

The Pro Region of BPTI Facilitates Folding

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Summary

The in vitro folding pathway of bovine pancreatic trypsin inhibitor (BPTI) has been described previously in terms of the disulfide-bonded intermediates that accumulate during folding of the protein. Folding is slow, occurring in hours at pH 7.3, 25°C. In addition, approximately half of the BPTI molecules become trapped as a dead-end, native-like intermediate. In vivo, BPTI is synthesized as a precursor protein that includes a 13 residue amino-terminal pro region. This pro region contains a cysteine residue. We find that, in vitro, both the rate of formation and the yield of properly folded BPTI are increased substantially in a recombinant model of pro-BPTI. The cysteine residue is necessary for this effect. Moreover, a single cysteine residue, tethered to the carboxy-terminal end of BPTI with a flexible linker of repeating Ser-Gly-Gly residues, is sufficient to assist in disulfide formation. Thus, the pro region appears to facilitate folding by providing a tethered, solvent-accessible, intramolecular thiol-disulfide reagent.

Introduction

Efforts to understand protein folding have been hampered by the ephemeral nature of kinetic folding intermediates (for review, see Kim and Baldwin, 1990). For the class of proteins in which folding is accompanied by the formation of disulfide bonds, however, folding intermediates with unformed and/or incorrect disulfide bonds can be trapped in a stable form. This has made it possible to identify and characterize intermediates that accumulate, both in vitro (e.g., Creighton and Goldenberg, 1984; Rothwarf and Scheraga, 1991; Weissman and Kim, 1991) and in vivo (e.g., Braakman et al., 1992; Bedows et al., 1992), during the folding of a number of disulfide-containing proteins.

The best characterized disulfide folding pathway (Creighton and Goldenberg, 1984; Weissman and Kim, 1991) is for the in vitro folding of bovine pancreatic trypsin inhibitor (BPTI). BPTI is a very stable 58 residue protein that has been the subject of numerous biophysical and structural studies. The native structure of BPTI (Deisenhofer and Steigemann, 1975; Wlodawer et al., 1984) consists of an amino-terminal 3_{10} helix, a highly twisted anti-parallel β sheet, and an α helix near the carboxyl terminus (Figure 1A). This native structure is stabilized by three disulfide bonds between residues 5–55, 30–51, and 14–38. Upon

reduction of these disulfide bonds, BPTI unfolds spontaneously, even in the absence of denaturants. The folding of BPTI is therefore linked thermodynamically to formation of the native disulfide bonds.

The native disulfide bonds of BPTI form spontaneously from reduced BPTI (R), provided that an oxidizing agent is present. Thus, the information necessary to specify the 3-D structure of BPTI is contained within its amino acid sequence. Several intermediates with different disulfide bond linkages accumulate transiently during the folding of BPTI (Creighton and Goldenberg, 1984; Weissman and Kim, 1991). The intermediates are denoted in brackets by the disulfide linkages of the Cys residues; native BPTI (N) is therefore [30–51; 5–55; 14–38]. Structural characterizations of these intermediates by nuclear magnetic resonance (NMR) or X-ray crystallography have been carried out (e.g., Stassinopoulou et al., 1984; States et al., 1987; Oas and Kim, 1988; Staley and Kim, 1990; Eigenbrot et al., 1990; Naderi et al., 1991; van Mierlo et al., 1991a, 1991b; Staley and Kim, 1992; Hurle et al., 1992; Darby et al., 1992; van Mierlo et al., 1992). The rates of interconversion between different disulfide-bonded intermediates have been measured (Creighton and Goldenberg, 1984; Goldenberg et al., 1989; Weissman and Kim, 1991, 1992; Kosen et al., 1992) and the pathway for the kinetically preferred route for folding of BPTI has been described (Figure 1B).

Three features of the BPTI folding pathway (Figure 1B) are particularly striking. First, all intermediates that are well populated at some point during folding contain only native disulfide bonds; that is, disulfide bonds that are present in native BPTI (Weissman and Kim, 1991). Second, BPTI does not fold by a simple sequential acquisition of native disulfide bonds (Creighton, 1977). Instead, an initially formed native two-disulfide intermediate, [30–51; 14–38], termed N' , must first undergo a slow intramolecular disulfide rearrangement to a more stable native intermediate [30–51; 5–55], termed $N\ddagger$. The intramolecular rearrangement of N' to $N\ddagger$ is the rate-limiting transition in the folding of BPTI. Third, during folding, approximately half of BPTI accumulates as a kinetically trapped, dead-end intermediate [5–55; 14–38], termed N^* (States et al., 1984). The N^* intermediate is unable to form a third disulfide bond because native structure in this intermediate buries the free thiols of Cys-30 and Cys-51, rendering these residues inaccessible to external oxidizing reagents (Creighton and Goldenberg, 1984).

The cDNA of BPTI (Creighton and Charles, 1987) encodes a 100 residue pre-pro-protein (Figure 2). This precursor polypeptide includes a 35 residue amino-terminal extension and a 7 residue carboxy-terminal extension. The latter contains some sequence similarity to sequences known to localize proteins to intracellular organelles (Creighton and Charles, 1987). The amino-terminal extension contains a pre region characteristic of signal peptides (von Heijne, 1985; Kaiser et al., 1987) that target proteins for translocation into the endoplasmic reticulum (ER), fol-

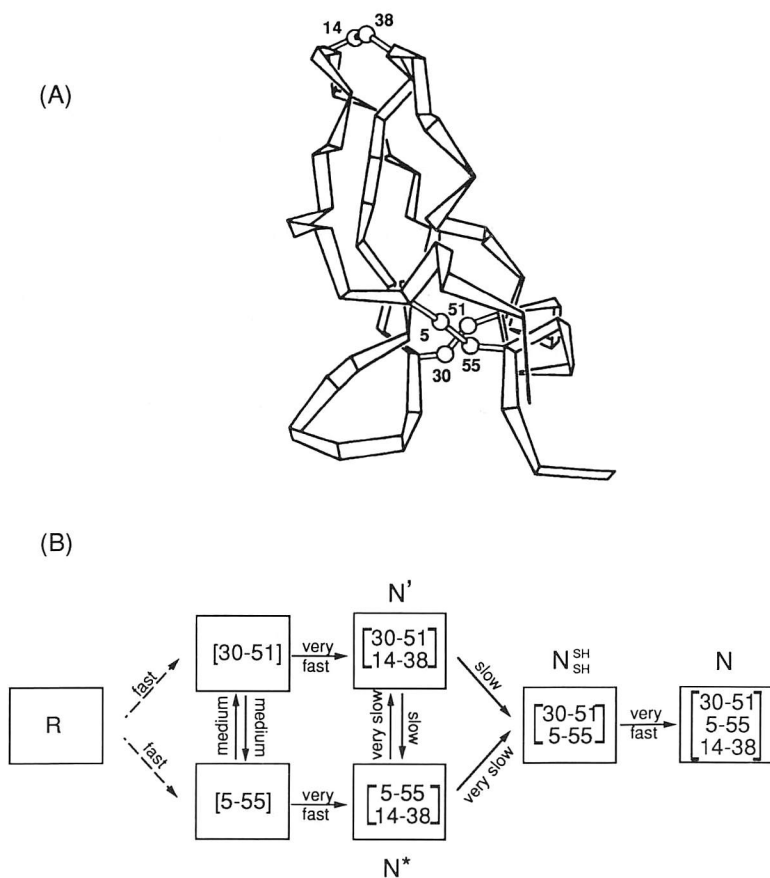


Figure 1. Structure and Folding Pathway of BPTI

(A) Schematic representation (adapted from Staley and Kim, 1990) of the crystal structure for BPTI (Deisenhofer and Steigemann, 1975; Wlodawer et al., 1984). The residues involved in disulfide bonds are labeled. The 14-38 disulfide bond is accessible to solvent, exposing ~50% of its total surface area, whereas the 30-51 and 5-55 disulfide bonds are inaccessible, exposing 0% of their total surface area (Lee and Richards, 1971).

(B) Schematic representation of the kinetically preferred pathway for the folding of BPTI at pH 7.3, 25°C (Weissman and Kim, 1991). R denotes the reduced protein. All of the well-populated intermediates contain only native disulfide bonds. Qualitative descriptions of the relative rates of the intramolecular transitions associated with each step are indicated (see Table 1). N* is a kinetically trapped intermediate that is stable for weeks under these conditions. The dotted arrows indicate that R is oxidized initially to a broad distribution of one-disulfide intermediates that then rearrange rapidly to [30-51] and [5-55].

lowed by a short pro region. Moreover, correctly folded BPTI with a 13 amino acid extension on the amino terminus has been purified recently from bovine platelets (J. Li, S. Olson, and D. A. Walz, personal communication).

