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Amino Acid Replacement That Eliminates Kinetic Traps in the Folding Pathway of Pancreatic Trypsin Inhibitor†

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ABSTRACT: The disulfide-coupled folding pathway of a bovine pancreatic trypsin inhibitor (BPTI) variant, in which Tyr 35 is replaced by Leu, was determined and compared with that of the wild-type protein. Two of the most highly populated intermediates in the refolding of the wild-type protein, [30–51,14–38] and [5–55,14–38], did not accumulate to detectable levels during folding of this variant. The absence of these native-like intermediates was associated with a substantially increased rate of overall folding, consistent with previous results indicating that these species act as kinetic traps. As in the folding of the wild-type protein, the kinetically preferred folding pathway for the mutant protein includes intramolecular rearrangements and intermediates with nonnative disulfide bonds. These results suggest that the predominance of the rearrangement mechanism is not simply the consequence of the stability of the kinetically trapped species. Rather, the rearrangements appear to arise because of conformational constraints in earlier intermediates.

The folding mechanism of bovine pancreatic trypsin inhibitor (BPTI)[1] has been extensively studied by trapping and characterizing disulfide-bonded intermediates, resulting in a detailed pathway defined in terms of the disulfides present at various stages of folding (Figure 1) (Creighton, 1978, 1992a,b; Creighton & Goldenberg, 1984; Weissman & Kim, 1991; Goldenberg, 1992a). During refolding of reduced BPTI, only a relatively small number of intermediates accumulate, indicating that nonrandom structure forms relatively early in folding. Disulfide formation does not take place by a simple sequential mechanism, however. Instead, the pathway includes intramolecular rearrangements and transient formation of intermediates containing disulfide bonds not present in the native protein. Although there is general agreement about most details of the pathway, there has been considerable controversy about the relative importance of the intermediates with native and nonnative disulfides. One view has emphasized the importance of both native and nonnative species, the latter as important intermediates in the rearrangements leading to the immediate precursor to the native protein, [30–51,5–55] (Creighton, 1978, 1992a,b). Others, however, have stressed the significance of native-like intermediates because they appear to be most stable and most highly populated during folding (Weissman & Kim, 1991, 1992a,b).

Analysis of mutant proteins can provide information about folding mechanisms complementary to that obtained from biophysical and kinetic studies (Fersht et al., 1992; Goldenberg, 1988a, 1992b; Jennings et al., 1991). Because of the ability to trap and analyze disulfide-bonded intermediates, the BPTI folding pathway is particularly well suited for mutational analysis (Goldenberg et al., 1989). In order to study the roles

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[1] Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; Y35L BPTI, BPTI variant with Tyr 35 replaced with Leu; N^{SH}_{SH}, native-like BPTI intermediate lacking the 14–38 disulfide bond; other disulfide-bonded folding intermediates are indicated by brackets enclosing the designations of the disulfide bonds present; CD, circular dichroism; GSSG, oxidized glutathione; DTT^{S}_{S} and DTT^{SH}_{SH}, disulfide and dithiol forms of dithiothreitol; IAEDANS, *N*-iodoacetyl-*N'*-(5-sulfo-1-naphthyl)ethylenediamine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediamintetraacetic acid; HPLC, high-performance liquid chromatography.

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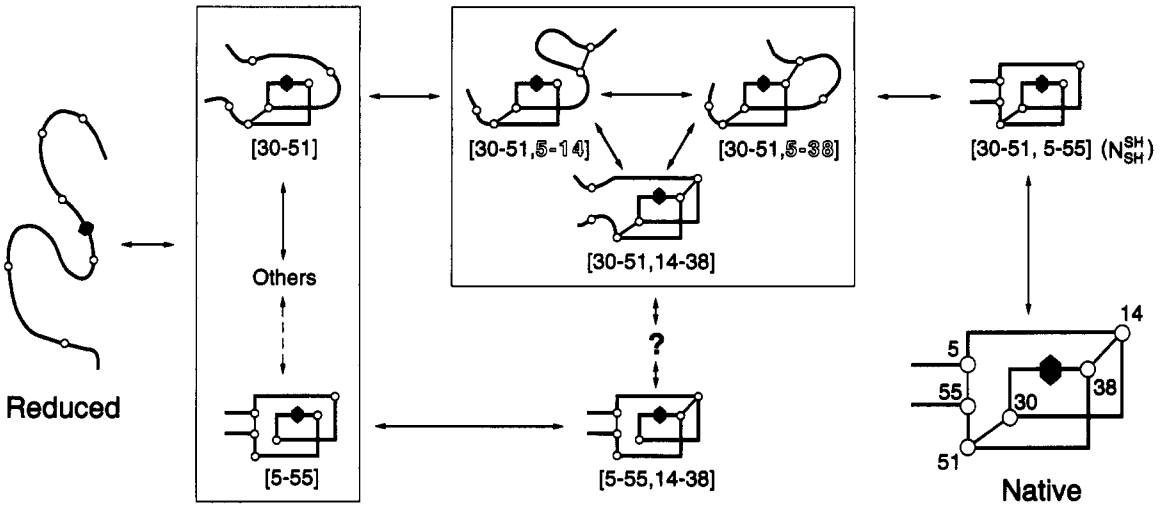


