Oxidation of Kinetically Trapped Thiols by Protein Disulfide Isomerase[†]

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ABSTRACT: The formation of a stabilized structure during oxidative protein folding can severely retard disulfide formation if the structure must be disrupted to gain access to buried cysteines. These kinetic traps can slow protein folding and disulfide bond formation to the extent that unassisted folding is too slow to be kinetically competent in the cell. Protein disulfide isomerase (PDI) facilitates the oxidation of a kinetically trapped state of RTEM-1 β-lactamase in which two cysteines that form the single disulfide bond in the native protein are buried and approximately 500-fold less reactive than exposed cysteines. Under second-order conditions, PDI-dependent oxidation of reduced, folded β-lactamase is 500-fold faster than GSSG-dependent oxidation. The rate difference observed between PDI and GSSG can be accounted for by the 520-fold higher kinetic reactivity of PDI as an oxidant. Noncovalent interactions between PDI (35 μM) and β-lactamase increase the reactivity or unfolding of β-lactamase in the steady-state by less than 3-fold. At high concentrations of PDI or alkylating agents, the reaction of β-lactamase cysteines approaches a constant rate, limited by the spontaneous unfolding of the protein (k_{unfold} = 0.024 ± 0.005 min^{-1}). PDI does not substantially increase the rate of β-lactamase unfolding; however, once β-lactamase spontaneously unfolds, PDI at concentrations greater than 44 ± 4 μM, oxidizes the unfolded substrate before it can refold (k_{fold} = 1.5 ± 0.2 min^{-1}). PDI also facilitates the glutathione disulfide-dependent oxidation of β-lactamase by approximately 1.7−3-fold, even at saturating GSSG concentrations; however, oxidation rates never exceed the rate of spontaneous β-lactamase unfolding. The high kinetic reactivity of PDI as an oxidant and the almost millimolar concentrations of PDI found in the endoplasmic reticulum provide one mechanism to avoid kinetic traps by making oxidation kinetically competitive with the formation of kinetic traps. In addition, PDI's high reactivity and its noncovalent interactions with unfolded proteins may minimize the significance of even relatively stable kinetic traps, as long as unfolding of the kinetic trap does not become rate-limiting. Chaperones, including the hsp70 chaperone of the endoplasmic reticulum, BiP, and the *Escherichia coli* chaperonin, GroEL, do not appear to increase the rate of β-lactamase unfolding. This suggests that the unfolding rate may help define those structures that the endoplasmic reticulum quality control system recognizes as native.

In the absence of folding catalysts and chaperones, the folding of disulfide-containing proteins is often slow (Goldberger *et al.*, 1963). Even under optimal conditions, the uncatalyzed oxidative refolding of reduced ribonuclease (RNase)[1] has a half-life of about 1.5 h (Lyles & Gilbert, 1990a), and bovine pancreatic trypsin inhibitor (BPTI) refolds even more slowly ($t_{1/2}$ = 8 h) (Weissman & Kim, 1993). In the endoplasmic reticulum, where disulfide-containing proteins are folded in eukaryotic cells, the time available for folding and exit from the ER is typically about 0.5−1.5 h (Lodish *et al.*, 1983). Clearly, spontaneous oxidative folding is often not fast enough to be kinetically competent *in vivo*. It was this realization that inspired the first isolation of a cellular catalyst of oxidative folding, protein disulfide isomerase (PDI) (Goldberger *et al.*, 1963).

During protein folding, cysteines that must eventually be oxidized to form disulfides in the native structure may become trapped in the reduced state by the formation of stabilized native or non-native structures, including those stabilized by other disulfide bonds in the molecule (Goto & Hamaguchi, 1981; States *et al.*, 1984; Creighton & Goldenberg, 1984; Weissman & Kim, 1993).

In order to complete folding, these kinetically trapped intermediates must unfold sufficiently to expose sterically shielded cysteine residues to oxidants (including intramolecular oxidants) or they must rearrange to species with accessible cysteines. Weissman and Kim (1993) reported that PDI accelerates the rearrangement of kinetically trapped BPTI folding intermediates with buried sulfhydryls by 4000- to 6000-fold compared to the approximately 140-fold rate enhancement observed in PDI-catalyzed folding of RNase (Lyles & Gilbert, 1990b). However, for both BPTI and RNase, the k_{cat}'s of the PDI-dependent reaction are similar, 0.3−5 min^{-1} (Weissman & Kim, 1993). Thus, the differences in the rate acceleration for these two proteins are largely due to differences in the spontaneous oxidative folding rates rather than the rate of the PDI-catalyzed reaction. The comparable rates of folding observed for the PDI-catalyzed reactions suggest that PDI might provide some mechanism for destabilizing the kinetic traps encountered during spontaneous refolding and minimizing their influence on slow refolding.

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[1] Abbreviations: PDI, protein disulfide isomerase; GSH, glutathione; GSSG, glutathione disulfide; βLa, RTEM-1 β-lactamase; $βLa_{red}$, reduced and refolded β-lactamase; ER, endoplasmic reticulum; RNase, bovine pancreatic ribonuclease A; BPTI, bovine pancreatic trypsin inhibitor; Gdn·HCl, guanidine hydrochloride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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PDI has several properties that might be useful in resolving potential problems with kinetic traps. PDI is a 55 kDa, ER resident that catalyzes disulfide bond formation and rearrangement (Freedman, 1989; Noiva & Lennarz, 1992). It is an abundant protein; concentrations in the ER have been estimated at near millimolar amounts (Lyles & Gilbert, 1990b; Zapun *et al.*, 1992). The two active sites of the molecule, including the sequence WCGHCK, are located in two thioredoxin-homology domains found near the N- and C-termini (Edman *et al.*, 1985). The disulfide bonds of PDI are exceptionally good oxidizing agents with a high redox potential (Lyles & Gilbert, 1990a; Hawkins & Freedman, 1991; Lundstrom & Holmgren, 1993) and a high kinetic reactivity with thiols (Gilbert, 1989; Darby *et al.*, 1994). In addition to the redox-active dithiol/disulfide centers at the active sites (Lyles & Gilbert, 1994; Vuori *et al.*, 1992), PDI interacts with unfolded proteins and peptides at a relatively weak binding site that exhibits little sequence preference (Morjana & Gilbert, 1991; Noiva *et al.*, 1993). PDI is an essential protein in yeast, but a mutant PDI with undetectable disulfide isomerase activity can rescue the null mutation, suggesting that some activity other than disulfide formation and rearrangement is essential for yeast viability (LaMantia & Lennarz, 1993).

