Hydrophobic Interactions Accelerate Early Stages of the Folding of BPTI

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ABSTRACT: Bovine pancreatic trypsin inhibitor (BPTI) has long served as an important model system for the studies of the protein folding process. Recently a kinetically important folding intermediate has been detected early on the oxidative folding pathway of BPTI [Dadlez, M., & Kim, P. S. (1995) *Nat. Struct. Biol. 2*, 674−679]. The intermediate, named [14−38], contains a single native disulfide bond between residues 14 and 38, and forms much faster than any other single-disulfide intermediate. A series of 24 mutants of BPTI has been studied here to detect amino acids which contribute to fast formation of [14−38]. Seven nonpolar or aromatic residues, distant from the cysteines by as many as eight residues, are found to accelerate the formation of 14−38 disulfide, without changing the reactivities of the cysteines. The acceleration is observed even in 8 M urea. It is concluded that in the early stages of the folding of BPTI and BPTI-like domains, the residual structure of the denatured state promotes native pairing of cysteines by way of interaction of hydrophobic residues. A similar mechanism may facilitate early steps in the folding of proteins in general.

Bovine pancreatic trypsin inhibitor (BPTI)[1] is a small protein with three disulfide bonds between residues 5−55, 14−38, and 30−51 (Figure 1). When these disulfide bonds are reduced, the protein unfolds. Starting from the reduced protein, re-formation of the disulfide bonds can be initiated at neutral pH in the presence of an oxidizing agent. The pathway of disulfide bond formation that accompanies the folding of BPTI has been studied for two decades (Creighton, 1974, 1977; Creighton & Goldenberg, 1984; Weissman & Kim, 1991, 1992a,b, 1993, 1995; Goldenberg, 1992; Dadlez & Kim, 1995). In the first step of reoxidation, intermediates containing a single-disulfide bond are formed. Since there are six cysteines in BPTI, 15 single-disulfide intermediates can possibly form at this stage of the refolding of BPTI. It has recently been shown (Dadlez & Kim, 1995) that an intermediate denoted [14−38], which contains a single-disulfide bond between residues 14 and 38, is prominent in the early stages of BPTI folding. It is a native-like intermediate because residues 14 and 38 are linked by a disulfide bond in the folded protein. Moreover [14−38] forms fast, so a substantial fraction of BPTI molecules fold by way of this intermediate; in other words [14−38] is a kinetically important intermediate in the folding pathway of BPTI. It is thus of interest to study factors contributing to fast formation of the 14−38 disulfide.

The intramolecular rate of formation of a disulfide bond may be influenced both by the intrinsic reactivities of the cysteines and by the structure of the polypeptide chain linking the two cysteines. For instance the intrinsic reactivities of the cysteines may be changed by the presence of charged residues in the immediate vicinity of cysteines (Zhang & Snyder, 1988). On the other hand, the structure of the polypeptide chain linking the two cysteines changes the spectrum of accessible conformations and thus may favor or hinder the formation of the disulfide bond due to entropic reasons. Transition state theory is sometimes employed to describe protein folding process (Fersht, 1995). Its formalism allows the kinetics of the disulfide formation to be linked with the thermodynamics of the transition state, i.e., protein conformation in which two cysteines are in contact, but the disulfide bond is not formed yet. In terms of the transition state theory the disulfide formation kinetics probes the influence of the structure of the unfolded protein on proper

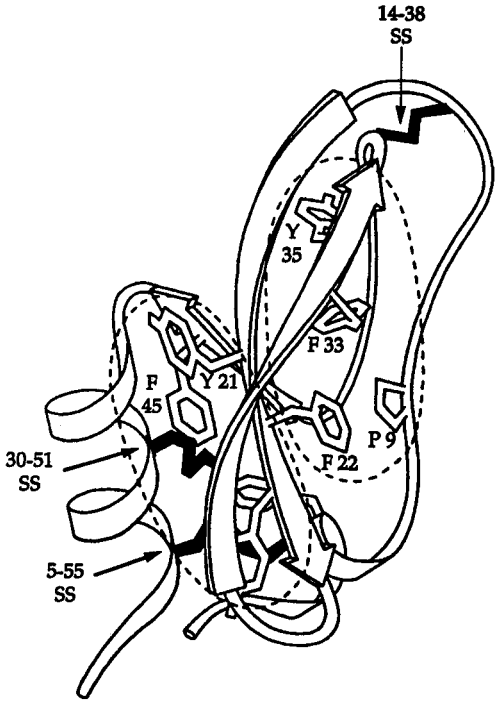

FIGURE 1: Schematic representation of the crystal structure of BPTI (Richardson, 1985). Disulfide bonds between cysteines, some aromatic side chains, and two hydrophobic cores are shown.

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[1] Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; WT, wild type; EDTA, ethylenediaminetetraacetic acid; HPLC, high-pressure liquid chromatography; TFA, trifluoroacetic acid; GSSG, oxidized glutathione; GSH, reduced glutathione; UV, ultraviolet; RED, reduced species; SMD, single mixed-disulfide species; DMD, double mixed-disulfide species; OXI, oxidized species; NMR, nuclear magnetic resonance; Abu, α-amino-*n*-butyric acid; NOE, nuclear Overhauser effect.

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pairing of residues in the folding process. Thiol groups are thus used here as reporter groups of the thermodynamics of the unfolded state in folding conditions, before the disulfide bond is formed, so the conclusions apply to all polypeptide chains not necessarily containing cysteines and not necessarily residing in oxidizing cell compartments. Moreover the kinetics measurements show directly how the residual structure of the unfolded state affects the kinetics of folding. Though the existence of conformational preferences in denatured proteins is well documented (Shortle, 1993; Evans & Radford, 1994), the experimental evidence of their role in folding is scarce (Fersht et al., 1994; Gronenborn & Clore, 1994; Frech & Schmid, 1995) and their significance sometimes questioned (Creighton, 1988; Privalov, 1989).

A qualitative conclusion of a fast formation of [14−38] in native BPTI has been confirmed by a more detailed study of the kinetics of formation of the first disulfide in a series of BPTI mutants and fragments (Dadlez & Kim, 1996). These studies allowed to separate local (intrinsic reactivity) and non-local (conformational) contributions to the accelerated formation of 14−38. Cysteines 14 and 38 have indeed been shown to possess higher intrinsic reactivities than cysteines 5, 30, 51, or 55, as the positively charged residues follow cysteines 14 and 38 in the BPTI sequence. Remarkably, the local effect, though significant, is not sufficient to account for fast formation of 14−38 disulfide. The data obtained for BPTI mutants suggest the existence of non-local factors (residual conformational preferences in the otherwise unfolded polypeptide chain), accelerating the formation of the disulfide bond linking cysteines 14 and 38 in BPTI at least 3−4-fold.