FIGURE 1: BPTI folding pathway. The fold of the polypeptide in the native protein is indicated schematically, and the intermediates are drawn to show qualitatively the degrees to which they contain native-like structure, as determined by NMR spectroscopy of analogs. Intermediates grouped together in boxes interconvert rapidly on the time scale of folding experiments and were treated as kinetically homogeneous populations in the kinetic analysis. The [5–55] intermediate interconverts more slowly than the other one-disulfide intermediates, as indicated by the dashed line. Nonnative disulfides in the intermediates are indicated by outline type. The approximate position of Tyr 35 in the polypeptide is indicated by the filled hexagon. Adapted from Figure 3 of Goldenberg (1992a).

of the four Tyr and four Phe residues that make up the hydrophobic core of native BPTI, we have constructed a series of mutant genes encoding proteins in which one of these residues is replaced with a smaller hydrophobic residue, Leu. We describe here the folding properties of one of these proteins, Y35L BPTI, for which the distribution of folding intermediates is severely altered. Comparing the folding mechanism of this mutant with that of the wild-type protein provides insights into not only the contribution of Tyr 35 to the stabilities of the various intermediates but also the roles of the intermediates in the folding mechanism.

EXPERIMENTAL PROCEDURES

Protein Purification. Y35L BPTI was produced in *Escherichia coli* HB101 carrying a plasmid encoding the mutant BPTI fused to the leader peptide of the *E. coli* Omp A protein (Goldenberg, 1988b). The leader peptide is cleaved from the BPTI sequence in vivo to yield correctly processed and folded BPTI. Bacteria were grown at 30 °C in 10 L of supplemented minimal media (Goldenberg, 1988) for 4 h, and then isopropyl β-D-thiogalactoside (IPTG) was added to a final concentration of 0.2 mM. After 22 h of growth, the bacteria were harvested to yield about 120 g of wet cell paste, which was stored at −70 °C.

The frozen bacterial pellet from a 10-L culture was resuspended at 4 °C in 400 mL of 0.05 M Tris-HCl, pH 8.0, 0.05 M NaCl, 5 mM EDTA, and 0.5 mM oxidized glutathione. After resuspension, the bacteria were lysed in a French pressure cell at 16 000 psi, and 100 mg of phenylmethanesulfonyl fluoride (dissolved in 10 mL of ethanol) was added. A 6-mL volume of 0.5 M $CaCl_2$ and 0.5 M $MgCl_2$ containing 4 mg of DNase I was then added, and the lysate was stirred at 4 °C for 1 h. The lysate was then diluted with 0.05 M Tris-HCl, pH 8.0, 0.05 M NaCl, and 5 mM EDTA to a total volume of 800 mL and centrifuged for 30 min at 12 000 rpm in a Beckman JA-17 rotor. The supernatant was applied to a ZetaPrep SP-100 ion exchange cartridge (CUNO Inc.) equilibrated in 0.05 M Tris-HCl, pH 8.0, 0.05 M NaCl, and 5 mM EDTA. After being washed with 1000 mL of the equilibration buffer, the BPTI was eluted with a 0.05–1 M NaCl gradient with a total volume of 1 L. The BPTI-containing fractions were pooled and concentrated to about 30 mL by ultrafiltration using a Millipore PCAC membrane with a nominal molecular weight cutoff of 1000. The concentrated BPTI was applied to a 3-cm diameter × 100-cm long column of Sephadex G-50 (fine) equilibrated and eluted with 0.1 M NH_4HCO_3. The BPTI fractions were then pooled and lyophilized. Final purification was obtained by reversed-phase HPLC using a Vydac C_{18} semipreparative column, which was eluted with a gradient of 12–60% acetonitrile in 0.1% trifluoroacetic acid.

Circular Dichroism Measurements. CD spectra were collected at 25 °C on an AVIV model 62DS circular dichroism spectrometer, using a bandwidth of 1 nm, a sampling step size of 0.2 nm, and signal averaging time of 0.5 s. For each sample, a minimum of three spectra were averaged. Protein concentrations were determined from absorbance at 277.5 nm, using extinction coefficients of 5720 M^{-1} cm^{-1} for the wild-type protein (Kosen et al., 1981) and 4290 M^{-1} cm^{-1} for the Y35L variant (estimated as 0.75 that of the wild-type protein, since the mutation replaces one of four Tyr residues). The validity of the extinction coefficient used for the mutant protein was confirmed by titration with a known concentration of bovine trypsin. Protein samples for CD spectroscopy were buffered with 10 mM Na_2HPO_4/NaH_2PO_4, pH 6.4.

