Effects of DsbA on the Disulfide Folding of Bovine Pancreatic Trypsin Inhibitor and α-Lactalbumin

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ABSTRACT: DsbA is a protein found in the periplasm of *Escherichia coli* that is required for the formation of disulfide bonds in secreted proteins. It contains only two cysteine residues, which can form reversibly a very unstable disulfide bond that has been proposed to be the oxidant that introduces disulfide bonds into secreted proteins. The present study investigates the effect of DsbA on the well-characterized disulfide-coupled refolding processes of BPTI and of α-lactalbumin. Disulfide-bonded DsbA in stoichiometric amounts proved to be a very potent donor of disulfide bonds to reduced BPTI but showed little catalytic activity at neutral pH in the presence of a glutathione redox buffer. In contrast to the related eukaryotic enzyme protein disulfide isomerase, DsbA did not substantially catalyze the usual intramolecular disulfide bond rearrangements of quasi-native folding intermediates of BPTI. Neither did DsbA catalyze the intramolecular rearrangements observed in the three disulfide-bonded "molten globule" form of α-lactalbumin at neutral pH. Thiol–disulfide exchange is normally very slow at acidic pH but occurs rapidly with DsbA; consequently, DsbA catalyzed the disulfide folding of BPTI under acidic conditions. It was then possible to detect some increase in the rates of disulfide rearrangements, but only with stoichiometric amounts of DsbA and on the hour time scale. These results suggest that the primary role of DsbA in the bacterial periplasm is to introduce disulfide bonds into newly secreted proteins.

The *Escherichia coli* gene *dsbA* (Bardwell et al., 1991; Kamitani et al., 1992) and its homologues in other microorganisms (Tomb, 1992; Yu et al., 1992) are required for efficient disulfide bond formation in secreted proteins [reviewed by Bardwell and Beckwith (1993)]. The gene product, DsbA, is a soluble, monomeric protein of 21 kDa that is found in the periplasm. It contains only two cysteine residues, in the sequence Cys30-His-Pro-Cys33, which can reversibly form a very unstable disulfide bond (Wunderlich & Glockshuber, 1993a; Zapun et al., 1993). Similar Cys-X-Y-Cys sequences are found in other structurally related thiol–disulfide oxidoreductase proteins, such as thioredoxin, glutaredoxin, and eukaryotic protein disulfide isomerase (PDI)[1] (Martin et al., 1993). The reduced and disulfide forms of DsbA are designated here $DsbA^{SH}_{SH}$ and $DsbA^{S}_{S}$, respectively.

In vitro studies have revealed the high oxidative power of Dsb^{S}_{S}, which is a consequence of the instability of its disulfide bond. The equilibrium constant for formation of the disulfide bond of $DsbA^{S}_{S}$ from that of GSSG is only 8×10^{-5} M at pH 7.5 (Zapun et al., 1993), a value 5 orders of magnitude lower than that of thioredoxin (Holmgren, 1981). The disulfide bond of $DsbA^{S}_{S}$ is more stable in the unfolded state than in the folded state. Consequently, the disulfide bond of DsbA destabilizes the folded conformation (Zapun et al., 1993; Wunderlich et al., 1993a). This contrasts with the stabilizing effect of most protein disulfide bonds, including that of thioredoxin (Kelley et al., 1987).

Of the two thiol groups of $DsbA^{SH}_{SH}$, that of Cys30 is solvent-exposed, reactive to alkylating reagents (Zapun et al., 1993, 1994), and fully ionized at pH values down to 4.0 (Nelson & Creighton, 1994). Only the Cys30 thiol group forms a mixed disulfide with glutathione, as the Cys33 thiol group is buried and generally unreactive (Zapun et al., 1993, 1994). The high reactivity of the disulfide bond of DsbA to external thiols, and that of its mixed disulfide to attack by either an external thiol group or that of Cys33, are features that make DsbA well suited to introduce disulfide bonds into proteins (Zapun et al., 1993, 1994). The kinetics of reaction of DsbA with glutathione were used to predict the likely reaction of DsbA with the cysteine residues of a protein or peptide (Zapun et al., 1993), but it is also possible that DsbA interacts more specifically with peptides and proteins and that it also catalyzes disulfide rearrangements.

Catalysts of protein thiol–disulfide exchange such as PDI and DsbA might potentially work in two different ways. They might function at catalytic levels (i.e., much less than stoichiometric to the substrate cysteine residues) in altering the rates of the chemical reaction between the protein and a thiol–disulfide reagent such as glutathione or DTT. The effect of a catalyst can be observed only if its contribution to the rate is at least comparable to the uncatalyzed rate, so the absence of an observable effect of DsbA on a particular step may be due to the rapidity of the uncatalyzed reaction. Also, a catalyst cannot increase the rate of a reaction in only one direction, so the absence of an effect in one direction of a reaction precludes an effect in the reverse direction.

Alternatively, the function of catalysts can be examined in the absence of such a reagent, but using stoichiometric or greater concentrations, when they are in effect the thiol or disulfide reagent. In the latter case, the initial form of the DsbA or PDI must be considered, i.e., whether the Cys-X-Y-Cys residues are in the dithiol or disulfide form. When

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[1] Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; CD, circular dichroism; $DsbA^{SH}_{SH}$ and $DsbA^{S}_{S}$, the protein DsbA, with free Cys30 and Cys33 thiol groups and with a disulfide bond between them, respectively; DTT, dithiothreitol; GSH and GSSG, the reduced and oxidized forms of glutathione, respectively; HPLC, high-pressure liquid chromatography; αLA, α-lactalbumin; N, native three disulfide-bonded BPTI; PDI, protein disulfide isomerase; R, fully reduced, unfolded BPTI; RNase, ribonuclease; UV, ultraviolet; 3SS-αLA, αLA with the disulfide bond between Cys6 and Cys120 reduced; [3SS]-αLA, 3SS-αLA in which the disulfide bonds have rearranged under conditions where the molten globule state is populated.

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catalytic amounts are used in the presence of greater concentrations of thiol–disulfide reagent, the thiol–disulfide status of the catalyst is generally determined by that of the reagent acting as a redox buffer, unless equilibration of the catalyst and the reagent is especially slow.

Two functions of PDI have been described. One is to catalyze the intramolecular rearrangement of protein disulfide bonds (Givol et al., 1964; Venetianer & Straub; 1964). The other is to catalyze the net formation or breakage of protein disulfide bonds (Creighton et al., 1980). The former usually requires transient breakage of at least one of the protein disulfide bonds, and the thiol form of a catalyst such as PDI is therefore required for this activity. On the other hand, the net formation of protein disulfide bonds is likely to require, at least transiently, a disulfide form of the catalyst, either the active site intramolecular disulfide or a mixed disulfide between the catalyst and another molecule like glutathione. The relative simplicity of the DsbA system and its known rate and equilibrium constants for reaction with GSH or GSSG (Zapun et al., 1993, 1994) make it possible to control its thiol–disulfide status.

The effects of DsbA on thiol–disulfide exchange reactions involving a variety of proteins have been studied *in vitro*. DsbA was shown to catalyze the reduction of insulin by DTT (Bardwell et al., 1991), a capability retained by a variant of DsbA lacking the thiol group of Cys33 (Zapun et al., 1994). When present in excess, DsbA was able to stimulate the regeneration of active RNase A from the scrambled form in the presence of GSH (Akiyama et al., 1992) or DTT (Yu et al., 1993), plus the oxidative refolding of RNase A and of alkaline phosphatase in the presence of GSSG (Akiyama & Ito, 1993). $DsbA^S_S$ was also shown to be a very efficient oxidant of the cysteine residues of reduced hirudin, when used in stoichiometric amounts, to incorporate many different disulfide bonds; it also appeared to catalyze the subsequent disulfide rearrangements (Wunderlich et al., 1993b). Catalysis by small amounts of DsbA of the disulfide refolding of hirudin in the presence of a glutathione redox buffer was found to be only marginal at alkaline pH but surprisingly efficient under acidic conditions (Wunderlich et al., 1993b). These observations have caused some to consider DsbA as the bacterial protein disulfide isomerase. In none of these cases is the complex overall process of disulfide bond formation and folding well characterized, however, so it is difficult to infer exactly what DsbA is or is not doing.