In order to explore mechanisms by which PDI might accelerate the oxidation of sterically inaccessible cysteines, we have exploited a simple model system, RTEM-1 β-lactamase (βLa). Native βLa is a monomer with only one disulfide bond, so reduction or isomerization of other disulfides in the molecule does not complicate the mechanism. The single disulfide contributes minimally to stability, and even under reducing conditions, βLa spontaneously refolds to a stable conformation (βLa_{red}) with near-native enzymatic activity (Laminet & Pluckthun, 1989; Zahn *et al.*, 1994b). The cysteines of βLa_{red} are very resistant to oxidation or alkylation, indicating that the native-like structure of βLa_{red} traps the cysteine thiols in a sterically inaccessible state. The X-ray crystal structure of native βLa (DeLucia *et al.*, 1980) shows that the single disulfide bond is buried in the interior of the molecule. The native-like activity and the resistance to alkylation suggest that the cysteine residues of βLa_{red} are inaccessible as well.

Our results suggest that PDI may provide multiple strategies for dealing with kinetic traps. Under second-order conditions, the PDI-dependent oxidation of βLa_{red} is approximately 500-fold faster than oxidation by GSSG. Most of this rate acceleration results from the high kinetic reactivity of PDI's disulfides that allows relatively low concentrations of PDI to efficiently trap βLa_{red} every time it spontaneously unfolds. Because of its enhanced reactivity and its high local concentration in the ER (Lyles & Gilbert, 1990b; Zapun *et al.*, 1992), PDI-facilitated disulfide formation may avoid kinetic traps by reacting with cysteines before they become trapped. If kinetic traps do form, PDI's high kinetic reactivity as an oxidant also diminishes the significance of unreactive, sterically shielded cysteines as long as unfolding does not limit the rate. Covalent and noncovalent interactions with PDI are insufficient to promote a significant extent of βLa_{red} unfolding. In addition, high concentrations of PDI or other chaperones do not increase the rate of βLa_{red} unfolding. If this is true in the cell, the inability to accelerate unfolding would imply that a sufficiently slow unfolding rate may help define a structure as "native".

MATERIALS AND METHODS

Materials. Recombinant rat PDI was expressed in *Escherichia coli* strain BL21(DE3) and purified to >95% homogeneity as described previously (Gilbert *et al.*, 1991). RTEM-1 β-lactamase was a generous gift of Dr. Timothy Palzkill (Baylor College of Medicine, Houston, TX). Glutathione (GSH), glutathione disulfide (GSSG), *N*-ethylmaleimide (NEM), iodoacetamide (IAM), dithiothreitol (DTT), bovine serum albumin (BSA), and lysozyme were from Sigma (St. Louis, MO); 2-mercaptoethanol was from J. T. Baker (Phillipsburg, NJ). Guanidine hydrochloride (Gdn·HCl) was from Pierce (Rockford, IL). The βLa substrate, nitrocefin, was obtained from Becton-Dickenson Microbiological Systems (Cockeysville, MD). GroEL/ES and recombinant hamster Grp78 (BiP) were obtained from Stress-Gen (Vancouver, BC).

Methods. βLa (0.25 mg/mL) was reduced and denatured in 100 mM Tris, pH 8.0, 6.4 M Gdn·HCl, and 20 mM 2-mercaptoethanol for 1.5 h at 37 °C. The reduced and denatured enzyme had less than 5% the activity of the native enzyme. Reduced, refolded β-lactamase (βLa_{red}) was produced by diluting the reduced, denatured enzyme 50-fold into 0.1 M Tris, pH 8.0, 25 °C. After 15 min, the refolded, active enzyme was further diluted into alkylation or oxidation reactions. Residual thiol (<0.06 mM) and Gdn·HCl (<0.01 M) had no effect on the rate of oxidation or alkylation since βLa_{red} in which the excess 2-mercaptoethanol and Gdn·HCl were removed by repeated dilution/concentration by ultrafiltration (Centricon 10) gave identical results. The specific activity of refolded βLa_{red} was consistently 60%−70% that of the original, native βLa. In the absence of other proteins, dilution of βLa or βLa_{red} to concentrations less than 20 nM resulted in a slow decrease in the enzyme activity with time (0.019/min) and decreased recovery of the activity during refolding and oxidation or alkylation. The presence of other proteins such as lysozyme (27 μg/mL) or BSA (0.7 mg/mL) eliminates this background loss of activity without affecting the rate constant for alkylation or oxidation. Therefore, BSA and/or lysozyme were included in the buffer during all experiments.

βLa activity was determined using the chromogenic substrate, nitrocefin (O'Callaghan *et al.*, 1972). Nitrocefin hydrolysis (80 μM final concentration) was observed at 486 nm, 25 °C, pH 7.0 (100 mM sodium phosphate). PDI concentration was determined by absorbance at 280 nm ($E^{0.1\%}$ = 0.94 (mg/mL)$^{-1}$ cm^{-1}) (Gilbert, 1989). All absorbance measurements were performed using a Beckman DU-70 spectrophotometer.

Folding of Reduced, Denatured βLa. The rate of activity regain during the refolding of reduced, denatured βLa was measured by diluting the reduced, denatured βLa (43 nM final concentration) into pH 8.0, 0.1 M Tris·HCl, 25 °C. Aliquots were withdrawn periodically and assayed for βLa activity using nitrocefin as substrate. The regain of βLa activity at the completion of the reaction was consistently between 60%−70% that of the native enzyme.

Alkylation of Reduced, Denatured βLa. The rate of sulfhydryl protection during βLa refolding was measured by diluting 1.7 μM reduced, denatured βLa 50-fold into 100 mM Tris·HCl, pH 8.0, 25 °C. At various times, aliquots of 20 μL were withdrawn and added to 20 μL of 100 mM NEM. After 60 s, 40 μL of 100 mM GSH was added to destroy

the NEM followed by 1 mL of 0.1 M Tris·HCl, pH 8.0. After a 15 min incubation to allow the unalkylated βLa to refold, βLa activity was measured. The zero-time point was determined by diluting the reduced, denatured βLa directly into NEM at a final concentration of 50 mM. The 100% activity was taken as the activity of reduced, refolded βLa exposed to the same conditions.