Such an effect, though thermodynamically not large, may be kinetically significant and facilitate channeling of the conformational search of the folding protein toward the native-like topology early in folding. The structure of the unfolded state may be thought to be helpful for folding if it accelerates the native-like pairing of residues in the folding intermediates. Here, the analysis of the interactions which accelerate the formation of the native 14−38 disulfide is presented. A BPTI mutant, with only two cysteines (14 and 38) in the sequence was used as a template (named pseudo-wild-type) for alanine scanning mutagenesis. A series of replacements by alanine on this pseudo-WT background was studied to detect which amino acid residues contribute to fast formation of the 14−38 disulfide bond. The results reveal some details of the folding code for BPTI and BPTI-like domains, showing that a hydrophobic cluster in the denatured state accelerates the formation of a kinetically important intermediate early in folding.

MATERIALS AND METHODS

General. The disulfide rearrangement reactions were carried out under nitrogen in a circulating water bath in order to maintain a constant sample temperature. Reactants were first dissolved in H_2O (containing glutathione or urea or both where necessary) acidified to pH 3 with formic acid. A small aliquot was withdrawn from the low-pH sample and analyzed by HPLC in order to obtain the elution profile of the protein species at time zero of the disulfide rearrangement. Disulfide rearrangement was initiated by adding to the remainder of the starting mixture (with vigorous stirring) 1/4th volume of 0.5 M phosphate buffer, 1 M NaCl, 5 mM EDTA, to give final conditions of 0.1 M phosphate buffer, pH 7.3, 0.2 M NaCl, 1 mM EDTA. For analytical reactions, the final protein concentration was 5−10 μM. At the appropriate times, aliquots of the refolding mixture were quenched to a final pH of ∼2 by addition of 1/10th volume of formic acid (or HCl in the case of urea-containing samples). The quenched samples were transferred to an automatic sample injector and analyzed by HPLC. The order in which the samples were injected was altered in order to check the efficiency of acid quenching. The pH of the remaining, unquenched portion of the folding reaction was checked at the end of each experiment. Urea was prepared fresh on a daily basis.

Reversed-phase HPLC was performed using Vydac C-18 columns immersed in a water bath heated to 35 °C. A linear gradient of solvent A (0.1% TFA in H_2O) and B (90% vol/vol acetonitrile in H_2O, 0.1% TFA) was used. Absorbance was monitored at 229 nm.

Species were assigned according to their molecular masses. Laser desorption mass spectrometry was performed with a FinniganMAT Lasermat. Samples were mixed with matrix (α-cyano-4-hydroxycinnamic acid) in 70% acetonitrile, 30% H_2O, 0.1% TFA.

Expression and Purification of BPTI Mutants. BPTI mutants were expressed as fusion proteins in *Escherichia coli* strain BL21 (DE3) pLysS, using the T7 system (Studier et al., 1990), as described previously (Staley, 1993; Staley & Kim, 1994). Briefly, the pAED4 plasmids (Doering, 1992) encoding a portion of the *E. coli* trp operon [trpLE 1413 polypeptide (Miozzari & Yanofsky, 1978)], which serves as a leader sequence, followed by a Met residue and the BPTI mutant gene, were prepared by oligonucleotide-directed mutagenesis (Kunkel et al., 1987). The gene sequence was confirmed for each mutant by dideoxynucleotide sequencing. Transformed cells were grown from overnight cultures in Luria broth (100 μg/mL ampicillin, 30 μg/mL chloramphenicol) to $A_{590} = 1$. Cells were induced with 0.4 mM isopropyl β-D-thiogalactopyranoside (IPTG) and harvested after 2.5 h by centrifugation. Cells were lysed by freezing, followed by sonication in 50 mM Tris, pH 8.7, 15% glycerol, 100 μM $MgCl_2$, 10 μM $MnCl_2$, 10 μg/mL DNase I. After centrifugation, the pellet was resuspended in 50 mM Tris, pH 8.7, 1% Nonidet (NP40), 1% deoxycholic acid, 1 mM EDTA, and then sonicated and centrifuged. Finally, the pellet was dissolved in 6 M GuHCl, 50 mM Tris, pH 8.7, 10 mM GSSG, and then sonicated and incubated at room temperature for 15 min. The resulting mixture was divided into 5 mL fractions, and each fraction was diluted 10-fold with H_2O and then centrifuged. The pellet was resuspended in 70% formic acid and ∼200 mg of cyanogen bromide was added to initiate cleavage. After 2.5 h, the mixture was centrifuged under vacuum for 4 h, diluted 10-fold with H_2O, and lyophilized. The sample was redissolved in 6 M GuHCl, 0.1 M dithiotreitol (DTT), 0.1 M Tris, pH 8.7, and dialyzed against 5% acetic acid. The dialysate was centrifuged and filtered prior to purification on a Vydac C-18 semi-preparative HPLC column. The molecular mass of the collected material as determined by laser desorption mass spectroscopy was within 2 amu from the expected value.

Replacement of the cysteines by alanine eliminates the side chain beyond the β-carbon, without introducing additional main-chain flexibility. Substitution of cysteines in BPTI by alanines has been shown previously to be least

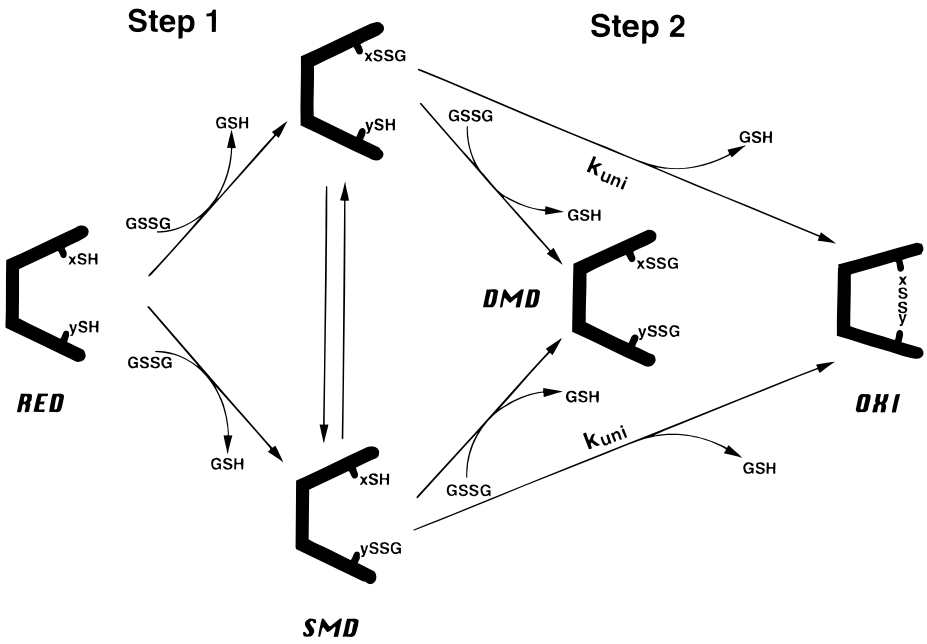

FIGURE 2: Scheme of the reaction of a protein mutant containing two cysteines with oxidized glutathione (GSSG). The reduced BPTI mutant (RED), dissolved in a neutral pH buffer containing an excess of GSSG, first undergoes oxidation (Step 1) to the single mixed-disulfide species (SMD), then (Step 2) to either the unimolecularly oxidized (OXI) or the double mixed-disulfide species (DMD). Although this reaction results in the formation of reduced glutathione (GSH), its concentration is always low and is therefore negligible.

disruptive of native structure (Staley & Kim, 1992; Marks et al., 1987; Hurle et al., 1992). It has also been shown that an additional substitution, M52L, introduced in the mutants, has no effect on BPTI stability (Yu et al., 1995), and is encountered in homologous proteins (Creighton & Charles, 1987).