The present study examined the effect of DsbA on the disulfide refolding of bovine pancreatic trypsin inhibitor (BPTI) and α-lactalbumin (αLA), which have been extensively characterized *in vitro* (Creighton, 1978; Creighton & Goldenberg, 1984; Darby & Creighton, 1993; Ewbank & Creighton, 1993a,b). Although BPTI and αLA are eukaryotic and not normal substrates for DsbA, BPTI has been observed to fold and to acquire rapidly its three correct disulfide bonds when expressed in *E. coli* and secreted into the periplasmic space (Marks et al., 1986; Goldenberg, 1988; M. Ostermeier and G. Georgiou, personal communication). The effect of eukaryotic PDI on the disulfide bond-coupled refolding of BPTI has been investigated (Creighton et al., 1980, 1993; Zapun et al., 1992; Weissman & Kim, 1993), so it was pertinent to compare the effects of DsbA.

The present study reveals important differences in the activity of DsbA and PDI, which probably reflect the different environments in which they normally function.

EXPERIMENTAL PROCEDURES

Proteins and Chemicals. GSH and GSSG from Sigma were used without further purification. All other chemicals were of reagent grade or better.

$DsbA^S_S$ was obtained as described previously (Zapun et al., 1993). $DsbA^{SH}_{SH}$ was prepared by incubation of $DsbA^S_S$ with 10 mM DTT in 50 mM MOPS (pH 7) prior to desalting by gel filtration on a Sephadex G-25 (Pharmacia) column equilibrated with 10 mM HCl.

BPTI (Trasylol) was a generous gift of Bayer AG. Bovine α-lactalbumin type III (calcium depleted) was from Sigma. PDI purified from bovine liver was a generous gift from R. B. Freedman (University of Kent, Canterbury, U.K.).

BPTI Refolding. Reduced BPTI was prepared by incubation of native BPTI (Trasylol) with 50 mM DTT in 6 M guanidinium chloride and 0.1 M Tris-HCl (pH 8) and desalted by reversed-phase HPLC. Aliquots were freeze-dried, kept under nitrogen at −80 °C, and dissolved in 10 mM HCl before use. Refolding and thiol–disulfide exchange reactions were carried out at 25 °C in either 0.2 M KCl, 0.1 M Tris-HCl (pH 7.5), and 1 mM EDTA or 0.2 M KCl and 0.1 M sodium acetate (pH 4.0), in the presence or absence of DsbA, with or without a glutathione redox buffer. The reactions were initiated by the addition of reduced BPTI or the appropriate isolated intermediate. At various time intervals, 200 μL of the reaction mixture was withdrawn and acid-quenched by addition to 50 μL of 0.2 or 0.1 M HCl for samples originally at pH 7.5 and 4.0, respectively, to lower the pH to ≈2. A very low pH is necessary to quench the thiol–disulfide exchange reactions that involve DsbA because it remains active under fairly acidic conditions (see Results). At pH 2, DsbA is unfolded and unreactive (Nelson & Creighton, 1994). A lower pH of quenching, however, resulted in a slow degradation of DsbA that generated additional peaks in the HPLC chromatograms. Samples were stored at 1 °C prior to loading on HPLC.

The acid-trapped samples were analyzed by HPLC on a Vydac C_{18} reversed-phase column, 0.45 × 25 cm, monitoring the absorbance at 220 nm, as described previously (Creighton et al., 1993). In experiments where the redox state of DsbA was also monitored, a fraction of the quenched mixture was further diluted 10-fold with 10 mM HCl and analyzed on the same column with a gradient of 44–50% (v/v) solvent B in 24 min. Solvent A was 0.1% (v/v) trifluoroacetic acid, and solvent B was 90% (v/v) acetonitrile/0.1% (v/v) trifluoroacetic acid.

Preparation of Folding Intermediates of BPTI. The folding intermediates (30–51), $(30–51,14–38)_N$, and $(5–55,14–38)_N$ were prepared by carrying out a refolding of reduced BPTI at pH 7.5 in the presence of 0.5 mM GSSG and 2 mM GSH. The reaction was quenched after 2 min to isolate (30–51) or after 10 min to isolate $(30–51,14–38)_N$ and $(5–55,14–38)_N$. The species were separated by reversed-phase HPLC, and the collected fractions were freeze-dried and stored like reduced BPTI. Aliquots were dissolved in 10 mM HCl before use.

α-Lactalbumin Rearrangements. The procedures were essentially those of Ewbank and Creighton (1993a). 3SS-αLA, in which the Cys6–120 disulfide bond is reduced, was prepared by incubating 100 μM αLA with 0.8 mM DTT and 250 μM $CaCl_2$ in 0.1 M Tris-HCl (pH 7.5) and 0.2 M KCl for 15 min at 25 °C. The disulfide-rearranged mixture, [3SS]-αLA, was prepared by dilution of the 3SS-αLA incubation mixture with $^3/_2$ volume of 10 mM EDTA in the same buffer for 10 min at 25 °C. Reduced DsbA and PDI were prepared just before use by incubation with 10 mM DTT. Each of

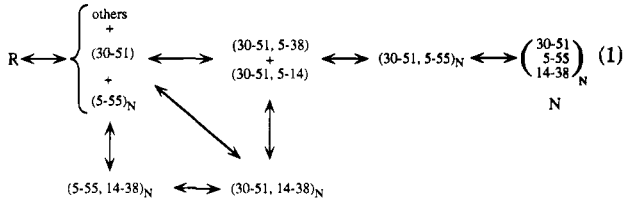
these proteins was isolated by gel filtration on a Sephadex G-25 (Pharmacia) column equilibrated with the appropriate buffer: 0.2 M KCl containing either 0.1 M sodium acetate (pH 4.0) or 0.1 M Tris-HCl (pH 7.5).

The 3SS-αLA to [3SS]-αLA transition at pH 4.0 was initiated by the addition of 3SS-αLA to a mixture containing EDTA or $CaCl_2$, with or without added $DsbA^{SH}_{SH}$ or DTT. Similarly, the rearrangement of [3SS]-αLA to 3SS at pH 4.0 or 7.5 was initiated by the addition of [3SS]-αLA to a mixture containing $CaCl_2$, with or without added reduced PDI, $DsbA^{SH}_{SH}$, or DTT. The reactions were quenched by the addition of $^3/_2$ volume of 30 mM HCl (to pH $\approx$2). The samples were analyzed by revered-phase HPLC on a Dynamax 300-A C_4 column, 0.46 × 25 cm, using a linear gradient of 25–50% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid, as described previously (Ewbank & Creighton, 1993a).

Carboxyamidomethylated 3SS-αLA, designated $3SS_{cam}$, was prepared, and CD spectra were recorded, as described by Ewbank and Creighton (1993b).

RESULTS

The disulfide folding pathway of BPTI can be summarized as



This pathway was determined by extensive kinetic analysis (Creighton, 1977a; Creighton & Goldenberg, 1984; Darby & Creighton, 1993) and is fully consistent with all of the available experimental evidence. R is the fully reduced and unfolded protein with six free thiols. The intermediate species are depicted by the residue numbers of the cysteine residues paired in disulfide bonds. Those that adopt the fully native-like conformation are denoted by the subscript N.

