Kinetic Analysis of the Folding and Unfolding of a Mutant Form of Bovine Pancreatic Trypsin Inhibitor Lacking the Cysteine-14 and -38 Thiols†

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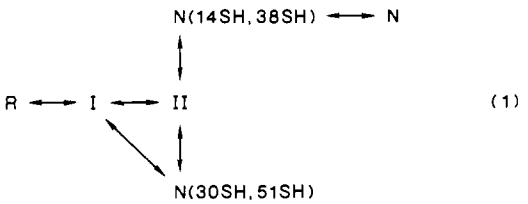
ABSTRACT: The kinetics of the disulfide-coupled unfolding–refolding transition of a mutant form of bovine pancreatic trypsin inhibitor (BPTI) lacking Cys-14 and -38 were measured and compared to previous results for the wild-type protein and other modified forms. The altered cysteines, which were changed to serine in the mutant protein, are normally paired in a disulfide in the native protein but form disulfides with Cys-5 in two-disulfide kinetic intermediates during folding. Although the mutant protein could fold efficiently, the kinetics of both folding and unfolding were altered, reflecting the roles of these cysteines in the two-disulfide intermediates with "wrong" disulfides. The intramolecular rate constant for the formation of the second disulfide of the native mutant protein was more than 10^3-fold lower than that for the formation of a second disulfide during the refolding of the wild-type protein. The observed rate of unfolding of the mutant protein was also lower than that of the wild-type protein, demonstrating that the altered cysteines are involved in the intramolecular rearrangements that are the rate-determining step in the unfolding of the wild-type protein. These results confirm the previous conclusion [Creighton, T. E. (1977) *J. Mol. Biol. 113*, 275–293] that the energetically preferred pathway for folding and unfolding of BPTI includes intramolecular rearrangements of intermediates in which Cys-14 and -38 are paired in disulfides not present in the native protein. The present results are also consistent with other, less detailed, studies with similar mutants lacking Cys-14 and -38 [Marks, C. B., Naderi, H., Kosen, P. A., Kuntz, I. D., & Anderson, S. (1987) *Science* (*Washington, D.C.*) *235*, 1370–1371].

The mechanism of folding of bovine pancreatic trypsin inhibitor (BPTI)[1] has been extensively studied by trapping and characterizing disulfide-bonded intermediates in the reductive unfolding of the native protein and the refolding of the reduced protein (Creighton, 1985; Creighton & Goldenberg, 1984). These experiments have resulted in the elucidation of a pathway, illustrated schematically in eq 1, that is currently



(1)

the most detailed description of a protein folding mechanism. The precursors to the native protein (N) include the following: R, the unfolded and reduced protein; I, a population of one-disulfide intermediates [predominantly (30–51) and (5–30)] that appear to be in rapid equilibrium; II, a mixture of three major two-disulfide species [(30–51,14–38), (30–51,5–14), and (30–51,5–38)]; two native-like species [N(14SH,38SH) and N(30SH,51SH)] that contain two of the three disulfides of the native protein.

Although the intermediates in the pathway are defined by their disulfide bonds, the specificity of disulfide formation in the intermediates is determined by the conformational properties of the polypeptide chain and can be largely eliminated by the presence of protein denaturants, such as urea or guanidinium chloride (Creighton, 1977c). In addition, spectroscopic studies of the trapped intermediates demonstrate that they have conformations that distinguish them from both the native and unfolded forms of the protein (Kosen et al., 1980, 1983; States et al., 1987). Thus, the formation of the disulfide bonds can be considered an experimental probe of the conformations that the polypeptide takes on during the process of folding or unfolding.

The rates of the individual steps making up the pathway have been measured under conditions where disulfide formation or reduction takes place by exchange with disulfide (RSSR) or thiol (RSH) reagents by the following two-step mechanism (Creighton, 1975; Synder, 1987):

$$P_{SH}^{SH} + RSSR \rightleftharpoons P_{SH}^{S\text{-}SR} + RSH \overset{k_{intra}}{\rightleftharpoons} P_{S}^{S} + 2RSH \quad (2)$$

The second step in the formation of a protein disulfide is intramolecular, with a first-order rate constant designated k_{intra}, and its rate reflects the tendency of the conformation of the polypeptide to bring the two cysteine thiols together. Two types of disulfide reagents have been used in the studies of BPTI, a cyclic intramolecular disulfide, oxidized dithiothreitol (DTT_S^S) (Cleland, 1964), and an intermolecular disulfide, oxidized glutathione (GSSG). Because the reduction of ox-

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[1] Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; R, fully reduced form of BPTI; N, native form of BPTI with three disulfide bonds; I, one-disulfide folding intermediates; II, two-disulfide folding intermediates; N(30SH,51SH), native-like form of BPTI lacking the 30–51 disulfide bond; N(14SH,38SH), native-like form of BPTI lacking the 14–38 disulfide bond; N(14Ser,38Ser), native state of the mutant form of BPTI in which Cys-14 and -38 are replaced with Ser residues; GSSG and GSH, disulfide and thiol form of glutathione, respectively; RSSR and RSH, disulfide and thiol form, respectively, of a general intermolecular thiol–disulfide reagent; DTT_S^S and DTT_{SH}^{SH}, disulfide and thiol form, respectively, of dithiothreitol (Cleland's reagent); P_S^S and P_{SH}^{SH}, disulfide and thiol form of a protein; IPTG, isopropyl β-D-thiogalactoside; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate. Other forms of BPTI are indicated by the numbers of the Cys residues linked in disulfide bonds, e.g., N is (30–51,14–38,5–55).

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idized glutathione results in a large increase in entropy, it is a much more potent oxidizing agent than is DTT^S_S, and the observed kinetics of disulfide formation or reduction are very different with the two types of reagent. However, it has been shown that the observed kinetics with the two types of reagent can be used to determine consistent values for k_{intra} for the individual steps in the scheme shown in eq 1 (Creighton & Goldenberg, 1984).

One of the most striking features of the BPTI folding pathway is the role of intramolecular rearrangements of disulfides during folding or unfolding. Of the various two-disulfide species that accumulate during refolding, only one, N(14SH,38SH), readily forms a third disulfide via disulfide exchange at 25 °C. However, as indicated in eq 1, this species is not readily formed from the one-disulfide intermediates. Instead, other two-disulfide species form and undergo intramolecular rearrangements to generate N(14SH,38SH).

One of the experiments that helped define the role of these rearrangements in the folding pathway was the use of chemically modified proteins in which the thiols of Cys-14 and -38 were alkylated (Creighton, 1977a). Since it was impossible for these proteins to form any of the species in "II" or N-(30SH,51SH), the rate of direct formation of the native-like species lacking the 14–38 disulfide could be measured. Creighton found that in the presence of DTT^S_S this reaction could not be detected and that in the presence of oxidized glutathione the intramolecular step for the formation of a second disulfide was much slower than that for the formation of the other two-disulfide species in the unmodified protein. Thus, this reaction does not contribute significantly to the folding of the unmodified protein and is not included in the scheme of eq 1, although there is not an absolute block between I and N(14SH,38SH). A potential complication to the interpretation of this experiment is the possibility that the blocking groups may interfere with the formation of the second disulfide. However, the kinetics of unfolding and refolding of the unmodified protein also demonstrate the importance of intramolecular steps in the pathway (Creighton, 1977a,b; Creighton & Goldenberg, 1984).