Concentration Measurements. Protein concentration was determined by measuring HPLC peak areas as follows. First, the response of the HPLC UV detector to the elution of a known amount of each species was measured. The response factor (Chau & Nelson, 1991) linking the sample amount injected with the peak area observed at 229 nm was determined by repeated HPLC analysis of aliquots of standard samples, for which the concentration had been measured by other methods. Standard sample concentrations were measured spectroscopically, assuming an extinction coefficient of 5720 M^{-1} cm^{-1} at 275 nm for BPTI (Kosen et al., 1981) and 1420 M^{-1} cm^{-1} for a tyrosine residue. The extinction of Tyr residue was subtracted in case of Tyr to Ala mutations. An automatic HPLC sample injector was used, ensuring a high degree of reproducibility in the sample amount injected.

Formation of a disulfide bond introduces additional absorption bands into the UV spectrum of mutants, but these bands are much weaker than the strong absorption bands of the proteins at 229 nm (Donovan, 1969). Accordingly, it is assumed here that formation of a disulfide bond does not change the HPLC detector response factors measured for proteins in the reduced state. The accuracy of concentration measurements does not influence the measurements of the rates of the intramolecular disulfide rearrangement.

Isolation of Single Mixed-Disulfide Species. The reduced form of each BPTI mutant was reacted with oxidized glutathione (GSSG) to obtain single mixed-disulfides with glutathione. Five species are expected to result from this reaction [the reduced form (RED), two single mixed-disulfides (SMD), one double mixed-disulfide (DMD), and the intramolecularly oxidized (OXI) species, see Figure 2]. In all mutants both single mixed-disulfide species could be isolated as separate peaks by HPLC. To isolate the single mixed-disulfides 2−3 mg of reduced mutant protein was dissolved in 0.2 mL of 6 M GuHCl, 0.1 M acetate buffer, pH 4, and added to 2 mL of 0.1 M oxidized glutathione, 6 M GuHCl, 0.1 M phosphate buffer, pH 7.3, 1 mM EDTA, with vigorous stirring and purging with N_2 at room temperature. After 2.5 min, reactions were quenched with 1/20th volume of HCl. The reaction mix was diluted 6-fold with solvent A and species were purified by HPLC, using a gradient of 0.1% solvent B per minute.

Measurements of k_{uni}. Rate constants for unimolecular disulfide formation in the protein mutants were measured by following the rearrangement of single mixed-disulfide species to oxidized species (Figure 3A). Mixed-disulfide species were incubated at 25 °C, pH 7.3 for different times, in the absence of external redox agents, and acid-quenched aliquots of the reaction samples were analyzed by HPLC. The gradient was 28−38% solvent B in 30 min. From the resulting elution profiles (Figure 3B), the peak areas and the concentrations of all three species accumulating during the course of the reaction (both single mixed-disulfides and the intramolecularly oxidized species) were measured. The decay of the sum of peak areas of both single mixed-disulfide species, [SMD](t), was monitored relative to total species (Figure 3C). This allows to measure the average rate of interconversion of single mixed-disulfide species to oxidized species (k_{uni}) by fitting the SMD decay data to a monoexponential decay curve. For each mutant, k_{uni} has been measured starting with the mixed-disulfide on either of the cysteines, in two separate experiments, since both single mixed-disulfide species could be isolated as separate peaks by HPLC during the reaction with GSSG. Similar results (within 10%) were obtained irrespective of which of the two SMD forms was used as a starting material, and the final result is the average of at least two such experiments. The

