 1) \times 10^{-3}$ and $K^U_{SSG} = 1.52 \pm 0.02$. The mixed disulfide of $DsbA^{30SSG}_{33OH}$ is about 40-fold less stable in the native protein than when unfolded; in other words, the native conformation destabilizes the mixed disulfide. The difference in free energies of the mixed disulfide in the native and unfolded forms is defined as

$$\Delta\Delta G_{SSG} = -RT \ln\left(\frac{K^N_{SSG}}{K^U_{SSG}}\right)$$

and gives the value of 2.17 ± 0.02 kcal mol^{-1} in this case.

No mixed disulfide was generated when $DsbA^{30OH}_{33SH}$ was incubated with GSSG under the same conditions, confirming that Cys33 is inaccessible.

Unfolding Equilibria. It is a thermodynamic requirement that the native conformation of a protein be stabilized (or destabilized) by a disulfide bond, either inter- or intramolecular, to the same extent as the disulfide bond is stabilized (or destabilized) by the protein conformation (Creighton, 1986). This relationship has been confirmed quantitatively in the case of the intramolecular disulfide bonds of thioredoxin (Lin & Kim, 1989) and of normal DsbA (Zapun et al., 1993) and of mixed-disulfides of variants of T4 lysozyme (Lu et al., 1992). That for a mixed disulfide is shown in Scheme 3. This linkage relationship predicts that the difference in free energy of folding of $DsbA^{30SH}_{33OH}$ and $DsbA^{30SSG}_{33OH}$ should be equal to the free energy of stabilization (or destabilization) of the disulfide bond by the native conformation of the protein.

$$\Delta\Delta G_{fold} = -RT \ln\left(\frac{K^{SSG}_F}{K^{SH}_F}\right) = \Delta\Delta G_{SSG}$$

The unfolding equilibrium curves for $DsbA^{30SH}_{33OH}$ and $DsbA^{30SSG}_{33OH}$ were determined by the CD ellipticity at 222 nm in solutions of varying concentrations of urea (Figure 4). Both denaturation curves were consistent with a two-state reaction, where only the native and the unfolded conformations are present at equilibrium, and were analyzed on that assumption. The midpoints for unfolding were 4.47 ± 0.03 M urea for $DsbA^{30SSG}_{33OH}$ and 5.10 ± 0.02 M urea for $DsbA^{30SH}_{33OH}$. The free energies of folding in the absence of urea (ΔG_{fold}) were determined by least-squares linear extrapolation of the transition region to 0 M urea (Pace, 1986) to be −9.8 ± 0.5 kcal mol^{-1} for $DsbA^{30SSG}_{33OH}$ and −12.5 ± 0.4 kcal mol^{-1} for $DsbA^{30SH}_{33OH}$. Therefore, the difference in conformational stability between the two species, $\Delta\Delta G_{fold}$, is 2.7 ± 0.9 kcal mol^{-1}, which agrees well with the value predicted from the thiol–disulfide exchange equilibrium values.

The linear extrapolations of free energies of folding to 0 M urea were over a long range, however, so very small differences in the slopes of the curves, known as the *m* values (Pace, 1986), cause very substantial differences in the extrapolated values. The *m* values for the various species of DsbA varied only slightly, with a mean and mean deviation of 2.27 ± 0.15 kcal mol^{-1} M^{-1}, but this small variation produced significant variation in ΔG_{fold}. In such circumstances, it is usually more accurate to assume that very closely related proteins have the same *m* value and to use the average value for all the proteins. When this is done, the value of $\Delta\Delta G_{fold}$ for $DsbA^{30SH}_{33OH}$ and $DsbA^{30SSG}_{33OH}$ is 1.5 kcal mol^{-1} (Table 1).

