Correlation between Disulfide Reduction and Conformational Unfolding in Bovine Pancreatic Trypsin Inhibitor†

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ABSTRACT: The native-like two-disulfide intermediate of bovine pancreatic trypsin inhibitor (BPTI), with the disulfide between Cys14 and Cys38 reduced, plays a particularly important role in the disulfide-coupled folding pathway of BPTI because of its participation in the rate-determining step of the reaction [Creighton & Goldenberg (1984) *J. Mol. Biol. 179*, 497−526; Weissman & Kim (1991) *Science 253*, 1386−1393]. In order to study directly the relationship between conformational stability and reductive unfolding kinetics, and to gain insight concerning the rate-limiting transition state in the thiol/disulfide-mediated folding/unfolding reaction of BPTI, BPTI variants based on a native-like two-disulfide analog of this intermediate, $BPTI^{Ala14}_{Ala38}$, were examined. The amino acid replacements introduced into $BPTI^{Ala14}_{Ala38}$ rendered it thermodynamically less stable. The kinetic stability, with respect to reduction by dithiothreitol, of the disulfides in these $BPTI^{Ala14}_{Ala38}$ variants was also decreased by the substitutions. The stabilization free energy (ΔG), obtained from chemical denaturation measurements, and the activation energy of the conformational transition ($\Delta G^{\ddagger}_{conf}$), from the reductive unfolding reaction for this series of variants, were highly correlated. The observed correlation implies a direct coupling of disulfide reduction to conformational stability in this set of protein variants. It also strongly suggests that the transition state in the rate-limiting step of the reductive unfolding reaction involves a highly unfolded conformation of the protein. These data are consistent with a conformation-coupled redox folding pathway for $BPTI^{Ala14}_{Ala38}$ involving two parallel paths with unfolded (30−51) and unfolded (5−55) as the reactive species. Furthermore, the results provide a theoretical explanation for the observed 1000-fold diminution in the rate of 5−55 disulfide bond formation, relative to that of 14−38 bond formation, from the one-disulfide (30−51) intermediate in the wild-type BPTI refolding reaction. The data fit a general paradigm for protein disulfide formation during protein folding whereby native-like structure in folding intermediates accelerates formation of solvent-exposed disulfides but inhibits formation of core disulfides. This model predicts that a "rearrangement" mechanism (i.e., with non-native disulfides involved in the rate-limiting step) to form buried disulfides at a late stage in the folding reaction may be a common feature of redox folding pathways for surface disulfide-containing proteins of high stability.

The high cooperativity of the protein folding reaction and the transient appearance of the diverse intermediates involved have made investigations of the conformations of the intermediates and the rate-determining transition states, as well as the forces involved in stabilizing these structures, extremely challenging (Kim & Baldwin, 1990; Matthews, 1993). Complementary to studies on the disulfide-intact folding process, disulfide bond formation/breakage has been used as a conformational probe to monitor the disulfide-coupled folding/unfolding reaction of several proteins, including bovine pancreatic trypsin inhibitor (BPTI),[1] ribonuclease A, and α-lactalbumin (Creighton, 1978; Creighton & Goldenberg, 1984; Weissman & Kim, 1991; Ewbank & Creighton, 1993; Rothwarf & Scheraga, 1993a,b; Li et al., 1995). Taking advantage of the relatively slow rate of the disulfide interchange reaction, the intermediates in the oxidative refolding process, mediated by thiol/disulfide

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[1] Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; N^{SH}_{SH}, a native-like two-disulfide intermediate in the BPTI folding pathway with the disulfide between Cys14 and Cys38 reduced; $BPTI^{Ala14}_{Ala38}$ or $BPTI^{Ser14}_{Ser38}$, BPTI variants with Cys14 and Cys38 replaced by alanines or serines; $BPTI^{SCam14}_{SCam38}$, a two-disulfide BPTI derivative in which cysteines 14 and 38 have been selectively reduced and carboxamidomethylated; ΔG, stabilization free energy of a protein; $\Delta G^{\ddagger}_{conf}$, activation energy of the conformational transition involved in the reductive unfolding reaction of a protein; DTT_{ox} and DTT_{red}, oxidized and reduced dithiothreitol; GuHCl, guanidine hydrochloride; EDTA, ethylenediaminetetraacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; IgG, immunoglobulin G; Z, IgG binding domain derived from the B domain in staphylococcal protein A; PCR, polymerase chain reaction; θ_{222}, ellipticity at 222 nm; CD, circular dichroism; NMR, nuclear magnetic resonance; S_N2, second-order nucleophilic substitution; I or II, one- or two-disulfide intermediates in the folding reactions of BPTI and its derivatives. Specific disulfide-bonded intermediates are also represented as numbers in parentheses showing the Cys residues involved in the disulfide bonds, e.g., (30−51, 5−55) represents the BPTI intermediate containing the Cys30−Cys51 and Cys5−Cys55 disulfide bonds.

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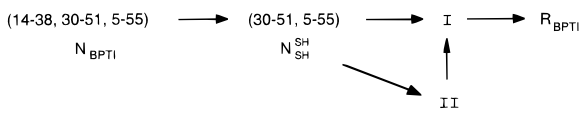
reagents, can be trapped by alkylating reagents or by low pH and further characterized by identifying the positions of the disulfide cross-link(s). From a kinetic analysis of the major and minor species interconverting during the reaction, information concerning the mechanism of the protein folding pathway can be acquired.

In contrast to the oxidative folding reaction, the reductive *unfolding* reaction generally does not fall into nonproductive kinetic traps, and the cooperativity after the rate-limiting step of the reaction often eliminates some populated intermediates observed in the folding reaction. Therefore, the unfolding reaction is less complicated though it contains less information than the folding reaction. Nevertheless, a study of unfolding can provide supplementary insights into protein folding.

The reactivity of a disulfide bond to reducing reagents in a protein is affected by factors including accessibility, local environment, dihedral angles of the disulfide, and the conformational energy of the protein (Creighton, 1975; Katz & Kossiakoff, 1986; Kuwajima et al., 1990; Ewbank & Creighton, 1993; Goldenberg et al., 1993; Li et al., 1995). Consequently, for proteins containing multiple disulfide bonds, the reduction rate for disulfides buried in a stable folded conformation is nearly always found to be slow and directly involved in the rate-determining step of the reductive unfolding reaction (Creighton, 1978; Kuwajima et al., 1990; Ewbank & Creighton, 1993; Li et al., 1995). The kinetics of the rate-limiting reductive process reflect the activation energy required for this step and can thus assist in illuminating the transition state of the specific reduction as well as that of the whole reductive unfolding reaction.