Alkylation of Reduced, Refolded β-Lactamase. Reduced, refolded βLa (22 nM final concentration) was added to 0.1 M Tris·HCl, pH 8.0, containing the indicated concentrations of NEM or IAM followed by incubation at 25 °C. Aliquots were withdrawn at various times and assayed for βLa activity. The decrease in βLa activity was measured over a 4 h time course. The rate constants for alkylation were determined by assuming complete activity loss at the end of the reaction. This assumption was confirmed by following the inactivation to completion in several experiments. Native βLa that had not been reduced showed no significant activity loss (<5%) in the presence of NEM or IAM.

Oxidation of Reduced, Refolded β-Lactamase. Oxidation of βLa_{red} was carried out at pH 8.0 (0.1 M Tris·HCl), 25 °C, in the presence of various concentrations of GSSG and/or PDI and a final concentration of 22 nM βLa_{red}. For PDI-assisted oxidation, PDI was incubated with GSSG for >15 min prior to addition of βLa_{red}. The extent of βLa_{red} oxidation was determined as previously described (Walker & Gilbert, 1994) by exploiting the observation that oxidized βLa is relatively insensitive (<35% activity loss) to the effects of 50 mM NEM in the presence of 1.5 M Gdn·HCl while the reduced, refolded βLa is completely inactivated (<5% residual activity). After initiating the reaction by the addition of βLa_{red}, aliquots were withdrawn at various times and diluted into Gdn·HCl at a final concentration of 1.8 M containing 50 mM NEM for 0.5 min and then diluted an additional 14-fold by the addition of buffer. After 15 min, βLa activity was determined. In all experiments, the initial rate of oxidation was measured by sampling multiple aliquots over a 2 h time course. The 100% activity was taken as the activity of the same amount of folded, reduced βLa.

RESULTS

Refolding of Reduced β-Lactamase. Native βLa has one disulfide bond located in the hydrophobic core of the molecule, but the formation of this disulfide is not essential for folding or for the regain of enzyme activity (Laminet & Pluckthun, 1989). Under reducing conditions, where formation of the single disulfide bond is not possible, regain of activity from the reduced, denatured protein is relatively rapid ($k = 0.52$ min^{-1}), and the specific activity of the folded βLa_{red} is 65% that of oxidized, native β-lactamase (Figure 1).

Once folded, the sulfhydryls of reduced βLa_{red} are very unreactive. Treatment of folded βLa_{red} with 20 mM NEM for 6 min has no significant effect (<9%) on βLa activity. However, NEM alkylation in the presence of 1.5 M Gdn·HCl results in rapid and complete activity loss. These observations are consistent with a disulfide-independent folding of βLa_{red} into an enzymatically active, native-like conformation (Laminet & Pluckthun, 1989) in which the two sulfhydryl groups that normally form the single disulfide bond are inaccessible and buried in the core of the molecule.

To determine how quickly the sulfhydryl groups become protected, reduced, denatured βLa was diluted into refolding

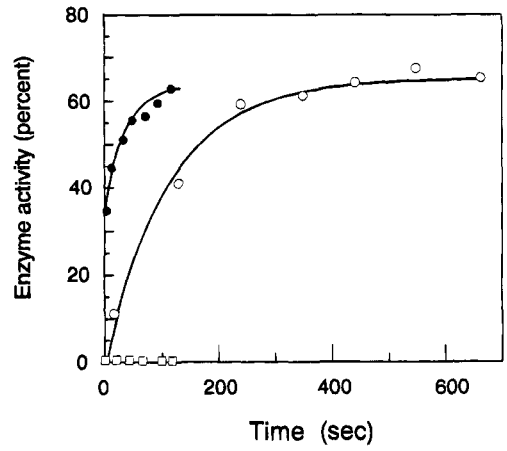


FIGURE 1: Regain of the enzyme activity of reduced, denatured βLa under reducing conditions compared to the rate of protection of the cysteines against alkylation by NEM. The increase in βLa_{red} activity (○) was determined at pH 8.0, 25.0 °C, at a concentration of 43 nM reduced, denatured β-La. The solid curve is an exponential function with a rate constant of 0.52 ± 0.05 min^{-1}, an activity of 65% ± 1% after completion of refolding, and an initial activity of 4% ± 3% at time zero. The time course for protection of the cysteines during the refolding of denatured, reduced βLa (●) was determined at pH 8.0, 25 °C, as described in the text. The curve drawn through the points is an exponential function with a rate constant of 1.5 ± 0.2 min^{-1}, a final recovery of βLa activity of 64% ± 1%, and a rapid protection of 36% ± 2% of the activity. Alkylation of reduced, denatured βLa by NEM in the presence of 1.8 M Gdn·HCl (□) shows that the amount of oxidized βLa in the preparation is negligible. For all experiments, the 100% activity represents the activity observed for the same amount of native βLa under the same conditions. Experimental points are from duplicate or triplicate independent determinations.

conditions. After various times, the reaction was exposed to a pulse of 50 mM NEM for 1 min. Under these conditions, βLa_{red} is insensitive to alkylation, but species with exposed sulfhydryl groups are alkylated and inactivated (Walker & Gilbert, 1994). The kinetics of NEM protection are biphasic (Figure 1). Approximately half of the βLa is rapidly protected even when the unfolded protein is diluted directly into an NEM-containing solution (zero-time point). The protection observed at early folding times is not due to residual oxidized βLa because adding NEM in the presence of denaturant completely inactivates the enzyme. Full protection against alkylation occurs with a rate constant of 1.5 min^{-1}, which is approximately 3-fold faster than the rate constant for regain of enzymatic activity (Figure 1).

