Catalytic Mechanism of DsbA and Its Comparison with That of Protein Disulfide Isomerase

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ABSTRACT: The mechanism of action of the bacterial periplasm protein DsbA in introducing disulfide bonds into proteins was studied by its action on a model disordered peptide containing only two cysteine residues. Most of the reactions between the various thiol and disulfide forms of the peptide and of DsbA could be measured directly. All those involving DsbA occurred 10^2−10^6 times more rapidly than is normally observed between other typical thiols and disulfides; DsbA apparently stabilizes the transition state of thiol−disulfide exchange. The reactions between DsbA and the peptide were even more rapid, and they were constrained to occur at only one sulfur atom of disulfide bonds involving the peptide. Both observations indicate that noncovalent binding interactions also occur between DsbA and the peptide, and the expected effect of binding between reactants on rates of reaction was quantified. Small quantities of DsbA had catalytic effects on the reaction between the peptide and glutathione, similar to those observed previously with the eukaryotic catalyst protein disulfide isomerase. The known reactions of DsbA could account quantitatively for these effects and indicated that the apparent catalysis was the result of the separate and sequential rapid reactions of the peptide and of glutathione at the active site of DsbA. DsbA did not catalyze the conformational changes involved in forming an intramolecular disulfide bond in the peptide; its catalytic effects were simply due to its rapid participation in thiol−disulfide exchange reactions. Protein disulfide isomerase is likely to function very similarly to DsbA.

Disulfide bond formation is an important part of the biosynthesis of many proteins that are exported from the cytosol; it is often essential for folding of the polypeptide chain, for the protein's biological activity, and for its resistance to degradation by proteolysis. The *in vitro* process of disulfide-linked folding is well understood in at least a few model proteins (Creighton, 1978, 1990), but the process *in vivo* is assisted by catalysts: protein disulfide isomerase (PDI) in the endoplasmic reticulum of eukaryotes [reviewed by Freedman (1992) and Freedman et al. (1994)] and several proteins in the periplasm of bacteria (Bardwell & Beckwith, 1993; Missiakas et al., 1994; Shevchik et al., 1994). Relatively little is known about the mechanisms of action of these catalysts, but they are being actively studied.

Major questions to be answered about *in vivo* disulfide formation in proteins are the identity of the oxidant that converts cysteine thiol groups to disulfide bonds, the mechanism of this reaction, the role of the conformation of the substrate protein, and the mechanism of action of the catalysts. Do the catalysts simply accelerate the chemical interconversion of thiol and disulfide forms of protein cysteine residues, or do they participate more actively in the folding process? What interactions occur between the catalysts and their substrate proteins?

In the bacterial periplasm, genetic methods have identified several proteins required for protein disulfide formation: DsbA, DsbB, and DsbC (Bardwell et al., 1991, 1993; Missiakas et al., 1993, 1994; Shevchik et al., 1994). From the phenotypes of bacterial cells deficient in these proteins, DsbA and DsbC appear to be involved directly in incorporating disulfide bonds into newly synthesized polypeptide chains, whereas DsbB appears to be a membrane protein that is involved in oxidizing DsbA. DsbA has only very limited ability to catalyze protein disulfide rearrangements (Zapun & Creighton, 1994), which may be carried out in the periplasm primarily by DsbC (Zapun et al., 1995). The single disulfide bond between the Cys residues in -Cys-Pro-His-Cys- of DsbA is ideally suited to be the direct oxidant of protein cysteine residues, for it is very unstable and reactive (Wunderlich & Glockshuber, 1993; Zapun et al., 1993, 1994), and DsbA has been shown to play this role *in vitro* (Wunderlich et al., 1993; Zapun & Creighton, 1994). Yet DsbA incorporates disulfide bonds into a target protein only if the conformation of that protein permits, in contrast to a reagent like GSSG, which will generate mixed disulfides with single cysteine residues that cannot readily form protein disulfide bonds with other cysteine residues.

The eukaryotic and bacterial catalysts PDI[1] and DsbA have certain similarities and differences. Both catalyze net protein disulfide bond formation, but PDI is much more active than DsbA in catalyzing disulfide rearrangements (Zapun & Creighton, 1994). Both are related structurally and function-

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[1] Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; $DsbA_{SH}^{SH}$ and $DsbA_{S}^{S}$, the dithiol and disulfide forms of protein DsbA, respectively; $DsbA_{33SH}^{30OH}$ and $DsbA_{33OH}^{30SH}$, DsbA with either Cys30 or Cys33, respectively, replaced by Ser; P_{SH}^{SH}, peptide substrate containing cysteine residues at positions 2 and 27; when necessary, the two thiol groups are distinguished by their residue numbers; P_{2OH}^{27SH}, peptide substrate with a single cysteine residue at position 27 and a Ser residue at position 2; the mixed disulfide forms of these peptides and of DsbA with glutathione are indicated by the sub- and superscript -SSG; GSSG and GSH, the oxidized and reduced forms of glutathione, respectively; HPLC, high-pressure liquid chromatography; PDI, protein disulfide isomerase.

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ally to thioredoxin: PDI has two putative domains that are clearly homologous to thioredoxin, plus at least two additional domains (Edman et al., 1985); DsbA is not detectably homologous in sequence to thioredoxin, other than its active site cysteine residues, but it has a thioredoxin-like structure, with an extra domain of 76 residues inserted into the polypeptide chain (Martin et al., 1993). Both PDI and DsbA appear to undergo reversible disulfide formation between the pairs of cysteine residues homologous to those in thioredoxin, although these disulfide bonds have very different stabilities (Zapun et al., 1993; Wunderlich & Glockshuber, 1993).

Elucidating the mechanisms of action of catalysts such as PDI and DsbA requires the use of simpler systems than disulfide formation in proteins with multiple cysteine residues. For that reason, we have devised a simple model system of a 28-residue peptide with only two cysteine residues, which permits monitoring the action of the catalysts at individual cysteine residues and at individual steps in disulfide formation, breakage, and rearrangement. The action of PDI on the reaction of this peptide with glutathione has been characterized (Darby et al., 1994), and similar studies are reported here for DsbA. The relative simplicity of DsbA makes it possible to measure all its reactions with the peptide and with glutathione, and these can explain quantitatively the catalytic effects of DsbA. The situation with PDI is probably similar, and the results explain many of the activities of these two catalysts and suggest reasons for the differences between them.

MATERIALS AND METHODS

Materials. The peptide substrate used in this study, P_{SH}^{SH}, is derived from residues 4−31 of BPTI and has the sequence FCLEPPYTGPSKARIIRYFYNAKAGLCQ, with the N- and C-terminal groups acetylated and amidated, respectively. A second peptide, P_{2OH}^{27SH}, in which Cys2 was replaced by a Ser residue, was also used. The peptides were prepared and quantified as described previously (Darby et al., 1994). Mixed disulfide forms of the peptide were isolated by reverse-phase HPLC analysis at acid pH, as used in analysis of the various reaction products described below; they were recovered by lyophilization.

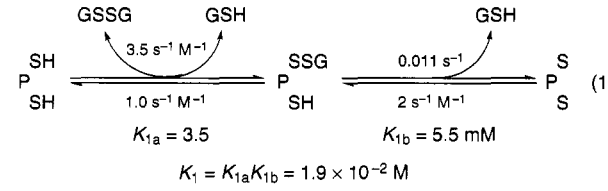
Normal DsbA; that with Cys30 or Cys33 replaced by Ser, $DsbA_{33SH}^{30OH}$ and $DsbA_{33OH}^{30SH}$, respectively; and the glutathione mixed disulfide form, $DsbA_{33OH}^{30SSG}$, were purified as previously described (Zapun et al., 1993, 1994). All of these forms were quantified using the same molar absorbance coefficient of 21 740 at 280 nm.

Thiol−Disulfide Exchange Reactions. All reactions between the thiol and disulfide forms of the peptide, glutathione, and DsbA were carried out at 25 °C in 0.2 M KCl and 1 mM EDTA buffered by either 0.1 M Tris-HCl (pH 7.4) or 0.1 M sodium acetate (pH 4.0). Reactions were quenched by addition of an aliquot of this mixture to 0.1−0.5 vol of 1 M HCl.

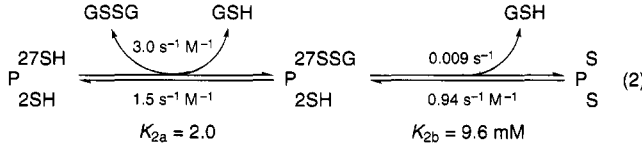
Analysis of Reaction Products. The oxidized, reduced, and glutathione mixed disulfide forms of the peptides and of DsbA were resolved by HPLC as described previously (Darby et al., 1994; Zapun et al., 1993). To resolve simultaneously the species P_{2OH}^{27SH}, P_{2OH}^{27SSG}, $P_{2OH}^{27S-S27}P_{2OH}$, $DsbA_{33SH}^{30SH}$, $DsbA_{33SH}^{30SSG}$, and $DsbA_{33SH}^{30S-S27}P_{2OH}$ (or the corresponding species of DsbA with Cys33 replaced by Ser), quenched reaction mixtures were analyzed on a Vydac 25 × 0.46 cm 218TP54 column at 38 °C at a flow rate of 1 mL min^{-1}, using the following gradient: 0 to 20 min, 27 to 33% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid; 20 to 25 min, 33 to 39%; 25 to 75 min, 39 to 43%; 75 to 80 min, 43 to 80%.