To investigate the role of the thiol groups in stabilizing the reduced form of DsbA, the unfolding equilibria of $DsbA^{30OH}_{33SH}$ and $DsbA^{30OH}_{33OH}$ were similarly examined (Figure 4). Both mutants were found to have similar conformational stabilities. The free energy of unfolding determined by linear extrapolation of each unfolding curve was −11.6 ± 0.2 kcal mol^{-1} for $DsbA^{30OH}_{33SH}$, with a midpoint at 4.84 ± 0.01 M urea, and −11.4 ± 0.3 kcal mol^{-1} for $DsbA^{30OH}_{33OH}$, with a midpoint at 4.74 ± 0.01 M urea. Assuming the same value of *m* as for the other forms of DsbA gave slightly different values (Table 1).

Kinetics of Reduction of $DsbA^{30SSG}_{33OH}$ by GSH. The pseudo-first-order rate constant (k_{obs}) for the reduction of $DsbA^{30SSG}_{33OH}$ by GSH (Figure 5) showed a linear dependence on the concentration of GSH between 10 and 40 μM. The second-order rate constant k_{red} was 560 ± 30 s^{-1} M^{-1}. This is substantially lower than the value of 1 × 10^4 s^{-1} M^{-1} measured in the normal protein (Scheme 1). Using the equilibrium constant K^N_{SSG} reported above, the rate of the

