Competition between DsbA-Mediated Oxidation and Conformational Folding of RTEM1 β-Lactamase[†]

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ABSTRACT: Similar to other proteins of the periplasm of *Escherichia coli*, TEM1 β-lactamase contains only a single disulfide bond. It can fold to its native conformation in both the presence and the absence of this disulfide bond. The GdmCl-dependent equilibrium unfolding of β-lactamase *in vitro* is well described by a N ⇌ I ⇌ U three-state model in which the native protein (N) first reacts to an intermediate of the molten globule type (I) and then to the unfolded state (U). We find that the disulfide bond of β-lactamase stabilizes I relative to U, but does not change the stability of N relative to I. The I ⇌ U transition is an extremely rapid reaction for both reduced and oxidized β-lactamase, but the N ⇌ I folding kinetics are slow and identical in the presence and the absence of the disulfide bond. This insensitivity of the N ⇌ I equilibrium and kinetics suggests that the region around the disulfide bond is already native-like folded and is presumably buried in the intermediate I, prior to the slow and rate-limiting events of folding. This was confirmed by measuring the stability of the disulfide bond, which, to a first approximation, is identical in N and I. In native, reduced β-lactamase, the thiol groups are inaccessible for oxidation by DsbA, but at the stage of the molten globule intermediate I oxidation is still possible, because I is in fast exchange with the unfolded protein U. The introduction of the disulfide bond into β-lactamase by DsbA competes with conformational folding at the stage of the final slow steps in the folding of the reduced protein. The major problem in the oxidation of proteins with one or two disulfide bonds (such as β-lactamase) is not the formation of incorrect disulfide bonds, but the premature burial of the thiol groups by the rapid conformational folding of the reduced protein. DsbA, the major thiol/disulfide isomerase of the bacterial periplasm, meets this problem. It is a very strong oxidant, and its reaction with cysteine residues in unfolded proteins is extremely fast.

Many secreted proteins are stabilized by disulfide bonds. In the course of protein folding, the formation of the native disulfide bonds is slow and coupled with the acquisition of the native chain conformation (Creighton, 1986; Weissman & Kim, 1991; Goldenberg, 1992; Darby & Creighton, 1993). *In vivo*, disulfide bond formation is catalyzed by thiol/disulfide oxidoreductases, such as protein disulfide isomerase (PDI) in the endoplasmic reticulum of eukaryotes and the Dsb[1] proteins in the periplasm of bacteria (Goldberger *et al.*, 1963; Bardwell *et al.*, 1991; Kamitani *et al.*, 1992; Zapun *et al.*, 1992). Enzyme-catalyzed disulfide bond formation is a two-step process (eq 1). In the first step, the disulfide

$$P_{SH}^{SH} + DsbA_{S}^{S} \rightleftharpoons P_{SH}^{S-S}DsbA_{SH} \rightleftharpoons P_{S}^{S} + DsbA_{SH}^{SH} \quad (1)$$

bond of the oxidoreductase is attacked by a cysteine thiol of the protein substrate, and an intermolecular mixed disulfide is formed. In the second step, this mixed disulfide is attacked by another protein thiol group in an intramolecular reaction to form the protein disulfide bond and liberate the oxidoreductase in the reduced state. Conformational chain folding should influence both steps. The reduced protein chains should not fold too rapidly in the first step to retain a good accessibility of the cysteines for forming the mixed disulfide with the oxidoreductase. In contrast, in the second step a tight coupling of intramolecular disulfide exchange with conformational folding would be of advantage to select and stabilize the correct disulfide bonds of the native protein (eq 1).

The number of disulfide bonds in secretory proteins can vary substantially. On the one hand, small proteins exist with many disulfide bonds, such as ribonuclease A, the pancreatic trypsin inhibitor, or the transforming growth factor. These proteins are from eukaryotes, and the disulfide bonds are absolutely necessary to convey them with a high conformational stability. In the absence of the disulfides, they are unfolded, and thus conformational folding is coupled with the formation of the correct native set of disulfide bonds. The folding of these proteins probably also involves extensive thiol/disulfide exchange to isomerize incorrectly formed disulfide bonds. Such reactions are well catalyzed by

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[1] Abbreviations: β-lactamase, TEM1-β-lactamase from *E. coli*; DsbA, product of the *dsb*A gene, a periplasmic thiol/disulfide oxidoreductase of *E. coli*; N and U, native and unfolded forms of a protein, respectively; I, folding intermediate; GdmCl, guanidinium chloride; DTT, dithiothreitol; GSH and GSSG, reduced and oxidized forms of glutathione, respectively; *m*, cooperativity parameter of a GdmCl-induced unfolding transition, $m = d\Delta G/d[GdmCl]$.

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eukaryotic PDI from the endoplasmic reticulum (Creighton *et al.*, 1980; Zapun *et al.*, 1992; Freedman *et al.*, 1994).

In contrast, prokaryotic secretory proteins generally contain only one or two disulfide bonds per monomer or per folding domain (Joly & Swartz, 1994). Alkaline phosphatase with two disulfide bonds and β-lactamase with a single disulfide bond represent well-known examples. Both enzymes are from the periplasm of *E. coli*. Unlike the small eukaryotic proteins with ≥3 disulfide bonds, TEM1 β-lactamase can fold to a native-like conformation even when the disulfide bond is not yet formed (Laminet & Plückthun, 1989; Schulz *et al.*, 1987), and thus the thiol groups of the reduced protein could become inaccessible before the disulfide bond has formed (Walker & Gilbert, 1994, 1995).

We investigated whether premature conformational folding of a reduced protein can indeed interfere with assisted disulfide bond formation and used the DsbA-mediated oxidative folding of β-lactamase as a model system. β-Lactamase folds through a partially-folded intermediate of the molten globule type both in equilibrium and in kinetic experiments (Georgiou *et al.*, 1994; Vanhove *et al.*, 1995; Zahn & Plückthun, 1994). The folding reactions that involve these intermediates can be studied individually for both the reduced and the oxidized forms. The simplest model to describe the equilibrium unfolding of both oxidized and reduced β-lactamase is shown in eq 2.

$$N \rightleftharpoons I \rightleftharpoons U \qquad (2)$$

In this study, we investigated how the conformational folding of the reduced protein, from the unfolded form (U) first to the molten globule-like intermediate (I) and then to the native state (N), interferes with the DsbA-mediated introduction of the disulfide bond. First, the folding transitions and the folding kinetics of the reduced and of the oxidized protein were measured to find conditions under which the native, the intermediate, and the unfolded species of β-lactamase exist and to determine the role of the disulfide bond for the conformational stability and dynamics. Then, oxidative folding was investigated with DsbA as the oxidant to elucidate the interrelationship between conformational folding and DsbA-mediated disulfide bond formation. The accessibility of the thiol groups for DsbA during folding was determined by competition experiments between conformational folding and DsbA-mediated oxidation, and by adding DsbA to the reduced protein at different times after the onset of its conformational folding.

We found that only the unfolded form of reduced β-lactamase is a good substrate for DsbA. It can no longer be oxidized by DsbA when it has reached a native-like folded conformation.