RESULTS

Reaction between the Model Peptide and Glutathione. The 28-residue model peptide used in this study is based on residues 4−31 of BPTI, including Cys5 and Cys30 but with Cys14 replaced by Ser. Consequently, the peptide has two cysteine residues at its positions 2 and 27 and is designated P_{2SH}^{27SH}. It is a simple model of an unfolded protein, as it adopts only local nonrandom conformations (Kemmink & Creighton, 1993) that do not affect formation and breakage of its disulfide bond. This peptide and a variant with Cys2 replaced by Ser, P_{2OH}^{27SH}, were used in a previous study of the effect of PDI on disulfide bond formation (Darby et al., 1994), using HPLC to resolve all the possible species. Its chemical thiol−disulfide exchange reactions with GSSG and GSH at pH 7.4 were extensively characterized in that and the present studies. Considering the two different mixed disulfide species together, the apparent rate and equilibrium constants are



The individual mixed disulfide species were distinguished by the HPLC separation, and that on Cys27 accumulated somewhat more than the other, probably due to weak electrostatic interactions of the acidic glutathione with neighboring charged residues on the peptide. Combined with the results on P_{2OH}^{27SH}, the rate and equilibrium constants via this intermediate are



Direct Reactions between the Peptide and DsbA. The normal function of DsbA is believed to be to transfer its disulfide bond to a reduced protein, such as P_{SH}^{SH}:

$$DsbA_S^S + P_{SH}^{SH} \overset{K_{3a}}{\rightleftharpoons} DsbA_{SH}^{S-S}P_{SH} \overset{K_{3b}}{\rightleftharpoons} DsbA_{SH}^{SH} + P_S^S \quad (3)$$

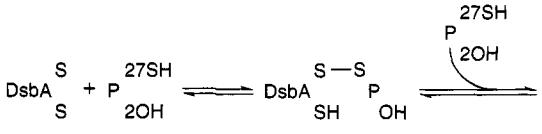
The mixed disulfides of DsbA always occur with the accessible sulfur atom of Cys30; the other cysteine residue of DsbA, Cys33, is buried and forms a disulfide bond only with Cys30 (Martin et al., 1993; Zapun et al., 1994).

The reaction between $DsbA_S^S$ and P_{SH}^{SH} was too rapid to be measured using manual mixing (data not shown), with a bimolecular rate constant > 10^5 s^{-1} M^{-1}. The reaction went to completion, as expected; the stabilities of the peptide and DsbA disulfide bonds relative to that of GSSG (eq 1; Zapun et al., 1993) indicate that the overall equilibrium constant

for the reaction should be[2]

$$K_3 = \frac{[\mathrm{DsbA}^{SH}_{SH}][\mathrm{P}^{S}_{S}]}{[\mathrm{DsbA}^{S}_{S}][\mathrm{P}^{SH}_{SH}]} = \frac{[\mathrm{DsbA}^{SH}_{SH}][\mathrm{GSSG}]}{[\mathrm{DsbA}^{S}_{S}][\mathrm{GSH}]^2}\,\frac{[\mathrm{P}^{S}_{S}][\mathrm{GSH}]^2}{[\mathrm{P}^{SH}_{SH}][\mathrm{GSSG}]} = \frac{1.9 \times 10^{-2}\ \mathrm{M}}{8.1 \times 10^{-5}\ \mathrm{M}} = (235) \quad (4)$$

The reactions between DsbA and the peptide are greatly simplified if one or both have only a single cysteine residue, so that the corresponding intramolecular disulfide bond cannot be formed. When the single-cysteine form of the peptide, P^{27SH}_{2OH}, was mixed with $DsbA^{S}_{S}$, a mixed disulfide complex between them was generated rapidly and reversibly (Figure 1b). Subsequently, the peptide dimer was formed by reaction of a second molecule of peptide with the mixed disulfide:



$$\mathrm{DsbA}^{SH}_{SH} + \mathrm{P}^{27S-S27}_{2OH}\mathrm{P}_{2OH} \quad (5)$$

The minimum or maximum value given for each measured equilibrium constant is that indicated by the maximum amounts of mixed disulfide complex measured here. The DsbA/peptide mixed disulfide complex was very unstable, however, and readily dissociated back to P^{27SH}_{2OH} and $DsbA^{S}_{S}$, even at pH 2. This is undoubtedly due to the intramolecular nature and the rapidity of this reaction at most pH values (Nelson & Creighton, 1994). Besides protonation of all thiol groups, acid trapping the mixed disulfide forms of DsbA also requires unfolding of the DsbA folded conformation, and the bound peptide probably stabilizes the DsbA structure in this case. Probably for that reason, the amount of complex measured varied with the trapping conditions; a maximum was reached with a final HCl concentration of 0.3 M, but higher acid concentrations resulted in poor recovery of the protein, so it was impossible to judge whether the reaction was adequately trapped.

Difficulties in trapping the mixed disulfide could also affect the value of the overall equilibrium constant for eq 5, although less dramatically, by overestimating the amounts of $DsbA^{S}_{S}$. However, the value measured, $2.1 \times 10^4\ M^{-1}$, is close to that measured for the equilibrium between DsbA and glutathione, $1.2 \times 10^4\ M^{-1}$ (Zapun et al., 1993); the two would be expected to have very similar values, as the disulfide bonds of the peptide and glutathione dimers have similar stabilities (as is confirmed in eqs 6 and 7 below).

Difficulties with trapping the mixed disulfides of DsbA are overcome if the buried Cys33 of DsbA is replaced by Ser, so that a disulfide bond can no longer be made in DsbA. Furthermore, when neither the peptide nor DsbA can form an intramolecular disulfide bond, the mixed disulfide bond between them need only compete with other intermolecular disulfides and should be much more apparent and readily quantified.

[2] Rate and equilibrium constants that were not measured directly but were calculated from other known rate and equilibrium constants are depicted within brackets.

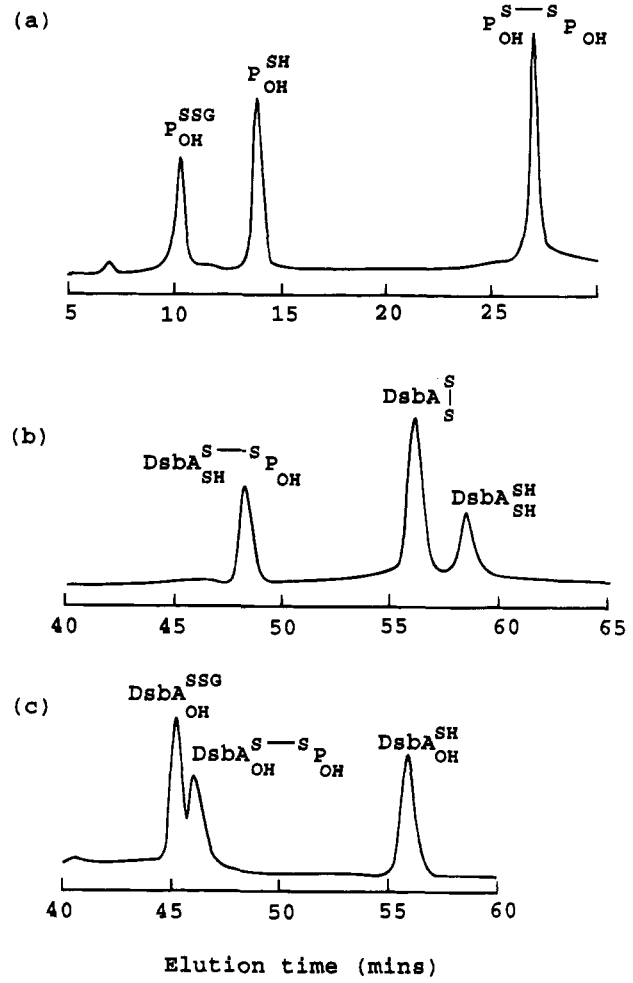


FIGURE 1: HPLC separation of the various disulfide forms of the peptide and of DsbA and of their mixed-disulfide complexes. (a) Separation of P^{27SH}_{2OH}, P^{27SSG}_{2OH}, and $P^{27S-S27}_{2OH}P_{2OH}$. (b) Products of the reaction of P^{27SH}_{2OH} with $DsbA^{S}_{S}$, including their mixed disulfide complex, $DsbA^{S-S27}_{SH}P_{2OH}$. (c) Products of the reaction of $DsbA^{SSG}_{OH}$ with P^{27SH}_{2OH}, including their mixed disulfide complex, $DsbA^{S-S27}_{OH}P_{2OH}$.

The equivalent of the reverse of the second step of eq 5 was measured by mixing $DsbA^{SH}_{OH}$ and the peptide disulfide dimer (data not shown). The mixed disulfide between them was generated rapidly and reversibly. The kinetics of the reaction in both directions were consistent with a simple bimolecular reaction, with the following rate constants:

$$\mathrm{DsbA}^{SH}_{OH} + \mathrm{P}^{27S-S27}_{2OH}\mathrm{P}_{2OH} \underset{8500\ s^{-1}\ M^{-1}}{\overset{55\,000\ s^{-1}\ M^{-1}}{\rightleftharpoons}} \mathrm{DsbA}^{S-S27}_{OH}\mathrm{P}_{2OH} + \mathrm{P}^{27SH}_{2OH} \quad (6)$$

The equilibrium constant implied by the rate constants, 6.5, is similar to that measured directly with equilibrium mixtures, 5.5 ± 1.0. The greater equilibrium constant measured here than for the analogous reaction in eq 5 (>1.4) is consistent with the DsbA mixed disulfide not being adequately trapped by acid in that instance.

Kinetic Restrictions on the Reaction between the Peptide and DsbA. The two sulfur atoms of the mixed disulfide between the peptide and glutathione, P^{SSG}, are very similar in their intrinsic chemical reactivities, and both react with model thiol groups at similar rates (Darby et al., 1994). With the thiol group of DsbA, however, they had very different reactivities.