Recently, Marks et al. (1987) produced genetically modifed forms of BPTI in which Cys-14 and -38 were replaced by Ala or Thr. These authors found that, like the alkylated proteins, these genetically modified proteins could refold in the presence of oxidized glutathione and that the rate of appearance of active trypsin inhibitor is only about 2–5-fold slower than that for the unmodified protein. However, in these studies there was no attempt to measure the rates of the individual steps in the refolding of the mutant or wild-type proteins. Thus, it was not possible to compare the rate of formation of the second disulfide in the mutant proteins with the rate of formation of a second disulfide in the wild-type protein.

In the present study, a similar mutant form of BPTI, in which Cys-14 and -38 have been mutated to Ser, has been studied in greater detail. The kinetics of both refolding and unfolding have been measured with techniques that allow the rates of interconversion of the native form of the mutant protein with the one-disulfide intermediates to be measured directly. It has been found that the intramolecular rate constant for the formation of the second disulfide of the mutant protein is (5×10^3)-fold slower than that for the formation of the second disulfide of the wild-type protein. These results are consistent with the earlier studies with chemically modified proteins and confirm that the rearrangement pathway illustrated in eq 1 is the preferred mechanism of folding of the wild-type protein. The observations of Marks et al. can be accounted for by the low rates of rearrangement steps in the folding of the wild-type protein, which cause the overall rate of folding of the wild-type protein to be similar to that of the mutant, even though the rates for forming the second disulfides in the two proteins are very different.

Experimental Procedures

Construction of the BPTI Expression Plasmids. The BPTI-encoding DNA sequence used in these experiments was derived from bovine genomic DNA isolated by Anderson and Kingston (1983) and generously provided by I. B. Kingston in the form of a 617 base pair fragment cloned into the *Sma*I site of M13 mp8. A 467 base pair subfragment of this insert was cloned into the *Stu*I site of M13 mp11fx (Nagai & Thogerson, 1984) to generate M13FXB1. This clone included 24 base pairs of bovine DNA that preceded the codon for Arg-1 of mature BPTI and 279 base pairs following the codon for the last residue of BPTI, Ala-58. These "extra" sequences include some of those that are involved in both RNA and protein processing during the synthesis of BPTI (Creighton & Charles, 1987).

Synthetic oligonucleotides were utilized to delete these sequences and to fuse the BPTI sequences to bacterial control sequences, according to the procedure of Zoller and Smith (1982). At the 5′ end of the BPTI sequence, deletion of the bovine sequences brought the codon for Arg-1 within 45 base pairs of the *Eco*RI recognition site of the M13mp11 polylinker. At the 3′ end of the BPTI sequence, the codon for Ala-58 (GCT) was fused to the sequence TAAGCT of the polylinker, thus generating an in-frame translational stop codon (TAA) and maintaining the *Hin*dIII recognition site (CTTAAG) of the polylinker.

The BPTI sequences were removed from this clone, M13FXB5, by cleavage with *Eco*RI and *Hin*dIII and inserted into the plasmid pINIII-*OmpA*-3 (Ghrayeb et al., 1984) that had been digested with the same enzymes. This expression plasmid, generously provided by Prof. M. Inoyue, includes bacterial transcriptional and translational start sequences and a sequence coding for the signal peptide of *Omp A*, an *Escherichia coli* outer membrane protein. The BPTI-coding sequence was inserted into the plasmid in the same orientation as the sequence coding for the signal peptide, but this construction, pTI101, included an additional 15 codons between the end of the signal sequence and the codon for Arg-1 of BPTI.

A fragment including both the *Omp A* and BPTI sequences was cleaved from pTI101, with *Xba*I and *Hin*dIII, and cloned into M13mp19, digested with the same enzymes, to generate M13OB1. A synthetic oligonucleotide was then used to direct a deletion resulting in a precise fusion between the last *Omp A* codon and the Arg-1 codon of BPTI. The fused *Omp A*–BPTI sequences were then removed from this clone, M13OB2, by cleavage with *Xba*I and *Hin*dIII and cloned into similarly digested plasmid prelilac A-3. This plasmid, also provided by M. Inoyue, contains the same transcriptional control sequences and restriction enzyme recognition sites as the pINIII-*OmpA*-3 plasmid, except that the promoter sequence has been mutationally altered to generate somewhat higher expression levels (Inouye & Inouye, 1985). The relevant region of the completed *Omp A*–BPTI expression plasmid, pTI103, is diagramed in Figure 1.

In order to produce BPTI in which Cys-14 and -38 were changed to Ser, the sequence in M13OB2 was mutated with synthetic oligonucleotides according to the method of Kunkel (1985). The *Xba*I–*Hin*dIII fragment containing the mutated *Omp A*–BPTI sequence was then introduced into the plasmid

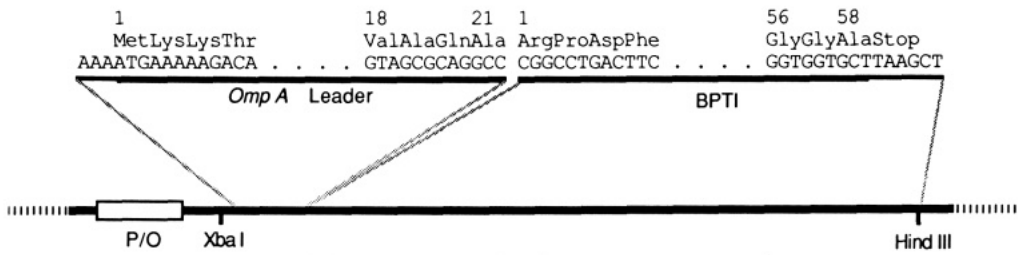


FIGURE 1: Region of the BPTI expression plasmid, pTI103, containing the *Omp A*–BPTI gene fusion. The DNA sequences coding for the leader peptide of *Omp A*, an *E. coli* outer membrane protein, were fused to the BPTI sequence so that in the resulting fusion protein the first amino acid of mature BPTI (Arg-1) directly follows the normal site of cleavage of pro-*Omp A* (Ala-21) by signal peptidase. The plasmid, which is derived from those of Ghrayeb et al. (1984) and Inouye and Inouye (1985), also contains an *E. coli* lippoprotein promoter and lactose operon operator sequences (indicated as P/O in the diagram) to allow regulated expression of the gene. The fusion gene is flanked by recognition sites for restriction endonucleases *Xba*I and *Hin*dIII.

prelilac A-3, to generate plasmid pTI106.

The sequence of each of the M13 clones generated during these constructions was determined by the dideoxy method of Sanger et al. (1977).

Purification of Wild-Type and 14,38-Ser-BPTI. *E. coli* HB101 (Boyer & Roullard-Dussoix, 1969) carrying pTI103 or pTI106 was grown at 37 °C in 10 L of supplemented minimal media containing 6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NH_4Cl, 0.2 g/L yeast extract, 3 g/L acid-hydrolyzed casein, 10 mg/L L-proline, 40 mg/L L-tryptophan, 2 mg/L thiamin, 2 g/L glucose, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, and 50 mg/L amplicillin. Four hours after inoculation with 200 mL of a saturated culture grown in the same media, isopropyl β-D-thiogalactoside (IPTG) was added to a final concentration of 0.5 mM to induce expression of the *Omp A*–BPTI gene, and L-cysteine was added to a concentration of 20 mg/L. The culture was grown for an additional 20 h with heavy aeration before harvesting by centrifugation. About 50 g of cell paste was recovered from a 10-L culture and stored at −20 °C.