Alkylation of Reduced β-Lactamase. Once folded, βLa_{red} is alkylated rather slowly by NEM. At higher NEM concentrations, inactivation becomes zero-order with respect to NEM, suggesting that an NEM-independent step limits the rate of alkylation. The apparent, first-order rate constant for alkylation at saturating NEM is 0.018 ± 0.005 min^{-1} (Figure 2). βLa_{red} is also inactivated by iodoacetamide (IAM) alkylation. At lower concentrations of IAM, inactivation of βLa_{red} is first-order in IAM concentration, but at higher concentrations, inactivation also saturates (Figure 2), suggesting a change in rate-determining step from an IAM-sensitive step to an IAM-independent reaction. At saturation, the observed first-order rate constant for IAM alkylation approaches a limiting value (k_{obs} = 0.024 ± 0.005 min^{-1}) that is insignificantly different from that observed with NEM. Since the intrinsic rate constants for alkylation of a typical, unhindered sulfhydryl group by NEM and IAM (k_{alk}) differ

Table 1: Rate Constants for Reaction of βLa_{red}

reactant	$C_{1/2}$[a] (mM)	k_{uni}[b] ($\times 10^3$ min^{-1})	k_{bi}[c] (M^{-1} min^{-1})	k_{RSH}[d] (M^{-1} min^{-1})
IAM	32 ± 4	24 ± 5	0.9	330[e]
NEM	<0.2	18 ± 5		1.2 × 10^4 [f]
GSSG	7 ± 2	1.8 ± 0.1	0.27	190[g]
PDI	0.044 ± 0.004	5.8 ± 0.3	130	5 x 10^4 [h]
PDI + 4 mM GSSG	0.011 ± 0.001	6.2 ± 0.2	620	
PDI + 40 mM GSSG	0.012 ± 0.003	5.2 ± 0.2	450	

[a] Concentration of reactant at which the initial velocity of the reaction with βLa_{red} is one-half of the maximal observed velocity at saturation. [b] First-order rate constant for the reaction with βLa_{red} at saturating concentrations of the reactant. [c] Second-order rate constant for the reaction of βLa_{red} at subsaturating concentrations of reactant (calculated from the ratio of $k_{uni}/C_{1/2}$). [d] Second-order rate constant for the reaction of the reagent with a typical exposed thiol of pK_a 8.6. Experimental values from the indicated references were adjusted to pH 8.0. [e] Creighton, 1984. [f] Torchinskii, 1994. [g] Szajewski *et al.*, 1980. [h] Gilbert, 1989.

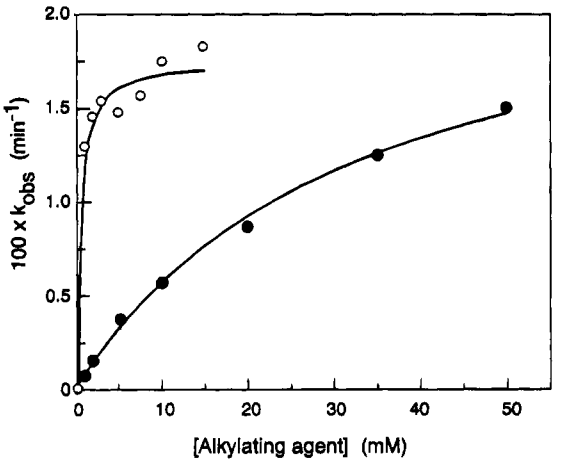


FIGURE 2: Saturation kinetics for alkylation of βLa_{red} by NEM and iodoacetamide. The apparent first-order rate constants for the inactivation of folded βLa_{red} by alkylation with NEM (○) or IAM (●) were determined at pH 8.0, 0.1 M Tris·HCl, 25 °C. The hyperbolic curves are drawn according to the kinetic constants shown in Table 1. Experimental points are from duplicate or triplicate independent determinations.

by over 40-fold (Table 1), the kinetic observations are consistent with the mechanism shown in eq 1 in which βLa_{red}

$$\beta\text{-La(SH)}_2 \underset{k_{fold}}{\overset{k_{unfold}}{\rightleftharpoons}} \underset{\text{SH}}{\beta\text{-La}} \xrightarrow{k_{alk}[Alk]} \underset{\text{S-Alk}}{\beta\text{-La}} \qquad (1)$$

is slowly converted to an unfolded form in which the sulfhydryls are exposed to alkylation. At high concentrations of alkylating agents, the rate-limiting step for sulfhydryl modification changes from alkylation (k_{alk}) to unfolding (k_{unfold}). The effects of βLa_{red} structure on the reactivity of cysteine sulfhydryls is also supported by the observation that 1.5 M Gdn·HCl increases the alkylation rate by >500-fold (data not shown).

GSSG-Dependent Oxidation. A convenient assay for βLa_{red} oxidation was developed on the basis of the observation that oxidized βLa is resistant to the effects of NEM alkylation in 1.5 M Gdn·HCl while βLa_{red} is completely inactivated (Walker & Gilbert, 1994). Using this assay, the effect of GSSG concentration on the initial rate of βLa_{red} oxidation was determined (Figure 3). As with alkylation, GSSG-dependent oxidation of βLa_{red} exhibits a change in rate-determining step with increasing GSSG concentration. However, the rate constant observed at saturating GSSG is 13-fold slower than the rate constant for spontaneous βLa_{red} unfolding (Table 1), suggesting that some GSSG-independent step other than βLa_{red} unfolding becomes rate-limiting at high

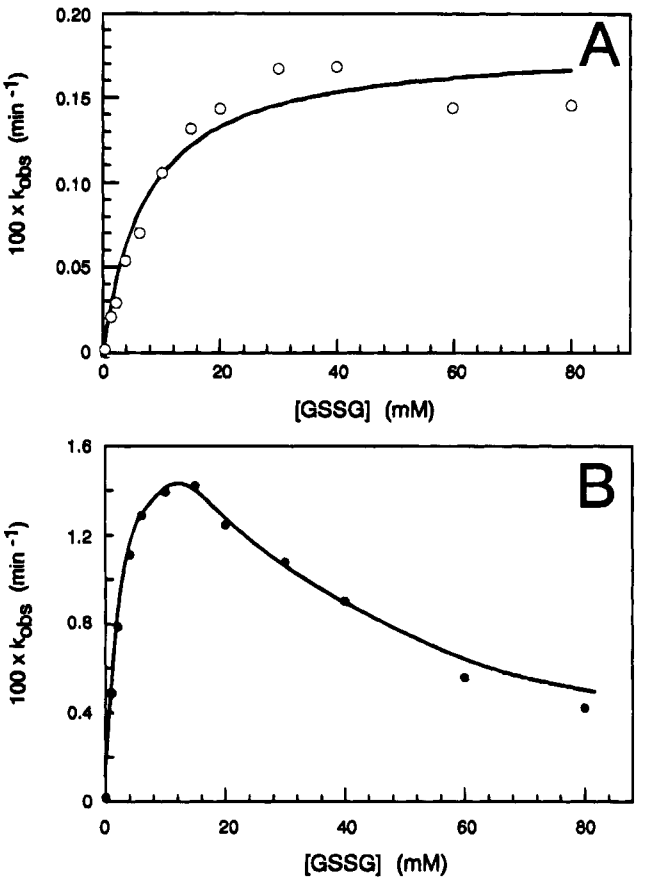


FIGURE 3: Effect of GSSG concentration on the rate of βLa_{red} oxidation in the presence and absence of Gdn·HCl. Experiments were performed at pH 8.0, 0.1 M Tris·HCl, 25 °C. A. β-Lactamase oxidation by GSSG in the absence of Gdn·HCl. The solid curve is drawn using the values shown in Table 1. B. βLa_{red} oxidation by GSSG in the presence of 0.2 M Gdn·HCl. Experimental points are from duplicate or triplicate independent determinations.