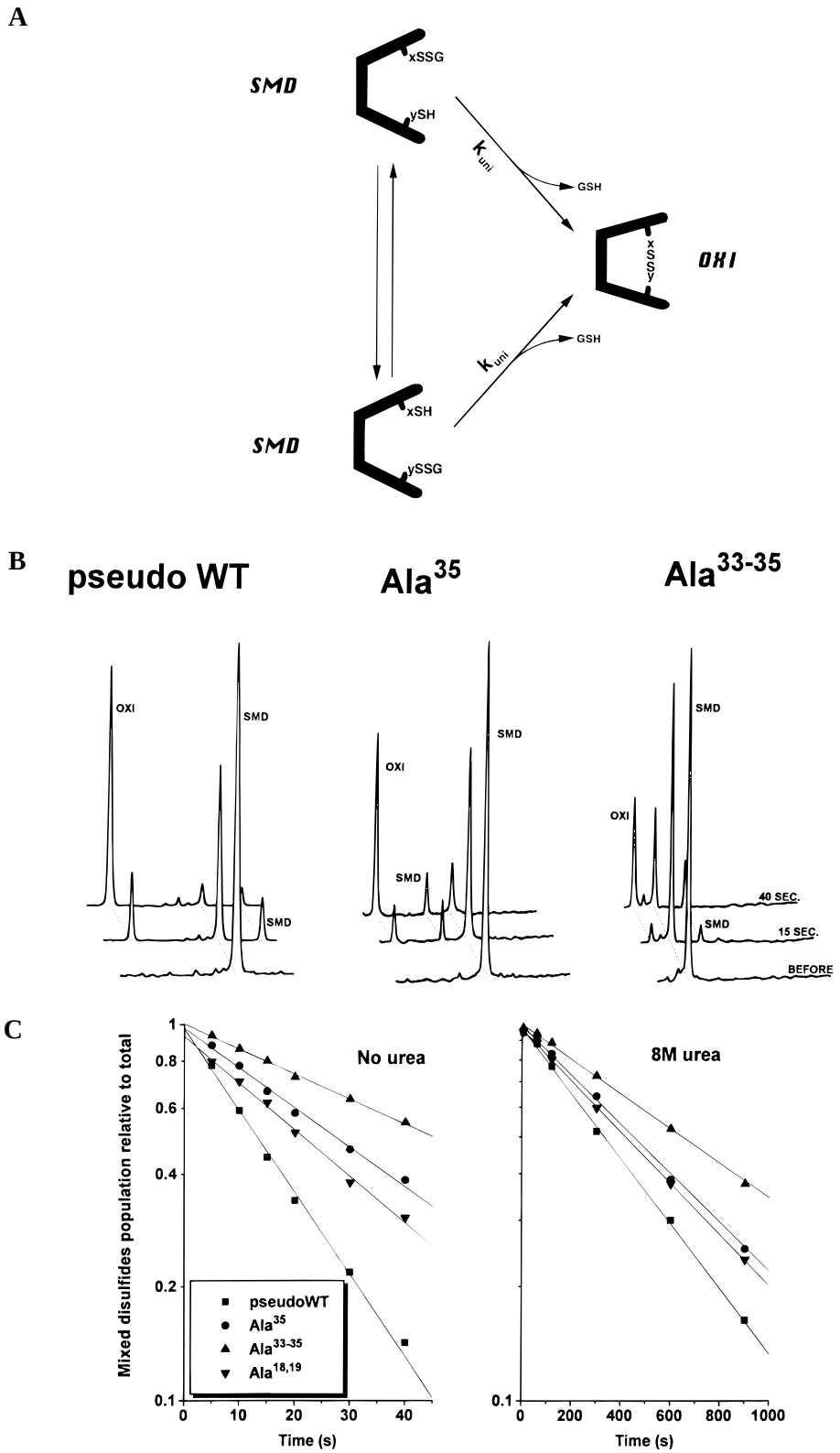

FIGURE 3: (A) Scheme of the rearrangement of a single mixed-disulfide species (SMD) to oxidized protein. Since the reaction starts with a mixed-disulfide species, no external oxidizing agent is needed, and the average unimolecular rate of oxidation (k_{uni}) can be measured directly from the rate of decay of the population of mixed-disulfides. (B) HPLC chromatograms showing the time course of the intramolecular disulfide rearrangement in three mutants of BPTI, pseudo-wild-type, Ala^{35}, and Ala^{33-35}. Each rearrangement started from a purified form of one of the two single mixed-disulfides (SMD) at pH 7.3, 25 °C, and has been quenched after a given time with acid. Obtained results were similar within 10% irrespective which of the two SMD forms had been used as a starting material. UV absorbance was monitored at 229 nm. (C) The disulfide rearrangement kinetics of SMD forms of four variants of BPTI, pseudo-wild-type, Ala^{35}, $Ala^{18,19}$, and Ala^{33-35} in the absence of urea (left panel) and in 8 M urea (right panel), at pH 7.3, 25 °C. The sum of both SMD peak areas is shown decaying in time, relative to the sum of peak areas of all species (oxidized and two single mixed-disulfides). The best fit to a monoexponential decay is shown. The sum of peak areas of all three species does not change during the course of the experiment by more than 10% (data not shown).

sum of the peak areas of all three species (two SMD and OXI) does not change by more than 10% during the time course of the experiment. The residual population of double mixed-disulfide form appears in the course of the experiment

indicating a slow intermolecular rearrangement. The measurements of the disulfide formation rate in pseudo-WT were repeated as a standard, with the reproducibility better than 10%.

Reactivity of Protein Cysteines toward Glutathione. In order to measure the relative intrinsic reactivities of the protein thiols, reduced protein mutants were reacted with 5 mM GSSG at 25 °C, pH 7.3. During this reaction, the reduced protein undergoes oxidation first to a single mixed-disulfide with glutathione, and then to either an intramolecularly oxidized species or a double mixed-disulfide with glutathione (Figure 2). Aliquots of the reaction mixture, quenched at different times, were analyzed by HPLC, using a gradient of 28−38% solvent B in 30 min. Because the sample contains an excess of GSSG, the formation of mixed-disulfides is a pseudo-first-order reaction. Thus, the population of reduced protein molecules decays monoexponentially with the slope equal to the sum of the intrinsic reactivities of both cysteines in the mutant. This reaction results in the formation of reduced glutathione (GSH), but its concentration is always low and therefore negligible.

Protein Sequence Database Search. A FASTA sequence homology search procedure of the GCG package has been used to search the Swissprot Protein Data Base for sequences homologous to BPTI sequence. A subset of 55 of these sequences of proteins or their domains, with invariantly spaced six cysteines has been analyzed to calculate the relative occurrence of different amino acids at different positions in the sequence.

RESULTS

The rates of formation of a disulfide bond between cysteines 14 and 38 have been measured in a series of mutants of BPTI. The 14−38 disulfide forms remarkably fast not only in the native BPTI (Dadlez & Kim, 1995), but also in a BPTI mutant in which the remaining cysteines 5, 30, 51, and 55 have been replaced by alanines (Dadlez & Kim, 1996). This mutant (named pseudo-wild-type) has been used here as a template for alanine scanning mutagenesis studies of the kinetics of formation of 14−38 disulfide. In each of the mutants of pseudo wild-type template a different amino acid residue (or residues) was replaced by alanine. Eighteen single-replacement mutants have been expressed in *E. coli*. In these single replacement mutants a single amino acid of the pseudo-WT sequence was replaced by alanine. Three mutants with multiple sequential replacements have been prepared. In these three mutants two or three neighboring amino acids were simultaneously replaced by alanines; they are denoted by indicating the positions of substitutions by alanine, for instance $Ala^{18,19}$ denotes a mutant in which Ile18 and Ile19 are both replaced by alanines. Accordingly, the remaining two multiple sequential mutants are Ala^{21-23} and Ala^{33-35}. Finally, three mutants with multiple non-sequential replacements $Ala^{18,19,33-35}$, $Ala^{20,33-35}$, and $Ala^{21-23,33-35}$ have been prepared. In these mutants the following replacements, $Ile^{18}Ile^{19}$ to Ala, Arg^{20} to Ala, and $Tyr^{21}Phe^{22}Tyr^{23}$ to Ala, respectively, were introduced in the Ala^{33-35} background. To facilitate the comparison with earlier results on the folding of BPTI, the disulfide formation experiments were carried out at 25 °C, pH 7.3, in 0.1 M phosphate buffer, 0.2 M NaCl, 1 mM EDTA. The effect of denaturant on the 14−38 disulfide formation rates was also studied by measuring the disulfide formation rates in 8 M urea.

In the presence of an oxidizing agent at neutral pH two cysteines readily form a disulfide bond. Since the oxidation by molecular oxygen, which is dissolved in water, is difficult to control (Creighton, 1986), the oxidation of thiols is usually carried out in anaerobic conditions in the presence of oxidized glutathione (GSSG). Glutathione-mediated formation of an intramolecular disulfide bond proceeds in two steps. First (Step 1 in Figure 2), a free thiol in the protein attacks the disulfide bond of oxidized glutathione, releasing a molecule of reduced glutathione (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 (Step 2 in Figure 2), a different protein thiol attacks the protein−glutathione mixed-disulfide, resulting in the formation of a disulfide bond between two protein Cys residues, and releasing a second molecule of GSH. In this work the rates of an intramolecular Step 2 were measured in a series of BPTI mutants.

Because the rate of the disulfide rearrangement is proportional to the population of thiolate anions, the rearrangement rate is very slow at low pH. This strong pH dependence permits to quench the reaction with glutathione by rapidly lowering the pH, and to separate and purify the populated species chromatographically (Weissman & Kim, 1991). After lyophilization, the disulfide rearrangement can be resumed by redissolving the trapped, purified single mixed-disulfide species in neutral pH buffer. This acid-quench method greatly facilitates the task of measuring the intramolecular rates of Step 2 disulfide rearrangement, which are of interest here. The validity of using acid-quenching and high-pressure liquid chromatography (HPLC) analysis to study the folding of BPTI has been established (Weissman & Kim, 1991).