The frozen bacterial pellet from a 10-L culture was resuspended at 4 °C in 150 mL of extract buffer containing 0.05 M Tris-HCl, pH 8 (measured at 25 °C), 0.05 M NaCl, and 0.005 M EDTA. After the bacteria were resuspended, 2 mL of 5 mg/mL hen egg white lysozyme and 1 mL of 10% Triton X-100 were added to promote lysis. After stirring for 3 h, 1 mL of 1 mg/mL DNase I and 2 mL of 0.5 M $CaCl_2$–0.5 M $MgCl_2$ were added, and the extract was stirred for an additional 3 h. An additional 350 mL of extract buffer and 15 mL of 0.1 M EDTA were then added, and the extract was centrifuged for 20 min at 10000 rpm in a Beckman JA-14 rotor at 4 °C. The supernatant was applied to a 1.5-cm diameter × 28 cm long column of Trisacryl M CM (LKB) equilibrated with 0.05 M Tris-HCl, pH 8, containing 0.1 mM EDTA, and the column was washed with 100 mL of the same buffer. After the sample was applied, and the column was washed at 4 °C, the column was brought to room temperature, where all subsequent steps were carried out. The column was eluted with a 500-mL linear gradient of 0–1 M NaCl in 0.05 M Tris-HCl, pH 8, containing 0.1 mM EDTA. The BPTI, identified by SDS–polyacrylamide gel electrophoresis, eluted from the column in about 0.45 M NaCl. The fractions containing BPTI were pooled and applied to a 2.5-cm diameter × 120 cm long column of Sephadex G-50 (superfine) equilibrated and eluted with 0.05 M ammonium acetate, pH 5. The BPTI-containing fractions were then applied to a 1.5-cm diameter × 27 cm long column of Trisacryl M CM that was equilibrated in 0.05 M ammonium acetate, pH 5. This column was eluted with a 500-mL linear gradient of 0.05–0.8 M ammonium acetate, pH 5. The BPTI eluted in 0.45 M ammonium acetate and was judged to be a least 95% pure by SDS–polyacrylamide gel electrophoresis and nondenaturing gel electrophoresis (Reisfeld et al., 1962). The protein was lyophilized and redissolved in water. A total of 10–15 mg of BPTI was typically recovered from a 10-L bacterial culture.

Measurement of Refolding and Unfolding Kinetics. The conditions and procedures used to study the kinetics of refolding and unfolding of wild-type and 14,38-Ser-BPTI were identical with those described previously (Creighton & Goldenberg, 1984). Folding or unfolding reactions contained 0.1 M Tris-HCl, pH 8.7, 0.2 M KCl, 1 mM EDTA, 30 μM protein, and the appropriate disulfide and thiol reagents. Commercial oxidized dithiothreitol (Calbiochem) was purified as described by Creighton (1977b) to remove less stable disulfides that can significantly alter the observed kinetics of protein disulfide formation. All reactions were carried out under a N_2 atmosphere in septum vials to minimize oxidation by molecular oxygen. Samples taken at times during folding or unfolding were quenched by the addition of 50 μL of the folding reaction mix to 12.5 μL of a quenching solution containing 0.5 M sodium iodoacetate and 0.25 M Tris-HCl, pH 6.8. Samples from refolding reactions were allowed to react with the iodoacetate for 2 min at room temperature. A total of 50 mg of solid urea was then dissolved in the samples, which were allowed to react for an additional 20 min and then put on ice. Samples from unfolding experiments were treated in the same way, except that the treatment with urea was omitted. The samples were electrophoresed through 15% polyacrylamide gels in the discontinuous buffer system of Reisfeld et al. (1962). The gels were stained with 0.1% Coomassie blue R-250 in 10% trichloroacetic acid–10% 5-sulfosalicylic acid. After the gels were destained by diffusion, the relative band intensities were measured on a Bio-Med Instruments soft-laser microdensitometer interfaced to a microcomputer. The densitometer peaks were integrated with software provided by Bio-Med Instruments. The bands corresponding to molecules with zero, one, or two protein disulfides were identified by their relative electrophoretic mobilities, on the basis of previous studies with the unmodified protein and other modified forms (Creighton, 1974, 1977a).

The kinetics of folding and unfolding were analyzed by comparing the observed time-dependent changes in the concentrations of the various species with those predicted by simulations of proposed models. The simulated kinetics were generated by numerical integration of the rate expressions making up the models, as described previously (Creighton & Goldenberg, 1984). The values of the rate constants were adjusted until the observed time dependencies of the various species were matched by the simulations.

RESULTS

Production of Wild-Type and 14,38-Ser-BPTI in E. coli. In order to facilitate the preparation of genetically modified

forms of BPTI, DNA sequences coding for the protein were introduced into a bacterial expression vector to generate the plasmid pTI103, diagramed in Figure 1. The BPTI sequences used were from an M13 phage clone isolated by Anderson and Kingston (1983) in the course of sequencing the genomic BPTI coding region. This clone included sequences coding for the entire mature protein as well as flanking sequences involved in RNA and protein processing. The BPTI sequences were introduced into a plasmid, described by Grahyeb et al. (1984), designed to produce proteins as fusions to the signal peptide of an *E. coli* outer membrane protein, *Omp A*. The bovine sequences flanking the BPTI-coding sequences were deleted by use of synthetic oligonucleotides to direct precise fusions of codon 1 of the BPTI sequence to the last codon of the signal peptide and of codon 58 to a stop codon (TAA).

When bacteria carrying plasmid pTI103 were induced with IPTG and lysed, active trypsin inhibitor was found in the bacterial extract but not in an identical extract prepared from bacteria carrying the parent plasmid lacking the BPTI gene. The level of trypsin inhibitor detected corresponded to about 2 μg of BPTI/mL of bacterial culture. Active BPTI was purified from the extract by ion-exchange and gel filtration chromatography and was found to be indistinguishable from authentic BPTI (Bayer Trasylol) by several criteria:

(1) electrophoretic mobility on SDS–polyacrylamide gels

(2) electrophoretic mobility of the native protein on nondenaturing polyacrylamide gels

(3) electrophoretic mobility of the reduced protein on nondenaturing polyacrylamide gels

(4) chromatography on C_{18} reversed-phase HPLC (Figure 2)

(5) amino acid composition

(6) sequence of the first six N-terminal amino acid residues

The correct N-terminal amino acid sequence indicates that the *Omp A* signal peptide was correctly cleaved from the protein, most likely by the bacterial signal peptidase. This result suggests that the protein was directed across the bacterial inner membrane to the periplasmic space (Dalbey & Wickner, 1985), where it would be expected to be able to fold and form its disulfides by air oxidation. However, it is possible that folding or processing may have occurred after the bacteria were lysed.

To produce BPTI in which Cys-14 and -38 were replaced by Ser, the *Omp A*–BPTI gene was subjected to oligonucleotide-directed mutagenesis, and the 14,38-Ser-BPTI was purified by the same protocol as used for the wild-type protein. The identity of the mutant protein was confirmed by conventional amino acid analysis and by the electrophoretic method of "counting" cysteine thiols (Creighton, 1980). The native mutant protein behaved identically with the wild-type protein during electrophoresis and ion-exchange chromatography. However, its elution from a C_{18} reversed-phase HPLC column was strikingly different from that of the wild-type protein (Figure 2). The reason for this difference is not known, but it may arise from conformational changes associated with the binding and elution of the proteins from the hydrophobic surface of the column (Katzenstein et al., 1986). Like the 14,38 Ala and Thr mutants prepared by Marks et al., 14,38-Ser-BPTI inhibited trypsin stoichiometrically at 1.3×10^{-7} M, consistent with previous observations that BPTI in which the 14–38 disulfide is selectively reduced retains its ability to bind to and inhibit trypsin (Kress & Laskowski, 1967).