GSSG concentrations. Intramolecular disulfide formation is a two-step process in which the GSSG-dependent formation of a mixed-disulfide intermediate (βLa[SH][SSG]) is followed by GSSG-independent intramolecular disulfide formation (Darby *et al.*, 1994; Gilbert, 1990). Rate-limiting intramolecular disulfide formation from a mixed-disulfide intermediate provides a second, slower step in the GSSG-dependent oxidation that could limit the rate at high GSSG concentrations (Figure 3A).

As with IAM alkylation, low concentrations of Gdn·HCl greatly increase the rate of GSSG-dependent oxidation (Figure 3B), consistent with the protection of βLa_{red} thiols by the folded structure of βLa_{red}. However, in the presence of Gdn·HCl, high concentrations of GSSG inhibit oxidation,

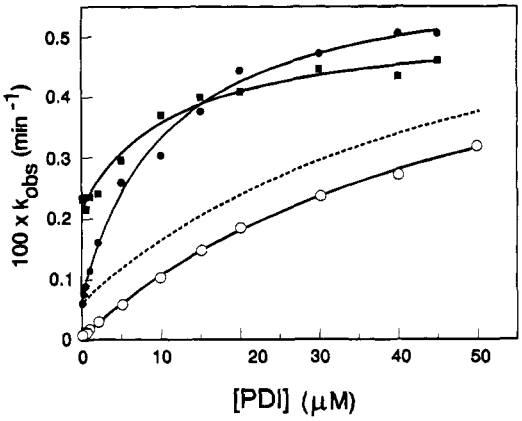


FIGURE 4: Oxidation of βLa_{red} in the presence of PDI and GSSG. Experiments were performed at pH 8.0, 0.1 M Tris·HCl, 25 °C: (○), 0 mM GSSG; (●), 4 mM GSSG; (■), 40 mM GSSG. Curves are drawn using the values shown in Table 1. Experimental points are from duplicate or triplicate independent determinations. The dashed curve represents the expected rate constants if the oxidation by 4 mM GSSG and PDI were simply additive.

most likely due to the formation of dead-end mixed disulfides with both βLa_{red} cysteines (βLa[SSG][SSG]) (Gilbert, 1990; Darby *et al.*, 1994). Since this inhibition is not observed without denaturant, the two sulfhydryl groups of βLa_{red} do not react with GSSG to form a dead-end species with two glutathione mixed disulfides (Figure 3A). This suggests that spontaneous unfolding of reduced β-lactamase does not expose both cysteines at the same rate or that the presence of Gdn·HCl changes the partitioning of the mixed-disulfide intermediate to favor reaction with additional GSSG rather than intramolecular disulfide formation.

PDI-Mediated Oxidation. PDI, even in the absence of exogenous oxidants, supports the oxidation of βLa_{red}. Under second-order conditions, PDI-dependent oxidation is approximately 500-fold faster than GSSG-dependent oxidation (Figure 4, Table 1). PDI-dependent oxidation also saturates at increasing PDI concentration (Figure 4). At saturation, the first-order rate constant ($k = 0.0058\ min^{-1}$) is 3.2-fold faster than GSSG-dependent oxidation and 4.1-fold slower than the first-order rate constant for spontaneous βLa_{red} unfolding.

PDI-dependent oxidation is at least a two-step process (eq 2). At saturating PDI concentrations, the rate-limiting, PDI-

$$\beta La(SH)_2 \underset{k_{fold}}{\overset{k_{unfold}}{\rightleftharpoons}} \beta La\begin{matrix}SH\\SH\end{matrix} + PDI \xrightarrow{[PDI]k_{mix}} \beta La\begin{matrix}S\text{-}SPDI\\SH\end{matrix} \xrightarrow{k_{intra}} \beta La\begin{matrix}S\\S\end{matrix} + PDI \quad (2)$$

independent step could be intramolecular disulfide formation, analogous to GSSG oxidation, or spontaneous unfolding of βLa_{red}, as with alkylation. If a step after βLa_{red} unfolding is rate-limiting, covalent or noncovalent complexes containing unfolded βLa and PDI should accumulate during the reaction. Several experimental approaches failed to detect significant accumulation of such intermediates. Quenching the PDI-dependent oxidation of βLa_{red} with NEM followed by SDS–PAGE and Western blotting with anti-βLa antibody showed no detectable covalent PDI–βLa complex (data not shown), although such intermediates may be difficult to trap (Darby & Creighton, 1995). If some step after βLa unfolding were rate-limiting, the βLa_{red} activity should initially fall as βLa_{red} unfolds ($k_{unfold} = 0.024\ min^{-1}$) and then increase ($k_{ox} = 0.0058\ min^{-1}$) as oxidation to native βLa occurs. However, incubating βLa_{red} with high concentrations (35 μM) of oxidized or reduced PDI for 20 min has no effect (<5%) on the βLa_{red} activity. On the basis of kinetic models that included rate-limiting steps after βLa_{red} unfolding, a 30% loss of activity would be expected. Thus, PDI-dependent oxidation of βLa_{red} to βLa occurs without the accumulation of detectable inactive species. This implies that the rate-limiting step at saturating PDI is βLa_{red} unfolding. The 4.1-fold slower rate of βLa_{red} unfolding observed for PDI-dependent oxidation may reflect a more extensive unfolding of βLa_{red} to allow PDI access to the buried cysteines.