Separation of Folding Intermediates. Reduced BPTI was prepared by incubating the native protein with 50 mM DTT_{SH}^{SH}, 6 M GuHCl, 0.1 M Tris-HCl, pH 8.7, 0.2 M KCl, and 1 mM EDTA for 1 h at room temperature. The reduced protein was purified by reversed-phase HPLC in the presence of 0.1% trifluoroacetic acid, dried by lyophilization, and then redissolved in 0.01 M HCl. Refolding reactions were carried out under an N_2 atmosphere and contained 30 μM reduced protein, 0.1 M Tris-HCl, pH 8.7, 0.2 M KCl, 1 mM EDTA, and 0.1 mM oxidized glutathione. The reactions were stopped by adding formic acid to a final concentration of 5%, and the products were fractionated on a reversed-phase HPLC column (Vydac C_{18}, 4.6-mm inside diameter × 25-cm long) eluted with a gradient of acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1 mL/min. The elution profiles were monitored by absorbance at 229 nm. For both wild-type and Y35L BPTI, the gradients were prepared by mixing two buffer solutions: A (0.1% trifluoroacetic acid) and B (0.1% trifluoroacetic acid and 90% acetonitrile). The gradient used for the wild-type

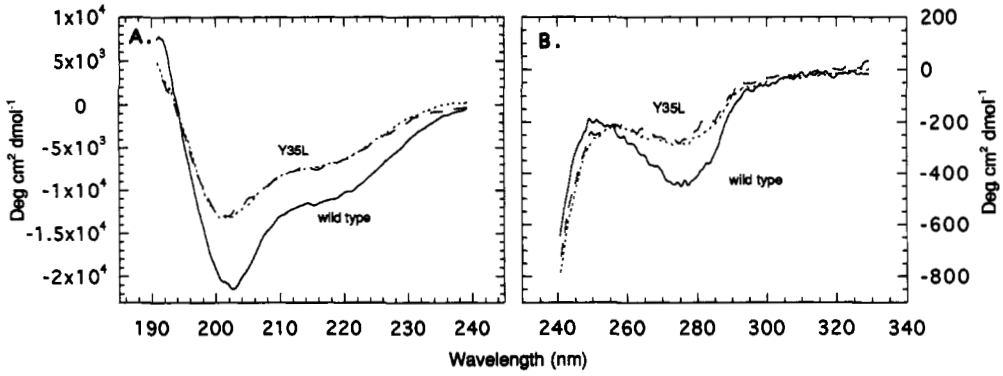


FIGURE 2: Far-UV (A) and near-UV (B) CD spectra of wild-type BPTI (solid line), native Y35L BPTI (short dashes), and refolded Y35L BPTI (long dashes). Spectra were collected at 25 °C, pH 6.4, as described under Experimental Procedures. The refolded protein was purified from a refolding reaction by reversed-phase HPLC, as illustrated in Figure 3.

protein began with 10% buffer B and was composed of four linear segments in which each segment ended with the following final composition of buffer B at the indicated total time: 25% B (15 min), 28% B (35 min), 29% B (40 min), and 32% B (105 min). The gradient used for the Y35L variant began with 10% B and was composed of the following linear segments: 25% B (5 min), 28% B (15 min), 32% B (50 min), and 33% B (110 min). The wild-type intermediates were separated at 37 °C and Y35L intermediates at room temperature.

Identification of Disulfides in the Folding Intermediates. The disulfide bonds in the isolated folding intermediates were identified by the method described by Weissman and Kim (1991). In brief, the free thiols of the intermediates were first blocked with 0.5 M iodoacetate and repurified by HPLC. The disulfides were then reduced with DTT_{SH}^{SH} and the resulting thiols reacted with *N*-iodoacetyl-*N'*-(5-sulfo-1-naphthyl)-ethylenediamine (IAEDANS), a fluorescent thiol-labeling reagent. The labeled protein was digested with thermolysin and the resulting peptides fractionated by reversed-phase HPLC (Vydac C_{18}). Peptides containing the Cys residues that were disulfide-bonded in the trapped intermediates were detected specifically by monitoring the absorbance of the column eluent at 340 nm, where the IAEDANS label absorbs strongly. These peptides were then identified by amino acid analysis and, in some cases, peptide sequencing.