Bovine pancreatic trypsin inhibitor (BPTI) is a small, globular protein composed of 58 amino acid residues with a conformation that is stabilized by 3 disulfide bonds cross-linking Cys5 and Cys55, Cys14 and Cys38, and Cys30 and Cys51 (Figure 1). The reductive unfolding mechanism of BPTI has been extensively studied (Creighton & Goldenberg, 1984; Mendoza et al., 1994) and determined to be:



where N_{BPTI} is native BPTI, N_{SH}^{SH} is the two-disulfide intermediate (30−51, 5−55) involved in the rate-limiting step, II represents the other two-disulfide intermediates formed from N_{SH}^{SH} by disulfide exchange, I represents the one-disulfide intermediates, and R_{BPTI} is reduced BPTI.

The disulfide between Cys14 and Cys38 is exposed on the surface of the protein molecule and is easily broken by reducing reagents (Kress & Laskowski, 1967; Creighton, 1975). Further reduction of either of the remaining disulfides, 5−55 and 30−51, protected in the hydrophobic core of the only populated intermediate, N_{SH}^{SH}, is very slow and is considered to be the rate-limiting step of the overall reaction. Two mechanisms by which the buried disulfides can be reduced are (i) intramolecular rearrangement involving the free thiol groups on Cys14 or Cys38 and (ii) direct reduction by reaction with reducing agents such as DTT. Even though intramolecular disulfide exchange within the polypeptide chain of the two-disulfide intermediate, N_{SH}^{SH}, is more favorable than direct reduction, the rate constants for both

FIGURE 1: Schematic representation of BPTI. The disulfide 14−38 located on the surface of BPTI is replaced by alanines in the reference molecule, [C14A/C38A]BPTI ($BPTI_{Ala38}^{Ala14}$). The disulfides 30−51 and 5−55 are buried in the core of BPTI.

interconversions are comparable, and it has been suggested that the conformations of the transition states for these two processes are quite similar (Creighton, 1978).

Kinetic studies on a set of BPTI mutants, aimed at probing the DTT concentration dependence of the reductive unfolding reaction, showed that parallel effects on the rates of both direct disulfide reduction and disulfide rearrangement were exerted by the substitutions (Mendoza et al., 1994). Furthermore, both processes were consistent with a transition state involving an extensively unfolded conformation (Mendoza et al., 1994). However, because of its extraordinarily high stability against thermal and chemical denaturation, the effects of mutations on the thermodynamic properties of BPTI have not been extensively investigated to establish a correlation with the reducing kinetics directly.

In the studies presented below, the correlation between the conformational stability and the reductive unfolding kinetics of a series of BPTI mutants was investigated. Use of the $BPTI_{Ala38}^{Ala14}$ variant as the "parent" molecule for the introduction of further mutations served two purposes. First, the absence of cysteines 14 and 38 in this series of mutants blocked the disulfide rearrangement pathway of unfolding, allowing the less efficient route involving DTT-mediated reduction of the 30−51 and 5−55 disulfides to be directly observed (Creighton, 1977; Mendoza et al., 1994). Second, the absence of the 14−38 disulfide in all variants destabilized them sufficiently to allow complete unfolding at neutral pH by guanidine hydrochloride (Hurle et al., 1990). This unfolding transition exhibited apparent two-state behavior (Hurle et al., 1990; Liu, 1994), making possible precise measurements of the relative stabilization free energies of the mutants.

The studies described here demonstrate that the rate of direct reduction of the disulfides in $BPTI_{Ala38}^{Ala14}$ varies dramatically with the amino acid replacements. The value of the activation energy of the conformational transition ($\Delta G^{\ddagger}_{conf}$) associated with the reduction reaction was found to be comparable to the stabilization free energy (ΔG) in this set of $BPTI_{Ala38}^{Ala14}$ variants. Our results suggest that the transition state in the reductive unfolding reaction of $BPTI_{Ala38}^{Ala14}$ involves a globally unfolded conformation.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis. [D3K/C14A/C38A]BPTI, [L6K/E7L/C14A/C38A]BPTI and [D3K/L6K/E7L/C14A/C38A]-BPTI, were prepared by mutating the sequence in pEZZB3:475, a plasmid encoding a fusion protein with two staphylococcal protein A IgG-binding domains, ZZ, and [C14A/C38A]BPTI (Nilsson et al., 1991), with synthetic oligonucleotides according to the α-phosphorothiolate method involving single-strand DNA (Taylor et al., 1985; Nakamaye & Eckstein, 1986).

The mutants [L6K/C14A/C38A]BPTI, [E7L/C14A/C38A]-BPTI, and [E7K/C14A/C38A]BPTI were constructed by the polymerase chain reaction (PCR) using mutagenic primers. The mutagenic primer contained the changes intended and an *Stu*I restriction site (located at the start of the BPTI gene); the other primer annealed to the end of the BPTI gene. *Stu*I and *Ava*I were used for the digestion of plasmid pEZY2:475 (Ma and Anderson, in preparation) and the PCR fragment after amplification. The digestion mixture of each fragment was cleaned by Qiaex (Qiagen) prior to ligation.

DNA sequences of the mutant plasmids were verified by resequencing the entire BPTI coding region using the dideoxy chain termination method (Sanger et al., 1977).

Protein Expression and Protein Purification. Competent cells of *Escherichia coli* strain RV308 (ATCC 31608) were transformed with vector series pEZYT2 or pEZY2 carrying the BPTI mutant genes (Ma and Anderson, in preparation). Seed cultures were grown at 30 °C with 2XTY media overnight. Fresh seed cultures were diluted 1000-fold into baffled flasks containing 2XTY media supplemented with 0.2% glycerol or glucose and 250 mg/L ampicillin. After 20−24 h, the cultures were centrifuged, and the cell pellets were kept in the freezer for at least 1 h.

Cell pellets were resuspended in 20 mL of ice-cold sucrose buffer (0.5 M sucrose, 0.1 M Tris-HCl, pH 8.2, 1 mM EDTA) for each liter of culture and incubated on an ice−water bath for 10 min; then 250 μL of hen egg white lysozyme (10 mg/mL) and 20 mL of ice-cold water were added to the suspension which was then incubated on ice for a further 5 min (Nilsson et al., 1991). Expressed protein in the periplasmic space was released into the buffer by a combination of cell wall lysis and osmotic shock. Supernatant obtained from centrifugation at 12000*g* was loaded onto either a chymotrypsin-Affi-Gel column, for [C14A/C38A]BPTI and [D3K/C14A/C38A]BPTI, or a trypsin-Affi-Gel column, for [L6K/C14A/C38A]BPTI, [E7L/C14A/C38A]-BPTI, [E7K/C14A/C38A]BPTI, [L6K/E7L/C14A/C38A]BPTI, and [D3K/L6K/E7L/C14A/C38A]BPTI, equilibrated in a buffer containing 0.1 M triethanolamine hydrochloride, pH 7.8, 0.3 M NaCl, and 10 mM $CaCl_2$. Both chymotrypsin- and trypsin-Affi-Gel were prepared by coupling 30−45 mg of protein/mL to Affi-Gel-10 (Bio-Rad) in 0.1 M MOPS (pH 7) followed by blocking unreacted groups with 1 M ethanolamine.