PDI also affects the rate of GSSG-dependent oxidation of βLa_{red}. At saturating GSSG concentrations (40 mM), increasing the concentration of PDI increases the rate of βLa_{red} oxidation by approximately 3-fold (Figure 4, solid-squares, Table 1), making the rate of βLa_{red} oxidation comparable to that observed at saturating PDI. A similar effect is observed at subsaturating GSSG (4 mM), and again, the limiting rate approaches that of spontaneous βLa_{red} unfolding. In addition to supporting direct oxidation, PDI also accelerates the glutathione-dependent oxidation, most likely by facilitating the intramolecular rearrangement of a mixed disulfide between glutathione and βLa_{red}, as observed with small peptide substrates (Darby *et al.*, 1994). However, the possibility cannot be rigorously excluded that PDI-dependent, GSSG oxidation of βLa_{red} avoids this mixed-disulfide intermediate. PDI and GSSG are not simply acting as independent oxidants during the oxidation of βLa_{red}, since the sum of their independent rates does not equal the observed rate in the presence of both PDI and GSSG (Figure 4).

Noncovalent Interactions. PDI that has been reduced and treated with NEM or iodoacetamide has no detectable disulfide isomerase activity, and alkylation has no effect on interactions of peptides with PDI's peptide-binding site (Noiva *et al.*, 1991). Alkylated PDI (20 μM) increases the rate of βLa_{red} alkylation at subsaturating concentrations of iodoacetamide (5 mM) by 1.7-fold; however, there is no detectable effect of PDI on the rate of alkylation at saturating iodoacetamide (50 mM), where βLa_{red} unfolding is rate-limiting (Figure 5). A similarly small, but significant effect of alkylated PDI is also observed at both saturating and subsaturating concentrations of GSSG (Figure 6), where the spontaneous unfolding rate is faster than subsequent oxidation and folding. Alkylated PDI (35 μM) increases the rate constant for GSSG-dependent βLa_{red} oxidation by 1.3- to 2.5-fold. These rate increases, albeit small, cannot be attributed to nonspecific protein effects; similar concentrations of other proteins (BSA and folded lysozyme) do not increase βLa_{red} alkylation or oxidation rates. The rate acceleration provided by alkylated PDI is observed only when the rate is not limited by spontaneous unfolding. Noncovalent interactions of βLa_{red} with PDI do not increase the rate of βLa_{red} unfolding, but they may increase the steady-state concentration of unfolded βLa_{red} or its reactivity with GSSG and IAM.

Other Chaperones. Other chaperones were examined for their ability to induce net unfolding of βLa_{red}. Incubation of βLa_{red} with BiP (grp78) at a concentration of 1.3 μM or GroEL/ES at a concentration of 0.2 μM (expressed as the concentration of tetradecamer) for 120 min did not result in

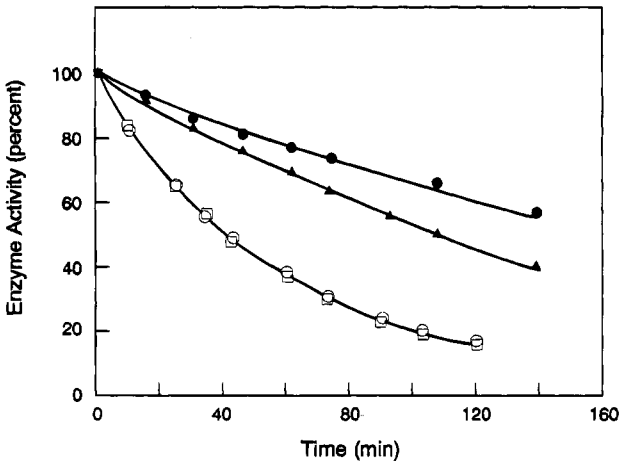


FIGURE 5: The effect of alkylated PDI on the inactivation of βLa_{red} by IAM. Experiments were performed at pH 8.0, 0.1 M Tris·HCl, 25 °C. Alkylation by nonsaturating IAM (5 mM) in the presence (▲) and absence (●) of 20 μM alkylated PDI. Alkylation by a near-saturating concentration of IAM (50 mM) in the presence (□) and absence (○) of 20 μM alkylated PDI. Experimental points are from duplicate or triplicate independent determinations.

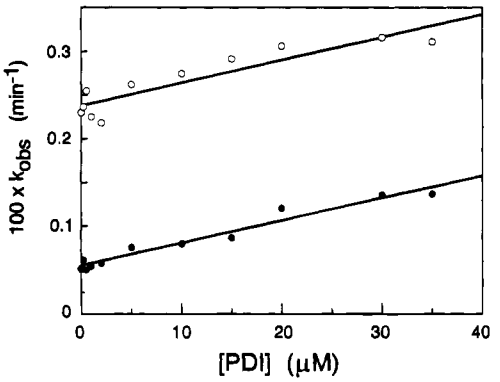


FIGURE 6: The effect of alkylated PDI on the GSSG-dependent oxidation of βLa_{red}. The observed rate constants for βLa_{red} oxidation by 40 mM (○), and 4 mM (●) GSSG were determined with various concentrations of NEM-alkylated PDI at pH 8.0, 0.1 M Tris·HCl, 25 °C. Experimental points are from duplicate or triplicate independent determinations.

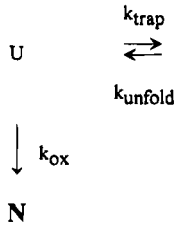
any detectable (<5%) decrease in the activity of βLa_{red}. Since equilibrium dissociation constants for BiP and GroEL binding to unfolded proteins are expected to be much lower than the concentrations of BiP and GroEL employed (Zahn *et al.*, 1994b), neither GroEL nor BiP appears to be capable of accelerating the rate of βLa_{red} unfolding. This is consistent with a previous report in which GroEL did not induce detectable unfolding of βLa_{red} (Laminet *et al.*, 1990).

DISCUSSION

During oxidative protein folding, rapid hydrophobic collapse and/or the formation of stabilized native or non-native structure can bury cysteine thiols and inhibit the access of oxidizing agents. If this interfering structure forms rapidly and unfolds or rearranges slowly, the folding protein will be kinetically trapped in a metastable, incompletely oxidized state. In the ER, stable kinetic traps pose a significant problem because folding and oxidation must be fast enough to allow effective protein secretion (Gething & Sambrook, 1992). For example, the oxidative folding of BPTI (three disulfides) proceeds very slowly in the absence of folding assistants because of the accumulation of two kinetically trapped, two-disulfide intermediates that are converted to native BPTI very slowly (Creighton & Goldenberg, 1984; Weissman & Kim, 1993). These kinetically trapped species oxidize or rearrange slowly because much of the stabilizing structure must be disrupted in the largely unfolded transition state (Zhang & Goldenberg, 1993; Mendoza *et al.*, 1994). Clearly, kinetic traps that retard folding to such an extent must be dealt with by the cellular folding machinery in order to make folding in the cell kinetically competent (Goldberger *et al.*, 1963).