The function of this 13 residue pro region is unknown. There is, however, a single cysteine residue in this region, suggesting that it might be important for folding and disulfide bond formation. In this study, we have examined the effect of the pro region on the *in vitro* folding of BPTI. We find that addition of the pro region to BPTI allows direct formation of three disulfide bonds, thereby circumventing the rearrangement process that is rate limiting in the folding of mature BPTI. This leads to a substantial increase in the overall rate and efficiency of folding.

Results

The Pro Region Facilitates Folding

To determine the effects of the pro region on the folding of BPTI, we constructed a recombinant model of pro-BPTI (Figure 2). This model is composed of the 13 residues that remain on the amino terminus after cleavage of the pre region followed by the 58 residues of mature BPTI. Since a methionine residue remains on the amino terminus following expression in *Escherichia coli*, this recombinant model is designated m-proBPTI.

A disulfide bond will not form spontaneously, even when two thiols are in close proximity, unless there is an appropriate oxidizing agent present. For these folding studies,

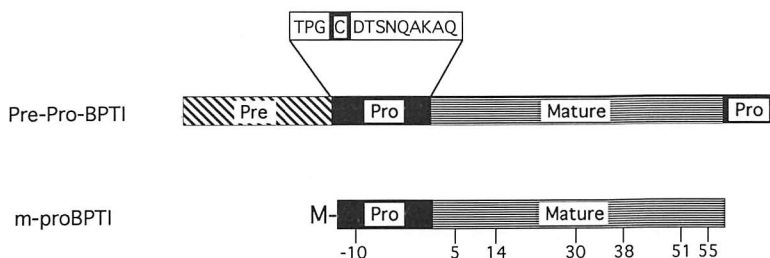


Figure 2. Schematic Representation of Pre-Pro-BPTI and m-proBPTI

The numbers indicate the location of the cysteine residues in m-proBPTI. The sequence of the pre region, assuming that translation is initiated at the first AUG codon, is MKMSRLCLSVALLVLLGTLAAS. The sequence of mature BPTI is RPDFCLEPPYTGPCAKRIIRYFYNAKAGLCQTFVYGGCRAKRNNFKSAEDCMRTC GGA, and the sequence of the carboxy-terminal pro region is IGPWENL.

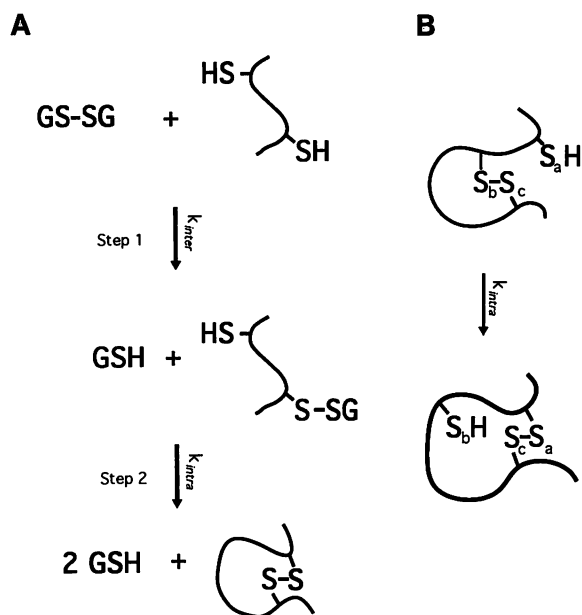


Figure 3. Diagram of Steps in the Formation and Rearrangement of Disulfide Bonds

(A) Formation of a protein disulfide bond by GSSG. Oxidation occurs in two steps. Step 1 is the intermolecular attack of a protein thiol on the glutathione–glutathione disulfide bond. Step 2 is the intramolecular attack of a second protein thiol on the protein–glutathione mixed disulfide.

(B) Intramolecular thiol–disulfide exchange between a protein thiol and an existing protein disulfide bond.

a redox buffer containing both the oxidized and reduced forms of glutathione was used to promote the formation and elimination of protein disulfide bonds. Reduced glutathione, abbreviated GSH, is the tripeptide Glu–Cys–Gly (the amide linkage to the Glu residue is through the γ carboxyl group). Oxidized glutathione, abbreviated GSSG, is a disulfide-bonded dimer of reduced glutathione. In vivo, GSH and GSSG are thought to be the principal redox reagents involved in disulfide bond formation (Hwang et al., 1992).

Formation of an intramolecular disulfide bond in a protein by GSSG proceeds in two steps (Figure 3A). First, a free thiol in the protein attacks the disulfide bond of GSSG, releasing a molecule of GSH. This step yields a species with a disulfide bond between a protein Cys residue and a glutathione molecule, referred to as a mixed disulfide. In a second step, a different protein thiol attacks the protein–glutathione mixed disulfide, resulting in the release of a second molecule of GSH and the formation of a disulfide bond between two Cys residues in the protein. Intramolecular rearrangement of existing disulfide bonds in a protein (Figure 3B) occurs by a mechanism similar to the second step in the oxidation reaction, except that during intramolecular rearrangement the protein thiol attacks a protein–protein disulfide bond instead of a protein–glutathione disulfide.

The folding reactions of mature and m-proBPTI were monitored as follows. First, folding was initiated by the

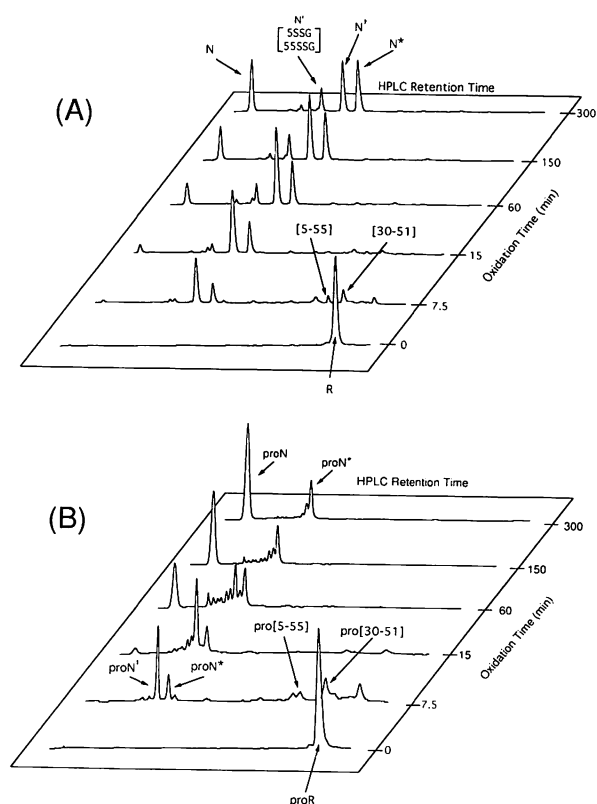


Figure 4. Time course of Folding of Mature BPTI and m-proBPTI (pH 7.3, 25°C)

(A) Folding of mature BPTI. A folding reaction was initiated by the addition of the redox buffer (0.5 mM GSSG, 2.0 mM GSH) to the fully reduced protein. At the indicated times, a portion of the sample was removed, quenched with acid, and analyzed by HPLC. N'[5SSG; 5SSSG] denotes the N' intermediate with a glutathione–protein mixed disulfide bond on Cys-5 and Cys-55.

(B) Folding of m-proBPTI.

addition of pH 7.3 redox buffer to fully reduced protein. This redox buffer contains 0.5 mM GSSG and 2.0 mM GSH. Second, at various times, a portion of the folding reaction was quenched with acid. Because the thiolate anion is the reactive species in thiol–disulfide exchange, protonation of the thiols prevents further oxidation or rearrangement of the intermediates. Third, the spectrum of intermediates present at each time point was determined by high pressure liquid chromatography (HPLC) carried out in acidic conditions (pH 2). The disulfide linkages of the observed intermediates were determined as described previously (Weissman and Kim, 1991; see also Experimental Procedures). As before, intermediates are denoted in brackets by the disulfide linkages of the Cys residues. The prefix “pro” is used to denote intermediates in the folding of m-proBPTI.

Addition of the pro region has little effect on the early time points in the folding of BPTI (Figure 4). For both mature BPTI and m-proBPTI, the fully reduced protein disappears rapidly. A set of single disulfide species, composed predominantly of the native intermediates [30–51] (or pro[30–51]) and [5–55] (or pro[5–55]), appears transiently.

Subsequently, two native two-disulfide intermediates accumulate: [30–51; 14–38] (termed N' or proN') and [5–55; 14–38] (termed N* or proN*).

In contrast, later time points in the folding of mature BPTI and m-proBPTI differ in three significant respects (Figure 4). First, m-proBPTI completes folding substantially faster than does mature BPTI. Second, the yield of properly folded protein (N or proN), compared to the nonproductive intermediate (N* or proN*) is increased substantially when the pro region is present. Finally, several intermediates are apparent in the folding of m-proBPTI that do not accumulate during the folding of mature BPTI.