In order to remove the Z fusion moiety attached to the bound BPTI, 0.2−0.5 mg of chymotrypsin was added to the chymotrypsin- or trypsin-Affi-Gel column, and the slurry was gently agitated overnight at room temperature. After washing with 5 column volumes of equilibration buffer (above) and 2 column volumes of 2 mM ammonium bicarbonate, the protein was eluted with 0.5 M acetic acid for the chymotrypsin-Affi-Gel column or dilute formic acid (pH 1.7) for the trypsin-Affi-Gel column. Fractions containing trypsin-inhibitory activity were lyophilized and further purified by cation-exchange HPLC (Aquapore, Brownlee). The buffer system for the separation consisted of 0.1 M ammonium acetate (buffer A) and 1 M ammonium acetate (buffer B). The major peak, containing the desired protein product, was collected and lyophilized. PD-10 columns (Pharmacia) were used for desalting or buffer exchange before all measurements.

All purified protein variants were subjected to N-terminal amino acid sequence determination (through the residues containing the introduced replacements), amino acid composition analysis, and mass spectrometry. All mutant proteins had the expected N-termini, amino acid compositions, and masses.

Guanidine Hydrochloride (GuHCl) Denaturation. The ellipticity at 222 nm (θ_{222}) of each protein was used to monitor equilibrium unfolding at various concentrations of GuHCl in 50 mM potassium phosphate, pH 7, 100 mM KCl, and 0.2 mM EDTA, at 25 °C. Measurements were made with an AVIV 60 DS CD spectrometer equipped with a temperature-control unit. A 1 mm path length cell was used. Protein concentrations were 15−20 μM. The total absorbance of the sample at 222 nm in 7.2 M GuHCl was less than 0.12. The GuHCl denaturation curves were analyzed by assuming a two-state unfolding reaction. The following equation was employed to fit the denaturation data:

$$\theta_{222} = \{(a_f[\mathrm{GuHCl}] + b_f) + (a_u[\mathrm{GuHCl}] + b_u) \times \exp[-(\Delta G - m[\mathrm{GuHCl}])/\mathrm{RT}]\}/\{1 + \exp[(\Delta G - m[\mathrm{GuHCl}])/RT]\} \quad (1)$$

where a_f, b_f, a_u, and b_u are the slopes and intercepts for the folded and unfolded base lines at 0 M guanidine hydrochloride, respectively. ΔG is the free energy of unfolding in the absence of denaturant, and m is the slope at the midpoint of the transition.

Kinetics of Protein Disulfide Reduction. Reductive unfolding reactions were carried out in 0.1 M Tris-HCl, pH 8.7, 0.2 M KCl, and 1 mM EDTA at 25 °C with a final protein concentration of 30 μM. At different times after the initiation of reductive unfolding by the addition of reduced DTT, samples were withdrawn and mixed with 0.5 M iodoacetamide (0.1 M final concentration) to quench the reaction by blocking any unreacted thiols. The rates of unfolding produced by reduced DTT concentrations ranging from 5 mM to 40 mM were quantitated by measuring the disappearance of inhibitory activity against trypsin. In the trypsin assay, each trapped sample was diluted 1:50 into a solution of 0.2 M triethanolamine, pH 7.8, 10 mM $CaCl_2$, and 20 μg/mL bovine trypsin and incubated for 15 min at 25 °C to allow trypsin−BPTI complex formation. *N*-α-Benzoyl-DL-arginine-*p*-nitroanilide was then added to a final concentration of 32 μg/mL, and substrate hydrolysis activity due to uncomplexed trypsin was measured by monitoring the change in A_{405} as a function of time.

RESULTS

Mutational Effects on Protein Stability. The protein variants derived from $BPTI^{Ala14}_{Ala38}$ are listed in Table 1. All were generated in a project aimed at studying the effects of charged residue substitutions on the BPTI surface (Ma and

Table 1: Stability of $BPTI^{Ala14}_{Ala38}$ Mutants Measured from Chemical Denaturation[a]

	GuHCl unfolding		
mutant[b]	C_m (M)	ΔG (kcal/mol)	$\Delta\Delta G$ (kcal/mol)
$BPTI^{Ala14}_{Ala38}$	5.01	7.2 ± 0.4	
D3K	4.84	6.5 ± 0.2	-0.7 ± 0.6
L6K	4.49	5.8 ± 0.1	-1.4 ± 0.5
E7L	4.55	5.5 ± 0.1	-1.7 ± 0.5
E7K	4.51	5.3 ± 0.2	-1.9 ± 0.6
[L6K/E7L]	3.79	4.6 ± 0.1	-2.6 ± 0.5
[D3K/L6K/E7L]	3.75	3.8 ± 0.1	-3.4 ± 0.5

[a] Measurements were performed in 50 mM potassium phosphate (pH 7.0), 100 mM KCl, and 0.2 mM EDTA, at 25 °C. C_m is defined as the GuHCl concentration at the midpoint of the unfolding transition. ΔG values were calculated by fitting GuHCl-induced denaturation data to the equation described under Experimental Procedures. Errors were obtained from the curve-fitting procedures. $\Delta\Delta G$ is the difference in ΔG between a mutant and the reference molecule, $BPTI^{Ala14}_{Ala38}$. [b] All variants include the [C14A/C38A] mutations of the reference molecule (first row in table); for conciseness, in other mutants (subsequent rows), only those substitutions that differ from the reference molecule are shown.

