

The structure of denatured bovine pancreatic trypsin inhibitor (BPTI)

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Abstract In the presence of denaturant and thiol initiator, the native bovine pancreatic trypsin inhibitor (BPTI) denatures by shuffling its native disulfide bonds and converts to a mixture of scrambled isomers. The extent of denaturation is evaluated by the relative yields of the scrambled and native species of BPTI. BPTI is an exceedingly stable molecule and can be effectively denatured only by guanidine thiocyanate (GdmSCN) at concentrations higher than 3–4 M. The denatured BPTI consists of at least eight fractions of scrambled isomers. Their composition varies under increasing concentrations of GdmSCN. In the presence of 6 M GdmSCN, the most predominant fraction of scrambled BPTI accounts for 56% of the total structure of denatured BPTI. Structural analysis reveals that this predominant fraction contains the *bead-form* isomer of scrambled BPTI, bridged by three pairs of neighboring cysteines, Cys5-Cys14, Cys30-Cys38 and Cys51-Cys55. The extreme conformational stability of BPTI has important implications in its distinctive folding pathway.

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Key words: Bovine pancreatic trypsin inhibitor; Urea; Guanidine hydrochloride; Guanidine thiocyanate; Denaturation; Unfolding; Scrambled protein; Unfolding intermediate

1. Introduction

Characterization of the molecular structure of denatured proteins is inherently difficult. Unlike native proteins, which usually adopt a well defined conformation [1], denatured proteins are composed of heterogeneous conformational isomers that under kinetic control exist in a state of rapid equilibrium [2,3]. Conventionally, structural analysis of denatured proteins has been limited to the measurement of the average property of the collective isomers [4–8] using various spectroscopic techniques, notably fluorescence and circular dichroism. Our laboratory has recently developed a disulfide scrambling technique [9] that is useful for structural characterization of denatured proteins as well as monitoring the pathway of protein denaturation [10,11]. In the presence of denaturant and thiol initiator, a disulfide containing protein denatures by shuffling its native disulfide bonds and converts to a mixture of scrambled isomers. For a protein that contains three disulfide bonds, there exist 14 possible scrambled isomers that are intra-crosslinked by different sizes of disulfide loops and adopt varied degrees of unfolding. A major advantage of this technique

is that the scrambled isomers can be trapped, purified, and structurally characterized [12,13]. This allows quantitative analysis of various denatured species and permits a more detailed identification and description of the structure of denatured proteins that has not been afforded with conventional methods [2,5,8].

Bovine pancreatic trypsin inhibitor (BPTI) is a small protein consisting of 58 amino acids and stabilized by three disulfide bonds [14]. BPTI is widely known for its disulfide folding pathway [15–19] which has been characterized by the predominance of stable folding intermediates containing mainly native disulfide bonds. The original model of Creighton [15–17] identified two 1-disulfide species and five 2-disulfide species, with 75% of their disulfide bonds being native. Specifically, the native disulfide of BPTI [Cys30-Cys51] was found to be a major and core component of both 1- and 2-disulfide intermediates. In follow up studies conducted by Weissman and Kim [18,19] five well populated intermediates, two 1-disulfide and three 2-disulfide species, were described and all of them were shown to contain only native disulfide bonds. The prevalence of the folding intermediates with native-like structures presents major implications for the unusual stability of BPTI and the mechanism as to how the native BPTI is being stabilized. In this respect, characterization of the conformational stability of BPTI is essential to support and understand the mechanism of BPTI folding. BPTI is indeed highly stable and very resistant toward denaturation with its disulfides still remaining intact [20–22]. Due to its extreme stability, the denaturation curves of BPTI have yet to be established. In the absence of denaturation curves, comparison of the conformational stability between BPTI and other homologous proteins is not feasible. To date, the majority of investigations of BPTI stability have applied the technique of reductive unfolding [23,24] and focused on mutant molecules lacking one of the three disulfides (typically Cys14-Cys38) [25,26]. A systematic study of BPTI mutants performed by Goldenberg and colleagues [27,28] has shown that the mechanism of reductive unfolding of BPTI is consistent with its reversed pathway of oxidative folding, and also revealed that the stability of disulfides in the native BPTI depends on both the local environment and the rest of the protein to favor their formation.

In this report, the conformational stability of native BPTI has been analyzed using the technique of disulfide scrambling. We demonstrate and discuss here (1) the denaturation curves and unusual conformational stability of the native BPTI; (2) the disulfide structure of denatured BPTI and (3) the implications of the BPTI stability for its distinctive folding pathway.

2. Materials and methods

2.1. Material

BPTI was obtained from Boehringer Mannheim (Germany). The

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Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; GdmCl, guanidine hydrochloride; GdmSCN, guanidine thiocyanate

protein was further purified in our laboratory by high performance liquid chromatography (HPLC). The purity was greater than 96% as judged by HPLC and N-terminal sequence analysis. Guanidine hydrochloride (GdmCl), guanidine thiocyanate (GdmSCN), urea and 2-mercaptoethanol were products of Sigma with grade purity of greater than 99%.

2.2. Denaturation of BPTI

The native BPTI (0.5 mg/ml) was dissolved in Tris-HCl buffer (0.1 M, pH 8.4) containing 0.25 mM of 2-mercaptoethanol and selected concentrations of denaturants (urea, GdmCl, GdmSCN). The reaction was allowed to reach equilibrium and was typically performed at 23°C for 24 h. 2-Mercaptoethanol (0.15 mM) was replenished twice at 4 h and 8 h. To monitor the kinetics of unfolding, aliquots of the sample were removed at time intervals, quenched with 4% trifluoroacetic acid and analyzed by HPLC. The denatured sample was subsequently acidified with an equal volume of 4% trifluoroacetic acid and stored at -20°C. The GdmSCN denatured samples were further purified by gel filtration (NAP-5 columns from Pharmacia AG) and eluted with 1% trifluoroacetic acid, prior to HPLC analysis.