The Rates of Individual Steps in the Folding Pathway of Mature BPTI Are Not Altered by the Presence of the Pro Region

An important advantage of acid quenching over quenching methods that modify protein thiols irreversibly, such as alkylation by iodoacetate, is that protonation is reversible. As a consequence, it is possible to purify acid-quenched intermediates by HPLC and subsequently to follow the rearrangement or oxidation of these species in isolation (Weissman and Kim, 1991).

Starting with purified acid-quenched intermediates, we examined the effect of the pro region on individual steps in the folding pathway of mature BPTI (see Figure 1B). Folding steps involving only the intramolecular rearrangement of existing disulfide bonds (e.g., the rearrangement of N' to N_{SH} and N*) were carried out in the absence of redox reagents to avoid formation or reduction of disulfide bonds. Folding steps involving formation of new disulfide bonds (e.g., oxidation [30–51] to N') were carried out in the presence of the circular disulfide reagent, oxidized dithiothreitol (DTT_{ox}). Unlike oxidation by GSSG, the rate of oxidation by DTT_{ox} is proportional to k_{intra} , the rate of the intramolecular transition that brings the two protein thiols together (step 2 in Figure 3A). This makes it possible to compare the rates of steps involving formation of a new disulfide bond with steps involving only the intramolecular rearrangement of existing disulfides. (For a more complete discussion see Experimental Procedures and Creighton, 1984.)

In the absence of the glutathione redox buffer, the pro region does not alter the rates of the intramolecular transitions associated with the individual folding steps (Table 1). This is true both for steps in which the rates are enhanced by native structure (e.g., the intramolecular transition in the oxidation of [30–51] to N') and for steps that are retarded by native structure (e.g., the rearrangement of N' to N* and N_{SH}). The only significant difference observed between m-proBPTI and mature BPTI is that the rate of the intramolecular rearrangement of pro[5–55] to pro[30–51] is about 2-fold faster than the rearrangement of [5–55] to [30–51]. The failure of the pro region to affect substantially the rates of the individual folding steps suggests that the pro region does not alter significantly the structure of these intermediates.

Mature BPTI and m-proBPTI Fold by Distinct Pathways

An apparent contradiction exists between the finding that

the pro region does not affect substantially any of the individual intramolecular rates for the folding of BPTI (Table 1) and the finding that the pro region facilitates the overall folding reaction (Figure 4). This apparent contradiction, however, can be resolved if the predominant folding pathway of BPTI is different in the presence of the pro region.

The rate-limiting transition in the folding of mature BPTI is the rearrangement of a native two-disulfide intermediate, N', to a more stable native two-disulfide intermediate, N_{SH} (see Figure 1B). Because the rearrangement of N' involves only intramolecular thiol–disulfide exchange, this transition is not influenced by the presence of external disulfide reagents like glutathione (Creighton, 1975). Although the pro region does not accelerate the rearrangement of N' to N_{SH} (Table 1), the overall rate of folding in the presence of the redox buffer is increased in m-proBPTI (Figure 4). These observations suggest strongly that the rate-limiting rearrangement in the folding of mature BPTI is circumvented in the presence of the pro region.

As a test of this proposal, we determined directly the effect of redox buffer on the productive folding of N' (and proN'), starting with purified, reversibly-trapped intermediates. For measurements made in the absence of redox buffer (where the number of disulfide bonds in an intermediate does not change), productive folding ends with the formation of N_{SH}, which is the immediate precursor to native BPTI (formation of the final 14–38 disulfide bond in N_{SH} occurs very rapidly in the presence of redox buffer; see Figure 1B). Thus, the rate of formation of N_{SH} (or proN_{SH}) in the absence of redox buffer is compared with the rate of formation of N (or proN) in the presence of redox buffer (Figure 5).

Addition of the redox buffer has no significant effect on the rate of productive folding of N' from mature BPTI (Figure 5A). This result is in agreement with the conclusion from earlier studies (Creighton, 1977) that the rate-limiting transition in the folding of mature BPTI is an intramolecular process.

In contrast, addition of the redox buffer to proN' leads to a substantial increase in the rate of productive folding (Figure 5B). There is also a substantial increase in the yield of proN, with a concomitant decrease in the yield of the dead-end intermediate proN*, in the presence of redox buffer (Figure 5B). These results demonstrate directly that the folding reactions of mature BPTI and m-proBPTI have distinct rate-limiting steps.

The pro region must be linked physically to the mature protein to accelerate folding. A peptide corresponding to the pro region of BPTI was produced by solid phase synthesis, and the effect of this peptide on the productive folding of N' from mature BPTI in the presence of the redox buffer was determined. Even at a 10-fold molar excess, the pro-peptide did not increase productive folding of N' (Figure 6).

A Single Cysteine Is the Only Residue Required for Acceleration of Folding by the Pro Region

The conclusion that, in the presence of the redox buffer, the pro region allows BPTI to circumvent the rate-limiting rearrangement step suggests that the cysteine residue in the pro region plays an important role in the folding of

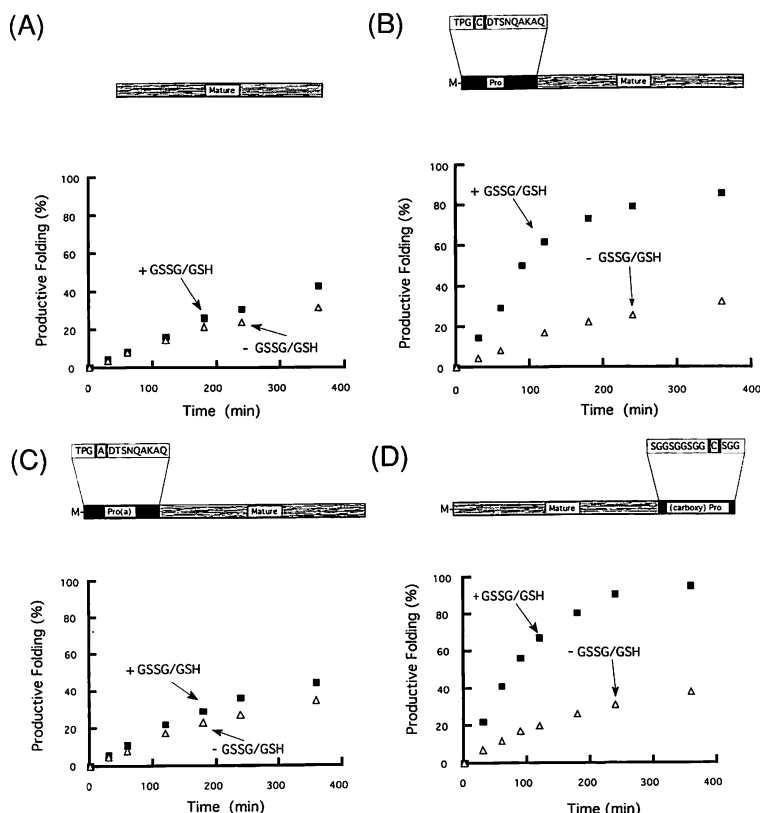


Figure 5. Effects of Redox Buffer on Folding of the N' Intermediates from Different Forms of BPTI

Acid-quenched N' was allowed to rearrange in the absence (triangles) or presence (squares) of the redox buffer. The concentration of N_{SH} produced during the rearrangement of N' in the absence of the redox buffer is compared with the concentration of N produced during the rearrangement of N' in the presence of the redox buffer (see text). (A) mature BPTI. (B) m-proBPTI. (C) m-pro(a)BPTI. (D) m-(carboxy)proBPTI.

m-proBPTI. To determine the role of this Cys residue, we studied two BPTI molecules with altered pro regions. The first, termed m-pro(a)BPTI, is identical to m-proBPTI except that the Cys residue in the pro region is replaced with an alanine. In the second, termed m-(carboxy)proBPTI, the pro region was replaced by a flexible amino acid tether containing a single cysteine residue but otherwise consisting of repeats of Ser-Gly-Gly. Moreover, this synthetic pro region was attached to the carboxyl terminus of mature BPTI (Figure 5D). The prefixes "pro(a)" and "(carboxy)pro" are used to denote intermediates in the folding of m-pro(a)BPTI and m-(carboxy)proBPTI, respectively.

Replacing the cysteine residue in the pro region with an alanine abolishes its ability to facilitate folding. As with N' and proN', in the absence of the redox buffer, pro(a)N' rearranges slowly to both the productive intermediate pro(a)N_{SH} and the kinetically trapped species pro(a)N*. In contrast with proN', however, addition of the redox buffer has little effect on either the rate or yield of productive folding of pro(a)N' (Figure 5C). Thus, as in mature BPTI, productive folding of pro(a)N' proceeds by intramolecular rearrangement to pro(a)N_{SH}.