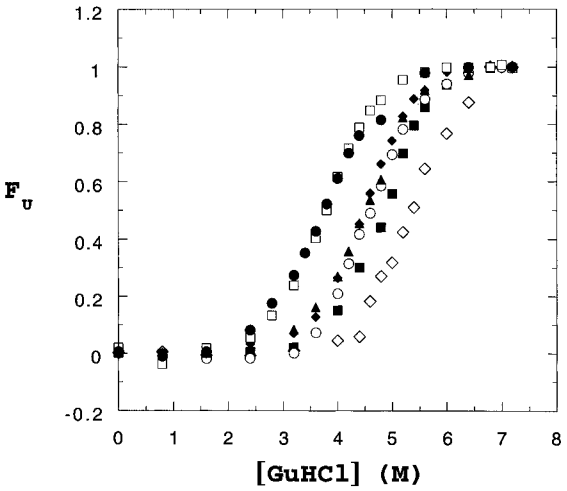


FIGURE 2: Guanidine hydrochloride-induced equilibrium unfolding of $BPTI^{Ala14}_{Ala38}$ and its mutants. (Open diamonds) $BPTI^{Ala14}_{Ala38}$; (filled squares) D3K; (filled diamonds) L6K; (open circles) E7L; (filled triangles) E7K; (open squares) [L6K/E7L]; (filled circles) [D3K/L6K/E7L]. Unfolding was monitored by the change in ellipticity at 222 nm. The base lines of the native and denatured states were dependent on [GuHCl]. The plot shows the data normalized to the fraction unfolded (F_u) after base line correction.

Anderson, in preparation). The reference mutant, $BPTI^{Ala14}_{Ala38}$, exhibited a stabilization free energy of 7.2 ± 0.4 kcal/mol (Table 1), in agreement with previous results for this mutant in which unfolding was monitored by the difference spectra at 287 nm (Hurle et al., 1990). All four $BPTI^{Ala14}_{Ala38}$ variants with single mutations, D3K, L6K, E7L, and E7K, showed reduced stability with respect to chemical denaturation (Table 1, Figure 2).

The destabilization caused by the substitutions at position 7 can possibly be attributed to the disruption of a hydrogen bond between the carboxylate oxygen on Glu7 and the backbone amide proton on Asn43 (Deisenhofer & Steigemann, 1975; Wlodawer et al., 1984). The decreases in the $BPTI^{Ala14}_{Ala38}$ stabilization free energy were 1.7 kcal/mol and 1.9 kcal/mol for E7L and E7K mutants, respectively. These values are similar to that obtained from the E7A mutation ($\Delta\Delta G = 1.5-1.8$ kcal/mol) in $[5-55]_{Ala}$, a BPTI derivative retaining the only 5−55 disulfide with the rest of the cysteines replaced by alanines (Yu et al., 1995). The destabilizing effects due to the amino acid replacement D3K were less significant (Table 1). The replacement L6K decreased the stabilization free energy by 1.4 kcal/mol. Lys6 in the structure of the BPTI homolog, protein K, is largely exposed to the solvent and disordered (Berndt et al., 1993); Lys6 in L6K-substituted BPTI mutant may also have a similar side-chain arrangement. The stability of the double mutant [L6K/E7L] was found to be lower than that of its parent single mutants (Table 1), but this effect was less than would be expected for full additivity (Wells, 1990). The triple mutant [D3K/L6K/E7L] had the lowest stabilization free energy, and the effect on ΔG from the mutations was additive with respect to that of the parent mutants, D3K and [L6K/E7L].

Mutational Effects on Reductive Unfolding. Kinetic studies of the reductive unfolding of two other two-disulfide BPTI derivatives, $BPTI^{SCam14}_{SCam38}$ (a BPTI derivative in which cysteines 14 and 38 have been selectively reduced and carboxamidomethylated) and $BPTI^{Ser14}_{Ser38}$, have shown that the rate-determining step is the reduction of one of the two buried disulfides, 30−51 or 5−55 (Creighton, 1977; Goldenberg, 1988). Hindered by the stable folded protein conformation, the approach of the reducing reagent to these completely buried disulfides requires at least partial unfolding, and the high activation energy for this conformational rearrangement is responsible for the slow rate of reduction.

The mechanism of protein disulfide reduction is analogous to the EX2 mechanism of amide proton exchange, which is also sensitive to conformational changes in proteins (Li et al., 1995). The rate-limiting step, reduction of the first disulfide, of the overall reductive unfolding reaction of $BPTI^{Ala14}_{Ala38}$

$$N_{2SS} \xrightarrow{k_r[DTT_{red}]} I \xrightarrow{k_R[DTT_{red}]} R_{4SH}$$

(where N_{2SS} is native $BPTI^{Ala14}_{Ala38}$, I represents one-disulfide intermediates, R_{4SH} is the reduced $BPTI^{Ala14}_{Ala38}$, k_r is the rate constant of the reduction reaction for native $BPTI^{Ala14}_{Ala38}$, and k_R is the rate constant of the reduction reaction for the one-disulfide intermediate) may be expressed as shown below when $k_r \ll k_R$ and the structure of the transition state involves at least partial unfolding:

$$N \underset{k_N}{\overset{k_{U^\ddagger}}{\rightleftharpoons}} U^\ddagger \xrightarrow{k_I[DTT_{red}]} I$$

where $U^\ddagger$ is the disulfide-exchange competent unfolded form of $BPTI^{Ala14}_{Ala38}$ with the disulfides intact, k_I is the rate constant for the reduction reaction involving $U^\ddagger$, $k_{U^\ddagger}$ is the rate constant of conformational unfolding, and k_N is the rate constant of conformational folding. Conformational folding from the unfolded state to the native state for $BPTI^{SCam14}_{SCam38}$ is extremely rapid: on the order of milliseconds as determined by stop-flow fluorescence studies (Jullien & Baldwin, 1981). Variants based on $BPTI^{Ala14}_{Ala38}$ in our studies also fold with rates similar to that of $BPTI^{SCam14}_{SCam38}$ (Hurle et al., 1990). In contrast to the fast rate of conformational folding, the value of $1/k_I[DTT_{red}]$ is on the order of seconds. Therefore, the equilibrium time between the folded and unfolded states is relatively fast compared to the rate of reduction, so the

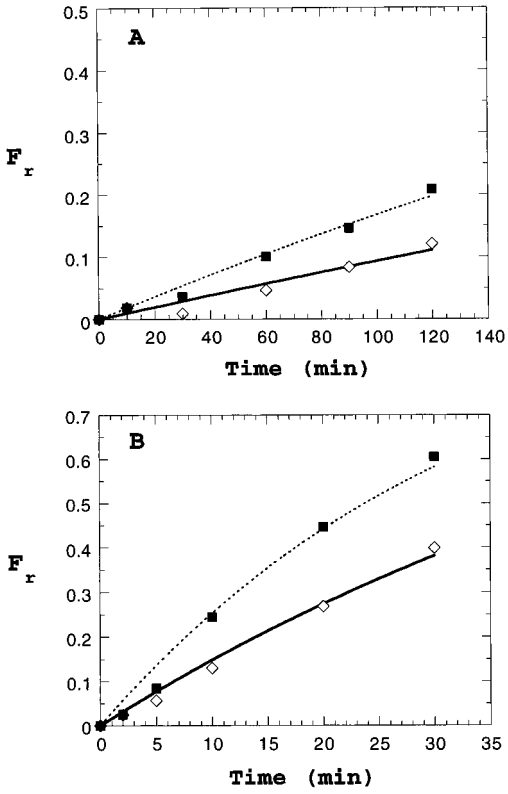


FIGURE 3: Kinetics of reduction by DTT at pH 8.7, 25 °C. (A) [L6K]BPTI$^{Ala14}_{Ala38}$; (B) [D3K/L6K/E7L]BPTI$^{Ala14}_{Ala38}$. Reduction reactions were performed as described under Experimental Procedures using 10 mM (open diamonds) or 20 mM (filled squares) DTT in (A) and 5 mM (open diamonds) or 10 mM (filled squares) DTT in (B). At the indicated times, samples were trapped with excess iodoacetamide, and the fraction of reduced protein (F_r) was measured.