For all mutants the rates of step 2 (k_{uni}) were measured directly, using purified single mixed-disulfide species with glutathione as the starting material for the disulfide rearrangement, in the absence of external oxidizing agents. First, mixed-disulfides with glutathione were prepared. Mutants were reacted with glutathione, reactions were acid-quenched at the appropriate times, the reversibly trapped mixed-disulfides were purified by HPLC and lyophilized. When redissolved in a neutral pH buffer, these protein mixed-disulfides rearrange spontaneously, in the absence of external oxidizing agents, to form unimolecularly oxidized species according to the scheme shown in Figure 3A. All three species could be separated on the HPLC column, as shown in Figure 3B, and their relative amount quantitated. This permits the calculation of the intramolecular rate of the disulfide rearrangement by fitting the parameters of a monoexponential decay to obtained data, as shown in Figure 3C.

Table 1 and Figure 4 illustrate the results of the measurements of the rates of formation of the 14−38 disulfide in single replacement mutants at 25 °C, pH 7.3, relative to pseudo-WT. Replacement of seven residues, all of them nonpolar or aromatic (Ile^{18}, Ile^{19}, Tyr^{21}, Phe^{22}, Phe^{33}, Val^{34}, Tyr^{35}), leads to a significant decrease in the rate of formation of 14−38 disulfide, with the strongest, 2-fold, decrease caused by the replacement of Tyr^{35} by Ala, corresponding to a change in activation energy of about 0.4 kcal/mol in the otherwise unfolded protein. For other residues the effect

Table 1: Rates of Formation of the 14−38 Disulfide in Selected Mutants Relative to the Disulfide Formation Rate in Pseudo-Wild-Type BPTI[a]

replacement by Ala		
position(s)	amino acid type	$k_{mutant}/k_{pseudo\text{-}WT}$
18	Ile	0.74
19	Ile	0.72
20	Arg	1.05
21	Tyr	0.75
22	Phe	0.74
23	Tyr	0.85
33	Phe	0.66 (0.67)[b]
34	Val	0.72
35	Tyr	0.54 (0.76)
18, 19		0.64 (0.81)
21−23		0.57
33−35		0.31 (0.57)
18, 19, 33−35		0.40
20, 33−35		0.33
21−23, 33−35		0.28

[a] Pseudo-wild-type is $C^{5}A$, $C^{30}A$, $C^{51}A$, $C^{55}A$, or $M^{52}L$ BPTI. Mutants contain single or multiple amino acid replacements by alanine in the pseudo-wild-type background. Disulfide rearrangement experiments were performed at 25 °C, pH 7.3, 0.1 M phosphate buffer, 0.2 M NaCl, 1 mM EDTA. Results in 8M urea are shown in parentheses. Repeated measurements for pseudo-WT show that the error of measurement of the disulfide formation rates is ±10%. [b] Numbers in parentheses denote rates measured at 25 °C, pH 7.3, 8 M urea, 0.1 M phosphate buffer, 0.2 M NaCl, 1 mM EDTA.

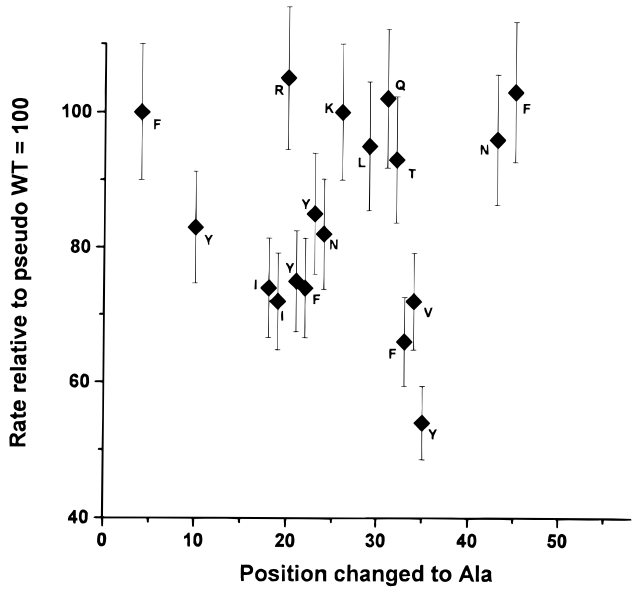

FIGURE 4: Rates of formation of the 14−38 disulfide bond in a series of single replacement BPTI variants relative to pseudo-wild-type. Position of each replacement by alanine is shown on the *X* axis. Letters adjacent to each point denote the type of amino acid replaced by alanine. Cysteines are located at positions 14 and 38. The sequence of native BPTI is the following: RPDFCLEP-PY^{10}TGPCKARIIR^{20}YFYNAKAGLC^{30}QTFVYGGCRA^{40}KRN-NFKSAED^{50}CMRTCGGA.

is weaker (Tyr^{10}, Tyr^{23}, Asn^{24}) or none (Phe^{4}, Arg^{20}, Lys^{26}, Leu^{29}, Gln^{31}, Thr^{32}, Asn^{43}, Phe^{45}). Hydrophobic residues in N- and C-terminal fragments and polar residues throughout the entire sequence bear no effect on disulfide formation.

It might be thought that these seven residues accelerate the disulfide formation by changing the intrinsic reactivities of the cysteines 14 or 38, and not *via* some non-local effect. To measure the effect of the replacements on cysteine intrinsic reactivities, a control experiment was carried out.