The first disulfide bond is formed randomly in a statistical manner (Darby & Creighton, 1993). The resulting one-disulfide species are, however, in rapid equilibrium, and (30–51) predominates, because it is stabilized by some structural native-like interactions (van Mierlo et al., 1992, 1993). In (30–51), Cys5, Cys14, and Cys38 are in flexible and unstructured parts of the molecule, and they can react to form any of the three possible disulfide bonds between them, to produce intermediates (30–51,5–14), (30–51,5–38), and (30–51,14–38)$_N$. The disulfide bond 5–55 is not formed readily in (30–51) or (30–51,14–38)$_N$. Instead, the latter species is in rapid equilibrium by intramolecular disulfide rearrangements with the (30–51,5–14) and (30–51,5–38) intermediates, which then rearrange intramolecularly to form (30–51,5–55)$_N$. The 14–38 disulfide bond is readily made in this last intermediate to form fully native BPTI.

The native conformation of BPTI is stable with any two of the native disulfide bonds (van Mierlo et al., 1991a), and the quasi-native species (5–55,14–38)$_N$ and (30–51,14–38)$_N$ are the predominant species during refolding at neutral pH. Even the intermediate (5–55)$_N$, with the disulfide bond that stabilizes the native conformation most, is relatively stable at neutral pH, where it tends to adopt a quasi-native conformation (van Mierlo et al., 1991b). Consequently, (5–55)$_N$ very rapidly forms the 14–38 disulfide bond, to generate the more stable

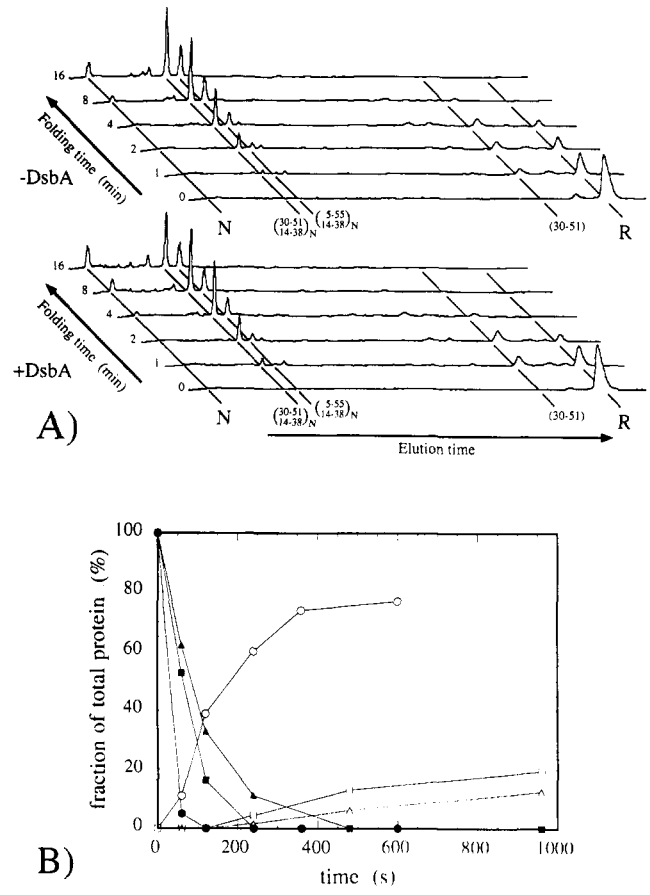


FIGURE 1: Effects of DsbA and PDI on the refolding of reduced BPTI at pH 7.5, 25 °C, with 0.5 mM GSSG and 2 mM GSH. The concentration of BPTI was 9.6 μM in the experiment with PDI, 12 μM in the others. The reaction was quenched by acidification at various times. (A) Reversed phase HPLC analysis of the species trapped in the absence and presence of 8 μM DsbA. (B) Quantification by integration of the absorbance peaks of the native, N (open symbols), and reduced, R (solid symbols), forms of BPTI: (△, ▲) in the absence of any catalyst; (□, ■) in the presence of 8 μM DsbA; (○, ●) in the presence of 1.6 μM PDI dimer. In both cases, the ratio of cysteine residues of BPTI to those of DsbA or to those of the two presumed active sites of PDI was 6:1. The data of refolding in the presence of PDI are from Creighton et al. (1993).

quasi-native (5–55,14–38)$_N$ which has the thiol groups of Cys30 and Cys51 buried in the hydrophobic core and unreactive (Creighton & Goldenberg, 1984; States et al., 1984). The exact mechanism of rearrangement of (5–55,14–38)$_N$ to (30–51,5–55)$_N$ is not known.

Catalytic Effects on the Refolding of BPTI. The effects of catalytic amounts of DsbA on the disulfide refolding of BPTI in the presence of 0.5 mM GSSG and 2 mM GSH were examined at pH 7.5 and 25 °C and compared to those observed previously with mammalian PDI under similar conditions (Creighton et al., 1980, 1993). The concentrations of reduced BPTI and of the Cys-X-Y-Cys active sites of DsbA and PDI were almost identical in each case. With these concentrations of GSH and GSSG, DsbA exists at equilibrium as 99% $DsbA^{SH}_{SH}$ and 1% $DsbA^{S}_{SO}$. The overall equilibration with glutathione in these experimental conditions should occur with a half-time of about 0.3 s (Zapun et al., 1993).

Very similar intermediates and time courses were observed with and without DsbA. Figure 1A compares HPLC separations of the various BPTI disulfide species in the presence and absence of DsbA. Under these conditions, the predominant intermediates are (30–51) and the quasi-native (30–51,14–38)$_N$ and (5–55,14–38)$_N$; HPLC separations emphasize these

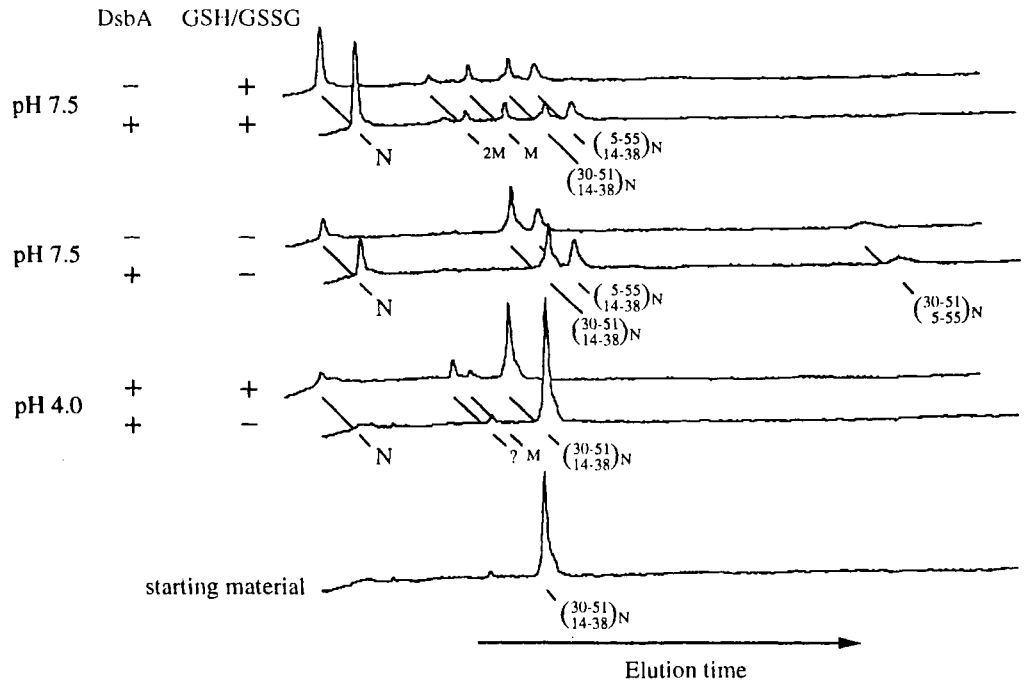


FIGURE 2: Effect of DsbA on the isolated BPTI intermediate (30–51, 14–38)$_N$. Five micromolar (30–51, 14–38)$_N$ was incubated for 2 h at neutral or acidic pH, with or without 0.5 mM GSSG and 2 mM GSH, in the presence or absence of 5 μM DsbA. When glutathione was omitted, DsbA was in the reduced form. The reaction was acid-quenched, and HPLC elution profiles of the resulting mixtures are shown. Their molecular weights indicated that M and 2M are single and double mixed disulfide species between the two free thiol groups of (30–51, 14–38)$_N$ and glutathione. The small peak generated at acid pH and labeled "?" had the molecular weight of normal BPTI and therefore is probably a disulfide isomer, but it could not be identified.

predominant intermediates, as the others that are substantially populated collectively, but not individually, are spread out over the elution profile. Figure 1B compares the kinetics of disappearance of the fully reduced unfolded BPTI (R) and the appearance of the native three disulfide-bonded proteins (N) in the absence and presence of either DsbA or PDI. The half-time for the disappearance of R was about 80 s in the absence of any catalyst. This was decreased substantially by PDI, to less than 25 s, and less so by DsbA, to 65 s.