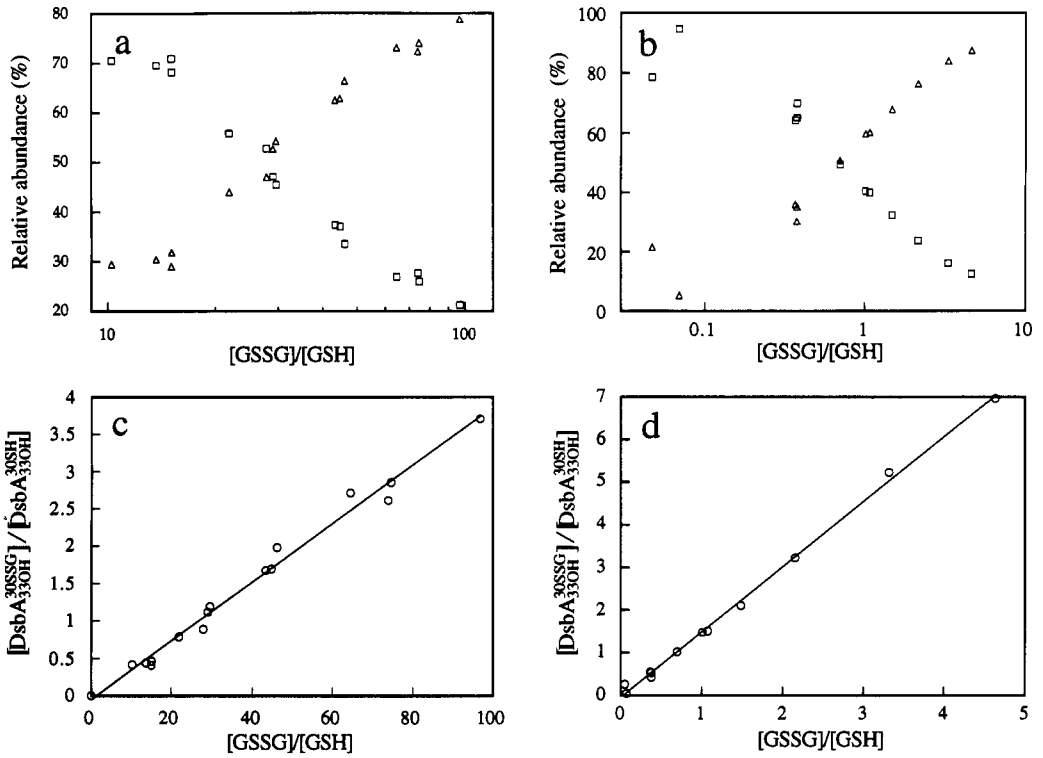


FIGURE 3: Measurement of the thiol–disulfide equilibrium of $DsbA^{30SH}_{33OH}$ with glutathione. The protein (≈13 μM) was incubated in mixtures containing various amounts of GSH and GSSG, 0.1 M Tris-HCl (pH 7.5), 0.2 M KCl, and 1 mM EDTA at 25 °C. The reaction mixtures were quenched by addition of HCl (to pH about 2), and the two forms of the protein, $DsbA^{30SH}_{33OH}$ (□) and $DsbA^{30SSG}_{33OH}$ (△), were separated by reverse-phase HPLC on a 25- × 0.46-cm Vydac TP518 column in 0.1% trifluoroacetic acid, eluted with a linear gradient of acetonitrile [38–48% (v/v) in 24 min]. The two DsbA species were measured by their absorbance at 215 nm. (a) In the absence of denaturant, the total concentration of glutathione was varied between 23 and 92 mM. (b) In 8 M urea, the total glutathione concentration was varied between 7 and 14 mM. (c and d) The plot of $[DsbA^{30SSG}_{33OH}]/[DsbA^{30SH}_{33OH}]$ *versus* [GSSG]/[GSH] should be linear, and the slope gives the equilibrium constant K^N_{SSG} or K^U_{SSG}, depending upon whether 8 M urea is absent (c) or present (d). The solid lines are linear fits to the data with the equilibrium constants given in the text.

Scheme 3

$$\begin{array}{ccc} U^{SH} + GSSG & \overset{K^U_{SSG}}{\rightleftharpoons} & U^{SSG} + GSH \\ \Big\| K_F^{SH} & & \Big\| K_F^{SSG} \\ N^{SH} + GSSG & \overset{K^N_{SSG}}{\rightleftharpoons} & N^{SSG} + GSH \end{array}$$

reverse reaction was calculated to be k_{ox} = 22 ± 2 s^{-1} M^{-1}; this is about half the value with the normal protein.

Reduction of Insulin. Wild-type DsbA had been shown to catalyze the reduction of insulin by DTT, as do other members of the thiol–disulfide oxidoreductase family, but with a rather low efficiency, more comparable to that of PDI than to that of thioredoxin (Bardwell et al., 1991). Figure 6 shows that $DsbA^{30SH}_{33OH}$ exhibited some activity in this assay in a concentration-dependent manner, whereas the two mutants $DsbA^{30OH}_{33SH}$ and $DsbA^{30OH}_{33OH}$ with no accessible cysteine residue were ineffective.

DISCUSSION

The present studies confirm the previous conclusion that only one of the cysteine thiols of $DsbA^{SH}_{SH}$ is reactive to alkylating reagents and to GSSG and that the other is presumably buried and inaccessible (Zapun et al., 1993). The reactive residue is shown to be the more N-terminal Cys30. These observations are confirmed by the recent crystal structure of $DsbA^{S}_{S}$ (Martin et al., 1993), which shows the cysteine residues in the appropriate environments. The two cysteine residues are part of a thioredoxin structural motif common to other members of the thiol–disulfide oxidoreductase family, which includes thioredoxin, PDI, and glutaredoxin. These three proteins had been shown to be similar in having only the more N-terminal cysteine residue of their active site accessible and reactive (Hawkins & Freedman, 1991; Bushweller et al., 1992; Kallis & Holmgren, 1980; Brandes et al., 1993). Consequently, DsbA can be considered a member of this family, although its thioredoxin fold is interrupted by a large insertion of 76 residues (Martin et al., 1993).

Kinetic characterization of the thiol–disulfide exchange reaction between DsbA and glutathione, summarized in Scheme 1, had shown that DsbA is well suited to introduce disulfide bonds directly into proteins secreted in the periplasm of *E. coli* (Zapun et al., 1993). The two key observations leading to this conclusion were the remarkable instabilities and reactivities of both the intramolecular disulfide bond of DsbA and of its mixed disulfide with glutathione. It is likely that these properties would also apply to the mixed disulfide with other thiol-containing molecules, including substrate proteins. With a protein, the mixed disulfide would be expected to be displaced rapidly by a second cysteine residue, either another in the substrate protein, if its conformation permits rapid formation of a disulfide bond, or otherwise by Cys33 of DsbA, to regenerate $DsbA^{S}_{S}$. In this way, $DsbA^{S}_{S}$ would incorporate disulfide bonds rapidly into proteins, but only when appropriate.