Simply attaching a cysteine residue to the end of mature BPTI by an unstructured peptide tether increases substantially both the rate and yield of productive folding of the N' intermediate (Figure 5D). As with the naturally occurring pro region, productive folding is enhanced only in the presence of the redox buffer. Interestingly, the yield of productively folded protein produced with the synthetic pro region was slightly greater than with the natural pro region (compare Figures 5D and 5B).

The Cysteine Residue in the Pro Region Forms a Mixed Disulfide with Glutathione during Folding

In N' from mature BPTI, the free thiols at residues 5 and 55 do not react readily with external disulfide reagents like glutathione (J. S. W. and P. S. K., unpublished data). This protection is almost certainly due to native-like structure in N'. NMR studies indicate that the N' intermediate folds to a structure that is very similar to that of native BPTI (Weissman and Kim, 1991; van Mierlo et al., 1991a). Moreover, a recent X-ray crystallographic analysis of a recombinant model of the N' intermediate has shown that the side chains of Val-5 and Ala-55 (replacing Cys-5 and Cys-55, respectively) are buried in the hydrophobic core of the molecule (A. A. Kossiakoff, personal communication).

In contrast with N', the proN' intermediate contains an additional free thiol at residue -10 that reacts readily with GSSG to form an intermediate (termed proN'[-10SSG]) containing a mixed disulfide between the cysteine residue in the pro region and glutathione (see Experimental Procedures). Strong evidence that the folding of proN' proceeds through proN'[-10SSG] is provided by the observation that both the redox buffer and a cysteine residue are required for enhanced folding.

The ability to produce reversibly trapped proN'[-10SSG] and examine the rearrangement in isolation made it possible to determine directly the role of this intermediate in the folding of m-proBPTI. The rate of disappearance of the proN'[-10SSG] intermediate is considerably faster (about 20-fold) than the rate at which proN' rearranges to proN* and proN_{SH} (Figure 7A and Table 1). During this transition, proN'[-10SSG] spontaneously and quantitatively forms

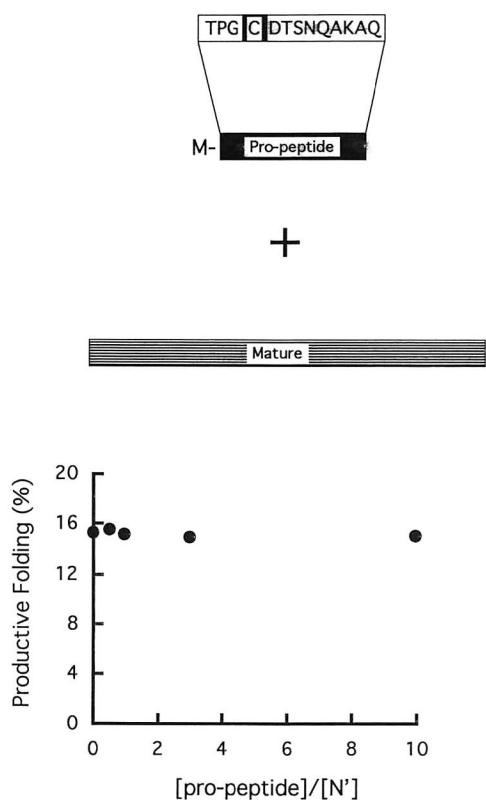


Figure 6. The Pro Region of BPTI Does Not Facilitate Folding In Trans. Acid-quenched N' (final concentration 3 μ M) was allowed to rearrange for 120 min at pH 7.3, 25°C in the presence of the redox buffer and the free pro-peptide. The yield of native protein is shown as a function of the molar ratio of pro-peptide to N' .

the final native state, proN, even in the absence of the redox buffer (Figure 7A).

In the presence of GSH, the transition of proN' to proN'[-10SSG] is reversible. Thus, when folding of m-proBPTI is carried out in the presence of the redox buffer, which contains both GSH and GSSG, proN' and proN'[-10SSG] should interconvert readily (Figure 8). The proN'[-10SSG] intermediate, however, undergoes productive folding much more rapidly than proN'. As a consequence, routes of folding involving the transition of proN'[-10SSG] to proN are preferred kinetically over routes involving rearrangement of proN' to proN* and proN \ddot{S} (Figure 8). This conclusion provides an explanation for the observation (see Figures 4B and 5B) that significant levels of proN* are not produced during the rearrangement of proN' in the presence of redox buffer.

Folding of m-proBPTI Proceeds Predominantly by Intramolecular Rearrangements at the Three-Disulfide Stage

During the transition of proN'[-10SSG] to proN, a set of distinct intermediates accumulates transiently (Figure 7A). By following the rearrangement of proN'[-10SSG] labeled with tritiated glutathione, we determined which of these intermediates contain mixed disulfides with glutathi-

Table 1. Rates of the Intramolecular Transitions Associated with Individual Folding Step at pH 7.3, 25°C

Transition	Mature BPTI (min ⁻¹)	m-proBPTI (min ⁻¹)
R→I	16 ± 1.5	18 ± 1.5
[30-51]→[5-55]	0.05 ± 0.01	0.06 ± 0.02
[5-55]→[30-51]	0.04 ± 0.01	0.09 ± 0.02
[30-51]→N'	300 ± 25	250 ± 25
[5-55]→N*	675 ± 25	775 ± 25
N'→N*	0.0012 ± 0.0001	0.0014 ± 0.0003
N'→N \ddot{S}	0.0014 ± 0.0002	0.0016 ± 0.0002
N \ddot{S} →N	675 ± 50	700 ± 25

These rates were determined directly with pure, reversibly trapped intermediates. For steps involving formation of a new disulfide bond, the rate of the intramolecular transition that brings the two protein thiols together, k_{intra} (step 2 in Figure 3A) was determined by oxidizing the intermediate with DTT \ddot{S} (see Experimental Procedures and Creighton, 1984). Each rate was measured 2 to 4 times. The mean values and deviations from the mean are reported.

one. Radioactive proN'[-10SSG] was allowed to rearrange for 30 min in the absence of the redox buffer, and the intermediates present were separated by HPLC. The concentration of protein present in the HPLC chromatogram was determined by UV absorbance and the concentration of glutathione was determined by scintillation counting.

As expected, the HPLC peak corresponding to proN'[-10SSG] contained stoichiometric quantities of glutathione. A second HPLC peak also contained stoichiometric quantities of glutathione (Figure 7B). The other major protein peaks, however, contained virtually no glutathione. Because the rearrangement was carried out in the absence of external disulfide reagents, the loss of glutathione indicates that these intermediates have formed an additional protein-protein disulfide bond (see Figure 3A). Thus, the absence of glutathione in the majority of intermediates demonstrates that, unlike N' from mature BPTI, proN'[-10SSG] is able to form readily a third disulfide bond.

Discussion

The Folding Pathway of m-proBPTI

Our results are summarized in the following pathway for the folding of m-proBPTI (Figure 9). As with mature BPTI, the fully reduced protein (proR) is converted rapidly into a set of single disulfide species, composed predominantly of the native intermediates pro[30-51] and pro[5-55]. These single disulfide species accumulate transiently and then form the native two-disulfide intermediates: pro[30-51; 14-38] (proN) and pro[5-55; 14-38] (proN*). On the time scale of these experiments, proN* does not oxidize or rearrange.

By contrast, the proN' intermediate is able to form a third disulfide bond. Oxidation of proN' occurs in two steps (Figure 8). First, the solvent-accessible cysteine residue in the pro region forms a mixed disulfide with glutathione to generate proN'[-10SSG]. Then, in an intramolecular process, proN'[-10SSG] forms a third protein-protein di-

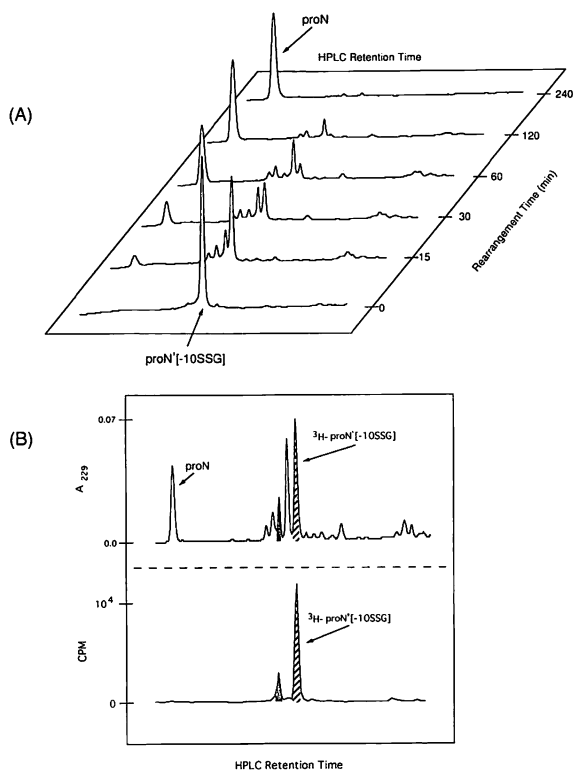


Figure 7. Quantitative Formation of ProN from ProN'[-10SSG]
(A) Time course of rearrangement of purified proN'[-10SSG] in the absence of redox buffer. (B) Detection of intermediates that contain mixed disulfide bonds with glutathione. Acid-quenched proN'[-10SSG] labeled with [³H]GSSG was allowed to rearrange at pH 7.3. After 30 min, the reaction was quenched with acid and analyzed by HPLC. The level of radioactivity present was determined by counting fractions in a scintillation counter. The molar ratio of glutathione to protein in each of the two shaded peaks is between 0.8 and 1.0. The absence of glutathione in the remaining intermediates indicates that these intermediates have formed a third protein-protein disulfide bond (see text and Figure 3A).