Stability of Folded, Reduced β-Lactamase. The more stable a kinetic trap becomes, the more difficult it will be to disrupt and the more it will slow productive folding. There are several independent ways to estimate the stability of the structure in βLa_{red} that protects the two cysteine residues against alkylation and oxidation. The equilibrium constant for forming the structure that protects the cysteine residues in βLa_{red} can be estimated from the ratio of the rate constant for folding into a structure in which the cysteines are protected (Figure 1) to the rate constant for unfolding the protective structure (Figure 2). This estimated equilibrium constant is 60, corresponding to a ΔG of stabilization of 2.4 kcal/mol. However, there is a folding process that is significantly faster than 1.5 min^{-1} (Figure 1) that leads to a protected but inactive βLa_{red} conformation(s). Consequently, the rate constant for folding into an NEM-protected state may be faster than 1.5 min^{-1} and the 2.4 kcal/mol would represent a minimum estimate of the ΔG. The rate constant for reaction of a typical sulfhydryl group with iodoacetamide (k_{alk}, eq 1) at pH 8.0 is 330 M^{-1} min^{-1} (Table 1). Under second-order conditions, where alkylation is rate-limiting, the observed rate constant for alkylation of βLa_{red} ($k_{obs} = k_{unfold}k_{alk}/k_{fold}$, eq 1) is 0.9 M^{-1} min^{-1}. The equilibrium constant for cysteine protection can be estimated from the ratio 330 M^{-1} min^{-1}/0.9 M^{-1} min^{-1} ($k_{fold}/k_{unfold} = K_U = 500$) corresponding to a stabilization free-energy of 3.5 kcal/mol. A comparable estimate (K_U = 380) is obtained using the corresponding rate constants for GSSG-dependent oxidation (Table 1). If the rate constants for alkylation and oxidation of unfolded βLa are actually lower than those of a totally exposed cysteine, the stability estimate of 3.7 kcal/mol would be an upper limit. Consequently, the stability of the structure hindering the sulfhydryl reactivity of βLa_{red} can be estimated as 2.4−3.7 kcal/mol. The urea-dependent equilibrium unfolding of the overall structure of βLa_{red} is complex and involves an inactive folding intermediate with fluorescence properties comparable to the native state (Zahn *et al.*, 1994b). This intermediate has been estimated to be 4.2 kcal/mol more stable than the fully unfolded protein and approximately 8 kcal/mol less stable than βLa_{red} (Zahn *et al.*, 1994b). Both the fully folded state and the folding intermediate appear to be more stable than the structure(s) protecting the cysteines from alkylation or oxidation, suggesting that cysteine exposure may not require complete disruption of the βLa_{red} structure.

Dealing with Kinetic Traps. The simple mechanism shown in eq 3 suggests three ways for cellular folding assistants to deal with kinetic traps. (1) Avoid them: foldases such as PDI or PPIase could avoid kinetic traps by increasing the rate of oxidation and productive folding (k_{ox}) until it exceeds that for forming the kinetic trap (k_{trap}). (2) Ignore them: kinetic traps can be tolerated as long as the observed rate of oxidation ($k_{ox}k_{unfold}/k_{trap}$) is fast enough to



(3)

be competent in the overall maturation of the protein in the ER. As long as unfolding of the kinetic trap does not become rate-limiting, a faster oxidation of the unfolded protein (k_{ox}) makes it possible to ignore more stable kinetic traps. (3) Unfold them: folding assistants might increase the rate constant for unfolding or increase the extent of unfolding of the kinetically trapped intermediate (k_{unfold}).

Avoiding Kinetic Traps. Under second-order conditions, PDI is 500-fold more effective than GSSG in trapping and oxidizing βLa_{red}. Most of this effect can be attributed to the high reactivity of PDI as an oxidant (Gilbert, 1989). The reaction of PDI's most reactive disulfide with GSH is approximately 260-fold faster than the reaction of a typical disulfide such as GSSG with GSH (Table 1). Given that there are two active sites per PDI monomer (55 kDa), the kinetic reactivity of PDI's active site disulfides can account for all of the 500-fold rate difference. The active site disulfides of PDI, like DsbA, the periplasmic oxidant from *E. coli*, are thermodynamically destabilized (Wunderlich *et al.*, 1993; Zapun *et al.*, 1993), and the reactivity of PDI's disulfides may result from the relief of the strain/destabilization in the PDI active site disulfides.

PDI is a very abundant protein; the concentration in the ER lumen has been estimated to be near-millimolar (Lyles & Gilbert, 1990b; Zapun *et al.*, 1992). A high local concentration along with high chemical reactivity as an oxidant favors a rapid second-order reaction with unfolded substrates, making oxidation competitive with initial folding. At an estimated ER concentration of 0.5 mM (1 mM active sites), the rate constant for PDI-dependent oxidation would be about 1 s^{-1}, comparable to the rates at which small proteins fold to the native state (Matthews, 1993). Using βLa_{red} as an example (k_{trap} = 1.5 min^{-1}), 0.5 mM PDI (k_{ox} $\approx$ 50 min^{-1}) would oxidize 97% of the unfolded βLa_{red} before it folds; however, 0.5 mM GSSG (k_{ox} = 0.16 min^{-1}) would oxidize only 9%. These values must be considered as upper limits since under the conditions used here PDI's active sites would be predominantly in the disulfide oxidation state. It is uncertain what fraction of PDI in the ER is oxidized, due to the uncertainty in the concentration of GSH and GSSG in the ER, the uncertainty in the redox potential of PDI, and the uncertainty of whether or not PDI is in redox equilibrium in the ER. Using the estimates for GSH and GSSG concentrations in the ER given by Hwang *et al.* (1992) and the redox potential estimates for PDI given by Lyles and Gilbert (1991a) and by Lundstrom and Holmgren (1993), PDI would be between 0.2% and 75% oxidized in the ER, assuming equilibrium conditions.