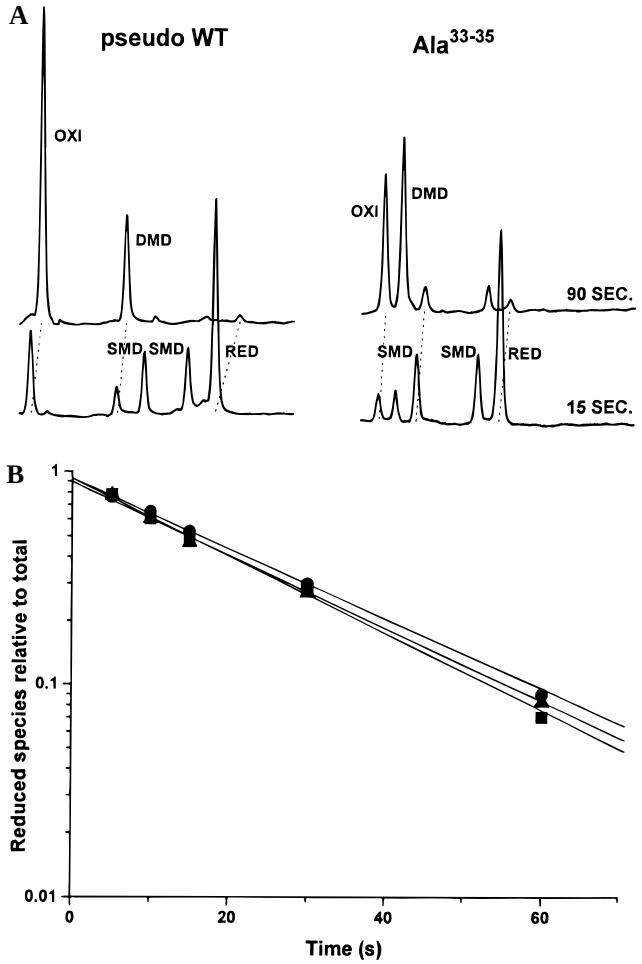

FIGURE 5: (A) HPLC chromatograms showing the time course of the reaction of 5 mM oxidized glutathione (GSSG) with two variants of BPTI: pseudo-wild-type and Ala^{33-35}. Each reaction started with a reduced form of the variant at pH 7.3, 25 °C, and was quenched at appropriate times with acid. Chromatograms of the samples quenched after 15 and 90 s are shown, along with peak assignments. As described in Figure 2 five species are expected to form [reduced (RED), two single mixed-disulfides (SMD), a double mixed-disulfide (DMD) and intramolecularly oxidized (OXI)], and all were well resolved on HPLC. Absorbance was monitored at 229 nm. (B) Kinetics of the reaction of oxidized glutathione with three BPTI variants, pseudo-wild-type, $Ala^{18,19}$, and Ala^{33-35}. The decay of the peak area of the reduced species is shown relative to the sum of the oxidized, single and double mixed-disulfide peak areas. The sum of peak areas of all species does not change significantly during the time course of the experiment (data not shown). The best fit to a monoexponential decay is shown.

In this experiment the reaction rates of oxidized glutathione with cysteines were measured in some of the BPTI mutants. The HPLC elution profiles show (Figure 5A) how during reaction with GSSG, the reduced protein (RED) undergoes oxidation first to mixed-disulfide species with glutathione (SMD), and then either to intramolecularly oxidized species (OXI) or a double mixed-disulfide with glutathione (DMD). The population of the reduced form, relative to total species was measured in different reaction times. Regardless of the replacement the reduced form decays with similar rate (Figure 5B) and both SMD species are equally populated, as is shown for pseudo-WT and Ala^{33-35} (Figure 5A). This indicates that the intrinsic reactivities of both cysteines remain unchanged in the mutants. In addition, in pseudo-wild-type BPTI, during reaction with glutathione 3-fold more OXI species forms than the DMD, whereas in case of

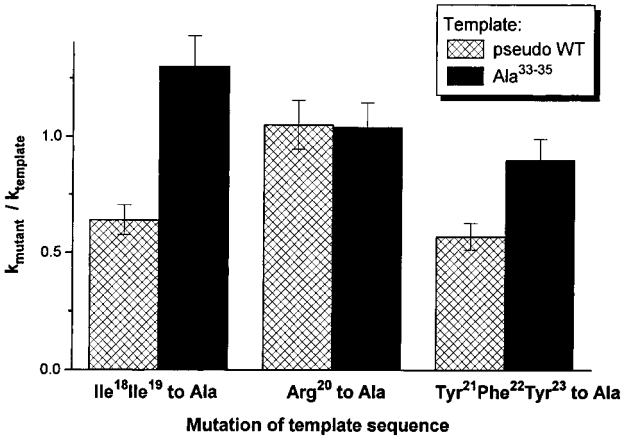

FIGURE 6: Comparison of the effect of the three mutations $Ala^{18,19}$, Ala^{20}, and Ala^{21-23} on the rates of formation of the 14−38 disulfide, introduced in the pseudo-wild-type background (hatched bars) or the Ala^{33-35} background (filled bars). Replacement of hydrophobic residues at positions 18, 19, 21, 22, and 23 decreases the rate of formation of the 14−38 disulfide only if hydrophobic residues Phe, Val, and Tyr occupy positions 33−35 (see text).

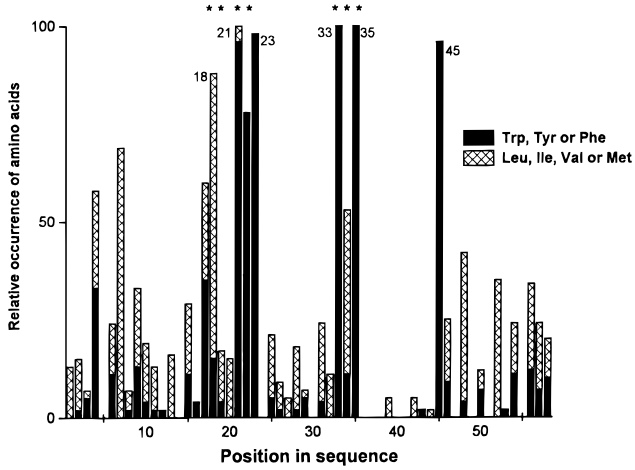

FIGURE 7: Statistics of amino acid type occurrence in different positions in a family of proteins homologous to BPTI. The analyzed database consisted of 55 sequences with invariant positions of cysteines, selected from the protein sequence database through a sequence homology search. Relative quantity of aromatic (Trp, Tyr, Phe, filled bars) or aliphatic residues (Leu, Ile, Val, Met, hatched bars) is shown at each position of the sequence. Positions 5, 14, 30, 38, 51, and 55 are invariant cysteines. Asterisks denote positions of the amino acids found in this work to accelerate the formation of a native 14−38 disulfide of BPTI.

Ala^{33-35} there is less OXI species than the DMD species (Figure 5A). This shows again that whereas the reactivities remain unchanged, k_{uni} is affected by mutation, indicating that some non-local, conformational factors must be involved.

Presented data indicate that the following residues, Ile^{18}, Ile^{19}, Tyr^{21}, Phe^{22}, Phe^{33}, Val^{34}, and Tyr^{35}, are involved in a net of interactions in the unfolded BPTI, which facilitate the formation of the disulfide between Cys 14 and Cys 38. These residues are located in two stretches of hydrophobic residues, one spanning positions 18−23 and the second positions 33−35 in BPTI. To check if residues from each stretch i.e., $Ile^{18}Ile^{19}$, $Tyr^{21}Phe^{22}Tyr^{23}$, or $Phe^{33}Val^{34}Tyr^{35}$, accelerate the formation of 14−38 due to interaction with close neighbors, three more mutants have been prepared. In these three multiple replacement mutants: $Ala^{18,19}$, Ala^{21-23}, and Ala^{33-35}, two or three subsequent residues have been replaced by alanines. As it is shown in Table 1 multiple replacements lead to further decrease of the disulfide formation rate. In Ala^{33-35} k_{uni} decreases to 31% of the pseudo-WT rate.

If residues 33, 34, and 35 act independently, their influence is additive, and the rate for Ala^{33-35}, as calculated from the results of single replacements Ala^{33}, Ala^{34}, and Ala^{35} from Table 1, is expected to be 26% of the pseudo-WT rate, close to the measured 31%. This indicates that each residue Phe^{33}, Val^{34}, or Tyr^{35}, increases the 14−38 formation rate independently, not by local interaction with its neighbors. A similar result has been obtained for Ile^{18} and Ile^{19} or Tyr^{21}, Phe^{22}, and Tyr^{23} residues (see Table 1).