sulfide bond, releasing a molecule of GSH. The oxidation of proN' results in the formation of a set of nonnative three-disulfide species designated III (i.e., intermediates that contain three protein-protein disulfide bonds, at least one of which is not found in native BPTI). These nonnative species then rearrange intramolecularly to the final native state, proN.

The pro region accelerates formation of a third disulfide bond in proN'. As a consequence, in the presence of the redox buffer, oxidation of proN' occurs more rapidly than rearrangement. Thus, m-proBPTI circumvents the rate-limiting transition in the folding of mature BPTI, intramolecular rearrangement of N' to N^{SH}. This leads to a significant increase in the rate of productive folding. Moreover, because the N' intermediate rearranges to the nonproductive intermediate N* as well as the productive intermediate N^{SH} (Figure 1B), circumventing this rearrangement step also suppresses formation of N*, leading to a substantial increase in the yield of productive folding.

Mechanism of Action of the Pro Region

The folding of proteins containing intramolecular disulfide

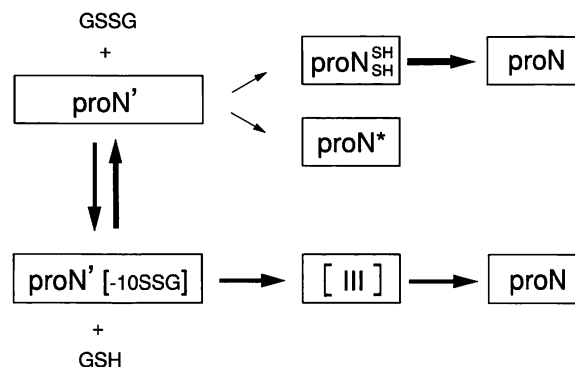
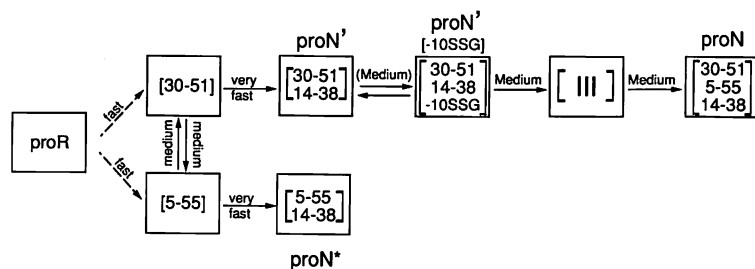


Figure 8. Diagram of the Final Steps in the Folding of m-proBPTI
The thickness of the arrows corresponds qualitatively to the relative rates of the given transition under the redox conditions used in these studies (0.5 mM GSSG, 2.0 mM GSH). The rate of rearrangement of proN'[-10SSG] to III is approximately 20-fold greater than the rate of rearrangement of proN' to proN^{SH} and proN*. As a consequence, folding of m-proBPTI proceeds predominantly through proN'[-10SSG].

bonds presents a unique problem (for example, see Gold-berg, 1992). On the one hand, it is native structure that stabilizes one set of disulfide bonds over the numerous alternate possibilities. On the other hand, native structure can inhibit formation of the native disulfide bonds by burying thiols and thereby inhibiting formation of protein-glutathione mixed disulfides (step 1 in Figure 3A), shifting the pKa of the cysteine thiol, thereby decreasing the rate at which the thiol reacts with glutathione (step 1 in Figure 3A), and constraining cysteines, thereby decreasing the rate of the intramolecular transition that brings the protein thiols together (step 2 in Figure 3A). As a consequence, intermediates with native-like structure, such as the N* and N' intermediates observed in the folding of BPTI, paradoxically can retard substantially the rate at which the final native state is reached.

By providing a solvent-accessible, intramolecular thiol-disulfide reagent, the cysteine residue in the pro region accelerates the rate of oxidation of the native-like, two-disulfide intermediate, proN'. Because the cysteine residue in the pro region of BPTI is accessible to solvent, proN', unlike N' from mature BPTI, can form a mixed disulfide with glutathione (yielding proN'[-10SSG]) in the presence of even moderate concentrations of GSSG. The effective concentration of this mixed disulfide relative to the remaining free thiols in proN' is expected to be approximately 100-fold higher than the concentration of the external oxidizing agent GSSG (0.5 mM). For example, the six cysteine residues in unfolded BPTI have, on average, an effective concentration of ~35 mM with respect to each other (Creighton, 1988), and two cysteine residues in unfolded thioredoxin have an effective concentration of ~50 mM (Lin and Kim, 1991). Because of this high effective concentration, the glutathione-mixed disulfide bond in proN'[-10SSG] is able to react readily with Cys-5 and/or Cys-55, yielding a set of nonnative three-disulfide species (III), despite the fact that the reactivity of these thiols is diminished by native structure.

The pro region also facilitates the intramolecular re-



ated in parentheses, is similar to the rate of the intramolecular transitions designated as "medium." proR denotes the reduced protein. III denotes a set of three disulfide intermediates containing at least one nonnative disulfide bond. One other mixed disulfide species is known to be present during the transition of proN'[-10SSG] to III (see Figure 7B). The relative flux of folding that proceeds through this species has not been determined. The dotted arrows indicate that proR is oxidized initially to a broad distribution of one-disulfide intermediates, which then rearrange rapidly to pro[30-51] and pro[5-55].

arrangement of these nonnative three-disulfide species. Because m-proBPTI contains seven cysteine residues, intermediates with three disulfide bonds also contain an additional free thiol (either in the pro region or in the mature protein). The remaining free thiol can act as an intramolecular reducing agent, thereby allowing the protein to undergo thiol-disulfide bond rearrangements, even in the absence of external reducing agents. Again, the effective concentration of the protein-thiol relative to the protein-disulfide bonds is expected to be much higher than the external reducing agent, GSH. Thus, intramolecular thiol-disulfide rearrangements involving the seventh protein thiol are expected to occur at a relatively rapid rate.

Moreover, the productive intramolecular rearrangement of the initially formed nonnative three-disulfide intermediates is not hampered by formation of kinetically trapped, native-like intermediates. ProN is the only three-disulfide species in m-proBPTI that contains only native disulfide bonds. By contrast, for mature BPTI there are two rapidly formed two-disulfide intermediates that contain only native disulfide bonds (N* and N'). The N* intermediate is stable for weeks under the experimental conditions used in these studies, and the N' intermediate must undergo a slow rearrangement involving the loss of large amounts of native structure before completing folding (Weissman and Kim, 1991, 1992). The absence of severe kinetic traps in the folding of m-proBPTI contributes substantially to an increased rate of folding.

Implications for In Vivo Folding

The finding that properly folded proBPTI can be isolated from bovine platelets (J. Li, S. Olson, and D. A. Walz, personal communication) demonstrates that folding of BPTI in vivo can occur prior to removal of the amino-terminal pro region. It is unlikely, however, that disulfide formation will be completed prior to the cleavage of the pre region (Figure 2), which targets the protein for translocation into the ER, because the pre region is cleaved by a signal peptidase during translocation of the nascent chain (Blobel and Dobberstein, 1975). Thus, it is likely that BPTI folds in the ER as the pro-protein.