Mixed disulfides of DsbA with another molecule or protein were impossible to isolate for further study, in part because of their reactivity with external thiol groups but primarily because they would always tend to be displaced by Cys33 to

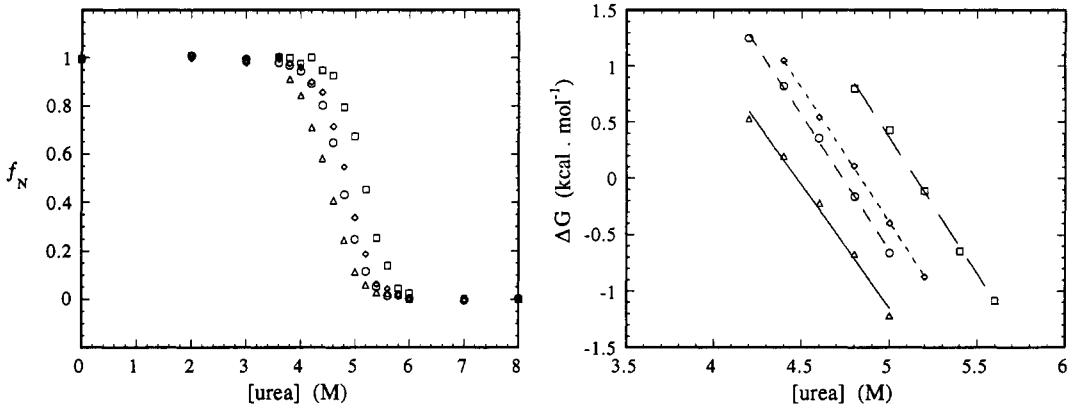


FIGURE 4: Urea-induced equilibrium unfolding transitions of $DsbA_{33OH}^{30SH}$ (□), $DsbA_{33OH}^{30SSG}$ (△), $DsbA_{33SH}^{30OH}$ (○), and $DsbA_{33OH}^{30OH}$ (◇). (a) The fraction of molecules fully folded (f_N) as a function of the urea concentration was measured by CD at 222 nm. The protein concentration was ≈14 μM in a cell with a path length of 0.1 cm, in 0.1 M Tris-HCl (pH 7.5), 0.2 M KCl, and 1 mM EDTA at 25 °C. (b) Linear extrapolation of the free energy of unfolding through the transition region. The free energy of unfolding is defined as $\Delta G_{unfold} = -RT \ln (1 - f_N/f_N)$.

Table 1: Midpoint of the Unfolding Transition and Free Energy of Unfolding of the Different Forms of DsbA[a]

	$[urea]_{1/2}$ (M)	ΔG_{fold} (kcal mol^{-1})[b]
$DsbA_{SH}^{SH}$ [c]	5.62 ± 0.11	−12.9
$DsbA_{33OH}^{30SH}$	5.10 ± 0.02	−11.8
$DsbA_{33SH}^{30OH}$	4.84 ± 0.01	−11.1
$DsbA_{33OH}^{30OH}$	4.74 ± 0.01	−10.9
$DsbA_{33OH}^{30SSG}$	4.47 ± 0.03	−10.3
$DsbA_{S}^{S}$ [c]	4.23 ● 0.16	−9.7

[a] Conditions were as given in legend to Figure 4. [b] $m = 2.3$ (kcal mol^{-1} M^{-1}) was used to calculate these ΔG_{fold}; the average of the m values obtained from the linear fits to the data was 2.27 ± 0.15 (kcal mol^{-1} M^{-1}). [c] Zapun et al. (1993); the error on these data published previously was overestimated.