MATERIALS AND METHODS

Materials. GdmCl (ultrapure) was from Schwarz/Mann (Orangeburg, NY); Hepes (sodium salt) and acetonitrile (HPLC grade) were from Sigma (St. Louis, MO). Dithiothreitol (DTT) and TFA were from Fluka (Buchs, Switzerland). All other chemicals were from Merck (Darmstadt, Germany). The concentrations of GdmCl were determined by the refraction of the solutions. The equation correlating the refractive index with the concentration of GdmCl is given by Pace (1986). The DsbA protein was expressed and purified as described (Wunderlich *et al.*, 1993).

Expression and Purification of β-Lactamase. TEM1 β-lactamase was purified from periplasmic extracts of *E. coli* BL21 (DE3) cells harboring a T7-expression plasmid (Studier *et al.*, 1990). Cells were grown at 25 °C in 10 L of LB broth, containing ampicillin (100 μg/mL), to an optical density at 550 nm of 0.7. After addition of isopropyl β-thiogalactoside to a final concentration of 1 mM, the cells were grown overnight and harvested by centrifugation. Cells were suspended in cold 200 mL of 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, and 5 mM EDTA containing 1 mg/mL polymyxin B (Sigma) and stirred for 1 h at 4 °C. After centrifugation, the supernatant was extensively dialyzed against 10 mM Tris/HCl, pH 8.0, and applied to a Whatman DE 52 anion exchange column (100 mL). β-Lactamase was eluted by a linear gradient from 0 to 0.5 M NaCl in 1 L. Fractions containing β-lactamase (between 100 and 150 mM NaCl) were pooled, mixed with 0.43 volume of 4.0 M ammonium sulfate, adjusted to pH 8.0 with 1 M Tris base, and applied to a phenyl-Sepharose HP column (60 mL) from Pharmacia. β-Lactamase was eluted by a linear gradient from 1.2 to 0 M ammonium sulfate in 1 L of 5 mM Tris/HCl, pH 8.0. Fractions containing pure β-lactamase (between 0.95 and 0.53 M ammonium sulfate) were combined, concentrated by ultrafiltration (Amicon YM 10), dialyzed against distilled water, and stored at −20 °C. Typically 800 mg of pure protein was obtained. As judged by reducing and nonreducing SDS−PAGE, all molecules were oxidized after purification. The correct molecular mass of TEM1 β-lactamase (28 949 Da) was verified by mass spectrometry, and the correct amino terminus was confirmed by Edman sequencing.

Spectroscopic Methods. For optical measurements, a Hitachi F4010 fluorescence spectrometer, a Jasco J-600A spectropolarimeter, and a Kontron Uvikon 860 spectrophotometer were used. The concentration of native β-lactamase was measured spectrophotometrically by using an absorption coefficient of $\epsilon_{280} = 30\,000\ M^{-1}\ cm^{-1}$ which was determined according to Gill and von Hippel (1989). For DsbA, $\epsilon_{280} = 21\,740\ M^{-1}\ cm^{-1}$ was used (Zapun *et al.*, 1993).

Preparation of Reduced β-Lactamase. About 5 mg of β-lactamase was dissolved in 500 μL of 50 mM DTT, 6 M GdmCl, 0.3 M Tris/HCl, and 2 mM EDTA, pH 7.5, and incubated at 25 °C for at least 3 h. The reduction was stopped by the addition of 10 μL of 3 M HCl, and the reduced protein was separated from the reagents by gel filtration over a fast desalting HR10/10 column (Pharmacia, Sweden), equilibrated with 5 mM ammonium acetate. Fractions containing the reduced protein were lyophilized and stored at −20 °C. The protein showed two free thiol groups in Ellman's assay (Riddles *et al.*, 1983) and gave a single peak in reversed-phase HPLC analysis.

Equilibrium Folding Transition. Reduced and oxidized β-lactamases were incubated at 25 °C in the presence of 0.1 M Hepes/NaOH, 2 mM EDTA, pH 7.0, and varying concentrations of GdmCl for at least 3 h. The fluorescence emission of the samples was measured at 340 nm (10-nm bandwidth) after excitation at 280 nm (1.5-nm bandwidth) in 1 × 1 cm cells at a protein concentration of 0.25 μM. The far-UV CD signal of the sample was measured at 220 nm (1-nm bandwidth) in a 0.5 cm thermostated cell at a protein concentration of 5 μM. The solutions of the reduced protein additionally contained 2 mM DTT. The fluorescence transition curves [$F_{obs} = f([D])$] were analyzed according to

eq 3 by assuming two-state transitions between the folded protein (N) and the intermediate (I) (Santoro & Bolen, 1988).

$$F_{obs} = \frac{(F_N + m_N[D]) - (F_U + m_U[D])}{1 + \exp[-(\Delta G^0_{NI} + m_{NI}[D])/RT]} + (F_N + m_N[D]) \quad (3)$$

The far-UV CD transitions [$\Theta_{obs} = f([D])$] were analyzed according to a three-state model ($N \rightleftharpoons I \rightleftharpoons U$), in which the accumulation of the intermediate state I is significant by using eq 4 (Barrick & Baldwin, 1993; Vanhove *et al.*, 1995):

$$\Theta_{obs} = \{(\Theta_N + m_N[D]) + \exp[a(\Theta_I + (\Theta_U + m_U[D]) \exp b)]\}/\{1 + \exp[a(1 + \exp b)]\} \quad (4)$$

$$\text{with } a = -(\Delta G_{NI}{}^0 + m_{NI}[D])/RT$$

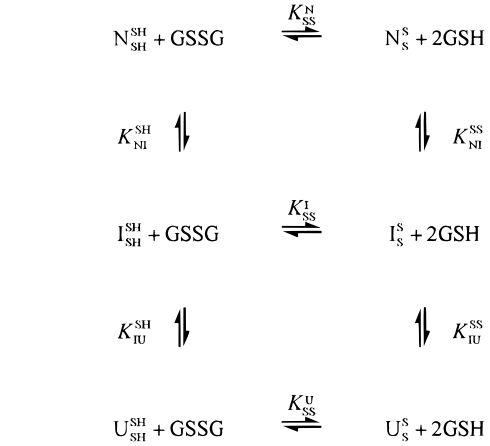
$$\text{and } b = -(\Delta G_{IU}{}^0 + m_{IU}[D])/RT$$

where $\Delta G_{NI}{}^0$ and $\Delta G_{IU}{}^0$ are the differences in free energy between N and I and between I and U, respectively, in the absence of denaturant. m_{NI} and m_{IU} are the slopes of the corresponding linear plots of $d(\Delta G_{obs})/d([GdmCl])$. The parameters m_N and m_U were introduced to account for the linear dependence of F_N, Θ_N, F_U, and Θ_U on denaturant concentration.

Refolding and Unfolding Kinetics. The refolding kinetics of reduced and oxidized β-lactamase were initiated by a 50-fold dilution of the unfolded proteins (in 6.0 M GdmCl, 0.1 M Hepes/NaOH, pH 7.0) to the final conditions of 0.1 M Hepes/NaOH, 2 mM EDTA, pH 7.0 at 25 °C, and 0.05−0.9 M GdmCl. The unfolding kinetics of reduced and oxidized β-lactamase were initiated by dilution of the native proteins to final concentrations of 0.9−2.3 M GdmCl in the same buffer. The final protein concentration was 0.25 μM. The kinetics were followed by the increase in fluorescence at 340 nm (10-nm bandwidth) after excitation at 280 nm (1.5-nm bandwidth) in 1 × 1 cm cells. The observed kinetic curves were analyzed as a sum of exponential functions by using the program GraFit 3.0 (Erithacus Software, Staines, U.K.).