After reduction and unfolding, the wild-type BPTI produced in *E. coli* was found to efficiently refold in the presence of either oxidized dithiothreitol or glutathione with kinetics similar to those observed previously with "authentic" BPTI under the same conditions. The kinetics of refolding and unfolding of 14,38-Ser-BPTI are described in the following sections and compared with the previous results with the unmodified protein.

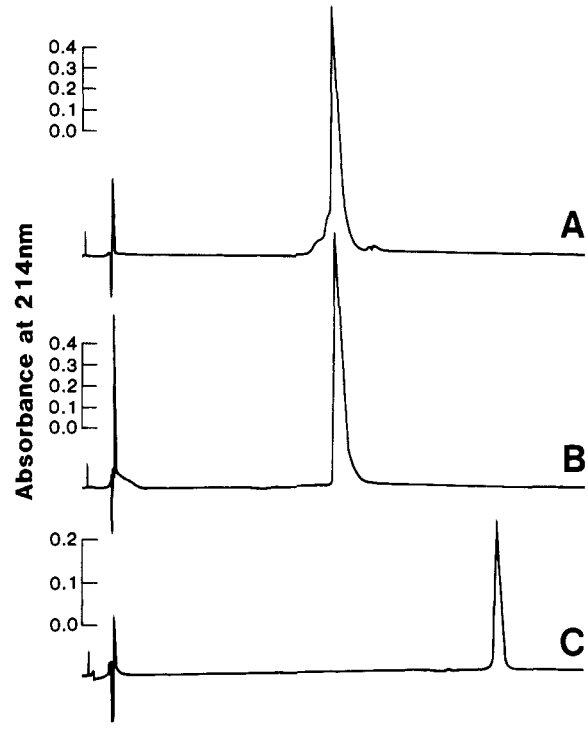
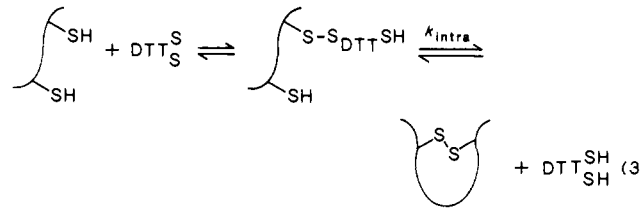


FIGURE 2: Reversed-phase high-performance liquid chromatography of (A) BPTI from bovine, (B) wild-type BPTI from *E. coli*, and (C) 14,38-Ser-BPTI from *E. coli*. The bovine protein was Bayer Trasylol, and the *E. coli* proteins were purified as described in the text. Approximately 40 μg (A and B) or 20 μg (C) of protein was applied to a Vydac C_{18} column equilibrated in 0.1% trifluoroacetic acid and eluted with a linear gradient of acetonitrile in the same buffer. Wild-type BPTI from bovine or *E. coli* eluted in 24% acetonitrile, while the 14,38-Ser-BPTI eluted in 36% acetonitrile. The mutant and wild-type proteins behaved identically upon ion-exchange chromatography, gel filtration chromatography, and gel electrophoresis.

Disulfide Formation in 14,38-Ser-BPTI in the Presence of Oxidized Dithiothreitol. Initial refolding experiments with 14,38-Ser-BPTI were carried out with oxidized dithiothreitol as the oxidizing agent. The use of this intramolecular disulfide reagent has the important advantage that the observed rate constant (k_{obsd}) for disulfide formation is related to the rate of the intramolecular step (k_{intra}) in a simple fashion. The mechanism of disulfide formation by exchange with DTT^S_S is



Because the intramolecular disulfide of DTT^S_S re-forms rapidly, the mixed disulfide intermediate is in rapid equilibrium with the reduced protein. Under the conditions used for these experiments, the equilibrium constant for the formation of the mixed disulfide is 3.3×10^{-3} M^{-1} (Creighton & Goldenberg, 1984). Therefore, the observed second-order rate constant for disulfide formation will be

$$k_{obsd} = k_{intra} \times 3.3 \times 10^{-3}\ M^{-1} \quad (4)$$

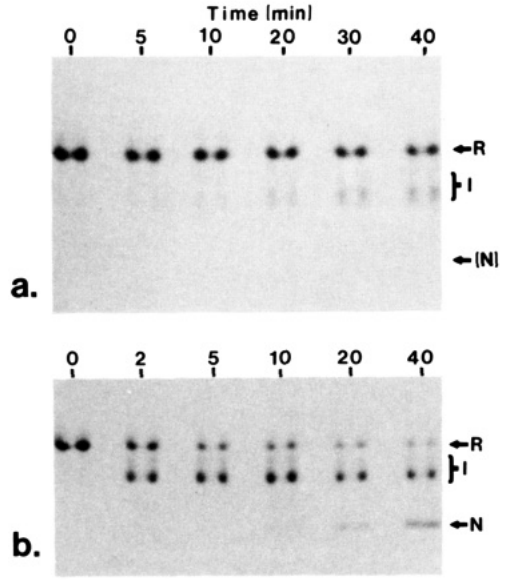


FIGURE 3: Gel electrophoresis of intermediates trapped in the refolding of reduced 14,38-Ser-BPTI in the presence of (a) 40 mM oxidized dithiothreitol or (b) 0.5 mM oxidized glutathione plus 10 mM reduced glutathione, at 25 °C, pH 8.7. At the indicated times, samples of the refolding reactions were quenched by the addition of iodoacetic acid and electrophoresed through polyacrylamide gels, as described under Experimental Procedures. The mobilities of the reduced protein (R), one-disulfide intermediates (I), and native protein (N) are indicated.

By use of this relationship, the intramolecular rate constants for all of the disulfide formation steps in the folding of wild-type BPTI have been measured (Creighton & Goldenberg, 1984).

The mutant protein was unfolded by reduction of its two disulfides and then placed under refolding conditions in the presence of DTT^S_S. At times during refolding, the reactions were quenched by irreversibly blocking all free thiols with iodoacetic acid, and the trapped molecules were separated by gel electrophoresis, as illustrated in Figure 3. Since each free thiol of the protein was blocked with a carboxymethyl group, the net charge of the basic protein was greater for species with more protein disulfides. In addition, the compactness of the molecules increases as disulfides form, thus causing the electrophoretic mobility to increase with greater numbers of disulfides.

As shown in Figure 3a, one-disulfide species readily formed from the reduced protein, but there was no detectable formation of a second disulfide after 40 min in the presence of 40 mM DTT^S_S (close to the limit of solubility). As found previously for unmodified bovine protein, wild-type BPTI produced in *E. coli* did refold efficiently in the presence of oxidized dithiothreitol (data not shown). As discussed in the following section, and illustrated in Figure 3b, 14,38-Ser-BPTI did refold in the presence of a stronger oxidizing agent, indicating that the failure to form a second disulfide in the presence of DTT^S_S was not due to any irreversible modification of the protein.