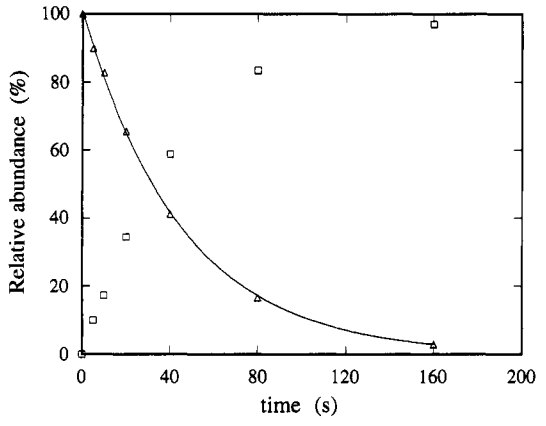


FIGURE 5: Kinetics of reduction of $DsbA_{33OH}^{30SSG}$ (≈1 μM) with 40 μM GSH. The conditions for the reaction, the acid quench, and the separation were as described in Figure 3. The symbols are as in Figure 3, and the solid curve was generated using a single exponential with $k_{obs} = 2.21 \times 10^{-2}$ s^{-1}.

generate $DsbA_{S}^{S}$. The production by site-directed mutagenesis of a DsbA variant in which Cys33 is replaced by a serine residue permitted the direct investigation of the elusive mixed disulfide.

The present work with $DsbA_{33OH}^{30SH}$ has shown that the mixed disulfide between Cys30 and glutathione is unstable, although 10-fold less so than measured with the normal protein (the equilibrium constants are 39 × 10^{-3} and 3.7 × 10^{-3}, respectively). At the kinetic level, the rate of reduction of $DsbA_{33OH}^{30SSG}$ by GSH (560 M^{-1} s^{-1}) was about 20-fold slower than observed with the wild-type protein (1.0 × 10^{-4} M^{-1} s^{-1}),

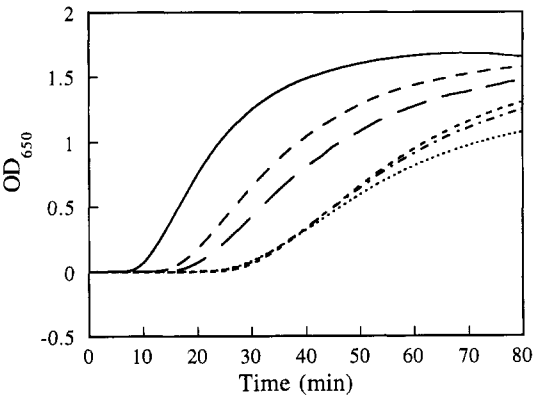


FIGURE 6: Reduction of 175 μM insulin by 1 mM DTT in the presence of 12 μM wild-type DsbA (—), $DsbA_{33SH}^{30OH}$ (- - -), $DsbA_{33OH}^{30OH}$ (···), $DsbA_{33OH}^{30SH}$ (– – –), or 6 μM $DsbA_{33OH}^{30SH}$ (— — —) and of buffer only (– · –). The buffer was 0.1 M potassium phosphate, pH 7, at 25 °C. The turbidity due to the precipitation of the B chain of insulin was monitored.

while the rate of formation of $DsbA_{33OH}^{30SSG}$ was 2-fold smaller than with the normal protein. These differences may be due to the replacement of Cys33 by Ser, or they could indicate that the previous studies underestimated the stability of the mixed-disulfide $DsbA_{33SH}^{30SSG}$. This species may be difficult to trap by acidification, as its conversion to $DsbA_{S}^{S}$ is rapid, even at acidic pH (unpublished observations). The equilibrium and rate constants measured with $DsbA_{33OH}^{30SSG}$ nevertheless differ by about 2 orders of magnitude from those expected for a normal disulfide bond (Zapun et al., 1993), indicating that $DsbA_{33OH}^{30SH}$ has retained most of the unusual properties of normal DsbA.

What is the physical reason for the instabilities of the disulfide bond of $DsbA_{S}^{S}$ and of its mixed disulfide with glutathione? The $DsbA_{S}^{S}$ disulfide is 4.5 kcal mol^{-1} less stable than when the protein is unfolded. The mixed disulfide is destabilized by 2.2 kcal mol^{-1} in $DsbA_{33OH}^{30SSG}$ and by 3.3 kcal mol^{-1} in the normal protein.