Preparation of Selectively Reduced and Alkylated Y35L BPTI. The 14–38 disulfide of Y35L BPTI was selectively reduced by incubating 250 μM native protein with 4 mM DTT_{SH}^{SH} for 1 min at 25 °C in the presence of 0.1 M Tris-HCl, pH 8.7, 0.2 M KCl, and 1 mM EDTA. The resulting thiols were blocked by adding either sodium-iodoacetate or iodoacetamide to a final concentration of 0.2 M and reacting for 2 min. The modified proteins were purified by reversed-phase HPLC, and the identities of the modified Cys thiols were confirmed by peptide mapping.

Measurement of Refolding and Unfolding Kinetics. The conditions and procedures used to study the kinetics of unfolding and refolding of Y35L BPTI were identical with those described previously (Creighton & Goldenberg, 1984; Goldenberg et al., 1989). Folding reactions were quenched with 0.1 M sodium-iodoacetate, and the trapped intermediates were separated by nondenaturing gel electrophoresis. The relative concentrations of the intermediates were determined by video densitometry of the Coomassie blue stained gels. Rate constants for the individual steps in folding and unfolding were estimated by comparing the observed time-dependent changes in the concentrations of the various species with those predicted by numerical integration of the rate expressions making up the folding model. In this analysis, most of the trapped intermediates were treated as populations of molecules with one or two disulfide bonds. Although some of the intermediates may undergo intramolecular rearrangements upon trapping with iodoacetate (Weissman & Kim, 1991), rearrangements among the components of these populations would not alter the apparent total concentrations of the populations and, thus, are not expected to affect the kinetic analysis.

RESULTS

In native BPTI, Tyr 35 is more than 95% buried (Wlodawer et al., 1987) and is rigidly fixed, as indicated by its low ring-flip rate (Wagner et al., 1987). In addition to extensive nonpolar interactions, the aromatic ring of this residue interacts with the amide hydrogen of Gly 37 and one of the side chain amide hydrogens of Asn 44 in what have been described as π-hydrogen bonds (Tüchsen & Woodward, 1987). The Y35L substitution was designed to destabilize the folded protein by removing a fraction of the buried surface area, recognizing that the loss of aromaticity and change in geometry of the γ-carbon could lead to greater structural perturbations than might be expected for a simple deletion of a few atoms.

Although the mutation may have caused significant structural changes at the atomic level, the native mutant protein appeared to have an overall conformation very similar to that of the wild-type protein. The native Y35L BPTI had an electrophoretic mobility on nondenaturing polyacrylamide gels that was indistinguishable from that of the wild-type protein, indicating that the two proteins have very similar hydrodynamic volumes. The near- and far-UV circular dichroism spectra of the Y35L mutant were also qualitatively similar to that of the wild-type protein, though less intense (Figure 2). This decreased intensity most likely arises from the removal of one of the aromatic side chains, which are believed to contribute to the rather unusual far-UV CD spectrum of wild type BPTI, as well as to the near-UV spectrum (Kosen et al., 1991; Manning and Woody, 1989). The mutant protein inhibited trypsin stoichiometrically at micromolar concentrations, further suggesting that the mutation does not greatly alter the overall conformation of the native protein.

Figure 3 illustrates a comparison of the distribution of disulfide-bonded intermediates generated during the refolding of Y35L BPTI with that observed for the wild-type protein. The two proteins were refolded under identical conditions (25 °C, pH 8.7) using oxidized glutathione (GSSG) as the oxidizing agent. After 5 min, the reactions were quenched by acidification, and the trapped intermediates separated by reversed-phase HPLC. The disulfide bonds in the separated

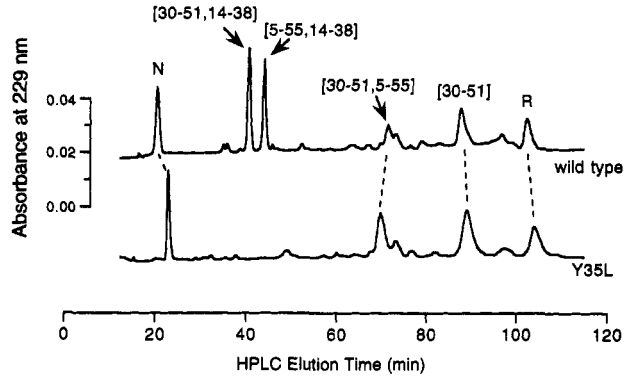


FIGURE 3: Reversed-phase HPLC separation of intermediates in the refolding of wild-type and Y35L BPTI. Reduced protein was refolded in the presence of 0.1 mM GSSG at 25 °C, pH 8.7, for 5 min and then trapped by the addition of formic acid to a final concentration of 5%. The intermediates were separated on a Vydac C_{18} column eluted with a gradient of acetonitrile in 0.1% TFA. The disulfide-bonded Cys residues in the separated intermediates were identified by peptide mapping, as described by Weissman and Kim (1991).

intermediates were identified by peptide mapping. The CD spectra of the refolded mutant protein with three disulfide bonds was identical to that of the starting native protein (Figure 2), indicating that the reformation of the disulfides was accompanied by formation of the native secondary and tertiary structure.