apparent rate of reduction $k_r[DTT_{red}]$ can be approximated as $(k_{U^‡}/k_N)k_I[DTT_{red}]$.

The change in free energy for the conformational unfolding associated with the reductive unfolding of BPTI$^{Ala14}_{Ala38}$ can then be derived by the following relationship:

$$\Delta G^{‡}_{conf} = -RT \ln (k_{U^‡}/k_N) = -RT \ln(k_r/k_I) \approx -RT \ln (k_r/k_{ex}) \quad (2)$$

where k_{ex}, which is assumed to be approximately equal to k_I, is the reduction rate constant for disulfides totally accessible to reducing agents [determined to be 10 s^{-1} M^{-1} at pH 8.7 for BPTI (Darby & Creighton, 1993)].

The rates of reduction for the two-disulfide BPTI variants were measured at two concentrations of reduced DTT and shown to be proportional to the concentration of the reducing agent and thus in agreement with the EX2 assumption (Figure 3). With dithiothreitol in excess, the rate of the reduction reaction was analyzed by assuming pseudo-first-order kinetics to obtain the apparent rate constants, k_r. The activation energies of the conformational transition associated with reductive unfolding ($\Delta G^{‡}_{conf}$) for this set of two-disulfide BPTI variants were calculated from eq 2 and are shown in Table 2.

Correlation between Conformational Stability and Reductive Unfolding Kinetics. The stabilization free energies of the mutants, determined by chemical denaturation experiments (Table 1), were plotted against the activation energies of the conformational transition involved in the reductive unfolding (Table 2). This plot also included a data point corresponding to the one-disulfide intermediate (5−55) based on the results of (5−55) analogs[2] (Kosen et al., 1992; Yu et al., 1995) and yielded a linear correlation with a slope of 1.03 (Figure 4). However, a small systematic difference, 0.4 ± 0.3 kcal/mol, existed between the two sets of values, with the $\Delta G^{‡}_{conf}$ values derived from reductive unfolding always being less than the ΔG values measured by guanidine hydrochloride denaturation. The different buffers used for the reduction reactions (pH 8.7) and the chemical denaturation experiments (pH 7) may account in part for this difference between the two values. However, the unfolded state accessed in the absence of denaturant in the reductive unfolding reaction may also not be identical to the denatured

Table 2: Apparent Rate Constants of Reduction (k_r) and Activation Energies of the Conformational Transition Involved in the Reductive Unfolding Reaction of BPTI$^{Ala14}_{Ala38}$ Variants[a]

mutant[b]	k_r[c] (s^{-1} M^{-1} × 10^{-4})	$\Delta G^{‡}_{conf}$ (kcal/mol)	$\Delta\Delta G^{‡}_{conf}$ (kcal/mol)
BPTI$^{Ala14}_{Ala38}$	1.0 ± 0.1	6.8 ± 0.1	
D3K	1.9 ± 0.1	6.4 ± 0.1	−0.4 ± 0.2
L6K	7.9 ± 0.5	5.6 ± 0.1	−1.2 ± 0.2
E7L	22 ± 1	5.0 ± 0.1	−1.8 ± 0.2
E7K	28 ± 1	4.9 ± 0.1	−1.9 ± 0.2
[L6K/E7L]	90 ± 8	4.2 ± 0.1	−2.6 ± 0.2
[D3K/L6K/E7L]	510 ± 20	3.1 ± 0.1	−3.7 ± 0.2

[a] $\Delta G^{‡}_{conf}$ is calculated according to eq 2. [b] See the legend to Table 1. [c] k_r is the average rate constant determined by reduction with two concentrations of DTT at pH 8.7, 25 °C. Errors were the average of the standard deviations obtained from curve-fitting procedures.

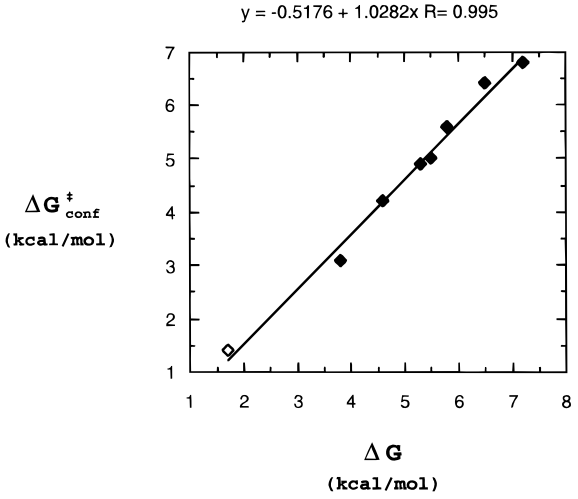


FIGURE 4: Correlation between the conformational stability, ΔG, and the activation energy, $\Delta G^{‡}_{conf}$, of the reductive unfolding reaction. Shown is a scatter plot of the ΔG values (Table 1) vs the $\Delta G^{‡}_{conf}$ values (Table 2) for each mutant. In addition, estimated ΔG and $\Delta G^{‡}_{conf}$ values were also plotted (open symbol) for highly destabilized variants of BPTI missing *both* the 14−38 and the 30−51 disulfide bonds.[2]

[2] The conformational stability (ΔG = 1.7 kcal/mol) of $[5-55]_{Ala}$ at 25 °C was calculated using the Gibbs−Helmholz equation (Becktel & Schellman, 1987): $\Delta G(T) = \Delta H - T\Delta S + \Delta C_p[T - T_m - T \ln (T/T_m)]$, using ΔC_p, T_m, ΔH, and ΔS values taken from Yu et al. (1995). The activation energy ($\Delta G^{‡}_{conf}$ = 1.4 kcal/mol) involved in the reductive unfolding of an analogous [5−55] variant, BPTI$^{SCam14,Ala30}_{SCam38,Ala51}$, with cysteines 14 and 38 blocked by carboxamidomethylation and cysteines 30 and 51 replaced by alanines (Kosen et al., 1992), was calculated by eq 2 using the apparent rate constant, k_r, of the reduction reaction (Kosen et al., 1992).

state present in solvents containing high concentrations of denaturant (Oliverberg & Fersht, 1996). Regardless of the interpretation, these results imply that the transition state for the reduction reaction of $BPTI^{Ala14}_{Ala38}$ is globally or nearly globally unfolded.