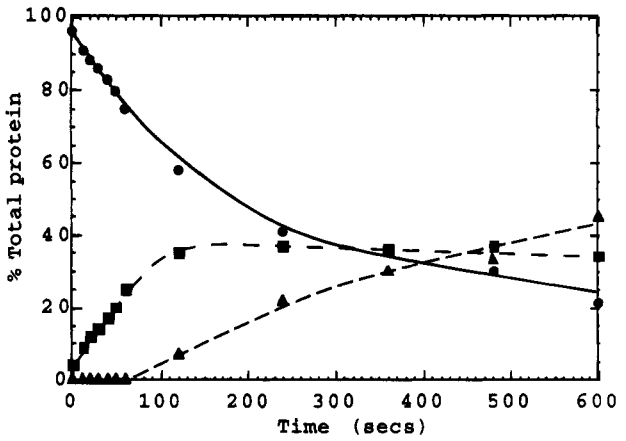


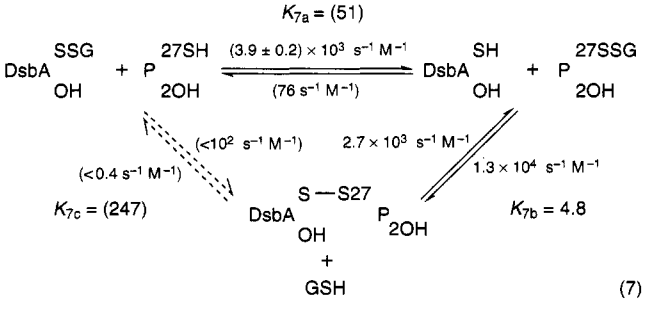
FIGURE 2: Kinetics of the reaction between $DsbA_{OH}^{SSG}$ and P_{2OH}^{27SH} at pH 7.4. The reaction between equal amounts (1 μM) of the two reactants was followed by acid trapping and HPLC analysis as in Figure 1. (●) $DsbA_{OH}^{SSG}$, (■) $DsbA_{OH}^{SH}$, and (▲) $DsbA_{OH}^{S-S27}P_{2OH}$. The curves were simply drawn through the data.

When P_{2OH}^{27SSG} was mixed with $DsbA_{OH}^{SH}$, the only products were $DsbA_{33OH}^{30S-S27}P_{2OH}$ and GSH (data not shown). The alternative products of $DsbA_{OH}^{SSG}$ and P_{2OH}^{27SH} were not detected. Therefore, the Cys30 thiol group of DsbA was constrained to react only with the sulfur atom of the peptide cysteine residue, not with that of glutathione.

When $DsbA_{OH}^{SSG}$ and P_{2OH}^{27SH} were mixed, the initial products were only $DsbA_{33OH}^{30SH}$ and P_{2OH}^{27SSG} (Figure 2), indicating that the peptide thiol attacked only the glutathione sulfur atom of the mixed disulfide, not that of Cys30 of DsbA. These initial products then reacted in a subsequent step, to generate the energetically favored products, $DsbA_{33OH}^{30S-S27}P_{2OH}$ and GSH. This second reaction to produce the $DsbA_{33OH}^{30S-S27}P_{2OH}$ complex occurred only after a lag period, as expected if the complex was not generated directly, but only from the products of the first reaction.

This remarkable series of kinetic reactions, in which the most stable products are not formed directly, is probably a result of (1) the low pK_a value of Cys30 of $DsbA_{SH}^{SH}$ (Nelson & Creighton, 1994), which makes it a much better leaving group than a normal sulfur atom (Szajewski & Whitesides, 1980) and would favor attack of the peptide thiol on the glutathione sulfur atom in the first step; (2) the structure of mixed disulfides of DsbA, which sterically prevents its Cys30 sulfur atom from reacting with external thiol groups and thereby limits such reactions to the other sulfur atom, such as that of glutathione in $DsbA_{33OH}^{30SSG}$.

These two sets of experiments gave the following rate constants for the reactions between the peptide and DsbA:



(7)

The rate constant of (76 s^{-1} M^{-1}) was calculated from the equilibrium constant for that reaction, which was calculated from the relative stabilities of each of the glutathione mixed disulfides of $DsbA_{OH}^{SH}$ and of the peptide relative to free GSH and GSSG (Zapun et al., 1994; eq 2):[2]

$$K_{7a} = \frac{[DsbA_{OH}^{SH}][P_{OH}^{SSG}]}{[DsbA_{OH}^{SSG}][P_{OH}^{SH}]} = \frac{[DsbA_{OH}^{SH}][GSSG]}{[DsbA_{OH}^{SSG}][GSH]} \frac{[P_{OH}^{SSG}][GSH]}{[P_{OH}^{SH}][GSSG]} = \frac{2.0}{3.9 \times 10^{-2}} = (51) \quad (8)$$

The reactions observed not to occur at significant rates are indicated in eq 7 by the dashed arrows. The value of the equilibrium constant, K_{7c}, could be calculated from K_{7a} and K_{7b}; the maximum values of the rate constants for this unobserved reaction are based on the value of the equilibrium constant and on the requirement, because it was not observed, that the largest rate constant be no more than 3% of that of the alternative reaction that did occur.

Equation 7 demonstrates that $DsbA_{33OH}^{30SH}$ reacts with the peptide/glutathione mixed disulfide with a 170-fold bias to attack the sulfur atom of the peptide cysteine residue rather than that of the glutathione. As these two sulfur atoms are chemically very similar and only 2.0 Å apart, this is most likely to occur because of steric constraints produced by the structure of the reactive complex between DsbA and the peptide.

The mixed disulfide between the peptide and glutathione has the stability expected for a normal intermolecular disulfide bond, like that of GSSG, with no other interactions between the peptide and the glutathione (eq 1). The same should be true of the peptide disulfide dimer, if there are no interactions between the two peptide molecules, and this is confirmed by the similarity of the equilibrium constants for forming the DsbA/peptide mixed disulfide measured in eqs 6 and 7. In contrast, the mixed disulfide between DsbA and glutathione or mercaptoethanol is very unstable (Zapun et al., 1993, 1994; Nelson & Creighton, 1994); eq 7 confirms that the mixed disulfide of $DsbA_{33OH}^{30SSG}$ is 50-fold less stable than a normal disulfide bond (Zapun et al., 1994). Strikingly, the equilibrium constants of eqs 6 and 7 indicate that the mixed disulfide between DsbA and the peptide is 150−400-fold more stable than that of $DsbA_{33OH}^{30SSG}$.

These observations indicate that there are binding interactions between the peptide and DsbA that restrict the reactions that take place and stabilize the complex between them.

Equilibrium and Rate Constants for the Reaction between the Peptide and DsbA. The rate measurements with $DsbA_{33OH}^{30SH}$ and P_{2OH}^{27SH} can be extrapolated to $DsbA_{SH}^{SH}$ and P_{SH}^{SH} if it is assumed that the Cys/Ser replacements have no effect on the reactivities of the other Cys residue of DsbA and the peptide. This assumption has been verified with the peptide, and it is only necessary to correct for the presence of one or two Cys residues (Darby et al., 1994). It is less satisfactory for $DsbA_{33OH}^{30SH}$, for the rates of reaction of its thiol group and its mixed disulfide with glutathione were observed to be altered somewhat by the replacement of Cys33 (Zapun et al., 1994; Nelson & Creighton, 1994). Correcting for these effects in various ways makes relatively little difference to the conclusions reached here, however, so the two forms of DsbA with Cys33 or Ser33 will be considered here to be equivalent.

Having measured the equilibria involving the mixed disulfide of DsbA and the peptide (eq 7), the known equilibria between the various forms of the peptide and glutathione (eq 1; Table 1) make it possible to calculate the equilibrium constants for the reaction between $DsbA_{SH}^{SH}$ and P_{SH}^{SH} (eq 3):[2]

$$K_{3a} = \frac{[DsbA_{SH}^{S}{}^{-S}P_{SH}]}{[DsbA_{S}^{S}][P_{SH}^{SH}]}$$

$$= \frac{[DsbA_{SH}^{S}{}^{-S}P_{SH}][GSH]}{[DsbA_{SH}^{SH}][P_{2SH}^{27SSG}]} \frac{[DsbA_{SH}^{SH}][GSSG]}{[DsbA_{S}^{S}][GSH]^2} \frac{[P_{2SH}^{27SSG}][GSH]}{[P_{SH}^{SH}][GSSG]}$$

$$= 4.8 \times 1.25 \times 10^4\ M^{-1} \times 2.0 = (1.2 \times 10^5\ M^{-1}) \quad (9)$$

where the equilibrium constants used in the calculation are from eq 7, Zapun et al. (1993), and eq 2, respectively. Likewise,

$$K_{3b} = \frac{[DsbA_{SH}^{SH}][P_{S}^{S}]}{[DsbA_{SH}^{S}{}^{-S}P_{SH}]}$$

$$= \frac{[DsbA_{SH}^{SH}][P_{2SH}^{27SSG}]}{[DsbA_{SH}^{S}{}^{-S}P_{SH}][GSH]} \frac{[P_{S}^{S}][GSH]}{[P_{2SH}^{27SSG}]}$$

$$= \frac{9.5 \times 10^{-3}\ M}{4.8} = (2.0 \times 10^{-3}\ M) \quad (10)$$

where the equilibrium constants are from eqs 2 and 7. The two partial equilibrium constants K_{3a} and K_{3b} are consistent with the overall equilibrium constant of (235) that was calculated in a similar way (eq 4).