During the refolding of wild-type BPTI, there was substantial accumulation of intermediates [30–51], [30–51,5–55], [30–51,14–38], and [5–55,14–38]. The intermediates [5–55] and [30–51,5–14] were also populated to detectable levels. This distribution of intermediates was identical to that determined by Weissman and Kim (1991) using the same methods. During the refolding of the mutant protein, however, there was no significant accumulation of intermediates [30–51,14–38] or [5–55,14–38]. The most highly populated intermediates in the folding of the mutant protein were [30–51] and [30–51,5–55]. A small amount of a species tentatively identified as [30–51,5–14] was also detectable. The same intermediates of Y35L BPTI were seen at either pH 8.7 or 7.3 and when either GSSG or oxidized dithiothreitol was used as the oxidant. During reductive unfolding of the mutant protein, the only intermediate to accumulate was [30–51,5–55].

The limited spectrum of folding intermediates observed for Y35L BPTI might suggest that the mutant protein folds via a simple sequential pathway, such as

$$R \rightarrow [30\text{–}51] \rightarrow [30\text{–}51,5\text{–}55] \rightarrow N \qquad (1)$$

rather than the rearrangement pathway observed for the wild-type protein (Figure 1) (R and N represent the fully reduced and native protein, respectively). In order to determine whether [30–51,5–55] is formed directly from [30–51] during folding of Y35L BPTI, the folding and unfolding of modified forms, in which Cys 14 and 38 were chemically blocked with either iodoacetate or iodoacetamide, were examined. Since the thiols of Cys 14 and 38 would be required in any rearrangement pathway between [30–51] and [30–51,5–55], the kinetics of folding and unfolding of the modified proteins represent only the direct pathway, while those of the unmodified protein represent the sum of both direct and rearrangement routes. The same approach was used previously to establish the role of rearrangements in the folding of the wild-type protein (Creighton, 1977; Goldenberg, 1988b). In the presence of oxidized dithiothreitol, where the observed rates

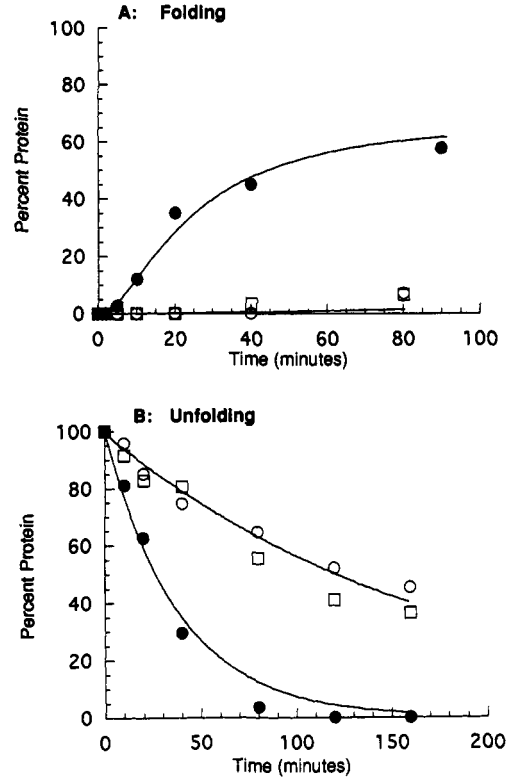


FIGURE 4: Refolding and unfolding kinetics of unmodified Y35L BPTI (●) and modified forms in which the thiols of Cys 14 and 38 were blocked with iodoacetate (○) or iodoacetamide (□). Refolding was carried out in the presence of 80 mM DTT_S^S at pH 8.7, 25 °C, while unfolding reactions were carried out in the presence of 2 mM DTT_{SH}^{SH} under the same conditions. For the modified protein, the indicated percent protein folded represents the concentration of [30–51,5–55], while for the unmodified protein, the percent folded represents the sum of N_{SH}^{SH} and N. The curves were generated by simulations based on the scheme shown in eq 2 and the rate constants in Table I.

of disulfide formation reflect the intramolecular step in the process, the modified proteins were found to form a second disulfide 200-fold more slowly than the unmodified protein (Figure 4). The unfolding of the modified proteins were also substantially slower than that of the unmodified Y35L BPTI. As observed previously in experiments with the wild-type protein (Creighton, 1977; Goldenberg, 1988b), the nature of the blocking group did not significantly affect the folding kinetics, most likely because the modified sulfur atoms are at or near the surface of the folded protein.