Determination of the Equilibrium Constant for Disulfide Bond Formation in β-Lactamase and Analysis by HPLC. Oxidized β-lactamase (23 μM) was incubated in a mixture of GSH and GSSG in 0.9−5 M GdmCl, 0.1 M Hepes/NaOH, and 2 mM EDTA, pH 7.0 at 25 °C, for 15−20 h. The GSH concentrations ranged from 4 to 28 mM, and the GSSG concentration was 2 mM. Thiol/disulfide exchange was stopped by adding 0.1 volume of 3 M HCl, and the reaction products were separated by reversed-phase HPLC at 40 °C on a Vydac C18 218TP54 25 × 0.46 cm column using a linear gradient from 52 to 46% solvent A in 20 min at a flow rate of 1 mL/min. Solvent A was 0.1% (v/v) trifluoroacetic acid in water, and solvent B was 0.1% trifluoroacetic acid in a 1:9 mixture of water and acetonitrile. The oxidized and reduced forms of β-lactamase were detected and quantified by the absorbances at 215 nm and at 229 nm. The apparent equilibrium constant for disulfide bond formation, K^{app}_{SS}, was calculated on the basis of the mechanism in Scheme 1 by using eq 5.

Scheme 1: Thermodynamic Linkage between the Reaction of β-Lactamase with Glutathione and the Reversible Three-State Protein Unfolding Reactions of the Reduced and the Oxidized Forms of the Protein[a]



[a] SH subscripts and superscripts denote the reduced forms of the species. N, I and U, S subscripts and superscripts denote the respective oxidized forms.

$$K^{obs}_{SS} = \frac{[P^S_S]}{[P^{SH}_{SH}]}\frac{[GSH]^2}{[GSSG]} = \frac{[N^S_S] + [I^S_S] + [U^S_S]}{[N^{SH}_{SH}] + [I^{SH}_{SH}] + [U^{SH}_{SH}]}\frac{[GSH]^2}{[GSSG]} \quad (5)$$

Oxidation of Reduced β-Lactamase and Analysis by HPLC. The oxidative folding of reduced β-lactamase was initiated at 25 °C by a 40-fold dilution of the reduced protein to a final concentration of 10 μM β-lactamase in 0.1 M Hepes/NaOH, 2 mM EDTA, pH 7.0, and 11−370 μM DsbA. Thiol/disulfide exchange was stopped by adding 0.1 volume of 3 M HCl, and the reaction products were analyzed by reversed-phase HPLC on a Vydac C18 column as described above. In the two-step experiments, the reduced protein was allowed first to refold for variable periods of time before in the second step 100 μM DsbA was added.

RESULTS

Conformational Stabilities of Reduced and Oxidized β-Lactamase. The GdmCl-induced unfolding transitions of reduced and oxidized β-lactamase are shown in Figure 1. Unfolding was followed by the increase of the amide circular dichroism at 220 nm (Figure 1A,B), which is sensitive to the changes in secondary structure upon unfolding, and by the decrease in tryptophan fluorescence at 340 nm, which is sensitive to changes in tertiary structure (Figure 1C,D). Very similar unfolding transitions were obtained by CD for the reduced and the oxidized forms of β-lactamase. In both cases, the curves are biphasic with a first transition centered around 0.9 M GdmCl. This transition is virtually identical for the reduced and the oxidized protein. Then a small plateau is reached near 1.2 M GdmCl, before a second, broad transition occurs. Above 4 M GdmCl, the CD signal of the unfolded protein is reached. The second transition is sensitive to the presence of the disulfide bond. It shows a midpoint near 2.0 M GdmCl for the oxidized protein and near 1.6 M GdmCl for the reduced protein. These results suggest that the unfolding of both reduced and oxidized β-lactamase occurs in two stages as shown in eq 1. In the first transition (between 0 and 1.2 M GdmCl), the native protein N unfolds to a partially folded intermediate I which

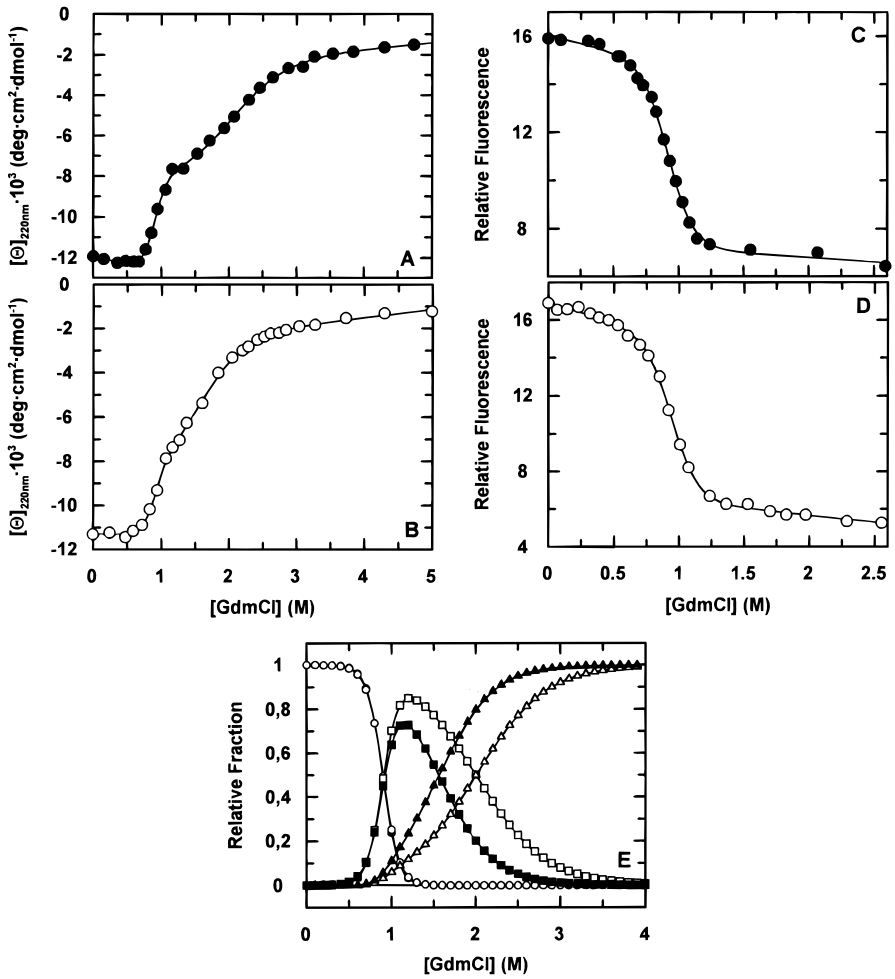


FIGURE 1: GdmCl-induced unfolding transitions of oxidized (●) and reduced (○) β-lactamase in 0.1 M Hepes/NaOH, 2 mM EDTA, pH 7.0 at 25 °C. (A, B) Unfolding monitored by the decrease of the far-UV CD signal at 220 nm. The protein concentration was 5 μM. The lines represent an analysis of the data according to a three-state model (eq 4) as described under Materials and Methods. (C, D) Unfolding monitored by fluorescence at 340 nm. The protein concentration was 0.25 μM. The lines represent an analysis of the data according to a two-state model (eq 3). (E) Three-state analysis of (○, □, △) oxidized and (●, ■, ▲) reduced β-lactamase. The fractions of (●, ○) the native protein, (■, □) the intermediate, and (▲, △) the unfolded protein are given as a function of the GdmCl concentration. The lines were calculated using the data given in Table 1.

has lost about half of its amide CD. Then, in the second transition (1.2−3 M GdmCl), the intermediate is converted to the fully unfolded state U.