DISCUSSION

The Unfolded Conformation Is Required for the Reductive Unfolding Reaction of $BPTI^{Ala14}_{Ala38}$. In a folding reaction, it is important to characterize the transition state of the rate-limiting step since the rate-determining transition state may be considered to be the transition state for the whole reaction. The formation of the second disulfide bond in one-disulfide intermediates has been shown to be the rate-limiting step of the folding reactions of (30−51, 5−55) analogs (Creighton, 1977; Marks et al., 1987; Goldenberg, 1988). Conformational unfolding in the transition state has been suggested by results indicating that factors which disrupt the folded structure, including heat and destabilizing mutations, increase the rate of formation of (30−51, 5−55) (Marks et al., 1987; Zhang & Goldenberg, 1993). Furthermore, the results of mutation studies on the rates of disulfide breakage as a function of dithiothreitol, via both direct reduction and disulfide rearrangement pathways in (30−51, 5−55), have indicated that each of these mechanisms involves extensive conformational unfolding (Mendoza et al., 1994). However, a direct correlation between the stabilization free energies of BPTI mutants and their respective rates of reductive unfolding has not previously been established.

Our studies have focused on studying the mechanism of direct reduction of (30−51, 5−55), using $BPTI^{Ala14}_{Ala38}$ as the reference molecule, to investigate how destabilizing mutations affect the kinetics of the reaction as well as protein stability. In the native conformation of BPTI, the 30−51 and 5−55 disulfide bonds are completely buried in the hydrophobic core of the protein; i.e., the sulfur atoms of Cys30, Cys51, Cys5, and Cys55 have zero solvent accessibility.[3] A high-resolution structure of $BPTI^{Ala14}_{Ala38}$ is not available, but its folded conformation is clearly very similar to that of wild-type BPTI based on its strong inhibitory activity against trypsin (Marks et al., 1987) and NMR characterization of the solution structure (Naderi et al., 1991). Therefore, it is not unexpected that the 30−51 and 5−55 cross-bridges in $BPTI^{Ala14}_{Ala38}$ would be shielded from reducing agents except when conformational fluctuations expose the disulfides. The rate constants for direct reduction, k_r, determined for $BPTI^{Ala14}_{Ala38}$ (1.0×10^{-4} s^{-1} M^{-1}), the two-disulfide intermediate, N^{SH}_{SH}, of wild-type BPTI (8.4×10^{-5} s^{-1} M^{-1}), $BPTI^{Ser14}_{Ser38}$ (1.2×10^{-4} s^{-1} M^{-1}), and $BPTI^{SCam14}_{SCam38}$ [1.4×10^{-4} s^{-1} M^{-1}, estimated from Figure 4, Creighton (1977)] are all identical within experimental error and are indicative of an extremely slow process (Creighton, 1977; Goldenberg, 1988; Mendoza et al., 1994).

The observed increases in reactivity of the buried disulfides toward reducing reagents resulting from the mutations in this series of $BPTI^{Ala14}_{Ala38}$ variants ranged from 2- to 500-fold (Table 2). For the same series of variants, the stabilization free energies determined by guanidine hydrochloride denaturation (ΔG) and the activation energies for the conformational transition associated with reductive unfolding ($\Delta G^{\ddagger}_{conf}$) were linearly correlated with a slope very close to 1 (Figure 4). These results thus strongly support the hypothesis that global conformational unfolding is required for direct reduction of the 30−51 and 5−55 disulfides (Mendoza et al., 1994). However, the possibility that vestigial native-like interactions remain in the conformation of the transition state cannot be excluded.

The EX2 mechanism for amide proton exchange has been applied to analyze the transition state of the disulfide reduction reaction in ribonuclease A (Li et al., 1995). For $BPTI^{Ala14}_{Ala38}$, if the transition state of the reduction reaction adopts a globally unfolded conformation, both the 30−51 and 5−55 disulfides should be approximately equally exposed and available for reduction. This implies that the reductive unfolding reaction for $BPTI^{Ala14}_{Ala38}$ could occur through two parallel pathways, one for each of these disulfides.

Implications for the Folding Reaction of $BPTI^{Ala14}_{Ala38}$. The principle of microscopic reversibility can be applied to the reductive unfolding and oxidative refolding reactions of proteins with disulfide bonds. Under identical reaction conditions, with only the redox potential being varied by adjusting the concentrations of thiol and disulfide reagents, the reducing and oxidizing reactions must share the same transition states for the interconversions between one-disulfide intermediates and the native conformation. The instantaneous conformations of the one-disulfide intermediates containing one mixed-disulfide resulting from the reduction of the unfolded form of $BPTI^{Ala14}_{Ala38}$, as deduced above, should likewise be unfolded, but these would be expected subsequently to rapidly equilibrate with the partially native-like ground state conformations of (30−51) and (5−55). Even though the transition state for each pathway may be structurally distinct, these two highly unfolded structures should be comparable energetically. Therefore, the transition states involving (30−51) and (5−55) in the oxidative refolding of $BPTI^{Ala14}_{Ala38}$ should also adopt extensively unfolded conformations similar to those for the reductive unfolding reaction. A proposed mechanism by which reduced and native-like $BPTI^{Ala14}_{Ala38}$ intermediates may interconvert with each other is shown in Figure 5.

The ordered structures in both $(30-51)_{NL}$ and $(5-55)_{NL}$ (Figure 5; van Mierlo et al., 1991, 1993; Staley & Kim, 1992, 1994) may act as kinetic traps that impede the formation of the next disulfide in the refolding reaction by lowering the concentration of the reactive (i.e., unfolded) species. Similar conclusions, based on trapping experiments with wild-type BPTI and disulfide-intact mutants, have also been reached by others (Weissman & Kim, 1991; Zhang & Goldenberg, 1993; Dadlez & Kim, 1995).