The redox buffer used for these in vitro experiments (2.0 mM GSH, 0.5 mM GSSG) is similar to that found in the ER. Glutathione is the principle redox buffer in the ER and

Figure 9. Schematic Representation of the Kinetically Preferred Pathway for the Folding of m-proBPTI at pH 7.3, 25°C

Qualitative descriptions of the relative rates of the individual folding steps are indicated (see Table 1). All of the rates denoted, except for the transition between proN' and proN'[-10SSG], refer to intramolecular transitions and, therefore, are independent of the redox buffer. Under the redox conditions used in these folding studies, the pseudo first-order rate constant for the transition between proN' and proN'[-10SSG], indicated in parentheses, is similar to the rate of the intramolecular transitions designated as "medium."

proR denotes the reduced protein. III denotes a set of three disulfide intermediates containing at least one nonnative disulfide bond. One other mixed disulfide species is known to be present during the transition of proN'[-10SSG] to III (see Figure 7B). The relative flux of folding that proceeds through this species has not been determined. The dotted arrows indicate that proR is oxidized initially to a broad distribution of one-disulfide intermediates, which then rearrange rapidly to pro[30-51] and pro[5-55].

there is, on average, an approximately 3-fold excess of GSH over GSSG throughout the secretory pathway (Hwang et al., 1992). While the concentration of glutathione in the ER is unknown, the total concentration of glutathione in the cell is in the low millimolar range (Gilbert, 1990; Hwang et al., 1992). Despite the similarity between the conditions used in these in vitro experiments and those thought to prevail in the ER, the overall rate of folding of m-proBPTI in these studies is slow, especially when compared with the rate of protein synthesis. This suggests that cellular factors (for review, see Gething and Sambrook, 1992) like protein-disulfide isomerase (PDI) might play an important role in increasing the rate of folding and disulfide formation in vivo. PDI is an abundant protein in the ER that has been shown to catalyze disulfide formation and rearrangement both in vitro (Freedman, 1991) and in cell-free extracts of canine pancreatic microsomes (Bulleid and Freedman, 1988). Earlier investigators found that PDI led to only a small increase (approximately 2.5-fold) in the rate-limiting rearrangement of the N' intermediate in mature BPTI and that the enzyme had no effect on the rate of disulfide oxidation by glutathione (Creighton et al., 1980). The effect of PDI on the folding of pro-BPTI, however, has not been determined.

Knowledge of the folding pathway of pro-BPTI will allow direct comparison with in vivo folding studies. The methodologies developed for separating and identifying BPTI folding intermediates should be applicable to such studies. Comparison between the folding pathway of pro-BPTI in vivo and in vitro should aid in the identification and characterization of cellular factors important for in vivo folding and disulfide formation.

Comparison with Other Pro Regions

Insulin provides the classic example of a protein that folds with the assistance of a pro region (Steiner and Clark, 1968). In this case, the mature protein is a disulfide-linked heterodimer, and the pro region serves to link the two peptides so that the molecule folds as a monomer.

There are now several other examples in which the pro region of a protein assists in folding or assembly (e.g., Verweij et al., 1987; Zhu et al., 1989; Silen and Agard, 1989; Gray and Mason, 1990; Winther and Sørensen, 1991). The best characterized of these examples are for

the proteases subtilisin BPN' (Zhu et al., 1989), α -lytic protease (Silen and Agard, 1989), and carboxypeptidase Y (Winther and Sørensen, 1991). For these proteins, the pro region forms a specific and tight complex with the folded protein and the pro regions do not require a physical linkage to the mature protein to assist folding. This suggests that the pro regions accelerate formation of the native conformation by acting as a specific scaffold that binds to and stabilizes a high energy intermediate of the folding protein. Thus, while pro peptides of this type can have dramatic effects on the rate of folding (e.g., Baker et al., 1992) these pro peptides are not expected to facilitate the folding of unrelated proteins.

By contrast, we find that the pro region of BPTI facilitates folding solely by providing a solvent-accessible, intramolecular, thiol-disulfide reagent. Moreover, a single cysteine residue attached to the end of BPTI by an unstructured peptide tether is sufficient to accelerate disulfide formation and rearrangement. This conclusion suggests that cysteine-containing pro regions similar to that found in BPTI could be useful in facilitating the folding of other disulfide-containing proteins.

Experimental Procedures

Plasmid Construction

All plasmids were constructed using standard cloning procedures (Sambrook et al., 1989) and the sequences were confirmed by DNA sequencing (Sanger et al., 1977). The genes encoding m-proBPTI and m-(carboxy)proBPTI were produced by oligonucleotide site-directed mutagenesis (Kunkel et al., 1987) of a gene encoding mature BPTI (Staley and Kim, 1992). The gene for mature BPTI was synthesized with convenient restriction sites and optimal codon usage for *E. coli* (Grosjean and Fiers, 1982) and ligated into the NdeI-BamHI site of a phagemid-T7 expression vector, pAED4 (Doering, 1992). The plasmid backbone of pAED4 is pUC-f1 (Pharmacia Fine Chemicals) and the T7 expression sequences of pAED4 are from the pET3a plasmid (Rosenberg et al., 1987). The plasmid encoding m-pro(a)BPTI was produced by oligonucleotide site-directed mutagenesis of the plasmid encoding m-proBPTI.

Protein Expression

To express protein, *E. coli* strain BL21(DE3)-pLys(S) was transformed with the appropriate vector. Freshly transformed cells were grown at 37°C in terrific broth and were induced, after reaching an OD_{550} of 0.8, by the addition of isopropyl β -D-thiogalactopyranoside to 0.4 mM. After 120 min, the cells were recovered by centrifugation at $2,000 \times g$ for 30 min.

Purification of Recombinant Protein

All of the variants of BPTI used in these studies form inclusion bodies when expressed in *E. coli*. The proteins were purified from the inclusion bodies as follows. Cell pellets were resuspended in ice-cold 50 mM Tris-HCl (pH 8.7), 15% (vol/vol) glycerol, and lysed by incubation with 100 μ g/ml lysozyme. Magnesium chloride was then added to 10 mM, manganese chloride to 1 mM, and DNAase I to 10 μ g/ml. After 30 min on ice, the inclusion bodies were recovered by centrifugation at $20,000 \times g$ for 20 min. The resulting pellet was sonicated in 50 mM Tris-HCl (pH 8.7), 1 mM EDTA, 1% Nonidet P-40, 1% deoxycholic acid and spun at $20,000 \times g$ for 20 min. The resulting pellet was dissolved by persistent vortexing in 6 M guanidine hydrochloride, 0.1 M Tris-HCl (pH 8.7), 50 mM reduced dithiothreitol (DTT) and dialyzed against 5% (vol/vol) acetic acid. The dialysate was cleared by centrifugation at $20,000 \times g$ for 20 min and purified on a Vydac C-18 preparative column (stock #218TP1022). As with all other HPLC separations, a linear acetonitrile-H₂O gradient in the presence of 0.1% trifluoroacetic acid was used. An unidentified BPTI-like impurity was not well resolved from m-proBPTI by reverse-phase HPLC. The level of this impurity was

determined by capillary zone electrophoresis, using a running buffer of 50 mM phosphate (pH 2.0), 25 mM NaCl. Fractions that were less than 95% pure were discarded. Protein sequencing and laser desorption mass spectrometry (Finnigan MAT) indicated that expression of m-proBPTI, m-pro(a)BPTI and m-(carboxy)proBPTI in *E. coli* resulted in the addition of a methionine residue to the amino terminus. Mature BPTI was purchased from FBA Pharmaceuticals and was purified in the reduced form on a Vydac C-18 preparative column.

Peptide Synthesis

The pro-peptide was synthesized on an Applied Biosystems Model 430A peptide synthesizer using standard Fmoc/HBTU cycles and acetic anhydride capping (Fields et al., 1991). The peptide was cleaved from the resin using standard Fmoc protocols. The sequence of the pro-peptide is MTPGCDTSNQAKAQ-CONH₂. The pro-peptide was purified by reverse-phase HPLC, and the molecular weight was confirmed by laser desorption mass spectrometry.

Purification of Intermediates

Acid quenched [5-55], [30-51], pro[30-51], and pro[5-55] were produced as follows. Reduced mature BPTI or m-proBPTI (final concentration 15 μ M) was oxidized for 25 min in folding buffer (150 mM NaCl, 100 mM sodium phosphate [pH 7.3], 1 mM EDTA) with 150 μ M GSSG. Components of the oxidation mixture were purified on a Vydac C-18 preparative column heated to 50°C. The peaks containing [30-51] and [5-55] were purified further on a Vydac C-18 semipreparative column (stock #218TP510) heated to 37°C. Acid-quenched proN' and (carboxy)proN' were produced by oxidizing reduced m-proBPTI or m-(carboxy)proBPTI, respectively, (final concentration 15 μ M) in folding buffer with 25 mM DTT for 2 hr and purifying on a Vydac C-18 preparative column. DTT was used as an oxidizing agent instead of GSSG to prevent the formation of proN'[-10SSG]. DTT was purified to remove contaminants with less stable disulfide bonds (Creighton, 1984). N' and pro(a)N' were produced by oxidizing reduced mature BPTI or m-pro(a)BPTI, respectively, (final concentration 15 μ M) in folding buffer with 150 μ M GSSG for 45 min, and purifying on a Vydac C-18 preparative column. Reversibly trapped proN'[-10SSG] was produced by reacting proN' with 10 mM GSSG in folding buffer for 4 min. In this procedure, 70% of the proN' was converted to proN'[-10SSG]. The proN'[-10SSG] was purified on a Vydac C-18 semipreparative column. To produce radioactive proN'[-10SSG], [³H]GSSG (New England Nuclear) diluted with nonradioactive GSSG to a specific activity of 40 μ Ci/ μ mol was used. All HPLC-purified intermediates were stored in a lyophilized form in a desiccator until a rearrangement reaction was initiated.