The rate constants for these two reactions can be estimated with certain assumptions. That for reaction of the Cys33 thiol group of DsbA with a mixed disulfide on Cys30, to form $DsbA_S^S$, is assumed to be the same, 90 s^{-1}, whether the mixed disulfide is with glutathione (Zapun et al., 1993) or the peptide. The overall rate of reaction of P_{SH}^{SH} with $DsbA_S^S$ is taken from the competition of P_{SH}^{SH} and GSH for $DsbA_S^S$ to be 3.2×10^5 s^{-1} M^{-1} (see Figure 4a below). These assumptions produce the following kinetic scheme for the reaction between $DsbA_S^S$ and P_{SH}^{SH}:

$$DsbA_S^S + P_{SH}^{SH} \underset{(90\ s^{-1})}{\overset{(1.1 \times 10^7\ s^{-1}\ M^{-1})}{\rightleftharpoons}} DsbA_{SH}^{S}{}^{-S}P_{SH} \underset{(1.4 \times 10^3\ s^{-1}\ M^{-1})}{\overset{(2.7\ s^{-1})}{\rightleftharpoons}} DsbA_{SH}^{SH} + P_S^S \quad (11)$$

These rate and equilibrium constants are consistent with the experimental observations made directly for this reaction. The rate constant inferred for the reaction of $DsbA_{SH}^{SH}$ with P_S^S, (1.4×10^3 s^{-1} M^{-1}), is nearly the same as that measured at pH 4 (see eq 15 below); this pH independence of the rate of the reaction is also observed in the reaction of $DsbA_{SH}^{SH}$ with GSSG and occurs because the Cys30 thiol group of $DsbA_{SH}^{SH}$ is fully ionized at both pH values (Nelson & Creighton, 1994).

Catalysis by DsbA of the Reaction between the Peptide and Glutathione. Small amounts of DsbA were observed to increase the rates of the reactions between the peptide and glutathione (eq 1). These catalytic reactions differ from those described above in that only small quantities of DsbA are present, while both glutathione and the peptide are present simultaneously. Similar catalytic effects were observed previously with PDI (Darby et al., 1994), but it was not possible to elucidate the mechanism of the catalysis. The information obtained here about the direct reactions of DsbA with the peptide and with glutathione should make it possible to determine the mechanism. One possibility is that the catalytic activity was the result of the thiol−disulfide exchange reactions of DsbA with the peptide and with glutathione occurring at rates 10^3-10^4 times greater than those directly between the peptide and glutathione; small amounts of DsbA might thereby increase the apparent rate of reaction between them by providing alternative, more rapid reaction mechanisms. Alternatively, DsbA could conceivably catalyze a direct reaction between the peptide and glutathione, as PDI appeared to do (Darby et al., 1994).

The experiments were performed as previously with PDI, to permit quantitative comparison of the two catalysts (Table 1). Those with PDI used a constant thiol/disulfide redox potential, so as to keep the uncertain proportions of dithiol and disulfide forms of this catalyst constant. The ratio $[GSH]^2/[GSSG]$ was kept constant at 4 mM, but the absolute concentrations of GSSG and GSH were varied somewhat. These particular concentrations of GSH and GSSG produce equilibrium mixtures of 98% $[DsbA_{SH}^{SH}]$, 2% $[DsbA_S^S]$, and <0.14% $DsbA_{33SH}^{30SSG}$.

The overall rate of reaction between the peptide and millimolar concentrations of glutathione was increased significantly by, and in proportion to, micromolar concentrations of DsbA (Figure 3). In each case, the kinetics of the reaction were consistent with apparently simple bimolecular reactions between the peptide and glutathione, proceeding through the mixed disulfide between them. There were no indications of saturation of DsbA with GSSG, consistent with previous indications that there is no significant binding interaction between them (Zapun et al., 1993). The velocity of the catalyzed reactions was, however, independent of the concentration of P_{SH}^{SH} over the range 2−20 μM. These observations are very similar to those made previously with PDI (Darby et al., 1994).

These results could be simulated as if the DsbA were catalyzing the direct reactions between the peptide and glutathione, but the observations can be explained quantitatively by the individual reactions of DsbA with each elucidated above. The rate-limiting step was not the reaction (eq 11) of the reduced peptide with the small amounts of $DsbA_S^S$ that would be present at equilibrium. The rate of this reaction would be independent of the glutathione concentration and have a half-time of 84 s with 1.3 μM DsbA. The catalyzed reaction was observed to be proportional to the glutathione concentration and was considerably slower, with half-times between 150 and 600 s; therefore, another step must have been rate limiting. That step appeared to be the regeneration of $DsbA_S^S$ by reaction of $DsbA_{SH}^{SH}$ with GSSG. The observed initial velocity of the catalyzed reaction, 53 μM s^{-1} M^{-1} [GSSG] (=5.3 s^{-1} M^{-1} (10 μM peptide) [GSSG]) (Table 1), was identical to that predicted for the reoxidation of the $DsbA_{SH}^{SH}$ (53 μM s^{-1}

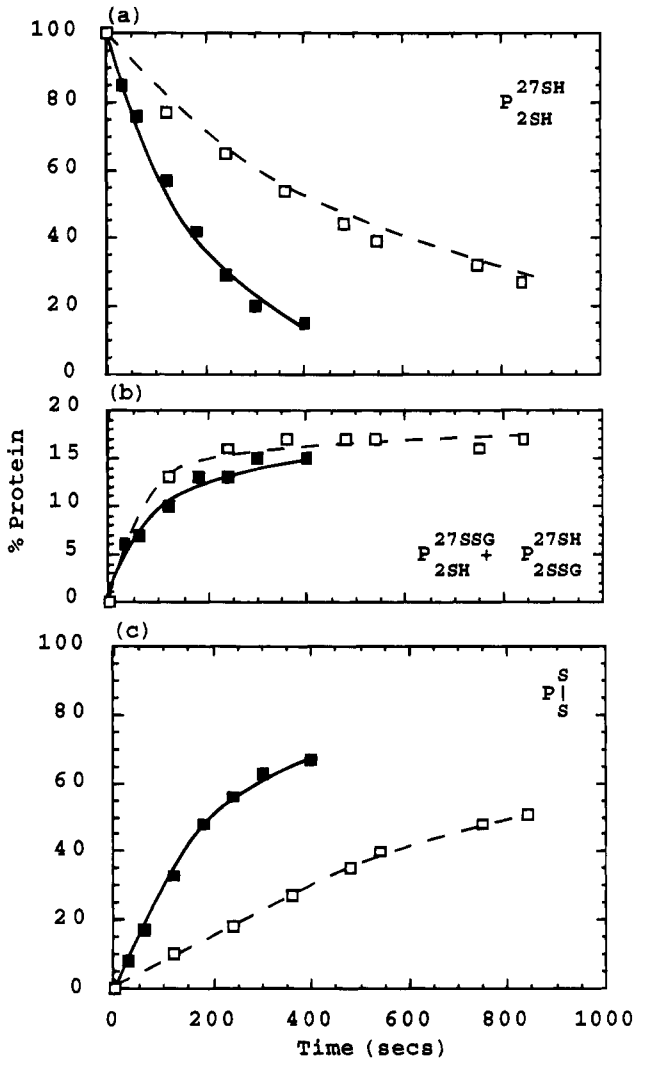


FIGURE 3: DsbA catalysis of the reaction of the reduced model peptide P_{2SH}^{27SH} with 0.5 mM GSSG and 1.4 mM GSH at pH 7.4. The kinetics of disappearance of P_{2SH}^{27SH} (a) and the appearance of the mixed disulfides P_{2SSG}^{27SH} plus P_{2SH}^{27SSG} (b) and of P_S^S (c) in the presence (■) and absence (□) of 1.3 μM DsbA. Under these conditions, the peptide reaches an equilibrium (eq 1) of 68% P_S^S, 14% P_{SH}^{SH}, and 18% P_{2SSG}^{27SH} plus P_{2SH}^{27SSG}. Very similar results were obtained with 0.25 mM GSSG/1.0 mM GSH and 0.125 mM GSSG/0.7 mM GSH, but the rates were correspondingly lower. The curves were simply drawn through the data.

M^{-1} [GSSG] = 41 s^{-1} M^{-1} (1.3 μM DsbA) [GSSG]) (Zapun et al., 1993). This explains why the increased rate was dependent upon the concentrations of GSSG and DsbA, but independent of the concentration of peptide, which did not participate in the rate-limiting step.

To confirm that the disulfide form of DsbA was responsible for this catalysis, the kinetics were measured at a constant GSSG concentration but varying concentrations of GSH. The rate of the forward noncatalyzed reaction of GSSG with the peptide or with $DsbA_{SH}^{SH}$ should be constant under these conditions, and the GSH should change only the reverse rate.