The kinetics of disulfide formation in the presence of DTT^S_S (with and without added reduced dithiothreitol) were analyzed by comparing the observed concentrations of reduced and one-disulfide species with those predicted by a simple kinetic model:

$$R \rightleftharpoons I \rightarrow N \qquad (5)$$

From these simulations, the rate constant for the formation of the one-disulfide species was estimated to be 5×10^{-3} s^{-1} M^{-1} and the rate constant for reduction was estimated to be 7.5 s^{-1} M^{-1}. The rate of forming a second disulfide was estimated to be no larger than 2×10^{-3} s^{-1} M^{-1}, indicating that k_{intra} for this step was less than 0.6 s^{-1}.

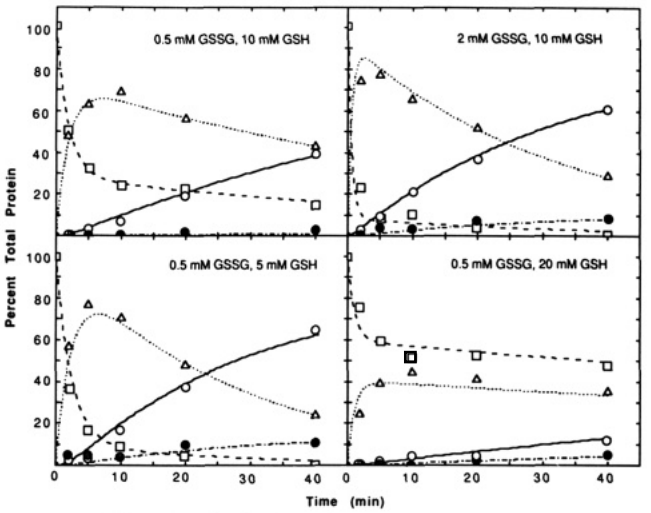


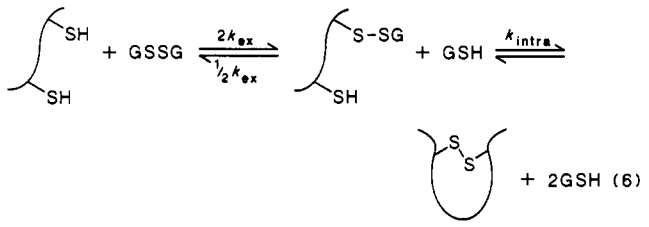
FIGURE 4: Kinetics of refolding of 14,38-Ser-BPTI in the presence of oxidized (GSSG) and reduced (GSH) glutathione at 25 °C, pH 8.7. Samples of refolding reactions containing the indicated concentrations of GSSG and GSH were quenched with iodoacetic acid at the indicated times and electrophoresed as in Figure 2. The relative concentrations of the reduced protein [R (□)], one-disulfide intermediates [I (△)], native protein [N (○)], and other two-disulfide species [II′ (●)] were determined by integrating microdensitometer traces of the Coomassie blue stained gels. The curves shown are those predicted by the scheme of eq 7 of the text, with the rate constants of Table I. The discontinuities in the predicted curves arose from the limited resolution of the computer plotting device used to generate the curves and not from the numerical integration procedure used to simulate the kinetics.

Refolding of 14,38-Ser-BPTI in the Presence of Glutathione. Because the refolding experiments with DTT^S_S provided only an upper limit for the rate of the formation of a second disulfide in 14,38-Ser-BPTI, the kinetics of refolding in the presence of a more potent oxidizing agent, glutathione, were measured, as illustrated in Figures 3b and 4. These experiments were carried out with mixtures of oxidized (GSSG) and reduced (GSH) glutathione, in order to minimize the irreversible accumulation of species with incorrect protein disulfides or mixed disulfides (Saxena & Wetlaufer, 1970). As in the experiments utilizing DTT^S_S, the folding reactions were quenched with iodoacetic acid and the trapped species separated by gel electrophoresis. Since glutathione carries a net negative charge, the formation of a mixed disulfide is expected to reduce the charge of the protein to an extent similar to that caused by carboxymethylation of the thiol by iodoacetic acid. Thus, molecules with the same protein disulfides are expected to migrate together, irrespective of whether the remaining thiols have formed mixed disulfides or were blocked by carboxymethylation.

The kinetics of refolding of 14,38-Ser-BPTI in the presence of four sets of concentrations of GSSG and GSH are shown in Figure 4. Under these conditions, there was a rapid conversion of reduced protein (R) to one-disulfide species (I) until R and I reached an apparent steady-state equilibrium. The steady-state level of I, relative to R, was favored by increased concentrations of GSSG and reduced concentrations of GSH. The subsequent conversion of the one-disulfide intermediates to the native protein (N) was much slower. Under some conditions, there was also a small accumulation of species with electrophoretic mobilities intermediate between those of the one-disulfide species and the native protein. These species

presumably have two nonnative disulfides.

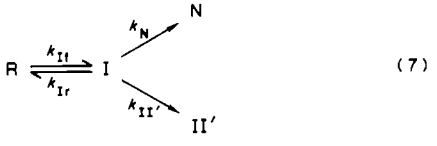
The mechanism of disulfide formation by exchange with an intermolecular disulfide reagent such as glutathione is expected to be



The rate of formation and reduction of the mixed disulfide intermediate is determined by the rate of a simple disulfide exchange reaction, with rate constant k_{ex}, which is about 20 s^{-1} M^{-1} under the conditions of these experiments (Creighton, 1975; Szajewski & Whitesides, 1980; Snyder, 1987). If there are two thiols available to form the mixed disulfide, the rate constant for forming the mixed disulfide is expected to be $2k_{ex}$, while the rate constant for reduction is $^1/_2k_{ex}$, since half of the exchange events will result in one glutathione moiety being exchanged for another. The first-order rate constant for the intramolecular step, k_{intra}, should be the same as that for the formation of the disulfide with DTT^S_S.

The observed rate of formation of the protein disulfide depends upon the relative rates of the formation and reduction of the mixed disulfide and its conversion to the protein disulfide. If k_{intra} is large relative to the rates of formation and reduction of the mixed disulfide, the observed rate of formation of the protein disulfide will be the rate of forming the mixed disulfide. However, if k_{intra} is small relative to the rate of reduction of the mixed disulfide, then there will be a steady-state equilibrium between the mixed disulfide and the reduced protein, and the observed rate of formation of the protein disulfide will be proportional to the concentration of the mixed disulfide and to k_{intra}.

The kinetic data shown in Figure 4 indicate that the formation of the first protein disulfide in the refolding of 14,38-Ser-BPTI was much faster than that of the second disulfide, suggesting that the rate of formation of the second disulfide was limited by a low value of k_{intra}. In order to analyze the kinetics of refolding, the following model was used to simulate the observed data:



The formation of I was assumed to depend on the concentration of GSSG with a second-order rate constant of k_{If}, and the reduction of I was assumed to depend on the square of the concentration of GSH, with third-order rate constant k_{Ir}. The formation of the native protein from the one-disulfide intermediates (including those containing mixed disulfides with glutathione) was assumed to be a first-order process with rate constant k_N. In these simulations, the effects of the concentrations of GSSG and GSH on the rate of formation of N from I were *not* accounted for explicitly. These concentrations would be expected to determine the fraction of I present as mixed disulfides and, therefore, the observed first-order rate constant, k_N, as discussed below. (Because the concentrations of both GSSG and GSH were considerably greater than that of the protein, the errors due to failure to account for changes in their concentration due to the formation of N were less than 5%.) The reduction of N was assumed to be insignificant, on the basis of results described in the following section. The formation of the other two-disulfide species, II', was also assumed to be first-order and irreversible, but the accumulation of these species was not sufficient to analyze their kinetics in detail.