Similarly, three additional mutants have been studied to check if the two stretches of hydrophobic amino acids at positions 18−22 and 33−35 interact with each other. In these three mutants the $Ile^{18}Ile^{19}$ to Ala, Arg^{20} to Ala, and $Tyr^{21}Phe^{22}Tyr^{23}$ to Ala replacements, respectively, are introduced in the Ala^{33-35} template, instead of pseudo-WT template, so that residues from both hydrophobic stretches are simultaneously replaced by alanines. As shown in Figure 6 and Table 1, whereas $Ile^{18}Ile^{19}$ to Ala mutation in the pseudo-WT background ($Ala^{18,19}$ mutant) decreases the rate 2-fold, increase is observed upon the same mutation in the Ala^{33-35} background (compare rates for mutants Ala^{33-35} and $Ala^{18,19,33-35}$). This indicates that the two isoleucines 18 and 19 accelerate the formation of 14−38 disulfide only when hydrophobic residues Phe, Val, and Tyr occupy positions 33−35. Thus the disulfide formation is accelerated due to the interaction of both groups of residues. Similarly, residues 21−23 seem to interact with residues 33−35 (compare rates for Ala^{21-23}, $Ala^{21-23,33-35}$, and Ala^{33-35}).

To measure the effect of denaturant on the observed differences in k_{uni} the rates of formation of 14−38 disulfide were measured in some of the mutants in the presence of 8 M urea. As it is shown in Figure 3C the rates of the disulfide rearrangement become much slower in 8 M urea, and the observed decrease of k_{uni} upon mutation is retained, though less pronounced (Table 1). For instance the triple-replacement Ala^{33-35} decreases k_{uni} 3-fold in the absence of urea and 2-fold in 8 M urea.

Nonpolar and aromatic character of amino acids at positions 18, 21, 22, 33, and 35 is highly retained in BPTI-like domains. A homology search of a Swissprot protein sequence database revealed 55 homologous sequences, with six cysteines in invariant positions in sequence. As illustrated in Figure 7, in these 55 proteins or domains, positions 21, 22, 33, and 35 are almost exclusively occupied by tyrosine or phenylalanine, and positions 18 and 34 are predominantly apolar aliphatic residues. This indicates that preferential formation of the disulfide 14−38 can be expected in the entire family of BPTI-like domains.

DISCUSSION

Seven residues (Ile^{18}, Ile^{19}, Tyr^{21}, Phe^{22}, Phe^{33}, Val^{34}, Tyr^{35}), have been found to contribute significantly to the preferential formation of the disulfide between cysteines 14 and 38 in BPTI. These residues, distant from cysteines by as many as eight residues, accelerate formation of the 14−38 disulfide due to the interaction of residues 18−22 with residues 33−35, and not by affecting the intrinsic reactivities of the cysteines. In 8 M urea the effect persists, but is diminished.

Three of the replacements studied here (Phe[33] to Ala, Tyr[35] to Ala, and Asn[43] to Ala) have previously been found not to affect the rate of formation of the first disulfide in reduced BPTI (Goldenberg & Zhang, 1993). However, the reported precision of measurement is comparable to the largest effect of a single-site mutation detected here (2-fold), so the decrease of the rate of formation of one of the disulfides, 14−38, upon mutation might have been difficult to detect.

In the native structure of BPTI, all seven above-mentioned residues, are located in either of the two strands of a central antiparallel β-strand composed of residues 18−35. As the X-ray or NMR studies show, nonpolar and aromatic residues form two hydrophobic cores stabilizing the native fold in BPTI (Deisenhofer & Steigemann, 1975; Berndt et al., 1992; Wlodawer et al., 1984), see Figure 1. The contribution of each residue to the stability of BPTI or its variants has been studied previously (Goldenberg et al., 1989; Kim et al., 1993; Yu et al., 1995). Remarkably, of seven residues mentioned above only Tyr[21] and Phe[33] are crucial for stability, the influence of the remaining five is moderate (Ile[19]) or weak (Ile[18], Phe[22], Val[34], Tyr[35]) (Yu et al., 1995). On the other hand, other residues highly stabilizing BPTI (Asn[43], Phe[45], Phe[4]) have no effect on the formation of 14−38.

All of the residues responsible for the acceleration of the formation of 14−38 disulfide are conventional hydrophobic residues. This suggests a hydrophobic interaction as a driving force for proper pairing of cysteines 14 and 38 early in folding of BPTI. Residual structure is abundant in denatured proteins and local hydrophobic interactions have been proposed as the most important factor stabilizing such structure in lysosyme (Evans et al., 1991; Broadhurst et al., 1991), RNase A (Beals et al., 1991), staphylococcal nuclease (Shortle et al., 1990), 434-repressor (Neri et al., 1992), plastocyanin (Dyson et al., 1992), dihydrofolate reductase (Garvey et al., 1989), and in peptide hormones (Boesch et al., 1978; Bundi et al., 1978).

Whatever the nature of the interaction between stretches 18−23 and 33−35, this interaction seems to accelerate the formation of 14−38 disulfide entropically. With these two stretches already in contact in reduced pseudo-WT or in BPTI, driving the two cysteines 14 and 38 in contact may be less entropically costly than in Ala[33−35], where the formation of a 14−38 disulfide restricts the accessible conformational space to a greater extent.

The possible nature of the first folding step has been described in numerous phenomenological models of the protein folding process. In the framework model (Ptitsyn, 1973, 1994; Kim & Baldwin, 1982, 1990; Baldwin, 1989) or diffusion−collision model (Karplus & Weaver, 1976, 1979, 1994; Bashford et al., 1988) small parts of sequence fold first to their fully native structure and then interact to yield the native structure of the entire protein. In sequential folding models (Levinthal, 1968; Wetlaufer, 1973; Kanehisa & Tsong, 1979; Go & Abe, 1981) small microdomains of native conformation are also expected to form first and the rest of the chain crystallizes around such folding nucleus in a process of growth toward the complete structure. Hydrophobic interactions have been suggested (Moult & Unger, 1991) as the main driving force for formation of such folding nuclei. On the other hand, in the hydrophobic collapse model (Levitt & Warshel, 1975; Dill, 1985) no nuclei at all are expected to form prior to collapse of the protein to the compact state; specific elements of structure are generated by change in the volume of the protein during collapse. More recently the nucleation process has been introduced to the collapse model in the form of hydrophobic zipping (Fiebig & Dill, 1993; Dill et al., 1993, 1995) in which neighboring hydrophobic residues interact and facilitate the interactions of more distant hydrophobic residues. The polar−nonpolar pattern in sequence generates structure. Finally, the nucleation−condensation model (Fersht, 1995) suggests that the coalescing folding nuclei are marginally stable and structurally heterogenous.