The catalyzed initial velocity of the reaction between P_{SH}^{SH} and 0.5 mM GSSG was maximal in the absence of GSH and declined monotonically with increasing GSH concentrations (Figure 4a). This is indicative of $DsbA_S^S$ being the primary catalytic species. The observed initial velocity of the catalyzed reaction, 0.11 μM s^{-1}, was

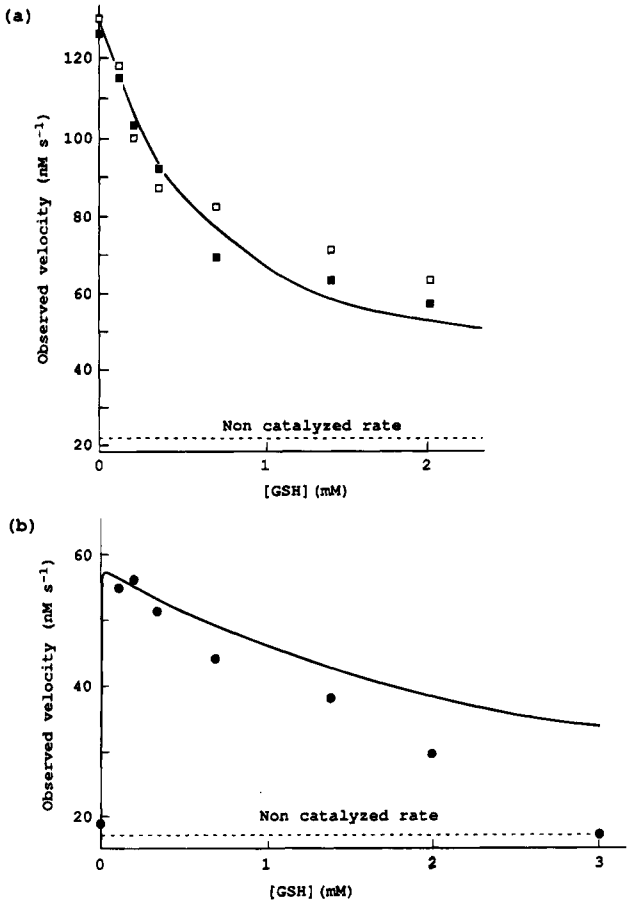


FIGURE 4: Catalysis of the reaction between GSSG and either P_{2SH}^{27SH} (a) or P_{2OH}^{27SH} (b) at a constant GSSG concentration of 0.5 mM and varying concentrations of GSH. The DsbA (2 μM) was initially equilibrated with the glutathione. The equilibrium concentrations of $DsbA_{SH}^{SH}$, $DsbA_{SH}^{SSG}$, and $DsbA_S^S$ vary markedly under these conditions, with only $DsbA_S^S$ being present in the absence of GSH, but $DsbA_{SH}^{SH}$ predominating in the presence of 2−3 mM GSH. The equilibrium concentration of $DsbA_{33SH}^{30SSG}$ would be at a maximal level of only 0.5% at 0.20 mM GSH. Upon addition of P_{2SH}^{27SH} or P_{2OH}^{27SH} to 10 μM, the velocity was measured by the initial rate of disappearance of P_{2SH}^{27SH}; the product in (a) was primarily P_S^S, although the mixed disulfide forms also appeared to their equilibrium concentrations, while in (b) the product was P_{2OH}^{27SSG}. The open and closed symbols in (a) represent two independent experiments. The solid curves in (a) and (b) are the expected results if GSH and the peptide compete for reaction with the $DsbA_S^S$ that is generated in the rate-limiting step by reaction of $DsbA_{SH}^{SH}$ with GSSG; they were calculated with the rate constants of eqs 7 and 11 and the rate and equilibrium constants for the reaction of DsbA with glutathione (Zapun et al., 1993). The maximal rate should be observed in (b) at about 15 μM GSH, where the rate of the reaction of GSH with the DsbA/peptide mixed disulfide reaches the rate at which the $DsbA_S^S$ is regenerated.

considerably less than the rate at which 2 μM $DsbA_S^S$ should react with 10 μM peptide, 6.4 μM s^{-1} (eq 11), and close to that at which $DsbA_S^S$ should be regenerated from 2 μM $DsbA_{SH}^{SH}$ by reaction with 0.5 mM GSSG, 0.041 μM s^{-1} (Zapun et al., 1993); this confirms that the reoxidation of DsbA was rate limiting. The decrease in rate with increasing GSH concentrations is due to the slowly generated $DsbA_S^S$ having the option of reacting with GSH rather than with the reduced peptide. Such a mechanism predicts the observed monotonic decrease in rate of reaction with the peptide.

The competition between the reaction of GSH and of the peptide with $DsbA_S^S$ gives an accurate value for the rate of

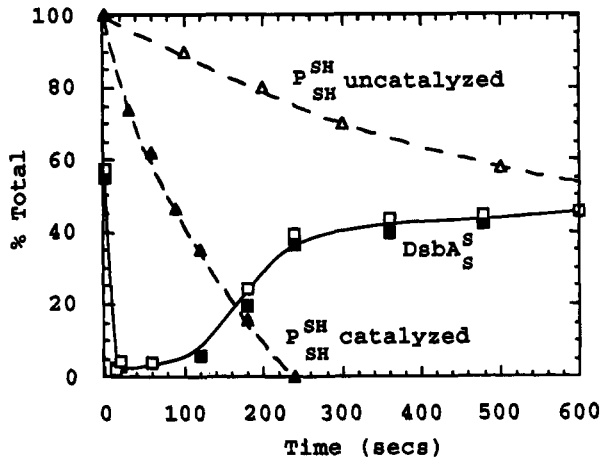
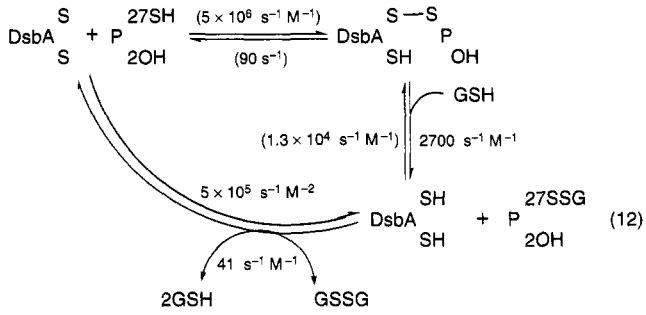


FIGURE 5: Relative amounts of P_{SH}^{SH} and $DsbA_S^S$ present during the reaction between P_{SH}^{SH} and GSH and GSSG in the presence of 3 μM DsbA at pH 7.4. DsbA was preincubated for 15 min with 0.5 mM GSSG and 0.2 mM GSH, and then P_{2SH}^{27SH} was added to 10 μM. At the indicated times, portions of the reaction mixture were acid trapped and the various forms of the peptide and of DsbA were analyzed by HPLC. The relative amounts of P_{SH}^{SH} (▲) and $DsbA_S^S$ (□, ■) are indicated as a function of time. The relative amounts of P_{SH}^{SH} in the absence of DsbA are indicated by the open triangles (△). The curves were simply drawn through the data.

the latter reaction. The catalyzed rate was halved at about 0.7 mM GSH, when the rate of reaction of $DsbA_S^S$ with GSH is 3.2 s^{-1}; this should be the same as the rate of reaction of $DsbA_S^S$ with 10 μM P_{SH}^{SH}, indicating an apparent second-order rate constant for the latter of 3.2×10^5 s^{-1} M^{-1}. This value is consistent with the direct measurements of the rate being $>10^5$ s^{-1} M^{-1} and has been used in eq 11; it accurately simulates the results of Figure 4a.

The kinetics of reaction of both the peptide and DsbA were monitored at GSH and GSSG concentrations where DsbA exists at equilibrium as nearly equal amounts of the dithiol and disulfide forms (Figure 5). When 10 μM P_{SH}^{SH} was added to this mixture of DsbA and glutathione, the peptide was converted to the disulfide form at a velocity of about 0.08 μM s^{-1}, much more rapidly than in the absence of DsbA. Monitoring the relative amounts of $DsbA_{SH}^{SH}$ and $DsbA_S^S$ indicated that the latter rapidly disappeared, presumably by reaction with the peptide, to a very low steady-state concentration and then slowly regained its equilibrium distribution as the reduced peptide disappeared. This is a direct demonstration that the regeneration of $DsbA_S^S$ by reaction with GSSG was rate limiting; the expected velocity of the reaction with 3 μM $DsbA_{SH}^{SH}$ and 0.5 mM GSSG (eq 11) would be 0.062 μM s^{-1}, very close to that observed. The steady-state level of $DsbA_S^S$ during the reaction would be expected to be about 0.7% of total DsbA, close to that observed at the minimum, before the reduced peptide disappeared.

Catalysis of Formation of the Peptide/Glutathione Mixed Disulfide. The above experiments indicated that DsbA increased the rate of disulfide bond formation in the peptide by transferring its disulfide bond from $DsbA_S^S$ (eq 11). This reaction occurs through a DsbA/peptide mixed disulfide, without involving the peptide/glutathione mixed disulfide P_{SH}^{SSG}, but this latter species also seemed to be generated by the DsbA-catalyzed reaction (Figure 3). That DsbA can catalyze formation of P_{SH}^{SSG} was confirmed with the reaction between GSSG and P_{2OH}^{27SH}, using catalytic amounts of DsbA and constant redox conditions, exactly as in Figure 3 for the dithiol form of the peptide (data not shown). Very similar rate enhancements were observed, and the increased rate again was proportional to the concentrations of both DsbA and GSSG (Table 1). The similarity in rates is again indicative that reoxidation of $DsbA_{SH}^{SH}$ by reaction with GSSG is the rate-limiting step. The most likely mechanism would be



The rate constants are from eqs 7 and 11 and Zapun et al. (1993), but take into account the presence of only a single cysteine residue on this peptide. The expected velocity of the upper reactions between 1.3 μM $DsbA_S^S$, 10 μM P_{2OH}^{27SH}, and 1.4 mM GSH would be about 6 μM s^{-1}, much faster than the observed catalyzed initial velocity of about 0.05 μM s^{-1}. This confirms that the reaction was limited by reoxidation of $DsbA_{SH}^{SH}$, which should occur with a velocity of about 0.03 μM s^{-1}, similar to that observed.