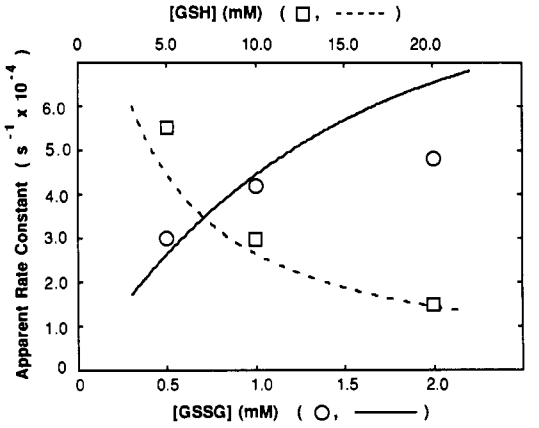


FIGURE 5: Dependence of the apparent first-order rate constant for formation of the second disulfide during the refolding of 14,38-Ser-BPTI on the concentration of oxidized (GSSG) and reduced (GSH) glutathione. The kinetics of refolding were measured in the presence of 10 mM GSH and varying concentrations of GSSG (○) or 0.5 mM GSSG and varying concentrations of GSH (□). The apparent first-order rate constants for the formation of native 14,38-Ser-BPTI (N) from the one-disulfide intermediates (I) were determined by fitting the observed kinetics of refolding of reduced 14,38-Ser-BPTI to the scheme of eq 7, as illustrated in Figure 4. Because the apparent rate constant depends upon the fraction of the one-disulfide intermediates present as mixed disulfides with glutathione, the rate constant increased with higher concentrations of GSSG and lower concentrations of GSH. The curves are those predicted by eq 10 and 11 of the text, with 1.6×10^{-3} s^{-1} for the value of k_{intra}.

Table I: Rate Constants for Refolding of 14,38-Ser-BPTI in the Presence of Oxidized and Reduced Glutathione

[GSH] (mM)	[GSSG] (mM)	F^a	k_N (s^{-1})^b	k_{intra} (s^{-1})^c	$k_{II'}$ (s^{-1})^b
5	0.5	0.28	5.5×10^{-4}	2.0×10^{-3}	1×10^{-4}
10	0.5	0.16	3.0×10^{-4}	1.8×10^{-3}	1×10^{-5}
10	1.0	0.28	4.2×10^{-4}	1.5×10^{-3}	4×10^{-5}
10	2.0	0.41	4.8×10^{-4}	1.2×10^{-3}	6×10^{-5}
20	0.5	0.091	1.5×10^{-4}	1.6×10^{-3}	5×10^{-5}

[a] The fraction of those molecules containing one protein disulfide that are expected to also contain a single mixed disulfide with glutathione was calculated from eq 11 of the text, assuming that the various species with one protein disulfide are in rapid equilibrium. [b] The apparent first-order rate constants for formation of native 14,38-Ser-BPTI (k_N) and nonnative two-disulfide species ($k_{II'}$) from the one-disulfide intermediates were determined by simulation of the observed kinetics, with the model indicated by eq 7 of the text. For all of the glutathione concentrations, the data were fit with the same values for the rate constants for formation and reduction of the one-disulfide intermediates, 13 s^{-1} M^{-1} and 23 s^{-1} M^{-2}, respectively. [c] The intramolecular rate constant for the formation of the second disulfide in native 14,38-Ser-BPTI was determined by dividing the apparent rate constant (k_N) by the fraction of one-disulfide intermediates expected to contain a single mixed disulfide with glutathione (F).

As shown in Figure 4, all of the kinetic data could be well fit with this model. The data for all of the different sets of GSSG and GSH concentrations could be fit using the same values for k_{If} and k_{Ir}, 13 s^{-1} M^{-1} and 23 s^{-1} M^{-2}, respectively. However, significantly different values for k_N were required to fit the data from the different sets of conditions. As shown in Table I and Figure 5, the apparent rate constant, k_N, increased at higher concentrations of GSSG and lower concentrations of GSH, as expected if the rate depends upon the fraction of the one-disulfide intermediates that are present as the mixed disulfide.

At steady state, the expected concentrations of one-disulfide species with one or two mixed disulfides with glutathione (I–G and G–I–G) relative to those without mixed disulfides (I_0) are given by

$$[I\text{–}G]/[I_0] = 4[GSSG]/[GSH] \quad (8)$$

$$[G\text{–}I\text{–}G]/[I_0] = 4([GSSG]/[GSH])^2 \quad (9)$$

The fraction of the one-disulfide species with a single mixed disulfide (F) is

$$F = [I\text{–}G]/([I] + [I\text{–}G] + [G\text{–}I\text{–}G]) \quad (10)$$

$$F = 4[GSSG][GSH]/(2[GSSG] + [GSH])^2 \quad (11)$$

Since only the molecules with a single mixed disulfide can form a protein disulfide, the observed rate constant, k_N, is expected to be

$$k_N = Fk_{intra} \quad (12)$$

The observed values of k_N were divided by the appropriate values of F, calculated from eq 11, to derive estimates of k_{intra}, as listed in Table I. These estimates ranged from 1.2×10^{-3} to 2.0×10^{-3} s^{-1} with a mean of 1.6×10^{-3} s^{-1}. The curves shown in Figure 5 indicate the expected values of the apparent rate constant calculated from eq 11 and 12 with 1.6×10^{-3} s^{-1} for the value of k_{intra}. The overall consistency between the observed and expected values for the apparent rate constant indicates that the assumptions made in the analysis are valid.

In contrast to this value of k_{intra} for the formation of the second disulfide of N(14Ser,38Ser), the intramolecular rate constant for the formation of the productive two-disulfide intermediates in the folding of the wild-type protein (II in eq 1) is 9 s^{-1}. Thus, the formation of these species [including (30–51,5–14), (30–51,5–38), and (30–51,14–38)] is about (5×10^3)-fold more rapid than the formation of N(14Ser,38Ser), and the direct formation of N(14SH,38SH) is not expected to play a significant role in the folding of the wild-type protein.

The calculations of k_{intra} for the mutant and wild-type proteins did not include corrections for the fraction of the various one-disulfide intermediates that actually participate in the formation of the second disulfide. Also, there was no correction for the fraction of the thiols that were un-ionized at pH 8.7. Both of these factors make the measured values of k_{intra} smaller than the microscopic rate constants for formation of the disulfide from the ionized form of the individual one-disulfide species. However, the rate constants for the mutant and wild-type protein were determined at the same pH and the distribution of one-disulfide intermediates should be similar (Creighton, 1977a), thus making the measured rate constants directly comparable. No attempt was made to interpret the kinetics of formation of the minor two-disulfide species.

Unfolding of 14,38-Ser-BPTI. The kinetics of unfolding of 14,38-Ser-BPTI, as well as the wild-type protein produced in *E. coli*, were measured under the same conditions used for the refolding experiments, except that the thiol–disulfide redox potential was made to favor the reduced unfolded form. In the presence of reduced dithiothreitol, the 14–38 disulfide of wild-type BPTI is rapidly reduced with a rate constant of 30 s^{-1} M^{-1}, but further reduction of the protein is much slower (Creighton, 1977b). Wild-type BPTI produced in bacteria was found to behave similarly (Figure 6). As found in previous experiments with bovine protein, the rate of appearance of the fully reduced protein was independent of the concentration of DTT^{SH}_{SH} (up to 20 mM), indicating that the rate of reduction was limited by an intramolecular process. Simulations of the unfolding kinetics indicated that this process had a rate constant of 1.2×10^{-5} s^{-1} [versus 2.0×10^{-5} s^{-1} measured previously (Creighton, 1977b)].