When unfolding is monitored by the decrease in tryptophan fluorescence (Figure 1C,D), only the first transition at 0.9 M GdmCl is detected. As in Figure 1A,B identical curves were obtained for the reduced and the oxidized protein. This confirms that the equilibrium between N and I is not affected by the disulfide bond. Also it indicates that in the intermediate I the tryptophan residues of β-lactamase are already exposed to the solvent.

Biphasic transitions have been found earlier for the oxidized forms of other β-lactamases and also for TEM1 β-lactamase under different conditions (Mitchinson & Pain, 1985; Goto & Fink, 1989; Uversky & Ptitsyn, 1994), and it was suggested that the folding intermediates that are present near 1.2 M denaturant show a conformation of the "molten globule" type, which is thought to occur generally in folding intermediates both *in vitro* and *in vivo* (Kuwajima *et al.*, 1987; Ptitsyn, 1992).

To determine the Gibbs free energy of stabilization, ΔG_{stab}, of the individual species, the three-state model in eq 2 was used to analyze the two fluorescence- and the two CD-detected folding transitions in Figure 1 (Barrick & Baldwin, 1993; Vanhove *et al.*, 1995). The results of this analysis are given in Table 1. For the first transition between the native protein and the molten globule intermediate, virtually identical values were obtained for the transition midpoints (between 0.90 and 0.95 M GdmCl) and for the cooperativity parameter m [between −25 and −27 kJ·K^{-1}·mol^{-1}]. As a consequence, identical values of $\Delta G_{stab} \approx -23$ kJ/mol could be calculated for the difference in free energy between N and I (at 0 M GdmCl) for both the reduced and the oxidized form of the protein. Also, the fluorescence- and the CD-detected transitions gave identical values for ΔG_{stab}. This confirms that the disulfide bond has no influence on the conformational stability of native β-lactamase. It neither stabilizes nor destabilizes the folded protein relative to the molten globule intermediate.

The second transition between the intermediate and the unfolded state could be measured only by CD (cf. Figure 1A,B). The midpoint of this transition increased from 1.6 M for the reduced protein to 2.0 M GdmCl for the oxidized protein, indicating that the disulfide bond stabilizes I relative to U. In parallel, the m value is reduced from −7.8 to −6.1 kJ/(K·mol)$^{-1}$. Under the assumption that the unfolding of β-lactamase follows the three-state mechanism in eq 2, we can calculate that the disulfide bond stabilizes I by about

Table 1: Thermodynamic Parameters for the Equilibrium Unfolding of β-Lactamase

form[a]	ΔG_{NI}^0 (kJ·mol^{-1})	m_{NI} (kJ·mol^{-1}·M^{-1})	$M_{1/2}$[b] (M)	ΔG_{IU}^0 (kJ·mol^{-1})	m_{IU} (kJ·mol^{-1}·M^{-1})	$M_{1/2}$[b] (M)	ΔG_{IU}^{2M}[c] (kJ·mol^{-1})
ox	24.2 ± 3.0[d]	-26.8 ± 3.3[d]	0.90[d]	12.2 ± 1.9[d]	-6.1 ± 0.9[d]	2.00[d]	0
	22.8 ± 3.1[e]	-24.8 ± 3.3[e]	0.92[e]				
red	23.1 ± 3.9[d]	-25.4 ± 4.4[d]	0.91[d]	12.1 ± 2.3[d]	-7.8 ± 1.1[d]	1.56[d]	-3.5
	23.2 ± 1.3[e]	-24.5 ± 1.3[e]	0.95[e]				

[a] ox, oxidized; red,reduced form of β-lactamase. [b] Midpoint of the transition. [c] ΔG_{IU} at 2.0 M GdmCl. [d] Parameters obtained after an analysis of the CD-detected transitions according to a three-state model. [e] Parameters from the two-state analysis (eq 3) of the fluorescence-detected transitions.

3.5 kJ/mol relative to U (Table 1). This value refers to the transition midpoint for the oxidized protein at 2.0 M GdmCl. It depends on the denaturant concentration, because the cooperativities of the two transitions (the *m* values) are different. Presumably, the three-species model (eq 2) is an oversimplification, because additional intermediates may be present between I and U (Uversky & Ptitsyn, 1994).

The analysis of the stability curves in Figure 1A−D results in profiles for N, I, and U as a function of the denaturant concentration (Figure 1E). The profiles for the native state are identical for the reduced and the oxidized protein, because they show identical N ⇌ I transitions. Above 0.5 M GdmCl, N unfolds to I. The concentration of I reaches a maximum near 1.2 M GdmCl and then declines because I is converted to U. The disulfide bond stabilizes I relative to U, and therefore more I and less U is populated between 1.5 and 3 M GdmCl when the protein is oxidized.

Kinetics of Folding of Reduced and Oxidized β-Lactamase. The two folding transitions of β-lactamase in Figure 1 show strikingly different kinetics. The equilibration between the molten globule intermediate and the unfolded protein in the second transition (I ⇌ U) is an extremely rapid reaction both in the presence and in the absence of the disulfide bond. Under all conditions, it was complete within the time of manual mixing (about 5 s).

In contrast, folding from the intermediate to the native protein in the first transition (N ⇌ I) is a slow process. The kinetics of this reaction were followed by fluorescence in the presence and in the absence of the disulfide bond in both the unfolding and the refolding direction. The unfolding reactions could be measured after diluting samples of the folded proteins to 0.9−2.3 M GdmCl. In all cases, the observed kinetics were well approximated by monoexponential processes which accounted for the entire change in fluorescence, as expected from the equilibrium transitions in Figure 1. With a time constant of about 1000 s, unfolding was slowest at the midpoint of the N ⇌ I reaction (at 0.9 M GdmCl). The rate of unfolding increased strongly with increasing GdmCl concentration (Figure 2), and at 2.3 M GdmCl, the time constant was about 10 s (Figure 2). The unfolding kinetics of the reduced and the oxidized protein were identical at all denaturant concentrations.