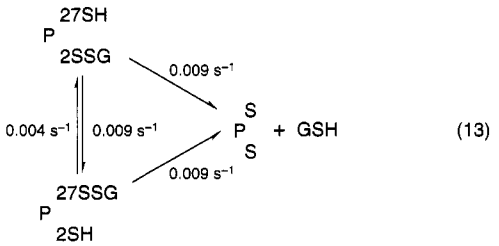
An alternative reaction mechanism would be that involving reaction between the $DsbA_{SH}^{SSG}$ mixed disulfide and P_{SH}^{SH} (eq 7). However, the amount of $DsbA_{SH}^{SSG}$ present should be less than that of $DsbA_S^S$, its rate of reaction with the peptide is only 0.004 times that of $DsbA_S^S$ (eq 11), and the predicted reaction velocity (0.07 nM s^{-1}) with the equilibrium amounts of $DsbA_{SH}^{SSG}$ expected is 3 orders of magnitude lower than the observed velocity. Therefore, the reaction via the $DsbA_{SH}^{SSG}$ mixed disulfide is unlikely to contribute substantially. This was confirmed with the mutant forms $DsbA_{33OH}^{30SH}$ and $DsbA_{33SH}^{30OH}$; only the former had any activity, and it was greatly decreased relative to that of normal DsbA (data not shown).

Another possibility would be that DsbA simply catalyzes the direct reaction between the peptide and GSSG, but this was ruled out by varying the GSH concentration while keeping that of GSSG constant, as in Figure 4a for P_{SH}^{SH}. In this case, the catalyzed velocity was negligible in the absence of GSH (Figure 4b). This indicates that GSH is the reactive species in the presence of DsbA, not GSSG, as in the noncatalyzed reaction. Therefore, $DsbA_S^S$ does not catalyze the direct reaction between the peptide and GSSG. The catalyzed rate increased dramatically at low GSH concentrations and then decreased monotonically. This indicates that $DsbA_{SH}^{SH}$ also did not catalyze the direct reaction between the peptide and GSSG, because the rate should then have increased with increasing GSH concentrations, as the fraction of DsbA present as $DsbA_{SH}^{SH}$ increased. The results of Figure 4b are those expected from the reaction occurring through a DsbA mixed disulfide, and only that with the peptide is sufficiently stable and reactive to account for the observed rate (eq 12). The catalyzed reaction requires GSH, but the presence of GSH also competes for reaction with

the $DsbA_S^S$ that is generated in the rate-limiting step. The known rate constants for the reactions of eq 12 could simulate the observed results very closely (Figure 4b).

The single-cysteine peptide just described, P_{2OH}^{27SH}, could form only the mixed disulfide with glutathione, but the mechanism and rate constants of eq 12 obtained with it explain why the peptide/glutathione mixed disulfide can also be generated during DsbA-catalyzed disulfide formation in P_{SH}^{SH}. Both reactions, to form P_S^S or P_{SH}^{SSG}, are predicted to occur in the DsbA/peptide mixed disulfide (eqs 11 and 12); P_S^S formation is predicted to occur with a first-order rate constant of (2.7 s^{-1}), whereas P_{SH}^{SSG} formation should occur at a rate proportional to the GSH concentration, with bimolecular rate constant 2.7 × 10^3 s^{-1} M^{-1} (eq 7). With GSH concentrations between 0.7 and 1.4 mM, the pseudo-first-order rate constant for the latter reaction will be 1.9–3.8 s^{-1}, so P_{SH}^{SSG} should be formed at a rate comparable to that of P_S^S. Therefore, P_{SH}^{SSG} will appear to be an intermediate in the overall reaction, although it arises because GSH reacts with the true intermediate, the DsbA/peptide mixed disulfide. The P_{SH}^{SSG} generated in this way does not accumulate to high levels, because it is rapidly converted to P_S^S, either nonenzymatically (eq 1) or by reaction with DsbA (see below).

Catalysis of Intramolecular Peptide Disulfide Formation. DsbA was also found to catalyze the second step in peptide disulfide formation, intramolecular conversion of the P_{SH}^{SSG} mixed disulfides to P_S^S, in the absence of disulfide and thiol reagents. The mixed disulfide glutathione can be either displaced to form the peptide disulfide bond or transferred to the other cysteine residue, with the following rate constants in the absence of catalysts:



(13)

All of these reactions involve attack of the free cysteine thiol group on the mixed disulfide bond and differ only in which sulfur atom of the disulfide bond is attacked. Consequently, they all occur with similar rates in the absence of catalyst.

Catalysis of peptide disulfide formation was demonstrated with the acid-trapped glutathione mixed disulfides, P_{2SH}^{27SSG} and P_{2SSG}^{27SH}; the former was a 5-fold better substrate than the latter (Figure 6). In contrast, exchange of the mixed disulfide glutathione between the cysteine residues was not increased in rate by DsbA, and the extent of this competing reaction was diminished as a consequence of the increase in rate of formation of P_S^S. The experimental data (not shown) could be simulated with no effect of DsbA on the rate of interchange of the mixed disulfide glutathione.

The reactions undergone by DsbA can predict the mechanism and the rate of these DsbA-catalyzed reactions. Each P_{SH}^{SSG} mixed disulfide is predicted to react with $DsbA_{SH}^{SH}$, to form the DsbA/peptide mixed disulfide, which can then form the peptide disulfide bond. For example,

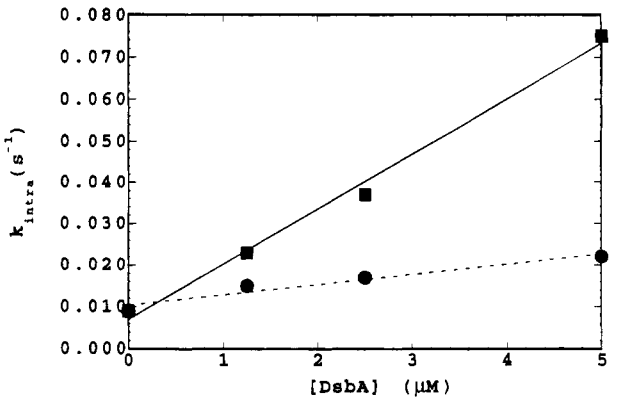
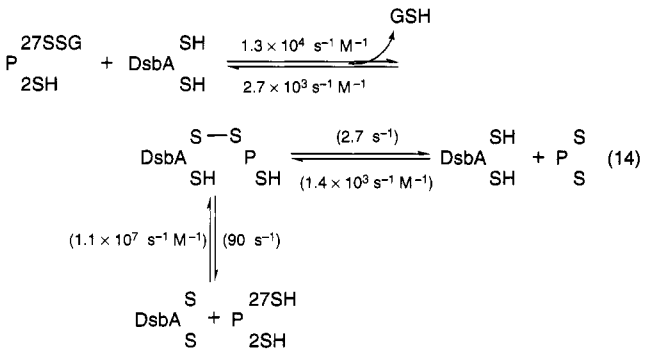


FIGURE 6: Catalysis by DsbA of the intramolecular reaction in the peptide mixed disulfide to form the peptide disulfide bond at pH 7.4. The individual mixed disulfide species P_{2SH}^{27SSG} (■) and P_{2SSG}^{27SH} (●) were acid trapped and purified by HPLC. Their intramolecular rearrangement of the mixed disulfide glutathione and formation of the peptide disulfide bond were followed as a function of time, in the absence of glutathione but in the presence of the indicated amounts of DsbA. The DsbA had been equilibrated initially with 0.5 mM GSSG and 1.4 mM GSH and isolated by gel filtration. The value of the rate constant for forming the peptide disulfide bond, k_{intra}, was obtained by simulation of the observed kinetics, on the basis that DsbA had no effect on the rate of the rearrangement between the two mixed disulfide forms (Table 1).



(14)

The rate constants are from eqs 11 and 12.

This mechanism accounts for the inability of DsbA to catalyze interchange of the glutathione mixed disulfide, as the glutathione is lost in the initial step. The rate-determining step would be the initial reaction between P_{SH}^{SSG} and $DsbA_{SH}^{SH}$ to form the mixed disulfide between them. The reverse step would be negligible in the absence of added GSH. The mixed disulfide intermediate would not be expected to accumulate to significant levels, because it would rapidly transfer the disulfide bond either to the peptide or to DsbA. $DsbA_S^S$ and P_{SH}^{SH} would be expected to be generated from the intermediate, but this reaction would be reversed rapidly. Consequently only small amounts of $DsbA_S^S$ and P_{SH}^{SH} should accumulate transiently; a maximum 2% of the P_{2SH}^{27SSG} should have accumulated transiently as P_{SH}^{SH} under the conditions in Figure 6, which would not be detectable. The primary products in the absence of GSH should be $DsbA_{SH}^{SH}$ and P_S^S, as observed.

The rate observed with P_{2SH}^{27SSG} (Figure 6) corresponds to a rate constant for reaction with $DsbA_{SH}^{SH}$ of 1.3 × 10^4 s^{-1} M^{-1}; this is exactly the rate measured with P_{2OH}^{27SSG} (eq 7). The slower rate observed with the other mixed disulfide indicates that it reacts more slowly with $DsbA_{SH}^{SH}$ and that there is some specificity in the reaction of DsbA with peptide cysteine residues.

Catalytic Activity of DsbA at Acidic pH. The thiol−disulfide exchange reaction is normally very slow at acidic pH, due to the very small quantities of the reactive ionized form of thiol groups. DsbA is surprisingly active at pH 4 (Wunderlich et al., 1993; Zapun et al., 1994), in part because its reactive Cys30 thiol group is ionized, having a very low pK_a value of about 3.5 (Nelson & Creighton, 1994). The rates of reaction between DsbA and glutathione are known at pH 4.0 (Nelson & Creighton, 1994); the greatest differences with neutral pH are the increased stability of the intramolecular and mixed disulfide bonds of DsbA and their somewhat slower reaction with external thiol groups, which are predominantly protonated at acidic pH. The catalytic actions of DsbA are usually much more apparent at acidic pH, and more amenable to study, because the noncatalyzed reactions are negligible.