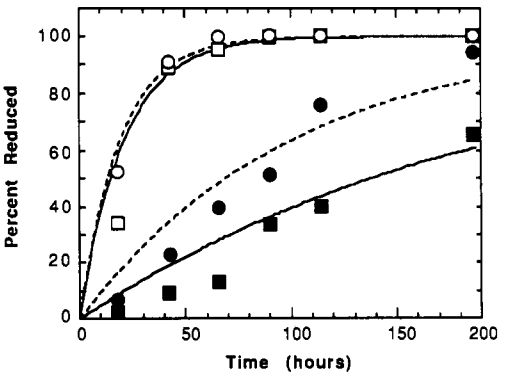


FIGURE 6: Kinetics of appearance of fully reduced protein during the unfolding of wild-type BPTI (open symbols) and 14,38-Ser-BPTI (filled symbols) in the presence of 10 mM (□, ■) and 20 mM (○, ●) reduced dithiothreitol at 25 °C, pH 8.7. The relative concentrations of the species present at the indicated times were determined by quenching samples of the reactions with iodoacetic acid, electrophoresing the samples through polyacrylamide gels, and determining the relative intensities of the resulting bands by densitometry of the Coomassie blue stained gels. Through the course of the experiment, the concentrations of thiols present were determined with Ellman's reagent (1959) and found not to vary more than 10%, indicating that oxidation by molecular oxygen was not significant. No intermediates were detected in the unfolding of 14,38-Ser-BPTI, and the kinetics were simulated by a model in which the rate of unfolding was determined by a single second-order process with a rate constant of 1.2×10^{-4} s^{-1} M^{-1} (lower two curves). During unfolding of the wild-type protein, one of the disulfides was rapidly reduced, but subsequent reduction was much slower, and there was no accumulation of one-disulfide intermediates. The kinetics of appearance of reduced wild-type protein were simulated (upper two curves) by a model in which the two-disulfide intermediate can either rearrange to other, rapidly reduced, two-disulfide species, with a first-order rate constant of 1.2×10^{-5} s^{-1}, or directly be reduced with a second-order rate constant of 1.2×10^{-4} s^{-1} M^{-1}. The independence of the observed rate of appearance of the reduced wild type protein on the concentration of DTT^{SH}_{SH} indicates that the direct mechanism of reduction does not contribute significantly under these conditions.

Under the same conditions, 14,38-Ser-BPTI unfolded considerably more slowly and the observed rate was proportional to the concentration of DTT^{SH}_{SH} (Figure 6). As in the refolding of the wild-type protein, no one-disulfide intermediates were observed during unfolding. The second-order rate constant for the appearance of the reduced protein was 1.2×10^{-4} s^{-1} M^{-1}.

These results are very similar to those obtained previously by Creighton (1977a) using the proteins with the Cys-14 and -38 thiols chemically blocked and indicate that these thiols are involved in the intramolecular step in the unfolding of the wild-type protein. The earlier experiments indicated that either thiol could serve this function.

The rate constants measured for the formation and reduction (k_f and k_r) of the second disulfide in N(14Ser,38Ser) can be used to calculate the equilibrium constant for its formation from the one-disulfide intermediates:

$$K_{eq} = ([N(14Ser,38Ser)][DTT^{SH}_{SH}])/([I][DTT^{S}_{S}]) = k_f/k_r \quad (13)$$

Although the rate constant for the formation of the second disulfide with DTT^{S}_{S} was too low to be measured directly, it can be calculated from the value of k_{intra} measured with GSSG (using eq 4) to be 5.3×10^{-6} s^{-1} M^{-1}. Thus, the equilibrium constant is 4.4×10^{-2}.

For the wild-type protein, the rate constants making up the rearrangement pathway

$$I \rightleftharpoons II \rightleftharpoons N(14SH,38SH) \quad (14)$$

have been measured [Creighton and Goldenberg (1984) and the present study for the rearrangement of N(14SH,38SH) to II]. These values can be used to calculate an overall equilibrium constant for the formation of N(14SH,38SH) from the one-disulfide intermediates:

$$K_{eq} = ([N(14SH,38SH)][DTT^{SH}_{SH}])/([I][DTT^{S}_{S}]) = 5 \times 10^{-2} \quad (15)$$

The consistency between these values demonstrates that the models used to interpret the kinetics of folding and unfolding of the wild-type and 14,38-Ser proteins satisfy the principle of microscopic reversibility and argues strongly that the interpretation of the kinetics is correct.

Discussion

The use of disulfide bond formation as a conformational probe has proven to be one of the most useful approaches to studying mechanisms of protein folding. However, elucidating the mechanism of a disulfide-coupled folding reaction is often quite difficult because of the large number of possible disulfide-bonded intermediates that may have distinct kinetic roles in the pathway. One method of testing the roles of specific cysteine residues and intermediates during folding is the use of modified proteins in which particular cysteine thiols have been removed, so that some of the potential steps are eliminated. In this way, the rates of alternative pathways can be determined individually. The recent development of genetic engineering techniques has made it possible to mutationally convert cysteine residues to other residues, thus causing smaller perturbations to the rest of the protein than might be generated by chemical modifications of the thiols. In the present study, Cys residues were changed to Ser, since this change seemed likely to maintain the polarity and size of the residues as well as possible.

In the case of BPTI, modified proteins have been used to determine the relative roles of direct and disulfide rearrangement pathways in folding and unfolding (Creighton, 1977a; Marks et al., 1987). The experiments presented here extend previous studies by utilizing a mutationally altered, rather than alkylated, protein and by making direct measurements of the rates of the individual steps in the formation and reduction of the native protein lacking the Cys-14 and -38 thiols.

The rate constant for the intramolecular step in the formation of the second disulfide in N(14Ser,38Ser) was determined to be 1.6×10^{-3} s^{-1}. This value is similar to that measured by Creighton, 5×10^{-3} s^{-1}, using a somewhat different method, for a protein in which the Cys-14 and -38 thiols were alkylated. In contrast, the intramolecular rate constant for the formation of the two-disulfide intermediates in the folding of the unmodified protein is 9 s^{-1}. Thus, the kinetically most accessible mechanism for the folding of the wild-type protein involves forming other two-disulfide intermediates that then undergo intramolecular rearrangements to form N-(14SH,38SH).

The unfolding of the wild-type protein also takes place via intramolecular rearrangements, as indicated by the independence of the rate of appearance of reduced protein on the concentration of DTT^{SH}_{SH}. The mutant protein unfolded considerably more slowly, and the rate depended upon the concentration of DTT^{SH}_{SH}, thus demonstrating that the intramolecular step in the unfolding of the wild-type protein involves Cys-14 or -38. (At much higher concentrations of DTT^{SH}_{SH}, the rate of reduction of the mutant would be expected to approach that of the wild-type protein, and the direct reduction mechanism would become significant in the unfolding of the wild-type protein.)