It is possible to determine whether the effect of a mixed disulfide on the stability of the protein is due to its effect on the folded or unfolded states, because mixed disulfide formation is a reversible modification that can be measured quantitatively in both the folded and unfolded states and compared to that for a typical accessible and normally reactive cysteine residue. Such an analysis has been applied to variants of T4 lysozyme containing single cysteine residues, by forming

a mixed disulfide with cystamine (Lu et al., 1992). The mixed disulfide of DsbA is a favorable subject for such an analysis, as it has large effects on the stability of DsbA. The equilibrium constant for the formation of a mixed disulfide between a standard protein thiol and glutathione (K_{model}) is expected and observed to be about 2 (Darby & Creighton, 1993). The measured values in the unfolded and folded states of $DsbA_{33OH}^{30SH}$ were 1.52 and 3.9×10^{-2}, respectively. Therefore, the mixed disulfide of $DsbA_{33OH}^{30SSG}$ is essentially normal in the denatured state and extremely unfavorable in the native conformation. The destabilization of the unfolded state compared to the standard protein thiol, given by $\Delta G^U = -RT(K_{SSG}^F/K_{model})$, is 0.2 kcal mol^{-1}. In the folded conformation, the free energy of destabilization compared to the model, given by $\Delta G^N = -RT(K_{SSG}^N/K_{model})$, is 2.3 kcal mol^{-1}. The same calculations can be made for the normal protein, using the values determined previously (Zapun et al., 1993). In this case, ΔG^U is 0.4 kcal mol^{-1} and ΔG^N is 3.7 kcal mol^{-1}. From this analysis, it appears that the destabilization of the mixed disulfide arises primarily from interactions in the folded state of DsbA.

These interactions are unlikely to involve primarily the disulfide bonds. The recently solved structure of $DsbA_S^S$ revealed that its active site can be superimposed on that of thioredoxin, with no detectable difference in geometry; the bond length and angles are close to optimal, and there are no indications of any conformational strain in either disulfide bond (Martin et al., 1993). Yet the disulfide bond of thioredoxin is 6.9 kcal mol^{-1} more stable than that of DsbA. Thus, the same protein fold can stabilize a disulfide bond in the case of thioredoxin (Lin & Kim, 1989) and destabilize it in the case of DsbA (Zapun et al., 1993; Wunderlich et al., 1993).

The crystal structure of $DsbA_S^S$ also gives no explanation for the instability of the mixed disulfide of $DsbA_{33OH}^{30SSG}$; Cys30 is on the surface of the molecule. No substantial conformation changes take place in DsbA upon reduction of either the inter- or intramolecular disulfide bonds, as detected by CD, and Cys30 is observed to be accessible and reactive (Figure 1; Zapun et al., 1993). The related thioredoxin also undergoes no substantial changes in conformation upon reduction of its disulfide bond (Stone et al., 1993).

Possible reasons for the instabilities of the disulfide bonds of DsbA are indicated by the effects on stability of the folded conformation of replacing the thiol groups (Table 1). Replacing Cys33 or Cys30 by Ser decreased the stability by 1.1 and 1.8 kcal mol^{-1}, respectively. The destabilizing effect of replacing Cys33 by Ser could be attributed to the placing of a more hydrophilic hydroxyl group in the interior of the protein (Blaber et al., 1993), but the greater destabilizing effect of replacing Cys30 cannot be explained in this way, as its thiol groups is accessible and reactive. Rather, the results suggest that the thiol groups, especially of Cys30, play very substantial roles in stabilizing the folded conformation of DsbA. Removing the thiol group, either by mutation or by forming a disulfide bond, either intramolecular or in a mixed disulfide, would therefore be energetically unfavourable in the folded state. The detailed explanation of this effect is unlikely to be simple, however, for the effects of replacing each thiol were not additive (Table 1) and there are probably interactions between them. Nevertheless, interactions of the thiol groups of the active-site cysteine residues with the folded structure of the protein are likely to be the explanation for the differences in stabilities of the disulfide bonds of DsbA, thioredoxin, and other members of this family.

The availability of the active-site mutants of DsbA should help to elucidate the detailed basis of this phenomenon.

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