Refolding was initiated by diluting the GdmCl-unfolded proteins, and the kinetics could be measured between 0.05 and 1.0 M GdmCl. At a very low concentration of denaturant (0.05 M GdmCl), refolding was a complex process; 37% of the regain of the native fluorescence occurred within the dead time of the experiment, and then two slow phases followed with time constants of 14 s (36% amplitude) and 140 s (27% amplitude), respectively. Such biphasic kinetics were found earlier for oxidized β-lactamase. They arise presumably from the parallel refolding of species

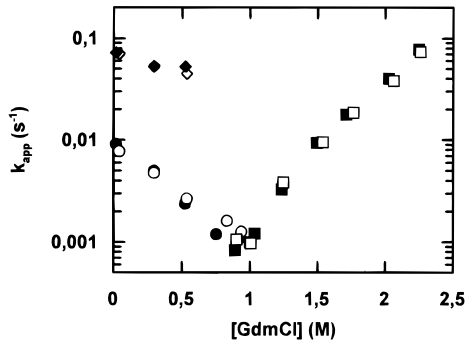


FIGURE 2: Dependence on GdmCl concentration of the refolding and unfolding kinetics of reduced (open symbols) and oxidized (closed symbols) β-lactamase. (■, □) Unfolding and (●, ○) refolding were measured in 0.1 M Hepes/NaOH, 2 mM EDTA, pH 7.0 at 25 °C, by the increase in fluorescence at 340 nm at a final protein concentration of 0.25 μM. Below 0.5 M GdmCl, refolding was biphasic. (●, ○) Apparent rate constant k_1 of the slow phase; (♦, ◊) apparent rate constants k_2 of the fast phase.

with different prolyl isomers (Vanhove *et al.*, 1995). The 14-s phase decreased in amplitude with increasing concentration of GdmCl and disappeared above 0.5 M GdmCl. Concomitantly, the slowest phase gained in amplitude, and its rate constant decreased strongly with increasing GdmCl concentration (Figure 2). The same rates were observed in unfolding and in refolding near the transition midpoint, as expected for reversible protein folding reactions. As in the unfolding experiments, identical kinetics were found for the refolding reactions of reduced and oxidized β-lactamase. In summary, the equilibrium and the kinetic results (Figures 1 and 2) demonstrate that the Cys77−Cys123 disulfide bond of β-lactamase neither affects the stability of the native protein relative to the molten globule intermediate nor affects the kinetics of the unfolding/refolding reactions that interconvert these two species.

Linkage between Conformational Folding and Disulfide Bond Formation. The unfolding transitions in Figure 1 suggest that the disulfide bond of β-lactamase slightly stabilizes the molten globule type intermediate relative to the unfolded state (in the I ⇌ U transition), but does not influence the stability of the folded protein relative to the intermediate (in the N ⇌ I transition). Disulfide bond formation and conformational folding are thermodynamically coupled (as shown in Scheme 1), and the increase in the stability of a protein caused by disulfide bond formation must reciprocally stabilize the disulfide bond to the same extent (Creighton, 1986). As a consequence, the changes in conformational stability brought about by a disulfide bond not only can be determined by unfolding experiments as in Figure 1 but also can be found by measuring the equilibrium constant for protein disulfide bond formation, K_{SS}, under conditions where the protein is in different conformational states (such as the native, the intermediate, and the unfolded

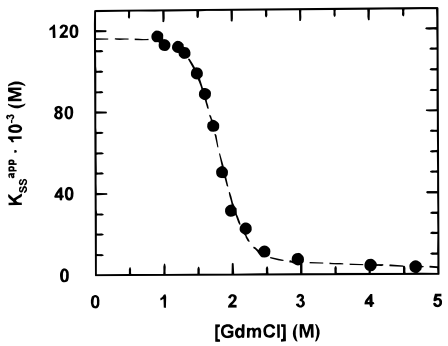


FIGURE 3: Dependence on GdmCl concentration of the measured equilibrium constants of β-lactamase with glutathione (K_{SS}^{app}) at pH 7.0, 25 °C. The values of (●) K_{SS}^{app} at the indicated concentrations of GdmCl were calculated by using eq 5 from equilibration experiments as described under Materials and Methods. The protein concentration was 23 μM, the GSSG concentration was 2 mM, and the GSH concentration was between 4 and 28 mM.

conformations of β-lactamase). Investigations on thioredoxin, T4-lysozyme, and DsbA have shown that the change in the conformational stability (ΔG_{stab}) of a protein upon disulfide bond formation can indeed be very well determined by measuring the stability of the disulfide bond in the folded and the unfolded protein (Lin & Kim, 1991; Lu *et al.*, 1992; Wunderlich *et al.*, 1993; Zapun *et al.*, 1993).

We used this method for β-lactamase to measure the influence of the conformational folding transitions on the apparent equilibrium constant for disulfide bond formation as shown in Scheme 1. In these experiments, the reduced and the oxidized forms of β-lactamase were allowed to equilibrate with different mixtures of reduced and oxidized glutathione at various concentrations of GdmCl for 20 h. Then the concentrations of reduced and oxidized β-lactamase were determined by reversed-phase HPLC, and K_{SS} was calculated. In the presence of $\geq$3 M GdmCl, β-lactamase is unfolded, and the equilibrium constant K_{SS}^{U} for disulfide bond formation with glutathione as the oxidant is 4 mM. Such a value is expected for an unfolded protein chain when the two cysteines are separated by 45 residues as in β-lactamase (Peng *et al.*, 1995; Darby & Creighton, 1993).

In total, the apparent value of K_{SS} (K_{SS}^{app}) was determined at 14 different concentrations of GdmCl. K_{SS}^{app} increased from 4 mM to about 110 mM when the denaturant concentration was lowered from 4.7 to 0.9 M GdmCl (Figure 3). This increase follows a sigmoidal curve, and it seems to level off near 1 M GdmCl. Measurements at lower concentrations of GdmCl were not possible. The native state N predominates under these conditions, and the equilibration with the glutathione system requires prior unfolding via I to U. With decreasing concentration of GdmCl, unfolding of N to I becomes slower (cf. Figure 2), and the I $\rightleftharpoons$ U equilibrium is shifted toward I. The combination of the two effects retarded the thiol/disulfide exchange reactions with glutathione so strongly that the equilibrium was not reached within a day.

From 4 M GdmCl, where the unfolded state U is populated, to 1.2 M GdmCl, where the intermediate I is maximally populated (cf. Figure 1E), K_{SS}^{app} increases from 4 to 110 mM. This suggests that upon formation of I from U the stability of the disulfide bond increases by RT ln (110/4) = 8.2 kJ/mol. Thermodynamic coupling (cf. Scheme 1) requires that the disulfide bond stabilizes I relative to U by the same amount. The true increase in the conformational stability (ΔG_{stab}) of I upon disulfide bond formation should be slightly higher than 8.2 kJ/mol, because about 10−20% of unfolded molecules are still populated at 1.2 M GdmCl (cf. Figure 1E). The stabilization derived from the difference in K_{SS} between 4 and 1.2 M GdmCl is significantly larger than the value of $\Delta G_{stab} = -3.5$ kJ/mol (at 2 M GdmCl), as derived from the three-state analysis of the unfolding transitions (cf. Table 1). This provides a further indication that the the I $\rightleftharpoons$ U transition is more complex than a two-state reaction (Griko & Privalov, 1994). As mentioned previously, there is evidence for additional equilibrium intermediates between I and U. The presence of such intermediates artificially broadens the I $\rightleftharpoons$ U transition, and the two-state analysis leads to apparent values of ΔG_{stab} which are too small (Carra & Privalov, 1995).

K_{SS}^{app} does not follow the N $\rightleftharpoons$ I transition. Rather, at the midpoint of this transition (at 0.9 M GdmCl, cf. Figure 1), it reached an asymptotic value of about 115 mM. This indicates that the K_{SS} values of I and N are indeed similar, as expected from the identical conformational stabilities of the reduced and oxidized forms of β-lactamase (in the N $\rightleftharpoons$ I transitions in Figure 1). This simple consequence of thermodynamic coupling (cf. Scheme 1) confirms that the disulfide bond is indeed equally stable in the N and I forms of β-lactamase and, vice versa, that the N $\rightleftharpoons$ I transition is unaffected by the disulfide bond.