The differences between PDI and DsbA were much more apparent in the rate of appearance of native BPTI. Only about 13% of the BPTI was fully refolded after 16 min in the absence of a catalyst, primarily because of the stabilities of the quasi-native species (30–51,14–38)$_N$ and (5–55,14–38)$_N$. In the presence of PDI, about 50% of the BPTI molecules were fully refolded after only 3 min (Creighton et al., 1993), representing a 27-fold increase in rate. In contrast, DsbA produced only a marginal, although real, 1.5-fold rate enhancement, with about 20% of the BPTI being in the native form after 16 min. The overall rate enhancement provided by PDI resulted both from faster disulfide bond formation (as reflected in the faster disappearance of R) and from faster intramolecular disulfide rearrangement of the quasi-native intermeidate (30–51,14–38)$_N$ and, to a lesser extent, of (5–55,14–38)$_N$.

Intermediate (30–51) both appeared and disappeared more rapidly in the presence of DsbA, indicating that DsbA was catalyzing disulfide bond formation. The quasi-native intermediates (30–51,14–38)$_N$ and (5–55,14–38)$_N$ were also formed somewhat more quickly in the presence of DsbA, but only (30–51,14–38)$_N$ seemed to disappear at an enhanced rate, albeit only slightly. This could be due to DsbA catalyzing either disulfide rearrangements of this intermediate or direct formation of the 5–55 disulfide bond.

To test more sensitively for an effect of DsbA on the rates of intramolecular disulfide rearrangements, isolated (30–51, 14–38)$_N$ was examined in the absence of any reagent (Figure 2, middle). The intermediate spontaneously rearranged slowly to (30–51,5–55)$_N$ and to (5–55,14–38)$_N$, as expected. The reaction was monitored over 2 h, and some air oxidation occurred, as indicated by the small amount of native BPTI generated from the (30–51,5–55)$_N$ intermediate that was formed by disulfide rearrangements. Very little effect of DsbA$^{SH}_{SH}$ was detected, even with an equimolar amount and after 2 h of incubation; the rearrangements were not increased in rate, nor were any BPTI disulfide bonds reduced. Under the same conditions, isolated intermediate (5–55,14–38)$_N$ was stable in the absence or presence of DsbA and underwent no disulfide bond formation, breakage, or rearrangement (data not shown). DsbA$^{SH}_{SH}$ would not be expected to reduce the disulfide bonds of either of these quasi-native intermediates, as their disulfide bonds are considerably more stable than that of DsbA$^{S}_{S}$.

When isolated (30–51,14–38)$_N$ was incubated in the presence of GSH and GSSG, it underwent the same rearrangements, and at the same rate, as expected (Figure 2, top), but the (30–51,5–55)$_N$ that was generated then rapidly formed the 14–38 disulfide bond by reaction with GSSG, to complete refolding. Some mixed disulfides of (30–51,14–38)$_N$ with glutathione, presumably on Cys5 and Cys55, were also observed to form, confirming that these thiol groups are not inaccessible even in the quasi-native conformation of (30–51,14–38)$_N$ (Creighton, 1977a). The presence of an equimolar amount of DsbA produced only a slight increase in the amount of N generated, even after 2 h of incubation.

These observations indicate that DsbA does not catalyze efficiently the intramolecular disulfide bond rearrangments that BPTI undergoes in its refolding (Creighton & Goldenberg, 1984). The primary effect of small quantities of DsbA was to increase slightly the rates of disulfide bond formation. Under the same conditions, PDI substantially catalyzed both disulfide

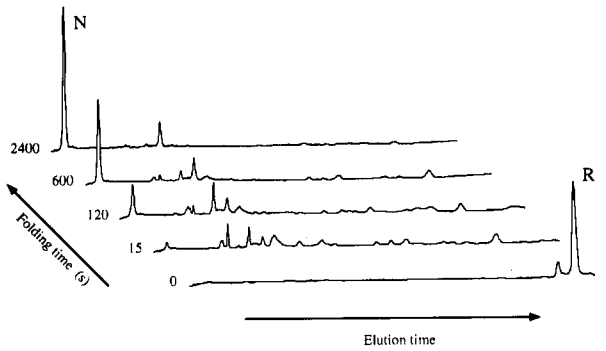


FIGURE 3: Refolding of reduced BPTI with $DsbA^S_S$ as oxidant. Reduced BPTI (25 μM) was mixed with 75 μM $DsbA^S_S$ at pH 7.5. Aliquots of the reaction mixture were acid-quenched at the various times and analyzed by reversed-phase HPLC. The only substantial peak other than N present after 2400 s is $(5–55, 14–38)_N$.

bond formation and rearrangement of the various intermediates (Figure 1; Creighton et al., 1980, 1993; Weissman & Kim, 1993).

Direct Effects of DsbA on the Refolding of BPTI. The above observations, plus the very high oxidative potential of $DsbA^S_S$ and the rapid rate of its thiol–disulfide exchange (Zapun et al., 1993), suggest that the primary function of DsbA is to introduce disulfide bonds directly into proteins. To test this, stoichiometric amounts of $DsbA^S_S$ were used as disulfide reagent to fold reduced BPTI (Figure 3). The reduced BPTI disappeared at a very rapid rate, which would correspond to a second-order rate constant greater than 4000 s^{-1} M^{-1}. Very many disulfide intermediates were generated initially, but native BPTI was generated more slowly and finally predominated, with only a minor amount of the protein being trapped in the two-disulfide form $(5–55,14–38)_N$. Rearrangements of the disulfide bonds of the initial intermediates must have been occurring, to lead primarily to these native-like forms. Such rearrangements of random one- and two-disulfide intermediates to the normal, most stable intermediates (30–51), $(30–51,14–38)_N$, $(30–51,5–55)_N$, and $(5–55,14–38)_N$ are known to be relatively rapid under these conditions (Creighton, 1977b), so there is no need to postulate that they were catalyzed by the DsbA. On the other hand, the final absence of the quasi-native intermediate $(30–51,14–38)_N$ in this experiment requires explanation.

One possibility is that $DsbA^S_S$ introduced the last disulfide bond directly into $(30–51,14–38)_N$. This was confirmed by mixing 5 μM $(30–51,14–38)_N$ (with an equimolar amount of $DsbA^S_S$. Three disulfide-bonded native BPTI, N, was produced rapidly, corresponding to a second-order rate constant of $k = 304 \pm 23$ s^{-1} M^{-1} for forming the 5–55 disulfide bond (Figure 4). A stoichiometric amount of $DsbA^{SH}_{SH}$ was generated, although N started to accumulate more rapidly than $DsbA^{SH}_{SH}$ at later times, probably due to air oxidation.

In the case of $(5–55,14–38)_N$ under the same conditions, no native BPTI or any other disulfide species was produced after 15 min of incubation (data not shown). Therefore, $DsbA^S_S$ was able to introduce the final disulfide bond directly into $(30–51,14–38)_N$, but not into $(5–55,14–38)_N$, which accounts for the accumulation in Figure 4 of the latter intermediate but not the former.