Identification of Intermediates

The disulfide bond linkages of the intermediates were determined as described previously (Weissman and Kim, 1991). Briefly, the free thiols in an acid-quenched intermediate were blocked by resuspending the lyophilized intermediate in 500 mM sodium iodoacetate, 6 M guanidine hydrochloride, 150 mM NaCl, 100 mM Tris-HCl (pH 8.7), 1 mM EDTA for 30 s, followed by purification on a Vydac C-18 analytical column (stock #218TP54). The thiols involved in disulfide bonds were then determined by the following method. The disulfide bonds in the blocked intermediate were reduced with DTT; the resultant thiols were labeled with a fluorescent iodoacetic acid derivative, IAEDANS (Molecular Probes); the protein was digested with thermolysin; and IAEDANS-labeled peptide fragments (indicating Cys residues that were originally involved in disulfide bonds) were separated by reverse-phase HPLC. The identity of the IAEDANS-labeled fragments was determined by amino acid sequencing. The IAEDANS-labeled fragments generated by thermolysin digestion of the intermediates of m-(carboxy)proBPTI, m-pro(a)BPTI, and m-proBPTI were identical to those observed for mature BPTI (Weissman and Kim, 1991), except that digestion of IAEDANS-labeled proN'[-10SSG] resulted in an additional fragment with the sequence MTPGCDTSNQ, corresponding to the cysteine residue in the pro region. The molecular weight of proN'[-10SSG] was confirmed by laser desorption mass spectrometry.

Folding of Purified Intermediates

Folding experiments were carried out as described previously (Weissman and Kim, 1991, 1992) with the following modifications. Unless stated otherwise, the redox buffer used in the folding experiments

consisted of 2.0 mM GSH and 0.5 mM GSSG. Folding experiments were carried out at 25°C in degassed folding buffer (150 mM NaCl, 100 mM sodium phosphate [pH 7.3], 1 mM EDTA) in an anaerobic chamber (Coy Laboratory Products). Folding reactions were quenched by the addition of 1/10th vol 3 M HCl. All samples were kept at 4°C prior to HPLC analysis.

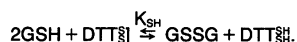
Determination of Rate Constants

Because individual folding steps were monitored directly using reversibly trapped, purified intermediates, the time course of all folding reactions could be described by a single exponential. The equilibrium constant for the transition between [30-51] and [5-55] is near unity. As a consequence, it was possible to determine both the rate of [30-51] → [5-55] and the rate of [5-55] → [30-51] by following the rearrangement of either [30-51] or [5-55].

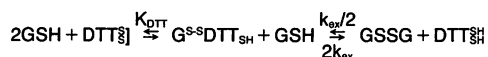
For folding steps involving the formation of an additional disulfide bond (i.e., oxidation of R, [30-51], [5-55], or N_{SH}), the rate of the intramolecular transition that brings together the two protein Cys residues, k_{intra} (step 2 in Figure 3A), was measured by oxidizing the appropriate intermediate with DTT_{SH}. The formation of a mixed disulfide bond between a protein thiol and DTT is unfavorable and is rapidly reversed. As a consequence, the observed second-order rate constant for formation of a protein disulfide bond, k_{obs} , in the presence of DTT_{SH} is given by:

$$k_{\text{obs}} = 2K_{\text{DTT}}k_{\text{intra}}$$

where K_{DTT} is the equilibrium constant for the formation of the mixed disulfide between a protein thiol and DTT (Creighton, 1984). The value of K_{DTT} can be determined from K_{SH} , the equilibrium constant between glutathione and DTT:



The equilibrium between glutathione and DTT can be described by the following equation:



where k_{ox} is the intrinsic rate of thiol-disulfide exchange, and $\text{G}^{\text{S-S}}\text{DTT}_{\text{SH}}$ is a disulfide-bonded heterodimer of glutathione and DTT. Thus, $K_{\text{DTT}} = 4K_{\text{SH}}$ (cf. Creighton, 1984). At pH 7.3, $K_{\text{SH}} = 0.005 \text{ M}^{-1}$ (Chau and Nelson, 1991; Rothwart and Scheraga, 1992; Lees and Whitesides, submitted). Determination of the rate of oxidation of purified [5-55] and [30-51] was complicated by the fact that these species also undergo intramolecular disulfide bond rearrangements. To maximize the rate of disulfide formation, the oxidation of these species was carried out in the presence of a high concentration (50 mM) of DTT_{SH}. Under these conditions, the rate of oxidation of [30-51] and [5-55] was more than 10-fold faster than the rate of rearrangement (Table 1).

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References

- Baker, D., Sohl, J. L., and Agard, D. A. (1992). A protein-folding reaction under kinetic control. *Nature* 356, 263-265.
- Bedows, E., Huth, J. R., and Ruddon, R. W. (1992). Kinetics of folding and assembly of the human chorionic gonadotropin β subunit in transfected chinese hamster ovary cells. *J. Biol. Chem.* 267, 8880-8886.
- Blobel, G., and Dobberstein, B. (1975). Reconstitution of functional rough microsomes from heterologous components. *J. Cell Biol.* 67, 852-862.
- Braakman, I., Helenius, J., and Helenius, A. (1992). Role of ATP and disulfide bonds during protein folding in the endoplasmic reticulum. *Nature* 356, 260-262.
- Bulleid, N. J., and Freedman, R. B. (1988). Defective co-translational formation of disulfide bonds in protein disulfide-isomerase-deficient microsomes. *Nature* 335, 649-651.
- Chau, M.-H., and Nelson, J. W. (1991). Direct measurement of the equilibrium between glutathione and dithiothreitol by high performance liquid chromatography. *FEBS Lett.* 291, 296-298.
- Creighton, T. E. (1975). The two-disulfide intermediates and the folding pathway of reduced pancreatic trypsin inhibitor. *J. Mol. Biol.* 95, 167-199.
- Creighton, T. E. (1977). Conformational restrictions on the pathway of folding and unfolding of the pancreatic trypsin inhibitor. *J. Mol. Biol.* 113, 275-293.
- Creighton, T. E. (1984). Disulfide bond formation in proteins. *Meth. Enzymol.* 107, 305-329.
- Creighton, T. E. (1988). On the relevance of non-random polypeptide conformations for protein folding. *Biophys. Chem.* 31, 155-162.
- Creighton, T. E., and Goldenberg, D. P. (1984). Kinetic role of a metastable native-like two-disulfide species in the folding transition of bovine pancreatic trypsin inhibitor. *J. Mol. Biol.* 179, 497-526.
- Creighton, T. E., and Charles, I. G. (1987). Sequences of the genes and polypeptide precursors for two bovine protease inhibitors. *J. Mol. Biol.* 194, 11-22.
- Creighton, T. E., Hillson, D. A., and Freedman, R. B. (1980). Catalysis by protein-disulfide isomerase of the unfolding and refolding of proteins with disulfide bonds. *J. Mol. Biol.* 142, 43-62.
- Darby, N. J., van Mierlo, C. P. M., Scott, G. H., Neuhaus, D., and Creighton, T. E. (1992). Kinetic roles and conformational properties of the non-native two-disulfide intermediates in the refolding of bovine pancreatic trypsin inhibitor. *J. Mol. Biol.* 224, 905-911.
- Deisenhofer, J., and Steigemann, W. (1975). Crystallographic refinement of the structure of bovine pancreatic trypsin inhibitor at 1.5 Å resolution. *Acta Crystallogr.* B31, 238-250.
- Doering, D. (1992). Functional and structural studies of a small f-actin binding domain. PhD thesis, Massachusetts Institute of Technology, Cambridge, Massachusetts.
- Eigenbrot, C., Randal, M., and Kossiakoff, A. A. (1990). Structural effects induced by removal of a disulfide-bridge: the X-ray structure of the C30A/C51A mutant of basic pancreatic trypsin inhibitor at 1.6 Å. *Protein Eng.* 3, 591-598.
- Fields, C. G., Lloyd, D. H., Macdonald, R. L., Otteson, K. M., and Noble, R. L. (1991). HBTU activation for automated Fmoc solid-phase peptide synthesis. *Peptide Research* 4, 95-102.
- Freedman, R. B. (1991). Protein-disulfide isomerase: an enzyme that catalyzes protein folding in the test tube and in the cell. In *Conformations and Forces in Protein Folding*, B. T. Nall and K. A. Dill, eds. (Washington: American Association for the Advancement of Science), pp. 204-214.
- Gething, M. J., and Sambrook, J. (1992). Protein folding in the cell. *Nature* 355, 33-45.
- Gilbert, H. F. (1990). Molecular and cellular aspects of thiol-disulfide exchange. *Adv. Enzymol.* 63, 69-172.
- Goldenberg, D. P. (1992). Native and non-native intermediates in the folding pathway of BPTI. *Trends Biochem. Sci.* 17, 257-261.