These results indicate that direct formation of [30–51,5–55] is much too slow to account for the folding rate of the unmodified protein. Thus, the predominant mechanism by which unmodified Y35L BPTI folds is one in which [30–51,5–55] is generated by rearrangement of other two-disulfide species, as is the case for the wild-type protein. The nature of the thiol–disulfide exchange reaction requires at least one intermediate with non native disulfides in this rearrangement mechanism. This role might be played by [30–51,5–14] or by other species that accumulate to only very low levels.

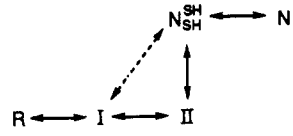
The rate constants for the individual steps in folding and unfolding of Y35L BPTI were determined by the same methods used previously for the wild-type protein and other mutants (Creighton & Goldenberg, 1984; Goldenberg et al., 1989). Folding kinetics were measured at pH 8.7, using different concentrations of oxidized dithiothreitol (DTT_S^S) in the presence or absence of reduced dithiothreitol (DTT_{SH}^{SH}). Similarly, unfolding experiments were performed with dif-

Table I: Rate Constants for Folding and Unfolding of Wild-Type and Y35L BPTI at pH 8.7, 25 °C

	$R \leftrightarrow I$ (s^{-1} M^{-1})	$I \leftrightarrow II$ (s^{-1} M^{-1})	$I \leftrightarrow N_{SH}^{SH}$ (s^{-1} M^{-1})	$II \leftrightarrow N_{SH}^{SH}$ (s^{-1})	$N_{SH}^{SH} \leftrightarrow N$ (s^{-1} M^{-1})
forward					
wild-type[a]	0.022	0.03	5.3×10^{-6}	5.0×10^{-3}	5.7
Y35L	0.02	0.013	7.1×10^{-5}	1.0×10^{-2}	0.13
reverse					
wild-type[a]	20	250	1.2×10^{-4}	1.2×10^{-5}	30
Y35L	25	250	4.8×10^{-2} [b]	3.5×10^{-4}	900

[a] The rate constants for the wild-type protein are from Creighton and Goldenberg (1984), except those for $I \rightarrow N_{SH}^{SH}$, $N_{SH}^{SH} \rightarrow I$, and $N_{SH}^{SH} \rightarrow II$, which are from Goldenberg (1988b). [b] Estimated from experiments using protein in which the 14–38 disulfide was selectively reduced and the resulting thiols were chemically blocked.

ferent concentrations of DTT_{SH}^{SH}, with and without added DTT_S^S. The data were analyzed by comparing the observed time-dependent concentration changes of the native, reduced, and intermediate species with those predicted by simulations based on the following model:



(2)

where N_{SH}^{SH} is the native-like two-disulfide species [30–51,5–55], I is the population of all intermediates with one disulfide (predominately [30–51]), and II represents the two-disulfide intermediate(s) that rearrange to form N_{SH}^{SH}. The data from all of the folding and unfolding experiments were fit to this model using a single set of rate constants (Table I). The rate constant estimated for direct reduction of N_{SH}^{SH} was consistent with that determined from the experiments using the modified protein with Cys 14 and 38 blocked. These results suggest that the folding pathway for the Y35L variant is very similar to that of the wild-type protein, except for the greatly reduced accumulation of [5–55,14–38] and [30–51,14–38] and a modest increase in the contribution of the direct formation of N_{SH}^{SH} (indicated by the dashed line in eq 2). The absence of [5–55], [5–55,14–38], and [30–51,14–38] may reflect either decreased rates of formation of these species or much greater rates of rearrangement to other intermediates.

The measured rate constants were then used to calculate the stabilities of the native protein and the intermediates, relative to the reduced and unfolded protein[2] (Goldenberg et al., 1989). Because of uncertainties in the rate constants and in estimating the compositions of the kinetic classes (I and II in eq 2), the relative stabilities calculated in this way have uncertainties of about 0.5 kcal/mol. Of the species that could be detected in the folding of the mutant protein, the native protein and N_{SH}^{SH} were destabilized by 6.5 and 2.3 kcal/mol, respectively, while the stability of [30–51] was not measurably altered and [30–51,5–14] appeared to be stabilized by 0.8 kcal/mol. We estimate that the two-disulfide species that could not be detected, [30–51,14–38] and [5–55,14–38], were destabilized by at least 2 kcal/mol.

The major consequence of destabilizing the native-like intermediates is that the rate of complete disulfide formation is actually increased substantially (Figure 5). During refolding of wild-type BPTI, [30–51,14–38], and [5–55,14–38] accumulate to high levels, especially at neutral pH (Creighton & Goldenberg, 1984; Weissman & Kim, 1991). Once formed, these species are very slow to form a third disulfide. At pH 8.7, [30–51,14–38] rearranges on the minute time scale to N_{SH}^{SH}, which can readily form the third disulfide, but at pH 7.3, [30–51,14–38] accumulates for more than 3 h. At either pH 8.7 or 7.3, rearrangements of [5–55,15–38] are extremely slow, leading to the accumulation of this species for several hours or even weeks. By destabilizing these native-like species, the Y35L substitution eliminates the kinetic traps lying between the one-disulfide intermediates and N_{SH}^{SH}, leading to more rapid formation of the three disulfide bonds found in the native protein.