When 5 μM native BPTI was incubated with the same amount of $DsbA^{SH}_{SH}$, no reduction of the BPTI was detected after 2.5 h (data not shown). This was to be expected, for the disulfide bonds of folded BPTI are all at least 10^5 times more

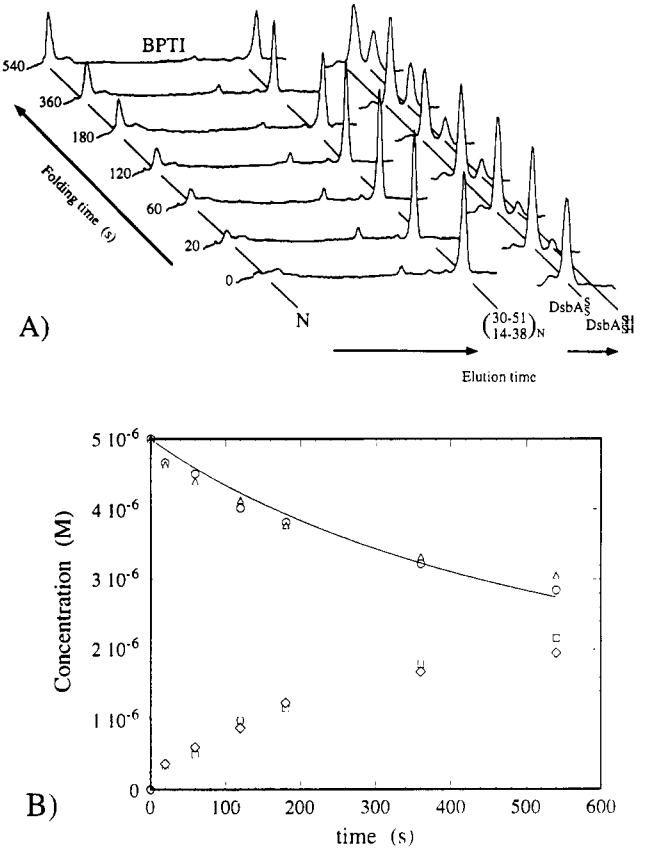


FIGURE 4: Direct oxidation of $(30–51, 14–38)_N$ (5 μM) to N-BPTI with 5 μM $DsbA^S_S$ at pH 7.5. (A) HPLC traces. (B) Quantification: (○) $(30–51, 14–38)_N$; (□) N; (△) $DsbA^S_S$; (◇) $DsbA^{SH}_{SH}$. The solid curve is that expected with a second-order rate constant of 304 s^{-1} M^{-1} for the reaction between $(30–51, 14–38)_N$ and $DsbA^S_S$, which was derived from a nonlinear least-squares fit to the data.

stable than is that of $DsbA^S_S$ (Creighton & Goldenberg, 1984).

Disulfide Bond Formation in BPTI at Acid pH. The surprising finding by Wunderlich et al. (1993b) that DsbA dramatically catalyzed the disulfide refolding of reduced hirudin in the presence of glutathione at pH 4.0, where normal thiol–disulfide exchange reactions are 3000-fold slower than at pH 7.5, prompted us to examine the refolding of reduced BPTI under similar circumstances. A major difference of DsbA at pH 4 is that its intramolecular disulfide bond and mixed disulfide with glutathione are substantially more stable than at pH 7.5 (Wunderlich & Glockshuber, 1993a; Nelson & Creighton, 1994). The equilibrium constant measured directly under similar conditions indicates that DsbA exists at equilibrium in the presence of 0.5 mM GSSG and 2 mM GSH at pH 4 as about 70% $DsbA^S_S$ and 5% mixed disulfide with glutathione. The half-time for reaction between DsbA and these concentrations of GSSG and GSH is 30 s (Nelson & Creighton, 1994).

Figure 5 shows the refolding of reduced BPTI at pH 4.0, with the same redox buffer and in the absence or presence of the same catalytic DsbA concentration as were used at neutral pH (Figure 1). Without DsbA, no reaction of reduced BPTI occurred on the hour time scale, as expected. In its presence, however, the reduced BPTI completely disappeared within 15 s. A broad spectrum of disulfide species was generated, which slowly evolved into native BPTI (N) and the quasi-native species $(30–51,14–38)_N$ and $(5–55,14–38)_N$. Three additional species were found to accumulate to a significant level, and two of them were shown by matrix-assisted laser desorption

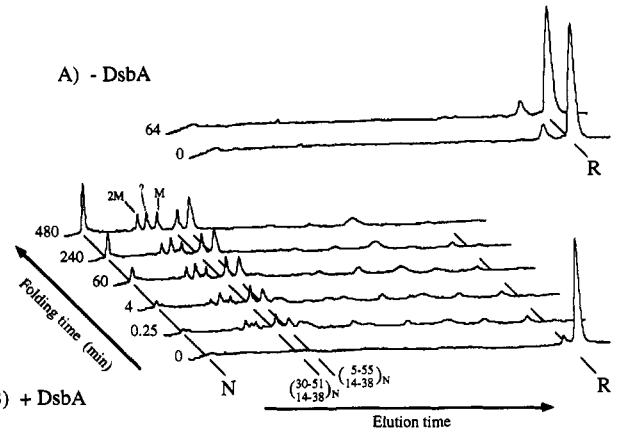


FIGURE 5: Refolding of BPTI at pH 4.0 in the presence of 0.5 mM GSSG and 2 mM GSH in the absence (A) and presence (B) of DsbA. The conditions were as in Figure 1, except that the buffer was 0.2 M KCl and 0.1 M sodium acetate (pH 4.0). The unidentified peak labeled "?" is apparently the same as that observed at acidic pH in Figure 2.

mass spectrometry to be single and double mixed disulfides with glutathione. Many of the BPTI species generated initially were spread out over the elution profile and only became evident as they were converted to a few predominant species. It was apparent that many species were formed very rapidly, perhaps almost randomly, and in very low individual amounts (there are 15 possible one-disulfide species, 45 two-disulfide species, and hundreds of mixed-disulfide species possible). Apparently, they subsequently rearranged slowly to more stable native-like species.

The uncatalyzed thiol–disulfide exchange reactions at both the one- and two-disulfide stage of BPTI refolding to the predominant intermediates occur on the second time scale at pH 8.7 (Creighton, 1977b) and would be expected to occur on the hour time scale at pH 4. That native and quasi-native species of BPTI resulted after a few hours does not require that their formation was being catalyzed by DsbA.

The effect of DsbA on the isolated stable intermediates was investigated at pH 4. In the absence of glutathione, DsbA^{SH}_{SH} did not cause any disulfide changes to $(30–51,14–38)_N$ (Figure 2, bottom). In the presence of GSH and GSSG, DsbA caused the slow formation of mixed disulfide species between glutathione and $(30–51,14–38)_N$, presumably involving Cys5 and Cys55. After 2 h of incubation with an equimolar concentration of DsbA, about 13% of the $(30–51,14–38)_N$ was found in a mixed disulfide form with glutathione and about 5% was converted to native BPTI.

To examine whether DsbA was able to catalyze disulfide rearrangements in less folded and stable species, similar experiments were performed with the isolated one-disulfide intermediate (30–51). When 3 μM (30–51) was incubated for 5 min with 0.8 μM DsbA in the glutathione redox buffer at pH 4.0, a spectrum of species was formed, including $(30–51,14–38)_N$ and $(30–51,5–55)_N$ (data not shown). When glutathione was omitted and DsbA^S_S was added in excess (10 μM), the same spectrum of species was obtained after 5 min of incubation. In contrast, no reaction occurred when DsbA^{SH}_{SH} was added in catalytic amounts (0.8 μM). The primary effects of DsbA^S_S appeared to be to introduce disulfide bonds directly into (30–51).