Clearly, the high kinetic reactivity of PDI as an oxidant would allow some kinetic traps to be avoided entirely. For some proteins, disulfide formation occurs cotranslationally, consistent with the suggestion that PDI-dependent oxidation can be competitive with synthesis and folding (Bergman & Kuehl, 1979). However, not all disulfides are formed cotranslationally. Human chorionic gonadatropin forms all its disulfides post-translationally (Huth *et al.*, 1992), and many of the disulfides formed in the maturation of influenza virus hemagglutinin (HA) are post-translational (Segal *et al.*, 1992). Because of PDI's high reactivity toward exposed cysteines, mechanisms may exist in the ER to inhibit cotranslational disulfide formation. These may include control of the redox state of PDI, the rapid and selective formation of structure that inhibits cysteine oxidation, or the interaction with other folding assistants of the ER. Segal *et al.* (1992) suggested that interaction of influenza virus hemagglutinin and BiP may actually inhibit disulfide formation until appropriate disulfides can be formed during the maturation of HA. This idea is consistent with the observation that BiP complexes of unfolded lysozyme are not substrates for PDI (Puig & Gilbert, 1994).

Ignoring Kinetic Traps. For a typical secreted protein, the half-life for maturation and exit from the ER is about 30 min (0.023 min^{-1}) (Lodish *et al.*, 1983). "Kinetic traps" that slow down oxidation could be tolerated as long as the half-life for assisted oxidation is kinetically competent on the time scale for exit from the ER. Since PDI (0.5 mM) can react rapidly with an exposed sulfhydryl group (50 min^{-1}), a kinetic trap could decrease the rate of PDI-dependent oxidation by 2200-fold (50 min^{-1}/0.023 min^{-1}), and oxidation would still be kinetically competent in the cell. As long as unfolding does not become rate-limiting, kinetic traps could be as stable as 4.5 kcal/mol without posing a problem. The ability to ignore such stable "traps" depends on PDI's reactivity and high local concentration. Uncatalyzed sulfhydryl oxidation by 0.5 mM GSSG would occur with a maximum apparent first-order rate constant of 0.08 min^{-1}. If uncatalyzed sulfhydryl oxidation were the only oxidation mechanism available, a kinetic trap that decreased the oxidation rate by only 4-fold (0.8 kcal/mol) would compromise timely exit from the ER.

Unfolding Kinetic Traps. Once kinetic traps form, folding assistants, including PDI, might provide mechanisms to unfold the stabilizing structures and/or increase the actual rate of unfolding (Zahn *et al.*, 1994b; Zahn & Pluckthun, 1994). PDI-dependent substrate unfolding would require a thermodynamically favorable interaction with the unfolded protein and high concentrations of PDI to drive the equilibrium (eq 4).

$$\beta La_{red} \underset{K_F}{\rightleftharpoons} U \overset{PDI}{\underset{K_{PDI}}{\rightleftharpoons}} U{\cdot}PDI \qquad (4)$$

The fraction of βLa_{red} that would be unfolded by PDI at equilibrium is given by eq 5

$$\frac{[U{\cdot}PDI]}{\beta La_{tot}} = \frac{P_t}{K_{PDI}K_F + K_{PDI} + P_t} \qquad (5)$$

where K_F is the equilibrium constant stabilizing βLa_{red} in a folded conformation, K_{PDI} is the dissociation constant for the complex between the unfolded protein and PDI, and P_t is the total concentration of PDI. In the absence of PDI, the fraction of protein that would be unfolded at equilibrium is given by $1/(1 + K_F)$. Alkylated PDI, which is capable only of noncovalent interactions with βLa_{red}, increases the rate of βLa_{red} alkylation or GSSG oxidation by 1.7−2.5-fold, consistent with an increased amount (or reactivity) of the

unfolded protein in the presence of PDI. If this effect were due completely to increasing the extent of βLa_{red} unfolding (K_F = 500) by noncovalent binding to PDI (at a 40 μM concentration), the K_d for the PDI−βLa interaction would only have to be 16−24 μM to account for a 1.7- to 2.5-fold increase in the extent of βLa_{red} unfolding. This is consistent with the relatively weak binding observed between PDI and peptides or unfolded protein substrates (Morjana & Gilbert, 1991; Lyles & Gilbert, 1990a). Since so little of βLa_{red} is unfolded at equilibrium in the absence of PDI (0.2%), a 2-fold increase in the alkylation/oxidation rate would only require an increase in the extent of unfolding to 0.4%, consistent with the inability to detect any decrease in βLa_{red} activity in the presence of PDI. PDI's ability to stabilize proteins (or protein domains) in an unfolded state could become more significant at the PDI concentrations in the ER. At 0.5 mM PDI, specific binding (K_d = 10 μM) of PDI to an unfolded protein would half-unfold a structure that is stabilized by a factor of 100 (2.8 kcal/mol).

The rate acceleration that can be derived from equilibrium binding of an unfolded protein to a folding assistant can be observed as long as spontaneous unfolding of the kinetic trap does not become rate-limiting. PDI is very effective in trapping the unfolded protein before it refolds, but PDI-dependent oxidation of βLa_{red} is never faster than spontaneous unfolding. The *E. coli* chaperone GroEL, which binds to unfolded proteins much more tightly than PDI (K_d's near 100 nM in the absence of ATP) (Zahn *et al.*, 1994b), can unfold pre-β-lactamase (but not mature βLa) by noncovalent interactions alone (Laminet *et al.*, 1990). Interestingly, the rate at which GroEL forms an inactive complex with pre-β-lactamase is much slower than expected for a simple binding reaction, suggesting that complex formation may be comparable to the rate of spontaneous pre-βLa unfolding. If mechanisms to increase the rate of unfolding are not present in the ER, kinetically trapped species that unfold slowly would be considered "native" even though the structure may not be the thermodynamically most stable one.

For kinetic traps that are stabilized by native or non-native disulfides, it is possible that PDI might destabilize the kinetic trap by covalent interactions with PDI's active site cysteines. The combined covalent interaction with PDI along with the decreased stability of the substrate due to the disruption of a stabilizing disulfide could also contribute to the unfolding of kinetic traps.

CONCLUSIONS

Kinetic traps that diminish the reactivity of protein sulfhydryl groups can significantly retard oxidation and folding. PDI, largely because of its high kinetic reactivity as an oxidant, can diminish the importance of kinetically trapped cysteines either by avoiding their formation or by providing for rapid oxidation once the stabilizing structure spontaneously unfolds. In addition, equilibrium binding to PDI may increase the extent of substrate unfolding. This effect, although small at the PDI concentrations employed in *in vitro* experiments, should be more significant at the much higher concentrations of PDI present in the ER lumen. The ability to deal effectively with kinetic traps provides at least one rationale for the presence of extremely high concentrations of PDI in the ER lumen.

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