DISCUSSION

The results presented here demonstrate that the accumulation of two of the most native-like intermediates in the BPTI folding pathway can be selectively eliminated by a single amino acid replacement. By preventing the accumulation of [30–51,14–38] and [5–55,14–38], the Y35L replacement leads to a substantially enhanced rate of complete disulfide formation. The mechanism of folding and disulfide formation does not appear to be greatly altered by the substitution, however, and, as for the wild-type protein, the kinetically preferred mechanism for the mutant protein involves intramolecular rearrangements. These results provide information about the contribution of the altered residue to the stabilities of the disulfide-bonded intermediates and about the origins of the intramolecular rearrangements in the folding pathway.

Role of Tyr 35 in Stabilizing the Folding Intermediates. The large destabilizations of [30–51,14–38], [30–51,5–55] and [5–55,14–38] by the Y35L replacement suggest that the altered residue plays a role in stabilizing the conformations of these species, consistent with NMR studies indicating that all of these intermediates have native-like conformations in the region of Tyr 35 (Naderi et al., 1991; van Mierlo et al., 1991a; Hurle et al., 1992). The relative insensitivity of [30–51] and [30–51,5–14] to the substitution, on the other hand, suggests that these intermediates are either unstructured in the Tyr 35 region or that they more readily accommodate the replacement, perhaps because of greater flexibility. This interpretation is supported by NMR studies of a [30–51] analog indicating that interactions between the ring of Tyr 35 and adjacent atoms, particularly the amide hydrogen of Gly 37, are weaker in this intermediate than in the native protein (van Mierlo et al., 1992, 1993). In addition, the ring of Tyr 35 is much more mobile in the [30–51] analog than in the native protein.

Role of Intramolecular Rearrangements in the BPTI Folding Pathway. The properties of the Y35L mutant also help illuminate the origins of the rearrangements in the wild-type folding pathway. Because [30–51,14–38] and [5–55, 14–38] must undergo intramolecular rearrangements in order to generate the native protein, it might appear that rear-

[2] The destabilizations of the native protein and intermediates were calculated using the rate constants in Table I and the relationship $\Delta\Delta G = -RT\ln(K_{mutant}/K_{wt})$, where K_{mutant} and K_{wt} are the equilibrium constants for forming the relevant species from the fully reduced protein. The equilibrium constants were calculated as the product of the forward rate constants divided by the product of the reverse rate constants. For the intermediates that were components of populations I or II, the equilibrium constants were calculated by multiplying the apparent equilibrium constant for forming the total population by the fraction representing the contribution of the individual intermediate to the population, as estimated by integration of the HPLC profiles. For both the wild-type and mutant protein [30–51] was estimated to represent 60% of the population of one-disulfide intermediates. For the wild-type protein, [30–51,14–38] was estimated to be 90% of II, while this species was estimated to represent less than 10% of this population for the mutant. [30–51,5–14] was estimated to represent 5% of II for the wild-type protein and 60% for the Y35L variant.