DsbA-Mediated Oxidation of Reduced β-Lactamase. During the *de novo* folding in the periplasm of *E. coli*, the disulfide bond is introduced into β-lactamase by the DsbA protein (Bardwell *et al.*, 1991; Kamitani *et al.*, 1992). Reduced and oxidized β-lactamases fold with identical rates to native-like conformations of similar stability. β-Lactamase is therefore an excellent model to investigate how conformational folding interferes with the DsbA-mediated introduction of a disulfide bond during oxidative folding.

First, we investigated whether the native-like folded form of reduced β-lactamase could be oxidized by DsbA. In these experiments, 10 μM reduced β-lactamase was incubated with 10−370 μM oxidized DsbA at pH 7.0, 25 °C. After various times, potential thiol/disulfide exchange was stopped by lowering the pH to 2, and the amounts of reduced and oxidized β-lactamase were determined by HPLC. This analysis showed that even after 1 h of incubation and at a 37-fold molar excess of DsbA β-lactamase remained fully reduced. Apparently, reduced β-lactamase is not a substrate for DsbA when it is native-like folded, because the thiol groups are inaccessible.

In the presence of 1.2 M GdmCl, oxidation was possible, and 10 μM β-lactamase was completely oxidized by 11 μM DsbA within about 200 s (Figure 4). The kinetic analysis of these data gave a second-order rate constant of 5.5×10^3 s^{-1} M^{-1} for this oxidation reaction. At 1.2 M GdmCl, reduced β-lactamase is composed of about 5% N, 70% I, and 25% U molecules (cf. Figure 1E). Since the equilibration between I and U is extremely rapid, it is not clear whether DsbA reacts with the thiol groups of β-lactamase directly in the intermediate or, after rapid unfolding, in the unfolded protein. Unfortunately, the rate of oxidation of the unfolded form of β-lactamase by DsbA cannot be measured, because DsbA becomes denatured at the high concentrations of denaturant that are required to unfold β-lactamase completely (cf. Figure 1). Darby and Creighton (1995b)

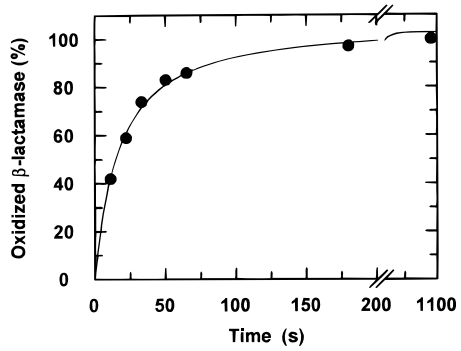


FIGURE 4: Oxidation of reduced β-lactamase by DsbA in 1.2 M GdmCl. Reduced β-lactamase was first incubated in 1.2 M GdmCl to populate the intermediate I. The reaction was started by the addition of DsbA. The final conditions were 10 μM β-lactamase, 11 μM DsbA in 1.2 M GdmCl, 0.1 M Hepes/NaOH, 2 mM EDTA, pH 7.0 at 25 °C. (●) Increase of the fraction of oxidized protein. The solid curve represents a nonlinear least-squares fit of the data for reoxidation to a second-order rate equation and gives a rate constant for oxidation of 5.5×10^3 s^{-1} M^{-1}.

found a rate constant of about 10^6 s^{-1} M^{-1} for the DsbA-mediated formation of a disulfide between two cysteines that are separated by 25 residues in an unstructured peptide (at pH 7.4). For unfolded RNase T1, a rate constant of 10^4 s^{-1} M^{-1} (at pH 7.0) was measured for the formation of a disulfide bond with 96 intervening residues (Frech & Schmid, 1995a). Based on these rates and assuming that they depend on $n^{-3/2}$ (where n is the number of residues between the two cysteines), the bimolecular rate constant for the reaction between DsbA and unfolded β-lactamase should show a value of 4×10^4 s^{-1} M^{-1}. At 1.2 M GdmCl, the equilibrium constant for the I ⇌ U reaction is about 0.3, and, therefore, if oxidation occurs in the unfolded protein, it should show a rate constant of 1.2×10^4 s^{-1} M^{-1}. This is reasonably close to the observed value of 0.55×10^4 s^{-1} M^{-1}.

Competition between Conformational Folding and DsbA-Mediated Oxidation. The previous experiments have shown that reduced β-lactamase cannot be oxidized by DsbA when it has reached a native-like conformation. This suggests that the formation of the disulfide bond has to compete with the conformational folding of the reduced protein. Oxidation is a second-order process, and therefore it should be accelerated and thus progressively precede folding when the concentration of DsbA is increased. To search for a competition between folding and oxidation, we folded reduced β-lactamase at 0.1 M GdmCl in the presence of increasing concentrations of DsbA. Folding of both the reduced and the oxidized protein is complete within 20 min under these conditions (cf. Figure 2). Therefore, the competition experiments were stopped after 40 min by acidification, and the amounts of reduced and oxidized β-lactamase were determined by HPLC. The results are shown in Figure 5. In the presence of a 2-fold excess of DsbA (20 μM) over β-lactamase (10 μM), folding of the reduced protein is apparently much faster than oxidation, and most molecules (95%) folded in the reduced state and thus escaped oxidation by DsbA. Only about 5% of all folded β-lactamase molecules contained the disulfide bond. In the presence of 100 μM DsbA, the fraction of oxidized protein increased to about 40%. Only at very high DsbA concentrations could oxidation compete efficiently with the folding of the reduced protein, and at 400 μM DsbA, about 80% of the refolding molecules were oxidized.

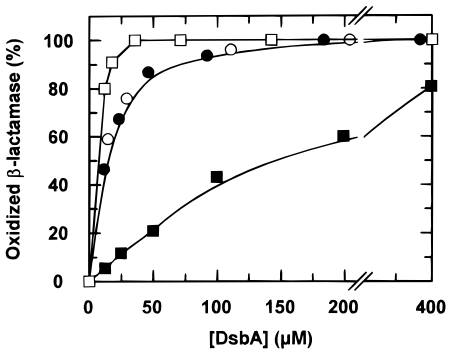


FIGURE 5: Oxidation of reduced β-lactamase in the presence of DsbA at different concentrations of GdmCl. The fraction of oxidized β-lactamase after 40 min of oxidative folding is shown as a function of the DsbA concentration. Reduced β-lactamase was first incubated in 4 M GdmCl and then diluted to the final conditions of 10 μM β-lactamase in 0.1 M Hepes/NaOH, 2 mM EDTA, pH 7.0 at 25 °C, and (■) 0.1 M GdmCl, (●) 0.4 M GdmCl, or (□) 0.6 M GdmCl and the indicated concentrations of DsbA. (○) Data for reduced β-lactamase, which was first incubated in 1.2 M GdmCl to populate I and then diluted to 0.4 M GdmCl.