The direct reactions between stoichiometric quantities of $DsbA_S^S$ and P_{SH}^{SH}, and the reverse between $DsbA_{SH}^{SH}$ and P_S^S, could be measured at pH 4, in the absence of GSH and GSSG (data not shown). Both appeared to be simple bimolecular reactions, and the mixed disulfide between them did not accumulate to significant levels. An equilibrium was reached that was fully consistent with the observed apparent rate constants:

$$DsbA_S^S + P_{SH}^{SH} \underset{1 \times 10^3\ s^{-1}\ M^{-1}}{\overset{2.5 \times 10^3\ s^{-1}\ M^{-1}}{\rightleftharpoons}} DsbA_{SH}^{SH} + P_S^S \quad (15)$$

These rates are compatible with those observed at pH 7.4 (eq 11); the forward rate constant is smaller at pH 4, due to decreased ionization of the peptide thiol groups; the rate is reduced by about the same extent as it is with GSH. The reverse rate constant is virtually the same, because the reactive Cys30 thiol group of DsbA is fully ionized at both pH values (Nelson & Creighton, 1994). The equilibrium constant of 2.5 indicates that the disulfide bonds of Dsb A_S^S and P_S^S have comparable free energies at pH 4. This is consistent with their similar overall equilibrium constants in reacting with GSH at pH 4: 1×10^{-2} M for DsbA (Nelson & Creighton, 1994) and about 3×10^{-2} M for the peptide (data not shown). These values would predict an equilibrium constant for eq 15 of 3, very close to the value of 2.5 measured directly. In contrast, this equilibrium constant has a value of (235) at pH 7.4 (eq 4); the pH dependence of the equilibrium and rate constants involving DsbA is due to the low pK_a value of Cys30 of $DsbA_{SH}^{SH}$ (Nelson & Creighton, 1994).

Noncatalyzed thiol−disulfide exchange reactions between the peptide and glutathione were negligible at pH 4, but occurred at substantial rates in the presence of small amounts of DsbA. Observations very similar to those presented here at pH 7.4 were made at pH 4 (data not shown), and the results were totally explicable on the same basis, differing quantitatively as expected from the different reactivities of DsbA at pH 4 (Nelson & Creighton, 1994).

DISCUSSION

DsbA is apparently able to catalyze the reactions between a model peptide and glutathione, similar to PDI under the same conditions (Darby et al., 1994). The action of PDI on a peptide in the presence of glutathione is probably of physiological significance, for GSH and GSSG concentrations like those used here are believed to be present in the endoplasmic reticulum (Hwang et al., 1992), where PDI functions. It is very unlikely, however, that DsbA encounters such levels of glutathione in the bacterial periplasm, which is connected to the cell exterior by pores that permit the passage of small molecules, so this situation is unlikely to be natural. Nevertheless, the comparison is useful for understanding the mechanisms of the two catalysts.

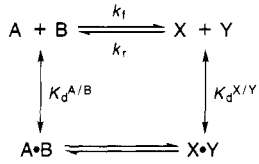
The relative simplicity of the DsbA molecule made it possible to characterize all its thiol−disulfide exchange reactions with the peptide and with glutathione. This information made it possible to predict the likely mechanism of action of small amounts of DsbA in the presence of both the peptide and glutathione, and these mechanisms could be tested by varying the GSSG and GSH concentrations individually (Figure 4) and by monitoring the thiol/disulfide state of the DsbA (Figure 5). The individual reactions could also predict with great accuracy the observed rate enhancements. These studies established that DsbA was not acting by catalyzing the direct reactions between the peptide and glutathione, but by reacting sequentially, first with one and then with the other (eq 14).

Interactions between DsbA and the Peptide. The catalytic activities of DsbA in the simultaneous presence of both glutathione and the peptide could be predicted from its individual reactions with each. This implies that the reaction of neither with DsbA was altered by the presence of the other, except when one of them was linked by a disulfide bond to DsbA and occupied its active site. Therefore, neither the peptide nor glutathione was likely to be bound substantially to the active site of DsbA in the absence of the disulfide link. There have been no indications that glutathione binds noncovalently to DsbA, at least with GSH and GSSG concentrations of up to 2 and 20 mM, respectively (Zapun et al., 1993).

Although noncovalent binding of the peptide to DsbA could not be directly demonstrated, there were numerous indications of additional binding interactions between DsbA and the peptide: (1) The $DsbA_{33SH}^{30S-S}P_{SH}$ mixed disulfide complex was 2500−4500-fold more stable than the $DsbA_{SH}^{SSG}$ mixed disulfide (eqs 6, 7, and 11). (2) The various forms of the peptide reacted with either $DsbA_{SH}^{SH}$ or $DsbA_S^S$ at rates 3×10^2 to 1.3×10^3-fold faster than did the corresponding forms of glutathione (eqs 6, 7, and 11). The larger the peptide, the faster the reaction (eqs 6 and 7). The reaction of $DsbA_S^S$ with P_{SH}^{SH} occurred with a rate constant of ($3 \times 10^5\ s^{-1}\ M^{-1}$), greater than the $5 \times 10^3\ s^{-1}\ M^{-1}$ predicted from the thiol/disulfide chemistry of the active site of DsbA [Scheme VII of Zapun et al. (1993)]; even greater rate constants of $>3 \times 10^6\ s^{-1}\ M^{-1}$ have been measured with reduced proteins (Wunderlich et al., 1993; Zapun & Creighton, 1994). (3) The reaction of P_{SH}^{SH} with $DsbA_{33SH}^{30SSG}$ (eq 7) was an exception, occurring at nearly the same rate as that of GSH; this suggests that the glutathione bound covalently to the active site Cys30 of DsbA prevents the binding interactions between the peptide and DsbA. (4) Cys30 of $DsbA_{SH}^{SH}$ was greatly biased to attack the cysteine sulfur atom of P_{2OH}^{27SSG}, rather than the equivalent glutathione sulfur atom only 2 Å away (eq 7); this is explained most readily by stereochemical restrictions imposed by the structure of a complex between DsbA and the peptide in the transition state of the reaction. This is also apparent in the greater stability of the DsbA mixed disulfide with the peptide, over that with glutathione, being due to its faster rate of formation; both mixed disulfide forms of DsbA react with extraneous thiol

groups in the reverse reaction at similar rates (eqs 6, 7, and 11). It should not be surprising that DsbA interacts with a peptide, for its three-dimensional structure was notable in having a cleft for a potential binding site near the cysteine residues (Martin et al., 1993). Such binding interactions appear to be weak with this peptide; nevertheless, if the disulfide bond contributes to the strength of the binding interactions, the binding interactions must contribute to the stability of the disulfide bond, and to the same extent.

Increased Rates of Reaction Due to Binding Interactions. The effect of binding to increase the rate of reaction between two molecules has long been recognized [e.g., Page and Jencks (1971)], but no quantitative analysis is available. Consider a reaction between groups on molecules A and B that can occur either as a normal bimolecular reaction, with second-order rate constant k_f, or intramolecularly in a noncovalent complex, A•B, with dissociation constant $K_d^{A/B}$:



(16)

Association of the reactants and products is assumed to be rapid relative to the rate of the chemical reaction. The rate of the forward bimolecular reaction, in the absence of any association, will be given by

$$\text{rate}_{bi} = k_f[A]_t[B]_t \quad (17)$$

where the subscript t indicates the total concentration of each reactant. When association occurs, the intramolecular reaction in the complex is assumed to occur by the same mechanism, so its rate constant will be given by the normal bimolecular rate constant, k_f, multiplied by the effective concentration of the reactive groups within the complex, designated here as $C_{eff}^{A/B}$:

$$\text{rate}_{comp} = C_{eff}^{A/B}k_f[A•B] = \frac{C_{eff}^{A/B}}{K_d^{A/B}}k_f[A]_f[B]_f \quad (18)$$

where the subscript f indicates the concentration of free A or B. The free concentrations can be readily converted to total concentrations if one reactant, say A, is present at considerably greater concentrations than the other, so that $[A]_f = [A]_t$. In such a situation, A is analogous to a ligand and B to a protein or enzyme to which it binds. In this case, the rate of the reaction via the complex from eq 18 will be given by

$$\text{rate}_{comp} = C_{eff}^{A/B}k_f\frac{[A]_t[B]_t}{K_d^{A/B} + [A]_t} \quad (19)$$

This equation is analogous to the Michaelis–Menten equation of enzymology. As would be expected, the rate of the reaction on the complex is predicted to be increased by increasing the effective concentration of the reactive groups in the complex and, when the concentration of A is low, by decreasing the dissociation constant of the complex.

The effect of binding on the rate of the reaction is given by the ratio of this rate (eq 19) to that when there is no binding (eq 17):

$$\frac{\text{rate}_{comp}}{\text{rate}_{bi}} = \frac{C_{eff}^{A/B}}{K_d^{A/B} + [A]_t} \quad (20)$$

This equation demonstrates that binding will increase the rate of the reaction so long as the effective concentration of the reacting groups in the complex is greater than both the dissociation constant of the complex and the concentration of the excess reactant, A. The greatest rate enhancements are expected at low concentrations of A, below the dissociation constant of the complex, where the rate enhancement is expected to be $C_{eff}^{A/B}/K_d^{A/B}$. At higher concentrations of A, most of it is in the free form and the bimolecular reaction is the more rapid.