Marks et al. (1987) recently reported that mutants of BPTI lacking Cys-14 and -38 refold to form active trypsin inhibitors at rates that are only 2–5-fold slower than that of the wild-type protein in the presence of 1 mM GSSG and 10 mM GSH. The apparent contradiction between their results and the conclusions of the present study, that the rate of direct formation of N(14Ser,38Ser) is more than 10^3-fold slower than the rate of formation of two-disulfide intermediates in the wild-type protein, arises because the overall rates of formation of native protein are limited by different steps for the mutant and wild-type proteins. As shown here, the rate of formation of N(14Ser,38Ser) under the conditions used in these experiments and those of Marks et al. is determined by the intramolecular step in the conversion of the one-disulfide intermediates to the native protein. Marks et al. observed that the kinetics of formation of active wild-type BPTI involved two distinct phases. The previously measured rate constants for the folding of wild-type BPTI in the presence of glutathione (Creighton & Goldenberg, 1984) indicate that under these conditions the formation of the one- and two-disulfide intermediates would be expected to be more rapid than either of the observed phases. The faster phase can be accounted for by the relatively slow rearrangements that form N(14SH,38SH) from the species included in II, and the slower phase would be expected to arise from the rearrangement of the off-pathway species N(30SH,51SH) to form other, productive, two-disulfide intermediates. [Although N(30SH,51SH) is an active trypsin inhibitor, the alkylation treatment used by Marks et al. to quench the folding reactions apparently led to inactivation of this species.] Thus, neither of the observed phases for the appearance of active wild-type protein reflects the rate of formation of the second disulfide, and comparison of the overall rates of folding of the mutant and wild-type protein tends to mask the importance of the Cys-14 and -38 thiols in the folding of the wild-type protein.

Marks et al. also observed that the difference in the overall rate of folding of the mutant and wild-type proteins was even less pronounced at temperatures greater than 25 °C and suggested that under these conditions the direct pathway becomes more significant in the folding of the wild-type protein. However, direct measurements of the rates of the individual steps in the folding of the two proteins are needed to assess the relative roles of the different mechanisms.

The intramolecular rate constants for disulfide formation in the mutant and wild-type proteins have been emphasized here because these rate constants reflect the tendency of the conformation of the polypeptide chain to favor the formation of the disulfide bonds. Although the observed kinetics for a disulfide-coupled folding reaction depend dramatically on the concentrations and nature of the thiol–disulfide reagents utilized, the intramolecular processes should, ideally, be independent of the reagents. Although it is possible that there would be some specific interaction between a protein and a thiol–disulfide reagent, it has been shown that for BPTI very consistent sets of intramolecular rate constants can be determined with glutathione and dithiothreitol (Creighton & Goldenberg, 1984). Thus, by comparing intramolecular rate constants, it is possible to use the kinetics of disulfide formation as a sensitive probe of the conformational properties of the polypeptide chain during folding.

The present studies confirm the earlier conclusion that the direct formation of N(14SH,38SH) is much slower than the other disulfide formation steps in the BPTI pathway. The

structural basis of the high energy of the transition state for this reaction is not yet known. It is possible that the transition state has a considerable fraction of the structure of the native protein, since the product of the reaction is nativelike. However, since the transition state must include three linearly arranged sulfur atoms (Szajewski & Whitesides, 1980), the structure of the region around the 5–55 disulfide would have to be significantly distorted from that in the native conformation. Thus, the factors that contribute to the stability of the native conformation might destabilize the transition state (Creighton, 1985; Goldenberg & Creighton, 1985). This may also be the cause of the high transition state energy for the intramolecular rearrangements between N(14SH,38SH) and the other two-disulfide intermediates. These possibilities are currently being investigated by examining the effects of mutations that destabilize the native conformation on the mechanism of folding and unfolding. Preliminary results demonstrate that destabilizing mutations in the region of the 5–55 disulfide greatly enhance the rate of unfolding of the protein (D. P. Goldenberg, R. W. Frieden, J. Haack, and T. Morrison, unpublished experiments). However, the relative effects of these mutations on the direct and rearrangement mechanism are not yet known. Further studies of mutationally altered forms of BPTI may be a powerful method of analyzing the origins of the conformational constraints on the polypeptide during folding and unfolding that are revealed by analysis of the kinetics of disulfide formation, reduction, and rearrangement.

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Registry No. BPTI, 9087-70-1; Cys, 52-90-4; glutathione, 70-18-8; oxidized glutathione, 27025-41-8.

REFERENCES

Anderson, S., & Kingston, I. B. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6838–6842.

Boyer, H. W., & Roulland-Dussoix, D. (1969) *J. Mol. Biol. 41*, 459–472.

Cleland, W. W. (1964) *Biochemistry 3*, 480–482.

Creighton, T. E. (1974) *J. Mol Biol. 87*, 579–602.

Creighton, T. E. (1975) *J. Mol. Biol. 96*, 767–776.

Creighton, T. E. (1977a) *J. Mol. Biol. 113*, 275–293.

Creighton, T. E. (1977b) *J. Mol. Biol. 113*, 295–312.

Creighton, T. E. (1977c) *J. Mol. Biol. 113*, 313–328.

Creighton, T. E. (1980) *Nature (London) 284*, 487–489.

Creighton, T. E. (1985) *J. Phys. Chem. 89*, 2452–2459.

Creighton, T. E., & Goldenberg, D. P. (1984) *J. Mol. Biol. 179*, 497–526.

Creighton, T. E., & Charles, I. G. (1987) *J. Mol. Biol. 194*, 11–22.

Dalbey, R. E., & Wickner, W. (1985) *J. Biol. Chem. 260*, 15925–15931.

Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70–77.

Ghrayeb, J., Kimura, H., Takahara, M., Hsiung, H., Masui, Y., & Inouye, M. (1984) *EMBO J. 3*, 2437–2442.

Goldenberg, D. P., & Creighton, T. E. (1985) *Biopolymers 24*, 167–182.

Inouye, S., & Inouye, M. (1985) *Nucleic Acids Res. 13*, 3101–3110.

Katzenstein, G. L., Vrona, S. A., Wechsler, R. J., Steadman, B. L., Lewis, R. V., & Middaugh, C. R. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 4268–4272.

Kosen, P. A., Creighton, T. E., & Blout, E. R. (1980) *Biochemistry 19*, 4936–4944.

Kosen, P. A., Creighton, T. E., & Blout, E. R. (1983) *Biochemistry 22*, 2433–2440.

Kress, L. F., & Laskowski, M., Sr. (1967) *J. Biol. Chem. 242*, 4925–4929.

Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488–492.

Marks, C. B., Naderi, H., Kosen, P. A., Kuntz, I. D., & Anderson, S. (1987) *Science (Washington, D.C.) 235*, 1370–1371.

Nagai, K., & Thøgerson, H. C. (1984) *Nature (London) 309*, 810–812.

Reisfeld, R. A., Lewis, U. J., & Williams, D. E. (1962) *Nature (London) 195*, 281–283.

Sanger, F., Nicklen, S., & Coulsen, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.

Saxena, V. P., & Wetlaufer, D. (1970) *Biochemistry 9*, 5015–5023.

Snyder, G. H. (1987) *Biochemistry 26*, 688–694.

States, D. J., Creighton, T. E., Dobson, C. M., & Karplus, M. (1987) *J. Mol. Biol. 195*, 731–739.

Szajewski, R. P., & Whitsides, G. M. (1980) *J. Am. Chem. Soc. 102*, 2011–2026.

Zoller, M. J., & Smith, M. (1982) *Nucleic Acids Res. 10*, 6487–6500.