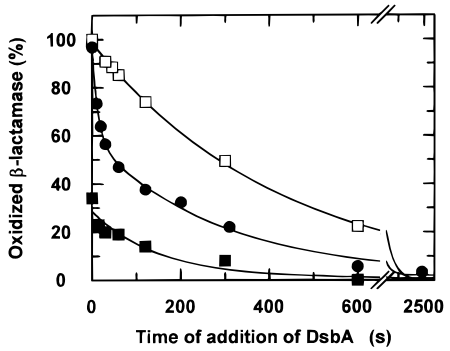


FIGURE 6: Competition between conformational folding and DsbA-mediated oxidation. The fraction of oxidized β-lactamase after 45 min of folding is shown. The final conditions were 0.1 M Hepes/NaOH, 2 mM EDTA, pH 7.0 at 25 °C, and (■) 0.1 M GdmCl, (●) 0.4 M GdmCl, or (□) 0.6 M GdmCl. At time point zero, conformational folding was started, and at the indicated times, DsbA was added at a final concentration of 100 μM. After 45 min, the fraction of oxidized β-lactamase was determined by RP-HPLC.

Reduced β-lactamase is in the native conformation between 0 and 0.6 M GdmCl (cf. Figure 1), but the rate of its refolding decreases strongly with increasing denaturant concentration (cf. Figure 2). If folding and DsbA-mediated oxidation of the reduced protein are indeed competing reactions, more protein molecules should become oxidized at increased denaturant concentration, because conformational folding is decelerated. This is indeed observed. When the competition experiments described above were performed at 0.4 M GdmCl instead of 0.1 M GdmCl, 50% oxidized molecules were observed already in the presence of 20 μM DsbA, and complete oxidation was achieved in the presence of 180 μM DsbA. At 0.6 M GdmCl, oxidation was strongly favored over conformational folding; 80% of the refolding β-lactamase molecules were oxidized already in the presence of 12 μM DsbA, and oxidation was complete when 30 μM DsbA was present.

Identical results were obtained in experiments in which the competition was initiated by diluting β-lactamase either from 4.0 M GdmCl, where the U state predominates, or from 1.2 M GdmCl, where the intermediate I is populated (Figure 5). This shows that the competition between folding and

Table 2: Comparison of the Rate Constants of Refolding of Reduced β-Lactamase As Obtained from the Competition Experiments with DsbA and from Fluorescence Experiments

[GdmCl] (M)	k_{1DsbA}[a] (s^{-1})	k_{2DsbA}[a] (s^{-1})	k_{1Fl}[a] (s^{-1})	k_{2Fl}[a] (s^{-1})
0.1	6.0×10^{-3}	−	6.4×10^{-3}	6.6×10^{-2}
0.4	3.1×10^{-3}	7.3×10^{-2}	4.0×10^{-3}	5.3×10^{-2}
0.6	2.4×10^{-3}	−	2.3×10^{-3}	5.6×10^{-2}

[a] k_{1DsbA} is the rate constant of the slow refolding reaction, and k_{2DsbA} is the rate constant of the fast reaction from the exclusion of DsbA from folding β-lactamase, as determined under the conditions of Figure 6. k_{1Fl} *and* k_{2Fl} are the respective rate constants from the fluorescence-detected refolding of β-lactamase under the same conditions.

DsbA-mediated oxidation occurs only during the final slow stage of folding, i.e., the I → N reaction.

Exclusion of DsbA from Folded Reduced β-Lactamase. The folding reactions which exclude DsbA from the thiol groups of the refolding β-lactamase molecules were identified in a series of two-step experiments. In the first step, the reduced protein was allowed to refold for variable periods of time before, in the second step, 100 μM DsbA was added. As in the previous experiments, we modulated the rate of refolding by varying the concentration of residual denaturant. Refolding in 0.1 M GdmCl is fairly fast, and 100 μM DsbA could introduce the disulfide bond only into about 30% of all molecules, even when it was present from the beginning of folding (Figuire 6). When refolding was allowed to proceed for increasing periods of time before adding DsbA, the percentage of oxidized molecules decreased further, and after about 600 s, all reduced β-lactamase molecules were folded and inaccessible for DsbA. During folding at 0.4 or 0.6 M GdmCl, β-lactamase is completely oxidized when DsbA is present already at the beginning of refolding. At both concentrations of denaturant, the formation of molecules that are resistant to oxidation by 100 μM DsbA followed a biphasic time course (Figure 6), and after about 2500 s, all molecules had folded and were thus resistant to oxidation by DsbA. Within experimental error, the time courses for the formation of oxidation-resistant molecules in Figure 6 parallel the rate-limiting steps of refolding as detected by fluorescence. Table 2 compares the rate constants of the fluorescence-detected refolding of reduced β-lactamase at 0.1, 0.4, and 0.6 M GdmCl with the rate constants of the reactions in which the refolding β-lactamase molecules became inaccessible for oxidation by DsbA. The latter data are less precise because they are based on a limited number of data points, and at 0.1 and 0.6 M GdmCl, only one of the two kinetic phases of refolding could be resolved. Generally, the kinetics as determined by fluorescence and by the resistance to DsbA-mediated oxidation coincide very well. This demonstrates that it is the final slow folding of reduced β-lactamase which renders the thiol groups inaccessible for DsbA. This enzyme can introduce the disulfide bond into reduced β-lactamase molecules only while they are in the unfolded U state or in the intermediate I state, which is still in rapid exchange with U.

DISCUSSION

β-Lactamase Folds in the Absence of Its Disulfide Bond. It was noted before that β-lactamase can reach its native, enzymatically active conformation while still in the reduced state and that in this folded state the two thiol groups are inaccessible for modification or oxidation by glutathione (Laminet & Plückthun, 1989; Walker & Gilbert, 1994, 1995). Equilibrium unfolding of β-lactamase occurs in two stages. Between 0.5 and 1.2 M GdmCl, the native protein is converted to a compact equilibrium intermediate of the molten globule type (in the N ⇌ I reaction), and then, between 1.2 and 3.0 M GdmCl, this intermediate unfolds in the I ⇌ U reaction. The N ⇌ I equilibrium is not affected by the disulfide bond; the I ⇌ U transition, however, becomes broader and is shifted to slightly higher concentrations of GdmCl when the disulfide is present in the oxidized protein. These changes are easiest explained by assuming that the Cys77−Cys123 disulfide bond lowers the entropy of the unfolded protein and thus destabilizes it (Betz, 1993). A covalent cross-link should favor more compact unfolded conformations and thus decrease the difference in solvent-accessibility between U and I (Myers *et al.*, 1995). This would explain why the cooperativity of the I ⇌ U reaction (the *m* value) decreases in the presence of the disulfide bond. These interpretations must remain tentative, however, because the I ⇌ U transition is probably more complex than a two-state process and additional intermediates might be populated between I and U (Uversky & Ptitsyn, 1994).

Surprisingly, the stability of the native state N relative to I and the cooperativity of the N ⇌ I transition are identical in the presence and in the absence of the disulfide bond. The equilibrium unfolding transitions of the reduced and the oxidized protein were superimposable, suggesting that the environment of the disulfide bond is already native-like when the compact intermediate I has formed and does not change further when I folds to N in the I ⇌ N transition. This was confirmed by the finding that the stability of the disulfide bond toward reduction by glutathione was very similar, if not identical, in the N and I states.

Not only the equilibrium between N and I, but also the kinetics of their interconversion are unaffected by the disulfide bond. Throughout the N ⇌ I transition (between 0 and 2.5 M GdmCl), the rates of unfolding and refolding are identical for the reduced and the oxidized forms of the protein. This confirms and extends the above conclusion that the region around the disulfide bond is already native-like folded and is presumably buried in the intermediate I, well before the slow and rate-limiting events of folding take place. Such a neutral role of a disulfide bond for both the stability and the folding kinetics has not been observed before. Normally, disulfide bonds stabilize proteins (unless the disulfide has a functional role as in DsbA) (Pace *et al.*, 1988; Vogl *et al.*, 1995), and they have strong effects on the folding kinetics (Mücke & Schmid, 1994; Jullien & Baldwin, 1981; Denton *et al.*, 1994).