When 5 μM reduced BPTI was mixed with 15 μM DsbA^S_S at pH 4.0, the reduced form completely disappeared within 10 min as a result of disulfide bond formation (data not shown); the second-order rate constant was apparently greater than 3400 s^{-1} M^{-1}. The native form was generated only slowly, however, in contrast to what was observed at neutral pH. Only about 10% of the molecules were in the native form after 4 h, while most of the material was present initially as very many disulfide intermediates, which then became trapped in the native-like two-disulfide species. Such a situation could have resulted from either the reaction reaching an equilibrium or the subsequent steps being extremely slow. An equilibrium situation was not expected, but this was checked by incubating native BPTI (5 μM) for 2.5 h with an equimolar amount of DsbA^{SH}_{SH}. No reduction of BPTI was observed, as at pH 7.5 and as was expected. Furthermore, when $(30–51,14–38)_N$ was incubated with a 10-fold excess of DsbA^S_S, the last disulfide bond was introduced only extremely slowly, with an apparent second-order rate constant of 5 ± 1 s^{-1} M^{-1}. In the experiment with stoichiometric amounts of DsbA^S_S and reduced BPTI, the concentration of DsbA^S_S dropped rapidly as it reacted with the BPTI, so it is not surprising that the quasi-native two-disulfide intermediates accumulated; the rates of introduction of the last disulfide bonds were very slow.

Catalytic Effects of PDI and DsbA on the Rearrangements in α-Lactalbumin at Neutral pH. α-LA binds Ca^{2+} and contains four disulfide bonds, one of which is readily cleaved by DTT (Iyer & Klee, 1973; Kuwajima et al., 1990). The resulting three disulfide-bonded protein, with two free cysteine residues, is stable in the presence of Ca^{2+} and is designated 3SS-αLA. In the absence of Ca^{2+} (upon addition of EDTA), this species adopts a "molten globule" state (Kuwajima, 1989); the three disulfide bonds rearrange spontaneously, and a mixture of disulfide "scrambled" species is formed, collectively designated [3SS]-αLA, that tend to retain the molten globule conformation (Ewbank & Creighton, 1991, 1993a,b). Upon addition of an excess of Ca^{2+}, [3SS]-αLA rearranges back into 3SS-αLA; Ca^{2+} binding by 3SS-αLA is fast, and the intramolecular disulfide rearrangements are rate-limiting. These transitions are suitable for examining the effects of DsbA on disulfide rearrangements involving protein molecules without fixed native-like conformations.

[3SS]-αLA was observed to rearrange back to 3SS upon addition of 10 mM $CaCl_2$, with a first-order rate constant of $(9 \pm 1) \times 10^{-5}$ s^{-1} at pH 7.5, 20-fold slower than at pH 8.7, as expected (Ewbank & Creighton, 1993a). In the presence of catalytic amounts of PDI, the rate was increased about 3-fold, to an apparent first-order rate constant of $(2.5 \pm 0.2) \times 10^{-4}$ s^{-1} (Figure 6B,D). In previous experiments, traces of DTT (≈20 μM) were kept in the reaction mixture because of the strong susceptibility of 3SS to be air-oxidized to native αLA in the presence of Ca^{2+}. Such a low concentration of DTT did not produce any significant reduction, as the intramolecular disulfide rearrangements were more rapid, and the resulting Ca^{2+}-bound 3SS-αLA is stable. The presence of both DTT and PDI, however, resulted in the rapid complete reduction of αLA (data not shown).

In contrast, DsbA at comparable concentrations in terms of active sites produced no detectable change in the spontaneous rearrangement of [3SS]-αLA to 3SS-αLA in the absence of DTT (Figure 6C,D). With 100 μM DTT, DsbA produced some net reduction to species 2SS-αLA and to fully reduced αLA. 2SS-αLA is an intermediate on the reduction pathway of αLA in the presence of Ca^{2+} that has both the 6–120 and 28–111 disulfide bonds cleaved but retains its two other native disulfide bonds (Ewbank & Creighton, 1993b). Reliable measurement of the rates was not possible, however, because fully reduced αLA eluted with DsbA (data not shown).

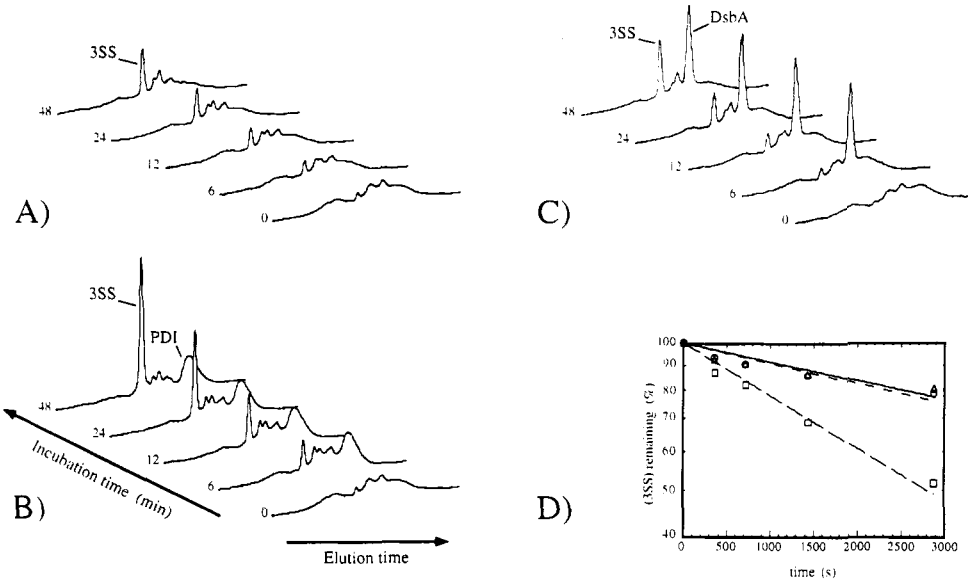


FIGURE 6: Effects of PDI and DsbA on the disulfide rearrangements of [3SS]-αLA to 3SS. [3SS]-αLA (4 μM) was incubated at 25 °C in 0.1 M Tris-HCl (pH 7.5), 0.2 M KCl, and 10 mM $CaCl_2$ and acid-quenched at various times, and the trapped species were separated by reversed phase HPLC. (A) With no catalyst; (B) with 0.25 μM reduced PDI dimer; (C) with 1 μM $DsbA^{SH}_{SH}$; (D) relative amounts of [3SS]-αLA remaining in (A) △, (B) □, and (C) ○. The lines were drawn using the first-order rate constants of 9×10^{-5}, 2.5×10^{-4}, and 9×10^{-5} s^{-1}, respectively.

Catalytic Effects of DsbA on the Rearrangements in α-Lactalbumin at Acidic pH. To investigate further whether DsbA is able to catalyze disulfide rearrangements in poorly structured polypeptides, the disulfide rearrangements between 3SS-αLA and [3SS]-αLA were investigated at pH 4.0 in the absence and presence of DsbA.

A form of 3SS-αLA with stable disulfide bonds, in which the two free thiol groups had been reacted with iodoacetamide, designated $3SS_{cam}$ (Ewbank & Creighton, 1993b), was used to characterize the conformational properties of 3SS-αLA at pH 4. CD spectra of $3SS_{cam}$ in the far- and near-UV demonstrated that it had similar conformational properties at neutral and acidic pH. In the presence of 10 mM Ca^{2+}, $3SS_{cam}$ had native-like CD spectra over the entire UV wavelength range at both pH 7.5 and 4.0. In contrast, $3SS_{cam}$ in the presence of 1 mM EDTA at both pH values lost its tertiary structure, as indicated by a loss of ellipticity in the near-UV, but retained most of its secondary structure, as exhibited in the far-UV. At both pH 7.5 and 4, the native conformation of 3SS-αLA is stable in the presence of Ca^{2+} and unstable in its absence, when it tends to adopt a molten globule-like structure. Consequently, disulfide rearrangements upon adoption of the molten globule conformation are expected to occur at pH 4.0 also, although they might be substantially slower than at alkaline pH.