The increases in the rates of the reactions between DsbA and the peptide attributed to binding were factors of only 10^2 to 10^3. If the peptide was not bound noncovalently, the dissociation constant for such a putative complex must be greater than the concentration of peptide, 10^{-5} M ($K_d^{A/B} > [A]_t$), and the increased rate due to binding will be given by $C_{eff}^{A/B}/K_d^{A/B}$. In this case, a rate enhancement of 10^2-10^3 fold requires that the effective concentrations of the thiol and disulfide groups in the noncovalent DsbA/peptide complex be only 10^2-10^3-fold greater than the dissociation constant of the complex. If the latter is $>10^{-5}$ M, the value of $C_{eff}^{A/B}$ need only be $>10^{-2}$ M. Effective concentrations of up to 10^{10} M are possible, and observed, when the reacting groups are held in the appropriate proximity and orientation for reacting (Page & Jencks, 1971); values of 10^2-10^5 M have been measured for thiol–disulfide exchange reactions in folded proteins (Creighton & Goldenberg, 1984). Therefore, the DsbA/peptide noncovalent complex need not be very stable to produce the observed rate enhancements and could have values of K_d too large to measure.

Implications for the Mechanism of Action of PDI. The active site of DsbA appears to be designed to cause thiol–disulfide exchange reactions to occur there more rapidly than in solution, even with a nonbinding substrate like glutathione; the structure of DsbA presumably stabilizes the transition state for the reaction (Nelson & Creighton, 1994). This rapidity of the reactions of DsbA with the peptide and with glutathione could account quantitatively for the ability of DsbA in effect to catalyze the reactions between the model peptide and glutathione. Although DsbA appeared to be catalyzing the direct reactions between P_{SH}^{SH} and GSSG (eq 1), it was actually providing alternative, more rapid mechanisms in which $DsbA_S^S$ first reacted with P_{SH}^{SH}, to generate $DsbA_{SH}^{SH}$ and P_S^S, and then $DsbA_{SH}^{SH}$ reacted with GSSG to regenerate $DsbA_S^S$. The second step was rate limiting, which explains why the catalyzed rate was proportional to the concentration of GSSG, but independent of the concentration of peptide. Nevertheless, all the kinetic data could have been simulated on the assumption that DsbA was acting like an enzyme and catalyzing the direct reactions between glutathione and the peptide. It would have been necessary only to assume that the DsbA was saturated with bound peptide. Only by characterizing all the direct reactions between DsbA, the peptide, and glutathione and by monitoring the state of DsbA (Figure 5) could the actual mechanism be elucidated.

Similar observations had been made previously on the catalytic actions of PDI (Darby et al., 1994). In that case, the PDI was concluded to be saturated with bound peptide (either reduced or as a mixed disulfide with glutathione) and

Table 1: Rate Enhancements of DsbA and PDI on the Rates of Disulfide Bond Formation in 10 μM Peptide by Reaction with Glutathione at pH 7.4 and 25 °C

obsd reaction	noncatalyzed rate constant	rel rate produced by[a] 1.3 μM DsbA	1.6 μM PDI[b]	rel catalytic enhancement, DsbA/PDI[c]
	$P_{SH}^{SH} + GSSG \leftrightarrow P_{SH}^{SSG_d} + GSH$			
forward	3.5 s^{-1} M^{-1}	2.5×[e]	10×	0.41
reverse	1.0 s^{-1} M^{-1}			
	$P_{SH}^{SSG_d} \leftrightarrow P_S^S + GSH$			
forward	0.011 s^{-1}	1.7×[e]	35×	0.05
reverse	2 s^{-1} M^{-1}			
	$P_{2OH}^{27SH} + GSSG \leftrightarrow P_{2OH}^{27SSG} + GSH$			
forward	3.0 s^{-1} M^{-1}	2.5×	10×	0.41
reverse	1.5 s^{-1} M^{-1}			
	$P_{2SH}^{27SSG} \leftrightarrow P_{2SSG}^{27SH}$			
forward	0.009 s^{-1}	1.0×	1.0×	
reverse	0.004 s^{-1}			
	$P_{2SH}^{27SSG} \leftrightarrow P_S^S + GSH$			
forward	0.009 s^{-1}	2.7×	44×	0.10
	$P_{2SSG}^{27SH} \leftrightarrow P_S^S + GSH$			
forward	0.009 s^{-1}	1.6×	37×	0.04

[a] The relative rate of the apparent reaction in the presence of the catalysts is indicated, even though the mechanism of the catalyzed reaction is very different. [b] Concentration of PDI monomer; from Darby et al. (1994). [c] Ratio of catalytic enhancements per active site, assuming that PDI has two active sites per monomer. [d] Considering the two cysteine residues of the peptide together. [e] Calculated from the effects of DsbA on the individual reactions.

to have a K_m of less than 3 μM, because the catalyzed velocity of the presumed reaction between the peptide and glutathione was independent of the peptide concentration. This conclusion is unlikely to be correct if PDI was acting similarly to DsbA, where the velocities were also independent of the concentration of the peptide. The independence of the rate on the peptide concentration would have resulted because the peptide was not involved in the rate-determining step. The potential complexity of the reaction mechanism indicates the need for care in extracting kinetic parameters in circumstances where the mechanism of the catalyzed reaction has not been determined.

The relative effects of DsbA and PDI on the peptide under the same conditions are compared in Table 1. The greatest difference between them is that the effects of PDI were somewhat greater in magnitude than those of DsbA. Detailed comparison is not yet possible, however, for the mechanism of action of PDI on the model peptide has not yet been elucidated, as the detailed properties of its active site disulfide bonds remain unclear (Darby et al., 1994); different steps may be rate limiting with the two catalysts. For example, it seemed likely that catalysis of the intramolecular step in forming the peptide disulfide bond occurred through a mixed disulfide between PDI and the peptide, and this mechanism was established here for DsbA (eq 14). However, the initial reaction between P_{SH}^{SSG} and $DsbA_{SH}^{SH}$ was rate limiting in eq 14, but the second step appeared to be rate limiting with PDI (Darby et al., 1994).

The catalytic activity of PDI in incorporating a disulfide bond into the reduced peptide was consistently 2.5-fold greater than that of DsbA, assuming that both active sites of PDI were active (Table 1). The difference in catalytic activities was much greater in the case of the intramolecular step of converting P_{SH}^{SSG} to P_S^S, being 10−25-fold greater with PDI than with DsbA. The greater activity of PDI correlates well with its much greater activity than DsbA of catalyzing disulfide rearrangements during protein folding (Zapun & Creighton, 1994). The two reactions are closely related, and they probably occur by similar mechanisms. Reduced forms of PDI or DsbA will initially react with one of the protein disulfide bonds, to form a mixed disulfide with one of the cysteine residues. In the case of a protein with several cysteine residues, they will all remain part of the same molecule and those with a free thiol group will be capable of reacting with this mixed disulfide bond, to regenerate a protein disulfide bond. Disulfide rearrangement will have occurred only if the cysteine thiol group that reacts is different from the one displaced from the original disulfide bond. A greater activity of PDI than DsbA in rearranging the P_{SH}^{SSG} disulfide bond would be expected to produce a greater activity in rearranging protein disulfide bonds also. PDI would also be expected to have an even greater advantage when all the original protein cysteine residues were paired in disulfide bonds. In this case, disulfide rearrangements require that a second disulfide bond be rearranged in the putative mixed disulfide with the catalyst; PDI has two active sites per monomer (Edman et al., 1985), and the more active DsbC is a dimer (Zapun et al. 1995).

Effects on Protein Conformational Transitions. Do PDI and DsbA participate directly in the polypeptide conformational changes that are involved in making and breaking disulfide bonds? This can now be answered in the case of DsbA. The relevant step is the intramolecular formation of the peptide disulfide bond from the mixed disulfide with DsbA; this has a rate constant of (2.7 s^{-1}), which is 250 times greater than that with the peptide mixed disulfide with glutathione, 0.011 s^{-1} (eq 1; Table 1). Is this just a consequence of the more rapid thiol−disulfide exchange reactions that occur in the active site of DsbA? This can be answered by comparing this difference in intramolecular rates to the corresponding difference when an extraneous thiol, such as GSH, reacts. The reactions of GSH with $DsbA_{SH}^{S-S}P_{SH}$ and with P_{SH}^{SSG} occur with rate constants of 2.7×10^3 s^{-1} M^{-1} (eq 7) and 1.0 s^{-1} M^{-1} (eq 1), respectively, a factor of 2.7×10^3 different. This is greater than the 250-fold difference in forming the peptide disulfide bond. Therefore, the greater reactivity of the $DsbA_{SH}^{S-S}P_{SH}$ mixed disulfide was even more pronounced in the intermolecular reaction with GSH than with the intramolecular peptide reaction. This indicates that the reaction with a second cysteine residue of a bound peptide is not favored by the DsbA active site, but is actually diminished by a factor of about 10, relative to the reaction with external GSH. This comparison can also be expressed as the effective concentration of the two cysteine residues in the peptide being 11 mM [=0.011 s^{-1}/1.0 s^{-1} M^{-1}] in solution and only 1 mM [=(2.7 s^{-1})/(2.7×10^3) s^{-1} M^{-1}] when bound to DsbA. This suggests that DsbA does not tend to bring together the two cysteine residues of a bound peptide. In contrast, the rate of the reaction on DsbA is somewhat lower than might be expected; presumably, the structure of the complex inhibits sterically the reaction of the thiol group of the second cysteine residue with the DsbA/peptide mixed disulfide.

Taken altogether, the results obtained thus far indicate that the catalytic activities of DsbA and PDI in protein disulfide bond formation and rearrangement can be understood, at least

to a first approximation, in their binding of peptide and protein substrates and in their rapid participation in thiol−disulfide exchange reactions.

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