DsbA-Mediated Oxidation and the Folding of the Reduced Protein Compete with Each Other. The thiol groups of reduced β-lactamase are inaccessible for oxidation by DsbA when the protein is native-like folded. At the stage of the molten globule intermediate I (at 1.2 M GdmCl), an oxidation

by DsbA is still possible, albeit at a somewhat lowered rate, when compared with DsbA-mediated disulfide bond formation in unstructured peptides or proteins. We assume that DsbA does not introduce the disulfide bond into I directly, because the thiol groups are already in a native-like environment in this intermediate. I can readily be oxidized by DsbA after fast unfolding, because it is only marginally stable relative to U, and because it equilibrates rapidly with this species.

We find that the introduction of the disulfide bond by DsbA competes with conformational folding at the stage of the final, slow steps of folding of the reduced protein (I → N). These steps exclude DsbA from the thiol groups of the refolding protein molecules. Unlike I, the native protein N cannot be oxidized by a mechanism in which oxidation is coupled to unfolding, because N is stable relative to I and, most importantly, because the equilibration between N and I (and U) is very slow. An extrapolation of the unfolding rates measured between 0.9 and 2.5 M GdmCl (Figure 2) to ≤0.5 M GdmCl suggests that under native conditions the N → I unfolding of the reduced molecules should occur with a rate that is smaller than 10^{-4} s^{-1}.

Premature conformational folding is also a problem in the oxidation of other proteins, such as the bovine pancreatic trypsin inhibitor and an immunoglobulin fragment. For both proteins, buried protein thiols were inaccessible for reaction with glutathione, but the incompletely oxidized forms were much less stable than the native proteins (Goto & Hamaguchi, 1981; Darby *et al.*, 1995; Creighton, 1978).

The consequences for the *de novo* folding of β-lactamase in the bacterial periplasm are clear. The disulfide bond should be introduced rapidly into the refolding molecules, well before the final slow steps of folding occur. DsbA is very well suited for this task. Its concentration in the periplasm is sufficiently high, and its reaction with free protein thiols is extremely rapid. The second-order rate constant for disulfide bond formation in a folding protein is about 10^3-10^4-fold higher with the oxidant DsbA relative to oxidized glutathione (Zapun *et al.*, 1993; Darby & Creighton, 1995b). Thus, the rapid formation of partially-folded intermediates with buried thiols does not seem to pose a problem for DsbA-mediated oxidative folding *in vivo*. As found here for β-lactamase, these intermediates are only mariginally stable and in a fast equilibrium with the unfolded state (Ptitsyn, 1994), which allows oxidation after rapid unfolding. In addition, DsbA can bind to unfolded chain regions and thus slightly stabilize the U state relative to I (Frech *et al.*, 1996).

Oxidative Folding Can Follow Different Mechanisms. There is increasing evidence now that oxidative folding *in vitro* as well as *in vivo* can follow different mechanisms. The major determinants for the mechanism are the number of disulfide bonds in a folding unit, the conformational stability and dynamics of the reduced protein, the accessibility for the oxidant of the protein thiols in the reduced state, and the ability of intermediates with wrong disulfides for disulfide reshuffling. The folding of β-lactamase represents a limiting case. It contains a single disulfide bond only; it can fold to a stable native conformation while still reduced, and in this folded state the thiols are inaccessible for oxidation. As a consequence, oxidation has to occur while the protein is still unfolded or in partially folded states, which are in a fast equilibrium with the unfolded state. DsbA is an excellent oxidant for β-lactamase, because it introduces disulfide bonds very rapidly. It is also conceivable that coupling with translocation into the periplasm decelerates folding (as GdmCl does *in vitro*) and thus allows more time for disulfide bond formation. For proteins such as β-lactamase, rapid conformational folding of the reduced protein thus seems to be an obstacle rather than an advantage for the formation of the disulfide bond. This scenario found for the folding of β-lactamase may also hold for other periplasmic proteins, because most of them contain only one or two disulfide bonds (Joly & Swartz, 1994).

The oxidative folding of a variant of ribonuclease T1 with a single disulfide bond follows a different mechanism. This small protein is only marginally stable when the disulfide bond is reduced, and the thiol groups remain partially accessible when the reduced protein folds to a native-like conformation (Frech & Schmid, 1995b). DsbA introduced the disulfide bond with equal efficiency into the folded and unfolded forms of the reduced protein. Thus, in the case of ribonuclease T1, DsbA-mediated oxidation seemed to be rather independent of conformational folding (Frech & Schmid, 1995a).

Proteins with several disulfide bonds, such as ribonuclease A and the pancreatic trypsin inhibitor, are usually unfolded when they are reduced, and molecules with incorrect disulfide bonds can form readily during oxidative folding. In these cases, an intricate interplay of conformational folding, disulfide bond formation, and disulfide bond isomerization is necessary to reach the correctly cross-linked stable native state (Creighton, 1990; Weissman & Kim, 1991).

This diversity in the mechanisms of folding and oxidation is reflected in a corresponding diversity of the thiol/disulfide oxidoreductases that mediate these reactions *in vivo*. Proteins with a high content of disulfide bonds, which require disulfide isomerizations and a tight coupling with conformational folding, occur predominantly in the secretory pathway of eukaryotes. Accordingly, PDI, the major thiol/disulfide oxidoreductase of the endoplasmic reticulum, is a large dimeric protein with four active sites (Freedman *et al.*, 1994; Kemmink *et al.*, 1995). PDI shows a high disulfide isomerase activity (Puig *et al.*, 1994; Darby & Creighton, 1995a) and an increased affinity for unfolded or partially folded proteins (Noiva *et al.*, 1993; Morjana & Gilbert, 1991; Cai *et al.*, 1994; Song & Wang, 1995; Quan *et al.*, 1995). This helps to recognize molecules with incomplete or incorrect disulfide bonding as substrates, to destabilize them by binding, and thus to facilitate the isomerization of incorrect, but shielded disulfides (Weissman & Kim, 1993; Zapun & Creighton, 1994; Walker & Gilbert, 1995).

Proteins with a low content of disulfide bonds (such as β-lactamase) occur predominantly in the bacterial periplasm. The major problem in their folding is not the formation of incorrect disulfide bonds, but the rapid conformational folding of the reduced molecules, which competes with the introduction of the disulfide bonds. The properties of DsbA, the most prevalent thiol/disulfide oxidoreductase in the periplasm of *E. coli*, reflect this problem. DsbA is a simple monomeric protein with a single active site (Bardwell *et al.*, 1991; Martin *et al.*, 1993; Wunderlich *et al.*, 1993). It is a very strong oxidant, because it is much more stable in the reduced than in the oxidzed form, and it reacts extremely fast with free protein thiols (Zapun *et al.*, 1993; Wunderlich *et al.*, 1995; Darby & Creighton, 1995b; Frech & Schmid,

1995a). If necessary, incorrect disulfides can be isomerized in the periplasm by the homodimeric DsbC protein, which resembles eukaryotic PDI in its isomerase properties (Zapun *et al.*, 1995).

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