In addition to allowing the cysteines access to the reducing agent, an extensively unfolded conformation for the transition state of the reductive unfolding reaction is possibly also necessitated by the nature of the second-order nucleophilic substitution (S_N2) reaction mechanism. The chemistry of thiol−disulfide exchange in small molecules has been studied thoroughly (Szajewski & Whitesides, 1980; Snyder et al., 1981; Snyder, 1984; Burns & Whitesides, 1990; Singh & Whitesides, 1990). The geometric constraints for the transition state of this S_N2 reaction require that the three sulfur

[3] Calculated using the program "Surfnew" by D. Kominos (1991) based on the algorithm developed by Lee and Richards (1971).

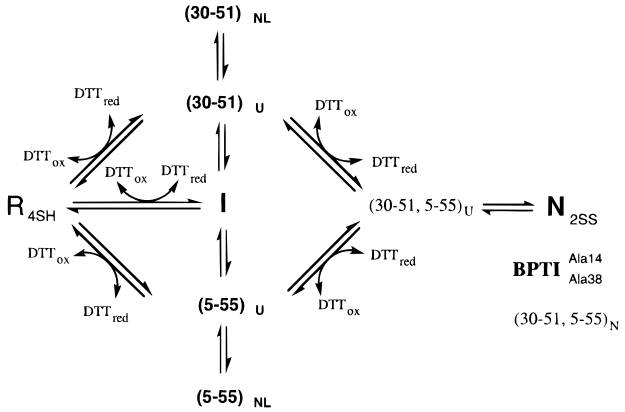


FIGURE 5: Proposed conformation-coupled folding pathway for $BPTI^{Ala14}_{Ala38}$. N_{2SS} [or $(30-51, 5-55)_N$] is native $BPTI^{Ala14}_{Ala38}$; $(30-51, 5-55)_U$ is globally unfolded $BPTI^{Ala14}_{Ala38}$; $(30-51)_U$ and $(5-55)_U$ are globally unfolded one-disulfide intermediates which are in equilibrium with the corresponding native-like species, $(30-51)_{NL}$ and $(5-55)_{NL}$, respectively; I represents other, presumably non-native-like, one-disulfide intermediates; R_{4SH} is reduced $BPTI^{Ala14}_{Ala38}$.

atoms involved in the displacement approach each other and align linearly.

Therefore, the S_N2 reaction of disulfide exchange is highly sensitive to steric effects even for exposed protein disulfides, and some degree of structural rearrangement *must* be required to allow buried disulfides in proteins to react with the attacking nucleophile. Furthermore, the ionized thiolate is a charged group, transfer of which to the hydrophobic core of a protein would be energetically unfavorable (Dao-pin et al., 1991; Stites et al., 1991). It has been estimated that the energy required to transfer a charged ion from the aqueous phase to the low-dielectric interior of a protein ranges from 19 kcal/mol for full burial to 4 kcal/mol for a half-exposed ion at the protein surface (Honig et al., 1986). Consequently, it is intuitively appealing that the rate of protein disulfide reduction should be closely related to the conformational flexibility and stability of the protein, especially for the disulfides that are protected in the interior of the protein. In order to accommodate the required geometric constraints of the thiol/disulfide exchange transition state and avoid the high energy cost associated with burying the charged thiolate ion, the exchange reaction to form core disulfides would be most favorable for an unfolded protein. This supports the idea that both the intramolecular and intermolecular disulfide exchange reactions involving N^{SH}_{SH} in BPTI occur in states having an extensively unfolded conformation (Mendoza et al., 1994), and it provides a mechanistic rationale for the observation that the energy required to unfold the native-like disulfide-bonded intermediates is linearly related to the activation energy of the rate-limiting step (Figure 4).

Implications for the Folding Reaction of Wild-Type BPTI. In the pioneering experiments conducted by Creighton and co-workers, the disulfide-linked folding mechanism of BPTI was first elucidated by studying the disulfide-bonded intermediates trapped at various stages during the oxidative renaturation of reduced BPTI (Creighton, 1977; Creighton & Goldenberg, 1984; Darby et al., 1995). A modified folding mechanism for BPTI has been proposed by Weissman and Kim (1991). A BPTI folding pathway incorporating results from both groups is shown in Figure 6A.

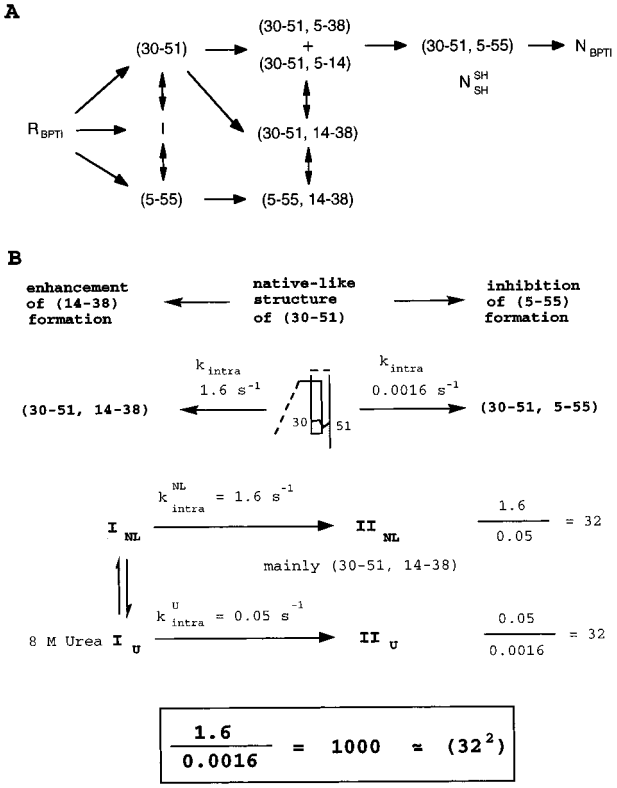


FIGURE 6: Consensus folding pathway of wild-type BPTI and the kinetics of 5-55 disulfide formation. (A) Shown is a schematic summary of the BPTI folding pathway derived from the results of Creighton and Goldenberg (1984) and Weissman and Kim (1991). R_{BPTI} is reduced BPTI; one- and two-disulfide intermediates are represented as I and II or as numbers in parentheses (representing the Cys residues involved in disulfide bonds); N_{BPTI} is native BPTI. (B) The extremely slow relative rate of 5−55 disulfide formation from the one-disulfide BPTI intermediate, (30−51), can be rationalized using the model shown in Figure 5 (see text). I_{NL} [mostly (30−51)] and I_U are the respective native-like and unfolded one-disulfide intermediates; II_{NL} [mostly (30−51, 14−38)] and II_U are the respective native-like and unfolded two-disulfide intermediates. Dashed-lined portions represent the nonordered structures observed in (30−51) analogs (van Mierlo et al., 1993; Staley & Kim, 1994).