- Goldenberg, D. P., Frieden, R. W., Haack, J. A., and Morrison, T. B. (1989). Mutational analysis of a protein-folding pathway. *Nature* 338, 127-132.
- Gray, A. M., and Mason, A. J. (1990). Requirement for activin A and transforming growth factor- β 1 pro-regions in homodimer assembly. *Science* 247, 1328-1330.
- Grosjean, H., and Fiers, W. (1982). Preferential codon usage in prokaryotic genes: the optimal codon-anticodon interaction energy and the selective codon usage in efficiently expressed genes. *Gene* 18, 199-209.
- Hurle, M. R., Eads, C. D., Pearlman, D. A., Seibel, G. L., Thomason, J. F., Kosen, P. A., Kollman, P., Anderson, S., James, T. L., and Kuntz, I. D. (1992). Comparison of solution structures of mutant bovine pancreatic trypsin inhibitor proteins using two-dimensional nuclear magnetic resonance. *Protein Sci.* 1, 91-106.
- Hwang, C., Sinskey, A. J., and Lodish, H. F. (1992). Oxidized redox state of glutathione in the endoplasmic reticulum. *Science* 257, 1496-1502.
- Kaiser, C. A., Preuss, D., Grisafi, P., and Botstein, D. (1987). Many random sequences functionally replace the secretion signal sequence of yeast invertase. *Science* 235, 312-317.
- Kim, P. S., and Baldwin, R. L. (1990). Intermediates in the folding reactions of small proteins. *Annu. Rev. Biochem.* 59, 631-660.
- Kosen, P. A., Marks, C. B., Fallick, A. M., Anderson, S., and Kuntz, I. D. (1992). Disulfide bond-coupled folding of bovine pancreatic trypsin inhibitor derivatives missing one or two disulfide bonds. *Biochemistry* 31, 5705-5717.
- Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987). Rapid and efficient site-specific mutagenesis without phenotypic selection. *Meth. Enzymol.* 154, 367-382.
- Lee, B., and Richards, F. M. (1971). The interpretation of protein structures: estimations of static accessibility. *J. Mol. Biol.* 55, 379-400.
- Lin, T.-Y., and Kim, P. S. (1991). Evaluating the effects of a single amino acid substitution on both the native and denatured state of a protein. *Proc. Natl. Acad. Sci. USA* 89, 10573-10577.
- Naderi, H. M., Thomason, J. F., Borgias, B. A., Anderson, S., James, T. L., and Kuntz, I. D. (1991). ^1H NMR assignments and three-dimensional structure of Ala14/Ala38 bovine pancreatic trypsin inhibitor based on two-dimensional NMR and distance geometry. In *Conformations and Forces in Protein Folding*, B. T. Nall and K. A. Dill, eds. (Washington: American Association for the Advancement of Science), pp. 86-114.
- Oas, T. G., and Kim, P. S. (1988). A peptide model of a protein folding intermediate. *Nature* 336, 42-48.
- Rosenberg, A. H., Lade, B. N., Chui, D. S., Lin, S. W., Dunn, J. J., and Studier, F. W. (1987). Vectors for selective expression of cloned DNAs by T7 RNA polymerase. *Gene* 56, 125-135.
- Rothwarf, D. M., and Scheraga, H. A. (1991). Regeneration and reduction of native bovine pancreatic ribonuclease A with oxidized and reduced dithiothreitol. *J. Am. Chem. Soc.* 113, 6293.
- Rothwarf, D. M., and Scheraga, H. A. (1992). Equilibrium and kinetic constants for the thiol-disulfide interchange reaction between glutathione and dithiothreitol. *Proc. Natl. Acad. Sci. USA* 89, 7944-7948.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).
- Sanger, F., Nicklen, S., and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74, 5463-5467.
- Silen, J. L., and Agard, D. A. (1989). The alpha-lytic protease pro-region does not require a physical linkage to activate the protease domain in vivo. *Nature* 341, 462-464.
- Staley, J. P., and Kim, P. S. (1990). Role of a subdomain in the folding of bovine pancreatic trypsin inhibitor. *Nature* 344, 685-688.
- Staley, J. P., and Kim, P. S. (1992). Complete folding of bovine pancreatic trypsin inhibitor with only a single disulfide bond. *Proc. Natl. Acad. Sci. USA* 89, 1519-1523.
- Stassinopoulou, C. I., Wagner, G., and Wüthrich, K. (1984). Two-dimensional ^1H NMR of two chemically modified analogs of the basic pancreatic trypsin inhibitor. Sequence-specific resonance assignments and sequence location of conformation changes relative to the native protein. *Eur. J. Biochem.* 145, 423-430.
- States, D. J., Dobson, C. M., Karplus, M., and Creighton, T. E. (1984). A new two-disulfide intermediate in the refolding of reduced pancreatic trypsin inhibitor. *J. Mol. Biol.* 174, 411-418.
- States, D. J., Creighton, T. E., Dobson, C. M., and Karplus, M. (1987). Conformations of intermediates in the folding of the pancreatic trypsin inhibitor. *J. Mol. Biol.* 195, 731-739.
- Steiner, D. F., and Clark, J. L. (1968). The spontaneous reoxidation of reduced beef and rat proinsulins. *Proc. Natl. Acad. Sci. USA* 60, 622-629.
- van Mierlo, C. P. M., Darby, N. J., Neuhaus, D., and Creighton, T. E. (1991a). (14-38, 30-51) double-disulphide intermediate in folding of bovine pancreatic trypsin inhibitor: a two-dimensional ^1H nuclear magnetic resonance study. *J. Mol. Biol.* 222, 353-371.
- van Mierlo, C. P. M., Darby, N. J., Neuhaus, D., and Creighton, T. E. (1991b). Two-dimensional ^1H nuclear magnetic resonance study of the (5-55) single-disulfide folding intermediate of bovine pancreatic trypsin inhibitor. *J. Mol. Biol.* 222, 373-390.
- van Mierlo, C. P. M., Darby, N. J., and Creighton, T. E. (1992). The partially folded conformation of the Cys-30 Cys-51 intermediate in the disulfide folding pathway of bovine pancreatic trypsin inhibitor. *Proc. Natl. Acad. Sci. USA* 89, 6775-6779.
- Verweij, C. L., Hart, M., and Pannekoek, H. (1987). Expression of variant von Willebrand factor (vWf) cDNA in heterologous cells: requirement of the pro-polypeptide in vWf multimer formation. *EMBO J.* 6, 2885-2890.
- von Heijne, G. (1985). Signal sequences. The limits of variation. *J. Mol. Biol.* 184, 99-105.
- Weissman, J. S., and Kim, P. S. (1991). Reexamination of the folding of BPTI: predominance of native intermediates. *Science* 253, 1386-1393.
- Weissman, J. S., and Kim, P. S. (1992). Kinetic role of the nonnative intermediates in the folding of BPTI. *Proc. Natl. Acad. Sci. USA* 89, 9900-9904.
- Winther, J. R., and Sørensen, P. (1991). Propeptide of carboxypeptidase Y provides a chaperone-like function as well as inhibition of the enzymatic activity. *Proc. Natl. Acad. Sci. USA* 88, 9330-9334.
- Wlodawer, A., Walter, J., Huber, R., and Sjölin, L. (1984). Structure of bovine pancreatic trypsin inhibitor. Results of joint neutron and X-ray refinement of crystal form II. *J. Mol. Biol.* 180, 301-329.
- Zhu, X. L., Ohta, Y., Jordan, F., and Inouye, M. (1989). Pro-sequence of subtilisin can guide the refolding of denatured subtilisin in an intermolecular process. *Nature* 339, 483-484.

1. The first part of the document discusses the importance of maintaining accurate records of all transactions and activities. It emphasizes that proper record-keeping is essential for financial transparency and accountability.

2. The second part of the document outlines the various methods and techniques used to collect and analyze data. It highlights the need for consistent and reliable data collection processes to ensure the validity of the findings.

3. The third part of the document describes the results of the study and the conclusions drawn from the data analysis. It provides a clear overview of the key findings and their implications for the field of study.

4. The fourth part of the document discusses the limitations of the study and the potential areas for future research. It acknowledges the constraints of the current study and suggests directions for further exploration.

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