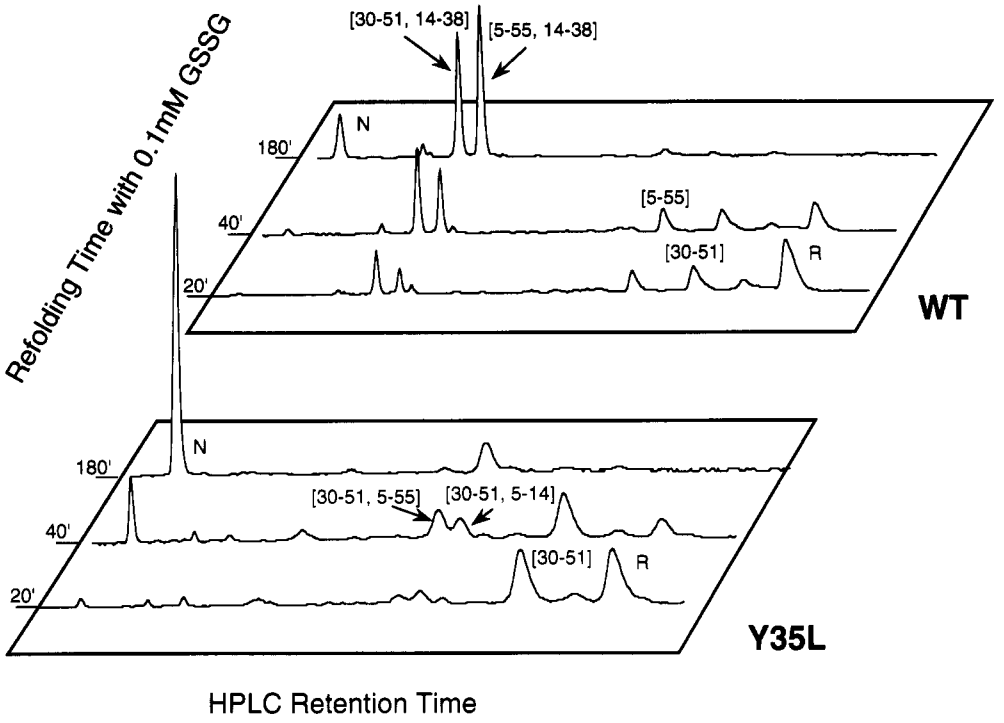


FIGURE 5: Reversed-phase HPLC separation of intermediates trapped at various times during the refolding of wild-type and Y35L BPTI at pH 7.3, 25 °C. Refolding experiments and HPLC separations were carried as in Figure 3, except that the reactions were buffered with 0.1 M MOPS, pH 7.3.

rangements arise only because of the accumulation of these native-like species (Weissman & Kim, 1992b). The results presented here demonstrate, however, that even if these intermediates are severely destabilized and do not accumulate to detectable levels, intramolecular rearrangements still represent the major folding mechanism. The predominance of the rearrangement mechanism in the folding of both the wild-type and Y35L mutant proteins may reflect conformational constraints in the one-disulfide intermediates. NMR studies of an analog of [30–51] indicate that this intermediate has much of the structure found in the native protein, except that residues 1–15 and 37–41 appear to be disordered (van Mierlo et al., 1992, 1993). Structure in [30–51] appears to inhibit the intramolecular step for formation of the 5–55 disulfide, but other disulfides, involving Cys 5, 14, and 38, can form readily. The other kinetically important one disulfide intermediate, [5–55], can fold to a fully native-like conformation, and this structure both promotes the formation of the 14–38 disulfide bond and disfavors the formation of the buried 30–51 disulfide (Creighton & Goldenberg, 1984; Staley & Kim, 1992; van Mierlo et al., 1991b). The Y35L substitution increases the rate of direct formation of N_{SH}^{SH} about 10-fold, perhaps by increasing the flexibility of [30–51], but the resulting rate is still about 200-fold slower than for forming other disulfides. As in the folding of the wild-type protein, the resulting two disulfide species (II in eq 2) must rearrange to N_{SH}^{SH} to yield the native protein.

Kinetic Traps in the BPTI Folding Pathway. A major goal of many experimental studies of protein folding is the identification and characterization of partially folded kinetic intermediates (Creighton, 1990; Kim & Baldwin, 1990; Matthews, 1993). By determining the structures of such intermediates, it is now becoming possible to construct detailed descriptions of the folding process. The intermediates most easily identified are usually those that are most highly populated during folding, i.e., those for which subsequent steps are relatively slow. Although it is tempting to assume that structure identified in an intermediate plays a role in directing further folding steps, perhaps by serving as a template for the formation of additional structure, it is also possible that the structure contributes to the kinetic barriers that makes identification of the intermediates possible. Destabilizing amino acid replacements may be a generally useful way of distinguishing these possibilities: If a substitution decreases the rate of subsequent folding steps, it would appear that structure in an intermediate promotes folding, while a substitution that increases the overall rate of folding may identify interactions that contribute to kinetic traps in the folding pathway (Kiefhaber et al., 1992).

The occurrence of kinetic traps in the BPTI folding pathway is most likely a direct consequence of the unusually high stability of this small protein (States et al., 1984; Weissman & Kim, 1991, 1992b), which can fold into a native-like conformation even in the absence of any one of the three disulfide bonds. In vivo, the effects of these traps are likely to be ameliorated by the enzyme protein disulfide isomerase, which has been shown to catalyze many of the intramolecular steps in the BPTI folding pathway (Creighton et al., 1980, 1993; Weissman & Kim, 1993). The presence of this enzyme in the endoplasmic reticulum of eukaryotic cells may be an important factor allowing the evolution of very stable disulfide-bonded proteins that would otherwise be very slow to form all of their disulfides. The propeptide present in the precursor to mature BPTI has also been shown to facilitate folding by providing an alternative rearrangement mechanism involving a Cys residue in the proregion (Weissman & Kim, 1992c).

Although kinetic traps may be particularly common in the folding of disulfide-bonded proteins, in which the disulfide bonds impose substantial steric constraints, they may also arise during the folding of other proteins if partially folded structures are sufficiently stable to inhibit conformational changes necessary to form the final native structure. As illustrated here, destabilizing mutations can be a useful experimental tool for eliminating kinetic traps, and, thereby

clarifying the analysis of a folding pathway.

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