Arguably, the most enigmatic feature of the BPTI folding pathway has always been the fact that the disulfide between Cys5 and Cys55 does not readily form once refolding has proceeded to the stage of either the one-disulfide intermediate (30−51) or the two-disulfide intermediate (30−51, 14−38). Instead, the most efficient kinetic route for regain of the native structure is through intramolecular disulfide rearrangements involving the non-native disulfide intermediates, (30−51, 5−14) and (30−51, 5−38) (Creighton, 1978). The slow rate of disulfide bond formation between Cys5 and Cys55 in (30−51) has been explained as a consequence of the ordered conformation of (30−51) hindering Cys55 from reaching Cys5, Cys14, or Cys38 (Creighton, 1990). The relative inaccessibility of the thiol group of Cys55 has also been suggested as a major factor preventing disulfide formation, based on NMR studies of a peptide model for (30−51) and a one-disulfide BPTI analog $[30-51]_{Ala}$, amide−proton exchange properties of a (30−51, 14−38) derivative, [C5V/C55A]BPTI, and reaction kinetics between glutathione and Cys5 and Cys55 in (30−51, 14−38) reversibly trapped by low pH (Oas & Kim, 1988; Staley & Kim, 1994; Schulman & Kim, 1994; Weissman & Kim, 1995).

The k_{intra} value for intramolecular 14−38 disulfide formation varies dramatically in intermediates (30−51), (5−55),

and (30−51, 5−55), ranging from 0.75 to 2300 s^{-1} (Creighton & Goldenberg, 1984; Kosen et al., 1992; Darby et al., 1995). This value can be considered an indicator of the folded conformation since it is proportional to the effective relative concentration of the Cys14 and Cys38 thiols in the different intermediates (Creighton, 1975). The rate of formation of the *second* disulfide, proceeding from the one-disulfide intermediates in the BPTI folding reaction, has been found to be enhanced 32-fold ($k_{intra} = 1.6\ s^{-1}$), presumably due to nonrandom and native-like conformational properties of the one-disulfide intermediates, compared to the rate measured in the presence of 8 M urea ($k_{intra} = 0.05\ s^{-1}$) (Darby & Creighton, 1993; Figure 6B). This result implies that the partially-folded conformations existing in the one-disulfide intermediates [mainly (30−51) (Creighton, 1978; Weissman & Kim, 1991)] cooperatively promote subsequent disulfide formation (especially between Cys14 and Cys38) by increasing the effective concentration of cysteine thiols. Concomitantly, the same native-like structure *inhibits* 5−55 disulfide cross-linking in (30−51) (Goldenberg, 1988), since the unfolded conformation is required for this reaction as discussed above. *Thus, the lowering of activation energy for formation of disulfides 14−38, 5−14, or 5−38 promoted by the conformational stability of (30−51) should be roughly equal in magnitude to the amount of energy required to unfold the (30−51) conformation.* The rate of 5−55 disulfide bond formation may thus be decreased by as much as a factor of 32 in folded (30−51) compared to the rate for unfolded (30−51). This is in agreement with the experimental finding that the k_{intra} value for disulfide 5−55 formation in (30−51) [k_{intra} value of $1.6 \times 10^{-3}\ s^{-1}$ (Goldenberg, 1988)] is 32-fold less than that for disulfide formation from unfolded one-disulfide intermediates [k_{intra} value of $0.05\ s^{-1}$ (Darby & Creighton, 1993)]. Therefore, the 10^3-fold ($\approx 32^2$) difference between the rates of second disulfide formation in (30−51) for surface vs buried disulfides may predominantly result from the nonrandom structure of the (30−51) intermediate favoring the formation of the solvent-exposed disulfides 14−38, 5−14, and 5−38 and disfavoring the formation of the core disulfide 5-55 (Figure 6A,B). The relatively stable, partially folded conformation adopted by (30−51) may thus be viewed as the primary reason for the slow rate of direct 5−55 disulfide bond formation in (30−51) compared to the rate of 5−55 formation via the intramolecular "rearrangement" route in the BPTI folding pathway.

These data suggest the following general paradigm for protein disulfide formation mediated by thiol/disulfide reagents. Oxidative folding initially involves random disulfide formation—which favors small, strainless cross-links between cysteines in linearly contiguous stretches of the polypeptide chain (Chan & Dill, 1988; Kosen et al., 1992; Darby & Creighton, 1993)—under kinetic control. Surface disulfide formation may occur preferentially, as observed for disulfide 14−38 in BPTI (Dadlez & Kim, 1995), especially at neutral pH where cysteine thiols are predominantly in the protonated form. Initial protein disulfide formation, caused by an intermolecular reaction with oxidant, is quickly following by intramolecular disulfide rearrangement. The rate of oxidation during rearrangement is enhanced by the relatively high effective concentrations of the thiol groups on the same chain, but it is still a rather random process. Stable intermediates containing core disulfides accumulate due to native-like noncovalent interactions, and this structure in turn also protects the buried disulfides from being accessed by intra- or intermolecular reducing agents.

Promoted by the compact structure of the intermediates containing core disulfides, surface disulfides in these intermediates then form with increased rates due to the higher effective concentrations of the participating cysteine thiols, and again another cycle of disulfide exchange in the polypeptide chain may occur and form additional buried disulfides. However, if the intermediates acquire stable, native-like structures *without* all the disulfides formed, such as (30−51, 14−38) or (5−55, 14−38) in the BPTI folding reaction, the formation of further core disulfides, 5−55 or 30−51, respectively, would require extensive unfolding of the intermediates in order to meet the geometric constraints of the S_N2 mechanism and avoid the presence of the charged thiolate ions in the hydrophobic interior of the folded conformation. Without unfolding, these intermediates would act as kinetic traps, leading to nonproductive pathways and greatly decreasing the rate at which the native folded structure is regained (Figures 5 and 6B). Getting out of such traps could constitute the rate-determining step of the overall reaction. Again, intramolecular disulfide exchange may be favored over direct protein disulfide formation, particularly at lower concentrations of oxidants (Weissman & Kim, 1995).

The high activation energy of the transition state is associated with the stability of the folded conformation of the quasi-native intermediates. The last steps of the refolding reaction mediated by the thiol/disulfide system involve the formation of the more exposed disulfides, and complete conformational disruption would thus not be required. The reverse reaction, reductive unfolding of the protein, would also be initiated from the more accessible surface disulfides, the reduction of which would have a lower activation energy and would be followed by disulfide exchange to reduce the buried disulfides. In summary, the proposed scheme envisions that the disulfide-linked folding reaction of proteins is kinetically controlled by the stability of disulfide-bonded intermediates with native-like conformations due to the unique geometric and electrostatic constraints inherent in the chemistry of thiol/disulfide exchange reactions.

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