No spontaneous disulfide rearrangements of 3SS-αLA occurred at pH 4 within 2 h in the absence of Ca^{2+}; the rate would be expected to be about 3×10^4 fold slower at pH 4 than at pH 8.7, so the half-time would be expected to be about 100 h at pH 4. In contrast, about 52% of 3SS-αLA rearranged in 2 h in the presence of 0.27 molar equiv of $DsbA^{SH}_{SH}$ (Figure 7A,B). When 100 μM DTT was included, about 50% of the 3SS-αLA was reduced to 2SS-αLA after 2 h (Figure 7C). In the presence of 10 mM Ca^{2+}, where 3SS-αLA retains the native conformation, very little reduction was observed after 8 h, even in the presence of both DsbA and 40 μM DTT (data not shown).

The [3SS]-αLA to 3SS transition is about 100-fold slower than the reverse reaction at pH 8.7 (Ewbank & Creighton, 1993a), and this is also expected at pH 4. In this case, even the catalyzed reaction would be expected to occur on the day time scale, and experiments to examine this reaction were not technically feasible.

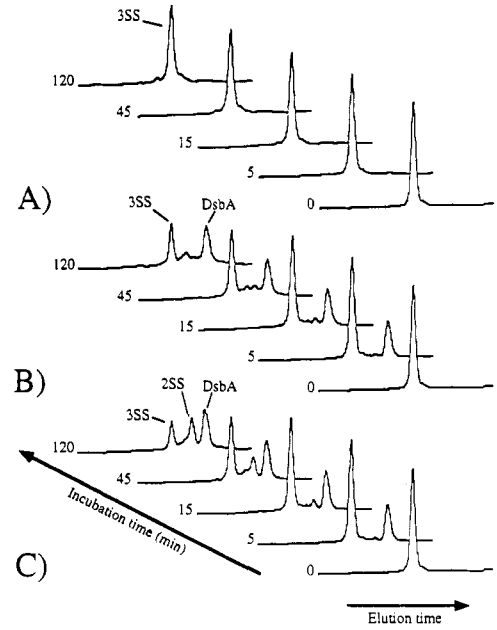


FIGURE 7: Effects of DsbA on the disulfide rearrangements of molten globule 3SS-αLA at pH 4. 3SS-αLA (7.5 μM) was incubated in 0.1 M sodium acetate (pH 4.0), 0.2 M KCl, and 1 mM EDTA at 25 °C; at the indicated times, the species present were trapped by acid and separated by reversed-phase HPLC. (A) With no additions; (B) with 2 μM $DsbA^{SH}_{SH}$; (C) with 2 μM $DsbA^{SH}_{SH}$ and 100 μM DTT.

These results indicate DsbA is able to catalyze some disulfide rearrangements in the molten globule form of αLA, but they are only apparent after long periods of incubation with high concentrations of DsbA under acidic conditions, when spontaneous thiol–disulfide exchange reactions are extremely slow.

DISCUSSION

The formation of disulfide bonds *in vivo* has long been known to be catalyzed. Eukaryotic PDI was identified 30 years ago

and shown to be able to isomerize the disulfide bonds of RNase A (Givol et al., 1964; Venetianer & Straub, 1964). Later, it was shown that PDI catalyzed the rates of virtually all of the steps in the BPTI pathway *in vitro*, including the net formation and breakage of protein disulfide bonds (Creighton et al., 1980), and it is now clear that PDI is involved in the net formation of disulfide bonds in the endoplasmic reticulum (Bulleid & Freedman, 1988; Creighton et al., 1993; LaMantia & Lennarz, 1993). Detailed mechanistic study of this enzyme has, however, been hampered by its complexity. It is a dimer of 57-kDa monomers, each containing two active site pairs of cysteine residues, plus three other domains and two additional cysteine residues of uncertain function, and it possesses other activities [for a review, see Freedman (1992)]. When the comparatively small and monomeric DsbA protein of *E. coli* was found to be required for disulfide bond formation, and its sequence revealed only two cysteine residues like those in the active sites of thioredoxin, glutaredoxin, and PDI (Bardwell et al., 1991), a simple disulfide isomerase appeared to be at hand for mechanistic investigation. The activities that DsbA exhibits on the reduction of insulin, on the refolding and unscrambling of RNase A, and on the refolding of hirudin (Bardwell et al., 1991; Akiyama et al., 1992; Wunderlich et al., 1993b) suggested that DsbA is the bacterial PDI.

The present work shows, however, that DsbA differs significantly from PDI in its effect on the disulfide refolding of two proteins, BPTI and αLA. Only a very marginal rate enhancement of disulfide bond formation in reduced BPTI was observed with catalytic amounts of DsbA in the presence of GSH and GSSG, in contrast to the large effect produced by PDI (Figure 1). The small effect of DsbA, which was nevertheless observed reproducibly, was probably the result of direct oxidation of BPTI, since about 1% of the DsbA molecules were in the disulfide form under the redox conditions of the assay (Zapun et al., 1993; Wunderlich & Glockshuber, 1993a).

More interestingly, DsbA did not catalyze the intramolecular disulfide rearrangements of the quasi-native BPTI species $(30\text{–}51,14\text{–}38)_N$ and $(5\text{–}55,14\text{–}38)_N$, whereas PDI has a dramatic effect on these reactions (Creighton et al., 1980, 1993; Weissman & Kim, 1993). This accounts for the main difference in the effects of DsbA and PDI on the refolding of reduced BPTI. These rearrangements occur in intermediates with folded conformations, however, so other examples involving less folded species were also examined, such as the one-disulfide intermediates in BPTI and the molten globule three-disulfide form of α-LA. DsbA was found to have no observable effect at neutral pH. Of course, DsbA might have been catalyzing these rearrangements at a slow rate, too slow to contribute substantially to the spontaneous uncatalyzed rate and to be apparent. In confirmation, an effect of DsbA on disulfide rearrangements could be observed at pH 4, where the uncatalyzed rate of disulfide interchange is 3000-fold slower than at pH 7.5. Even in these instances, however, catalysis of disulfide rearrangements was apparent only with stoichiometric amounts of DsbA and on the hour time scale. Although DsbA does have some apparent ability to catalyze disulfide interchange in unfolded protein species, that activity is usually insignificant and is much less than that of PDI.

DsbA has been concluded to catalyze disulfide rearrangements in the disulfide folding of hirudin (Wunderlich et al., 1993b). The rearrangements in hirudin occurred, however, also on the hour time scale and with a 3-fold molar excess of $DsbA_{SH}^{SH}$. Moreover, the substrate was scrambled hirudin with no free cysteine residues. The ability of DsbA to catalyze the reactivation of disulfide-scrambled forms is often taken as evidence of its ability to catalyze intramolecular disulfide interchange. The process with disulfide-scrambled forms undoubtedly requires a number of steps, however, such as initial reduction of at least one of the disulfide bonds, rearrangement of the remaining disulfide bonds, and re-formation of the missing disulfide bonds. Which step is rate-limiting and catalyzed by DsbA is usually not known. What appears to be disulfide rearrangements can actually occur by transient reduction of some disulfide bonds and re-formation of others, rather than by catalysis of intramolecular disulfide rearrangements. The activities of DsbA in forming and breaking protein disulfide bonds were observed here to be generally greater than that in rearranging protein disulfides.

The somewhat different actions of DsbA on BPTI and hirudin may also reflect differences in their folding pathways. BPTI adopts a very stable structure, which is stable even with only two of its three disulfide bonds, and some native-like structure is already present in the most prominent one-disulfide species (30–51). The disulfide bonds in BPTI are formed following a relatively well-defined order (Creighton & Goldenberg, 1984). On the contrary, the hirudin native conformation is not very stable (Otto & Seckler, 1991), and the correct set of all three disulfide bonds is required for it to acquire its native structure, which shows very little secondary structure and contains, in solution, a large unstructured C-terminal extension (Haruyama & Wüthrich, 1989). During its disulfide refolding, nonnative disulfide bonds are formed even to the three-disulfide stage, producing a disulfide-scrambled protein. Those bonds must then rearrange to the correct set (Chatrenet & Chang, 1993), but this is not inhibited by the presence of folded conformation. This folding mechanism is not unlike that exhibited by RNase A (Wearne & Creighton, 1988), and DsbA was also shown to catalyze the rearrangement of its scrambled form (Akiyama et al., 1992; Yu et al., 1993).