Present study describes a hydrophobic folding nucleus in BPTI, which, though thermodynamically not stable, clearly accelerates proper pairing of two thiols in the folding process. It consists of seven residues, located between positions 18−35 of BPTI sequence in two stretches which interact forming an intrachain loop. In the native protein this part of the chain forms a β-sheet and a turn at position 26, with seven hydrophobic residues forming two separate hydrophobic cores (Figure 1). No β-sheet is observed in reduced or oxidized pseudo-wild-type BPTI. In the folding nucleus the hydrophobic side chains contact most probably in a heterogenous, non-native fashion, but the main chain topology is native-like. Moreover the nucleus is resistant to urea denaturation, so it is not formed anew upon transition from unfolding to folding conditions but merely stabilized.

The structure of reduced, denatured BPTI has been studied in native BPTI and model molecules using a wide variety of methods, and it is believed either to be close to a random coil (Creighton, 1988) or to a collapsed form (Amir & Haas, 1988; Amir et al., 1992; Gottfried & Haas, 1992; Ittah & Haas, 1995; Ferrer et al., 1995). Circular dichroism or optical rotatory dispersion studies (Kosen et al., 1981; Gussakovsky & Haas, 1992; Ferrer et al., 1995), electrophoretic mobility studies (Creighton, 1979; Goldenberg & Zhang, 1993), and UV absorption studies (Kosen et al., 1980) suggest that reduced BPTI is devoid of structure.

Other methods detect conformational preferences and overall collapsed structure in reduced BPTI. Fluorescence transfer (Amir & Haas, 1988; Amir et al., 1992; Gottfried & Haas, 1992; Ittah & Haas, 1995) or disulfide formation rate measurements (Dadlez & Kim, 1996) indicate a collapsed structure. Also, recent reevaluation (Ferrer et al., 1995) of the earlier studies of binding of ANS to reduced BPTI (Gussakovsky & Haas, 1992; Darby & Creighton, 1993) suggests some hydrophobic clustering in reduced BPTI.

The most detailed information about the structure of reduced BPTI comes from nuclear magnetic resonance (NMR). NMR studies were carried out on native BPTI with cysteines reduced or carboxyaminomethylated (Roder, 1981; Kemmink et al., 1993), or on a BPTI variant with cysteines replaced by alanines (Lumb & Kim, 1994) or α-amino-*n*-butyric acid (Abu) (Pan et al., 1995). Also, short fragments of BPTI have been studied (Kemmink et al., 1993; Kemmink & Creighton, 1993; Lumb & Kim, 1994). In these studies significant deviations from the expected random coil chemical shifts are observed even in strongly unfolding conditions, in agreement with the studies of other proteins (Evans et al., 1991; Neri et al., 1992). The backbone NOE patterns are different in different parts of molecule, indicating different average conformational properties (Kemmink & Creighton, 1993). All the most significant deviations of the chemical shift can be attributed to local interactions of the

aromatic residues with residues distant by not more than three residues. The local nature of clusters $Tyr^{10}-Gly^{12}$, $Tyr^{21}-Ile^{19}$ has been confirmed by the studies on BPTI fragments in which non-random chemical shifts are fully retained. Studies of peptide fragments as short as four residues allowed to postulate a more general tendency for aromatic residues (Trp, Tyr, Phe) to interact with their neighbors (Kemmink & Creighton, 1993, 1995). No apparent long-range (>3 residues) interactions have been detected by NMR in reduced BPTI. However, data presented here suggest the existence of a more distant interaction of residues 18−22 with residues 33−35. It might have escaped detection by NMR because of a possible heterogeneity and flexibility of hydrophobic cluster thus formed.

Taken together previous studies show the existence of local interactions of aromatic groups either with amide group or with aliphatic residue, and, more generally, a collapsed structure in reduced BPTI. Local interactions may introduce a significant restraint on the conformation of the chain, leading to a more global deviation from the random coil behavior, as suggested recently (Pan et al., 1995). However, more distant residues also interact, most probably *via* hydrophobic interactions, and form the structure in the unfolded BPTI. These interactions accelerate formation of 14−38, and fix the overall main chain topology in a native like way, with two central β-strands 14−24 and 29−37 linked by a turn at positions 25−28 (Figure 1).

On the oxidative folding pathway of BPTI reduced state is followed by single-disulfide intermediates. Remarkably, a substantial degree of a native-like structure is formed during this first step of folding, much earlier than the final set of three disulfides. In many folding intermediates of BPTI, either with one or with two native disulfides a significant amount of native or native-like structure has been detected. Of two models of [14−38], the synthetic model, in which remaining four cysteines are replaced by α-amino-*n*-butyric acid, folds into a native-like molten globule (Barbar et al., 1995; Ferrer et al., 1995); however, the recombinant model, in which alanines replace four cysteines, does not show folded structure, as judged by circular dichroism (Dadlez and Kim, unpublished). The native-like structure is more pronounced in case of models of single-disulfide intermediates [30−51] and [5−55] in which disulfides link cysteines 30 and 51 and cysteines 5 and 55, respectively (States & Creighton, 1987; Oas & Kim, 1988; Staley & Kim, 1990, 1992, 1994; van Mierlo et al., 1991, 1993). The structure in the two-disulfide intermediates, [14−38, 5−55] and [14−38, 30−51], is essentially native (Creighton, 1977; Creighton & Goldenberg, 1984; Weissman & Kim, 1991, 1992a,b, 1993).

The native-like fold of the two-disulfide intermediates, attained before all three disulfides are formed, is very stable. Formation of the missing, third disulfide is hindered at the stage of two-disulfide intermediates [14−38, 30−51] or [14−38, 5−55], because the native-like structure buries the remaining two free thiol groups and blocks the formation of the last disulfide (Creighton, 1977; Creighton & Goldenberg, 1984; Weissman & Kim, 1991,1992a,b, 1993, 1995). The 14−38 disulfide which forms fast at every stage of BPTI folding has to be rearranged in these two kinetic traps *via* non-native disulfides, so the productive two-disulfide intermediate [30−51, 5−55] is formed. In [30−51, 5−55] exposed to solvent 14−38 again forms fast, which completes the BPTI folding pathway (Creighton, 1977; Creighton & Goldenberg, 1984; Weissman & Kim, 1991). Though the residual structure of the unfolded state in BPTI facilitates efficient search for the native fold early in folding, this native fold of intermediates hinders completion of all three disulfides later on the folding pathway of BPTI.

A similar fold, with the three, invariantly paired, disulfide bonds is shared by a family of proteins and protein domains (Creighton & Charles, 1987). Remarkably a nonpolar or aromatic character of positions 18, 21, 22, 33, and 35 is highly retained in this family. It is plausible that all proteins from the family retain not only a similar three-dimensional structure, but also a similar preference for forming a native-like chain topology early in folding. Early folding of BPTI and BPTI-like domains seems to be directed by the interactions of hydrophobic residues at positions 18−23 with residues at positions 33−35, which significantly accelerate the pairing of cysteines 14 and 38 in a native-like fashion. In general the interactions of aromatic residues in the denatured state in the folding conditions may significantly facilitate proper pairing of other residues (not necessarily cysteines) in the early phase of the folding process.

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