The results presented here confirm that the primary role of DsbA is to introduce disulfide bonds into newly synthesized proteins (Bardwell et al., 1993). This was initially suggested by the instability, and high oxidative power, of the disulfide bond of $DsbA_S^S$ and of its mixed disulfide (Zapun et al., 1993, 1994). The instability of the disulfide bond of $DsbA_S^S$ causes it to be highly reactive, and the instability of the intermediate mixed disulfide ensures that the disulfide bond will be transferred rapidly and efficiently to a protein molecule in which it will be more stable. Furthermore, $DsbA_{SH}^{SH}$ should be able to reduce only very unstable protein disulfide bonds. These expectations were confirmed here with BPTI and αLA. Stoichiometric amounts of $DsbA_S^S$ transferred the disulfide bond to reduced proteins, and $DsbA_{SH}^{SH}$ reduced only very unstable disulfide bonds. $DsbA_S^S$ rapidly introduced disulfide bonds into reduced BPTI, probably randomly as a result of the unfolded nature of reduced BPTI (Darby & Creighton, 1993; Kemmink & Creighton, 1993). The rearrangements of these disulfide bonds to native pairings, which are required for the eventual appearance of native BPTI, probably occurred spontaneously. In the case of the quasi-native species $(30\text{–}51,14\text{–}38)_N$, which normally rearranges intramolecularly to $(30\text{–}51,5\text{–}55)_N$ more rapidly than it can form the 5–55 disulfide bond directly (Creighton, 1977a), $DsbA_S^S$ bypassed the disulfide rearrangements by incorporating the 5–55 disulfide bond (Figure 4). PDI has also been shown to have such functional capabilities, even though it also catalyzes disulfide rearrangements (Creighton et al., 1980). In contrast, DsbA was not able to introduce the 30–51 disulfide bond directly

into the other quasi-native species, $(5\text{–}55,14\text{–}38)_N$. These differences are explicable: $(5\text{–}55,14\text{–}38)_N$ has a more stable folded conformation than does $(30\text{–}51,14\text{–}38)_N$ (States et al., 1987), and the free thiol groups of $(5\text{–}55,14\text{–}38)_N$ are not accessible (Creighton & Goldenberg, 1984), whereas those of $(30\text{–}51,14\text{–}38)_N$ are (Figure 2; Creighton, 1977a). $DsbA_S^S$ apparently can react with accessible cysteine thiol groups but cannot "unfold" a folded conformation to get access to buried groups.

The kinetics of reaction between DsbA and glutathione were used previously to predict the rate at which $DsbA_S^S$ would transfer its disulfide bond to a protein, P, by thiol–disulfide exchange (Zapun et al., 1993)

$$DsbA_S^S + {}^{HS}_{HS}P \underset{10^2\ s^{-1}}{\overset{10^4\ s^{-1}M^{-1}}{\rightleftharpoons}} DsbA_{SH}^{S-S}{}_{HS}P \underset{40\ s^{-1}M^{-1}}{\overset{10^4\ k_{intra}}{\rightleftharpoons}} DsbA_{SH}^{SH} + {}^{S}_{S}P \qquad (2)$$

where k_{intra} is the rate constant for the intramolecular step in forming the protein disulfide bond from a mixed disulfide with glutathione (Darby & Creighton, 1993). For concentrations of $DsbA_S^S$ less than 10^{-2} M, the observed rate constant should be

$$k_{obs} = \frac{10^4\ s^{-1}\ M^{-1}}{10^2\ s^{-1}} \times 10^4 k_{intra} = 10^6 k_{intra}\ M^{-1} \qquad (3)$$

The measured value of k_{intra} for forming all of the initial disulfide bonds in reduced BPTI is 2 s^{-1} at pH 8.7 (Darby & Creighton, 1993), which would be approximately 0.1 s^{-1} at pH 7.5. Therefore, the rate constant for forming the initial disulfide bonds using $DsbA_S^S$ should be 10^5 s^{-1} M^{-1}, which is compatible with the observed value of greater than 4×10^3 s^{-1} M^{-1}. These considerations can also account for the rate at which $DsbA_S^S$ introduced the 5–55 disulfide bond into $(30\text{–}51,14\text{–}38)_N$, in spite of its low value of k_{intra}; the observed rate would indicate from eq 3 a value of k_{intra} of 3×10^{-4} s^{-1}. $DsbA_S^S$ is able to introduce disulfide bonds into a protein so much more rapidly than a small molecule like GSSG because of the rapid rates at which it participates in thiol–disulfide exchange reactions (eq 2), as well as the instability of its disulfide bond. The validity of eq 3 needs to be established, however, as it does not include the possibility of the protein substrate binding to DsbA, as appears to be possible from its crystal structure (Martin et al., 1993). Also, DsbA was found to catalyze formation of mixed disulfides between the protein and glutathione (Figure 2), which is not accounted for by eq 2.

Some aspects of the different behaviors of DsbA and PDI are not too surprising. PDI acts within the lumen of the endoplasmic reticulum, a compartment that presumably preserves a stable environment with high concentrations of both GSH and GSSG (Hwang et al., 1992). In contrast, the periplasm of Gram-negative bacteria, where DsbA is found, is probably similar to the growth medium, because small molecules can diffuse freely through the outer cell wall (Decad & Nikaido, 1976). Small disulfide or thiol molecules can enter the periplasmic space and influence the formation of disulfide bonds (Wunderlich & Glockshuber, 1993b; Missiakas et al., 1993). *E. coli* are able to grow on minimal medium or under anaerobic conditions, so there is probably no small-molecule equivalent to the glutathione that is present in the ER of eukaryotes. $DsbA_S^S$ seems to be the direct oxidant of the secreted proteins and to play the role of the GSSG in the ER. How $DsbA_{SH}^{SH}$ is itself oxidized to participate in several rounds of disulfide bond formation is not certain, but the intramembranous protein DsbB is thought to play a role in this process (Bardwell et al., 1993; Missiakas et al., 1993). Disulfide bond rearrangements are frequently important in the oxidative folding of proteins, for there is a kinetic barrier to introducing disulfide bonds that will be buried in a stable folded conformation (Creighton, 1978). It remains to be established whether DsbA bypasses such rearrangements by incorporating disulfide bonds directly, as it did with $(30\text{–}51,14\text{–}38)_N$ of BPTI, or whether there is another catalyst catalyzing disulfide bond rearrangements.

The role of DsbA in the bacterial periplasm may be more important for growth under acidic conditions than at neutral pH, where thiol–disulfide exchange occurs spontaneously. The remarkable ability of DsbA to incorporate disulfide bonds at pH 4 into reduced hirudin (Wunderlich et al., 1993b) was confirmed here with BPTI (Figure 5). Part of the explanation for the activity of DsbA at acidic pH is that the Cys30 thiol group of $DsbA_{SH}^{SH}$ is fully ionized at pH 4 (Nelson & Creighton, 1994). Also, the disulfide bond of $DsbA_S^S$ is considerably more stable at pH 4 than at neutral pH (Wunderlich & Glockshuber, 1993a; Nelson & Creighton, 1994), making it more populated under any given redox conditions, yet it is nearly as reactive as at pH 7.5 (Nelson & Creighton, 1994). The many favorable properties of DsbA should make it feasible to understand the details of the thiol–disulfide exchange chemistry that is crucial to its function.

ACKNOWLEDGMENT

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