2.3. Structural analysis of scrambled BPTI

The predominant fraction of scrambled BPTI, X-BPTI-b (~20 µg) was isolated by HPLC and treated with 2 µg of thermolysin (Sigma, P-1512) in 30 µl of *N*-ethylmorpholine/acetate buffer (50 mM, pH 6.4). Digestion was carried out at 37°C for 16 h. Peptides were then purified by HPLC and analyzed by amino acid sequencing and mass spectrometry in order to identify the disulfide containing peptides.

2.4. Amino acid sequencing and mass spectrometry

Amino acid sequences of disulfide containing peptides were analyzed by automatic Edman degradation using a Perkin-Elmer Procise sequencer (Model 494) equipped with an on-line PTH amino acid analyzer. The molecular masses of disulfide containing peptides were determined by MALDI-TOF mass spectrometer (Perkin-Elmer Voyager-DE STR).

2.5. Plotting of the denaturation curves of BPTI

Denaturation of the native BPTI is defined by the conversion of the native structure to scrambled isomers. The denaturation curve was therefore determined by the fraction (%) of native BPTI converted to the scrambled isomers under increasing concentrations of a selected denaturant. On the other hand, unfolding describes the state of the denatured BPTI and is structurally defined by the composition of scrambled isomers.

Table 1

Structures of disulfide containing peptides derived from the thermolysin digestion of scrambled BPTI (X-BPTI-b)

Fraction	Sequence	Sequence positions	Cys-Cys	M+H obs.(exp.)
1	YN	23-24	none	n.o.(296.1)
2	RPD	1-3	none	387.3(387.2)
3	FK	45-46	none	748.5(748.3)
4	LCQT GGCR	29-32 36-39	[30-38]	853.4(853.4)
5	FKS	45-47	none	n.o.(381.2)
6	VY IIR SAEDC MRTCGGA	34-35 18-20 47-51 52-58	none none [51-55]	n.o.(281.1) n.o.(401.3) 1216.1(1216.4)
7	FC TGPK	4-5 11-15	[5-14]	771.1(771.3)
8	FYN LCQT VYGGCR	22-24 29-32 34-39	none [30-38]	n.o.(443.2) 1115.7(1115.5)
9	FC YTGPCK	4-5 10-15	[5-14]	934.5(934.4)
10	IIRY	18-21	none	564.3(564.4)
11	LEPPY	6-10	none	618.3(618.3)
12	FC LEPPYTGPCK	4-5 6-15	[5-14]	1370.8(1370.6)

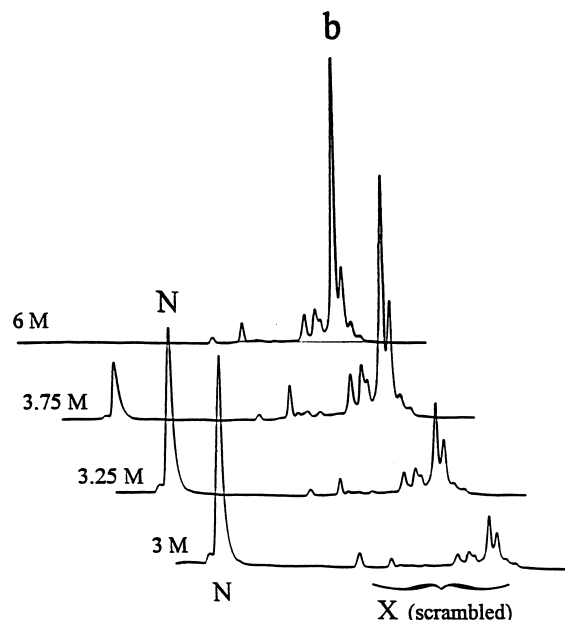


Fig. 1. Denatured states of BPTI under indicated concentrations of GdmSCN. The native BPTI (0.5 mg/ml) was allowed to denature in Tris-HCl buffer (0.1 M, pH 8.4) containing 2-mercaptoethanol (0.25 mM) and indicated concentrations of the denaturant. After the reaction reached equilibrium (24 h incubation at 23°C), the samples were acidified with 4% trifluoroacetic acid and analyzed by HPLC. 'N' indicates the native species and 'X' covers the elution positions of all scrambled species. The major fraction of scrambled BPTI is designated 'b'. Solvent A was water containing 0.05% trifluoroacetic acid. Solvent B was acetonitrile/water (9:1, by volume) containing 0.042% trifluoroacetic acid. The gradient was 10% B to 61% B in 60 min. The flow rate was 0.3 ml/min. Column was Zorbax C-18 for peptides and proteins, 4.6 mm, 10 µm. Column temperature was 23°C.

3. Results and discussion

3.1. Denaturation of the native BPTI in the presence of urea, GdmCl and GdmSCN

The native BPTI was allowed to denature and unfold in Tris-HCl buffer (pH 8.4) containing 2-mercaptoethanol (0.25 mM) and varied concentrations of urea, GdmCl and GdmSCN. Under these conditions, denatured BPTI was recovered as a mixture of 3-disulfide scrambled isomers which can be well separated from the native BPTI (Fig. 1). The presence of fully oxidized scrambled isomers is supported by two pieces of experimental evidence: (1) the HPLC profiles of the denatured BPTI remain indistinguishable regardless of whether the sample is quenched by acidification (4% trifluoroacetic acid) or by alkylation (iodoacetic acid); (2) denatured samples quenched by alkylation were further analyzed for their content of disulfide bond and free cysteine by the dabsyl chloride method [29] and MALDI mass spectrometry. Both methods confirm that the denatured BPTI contains 3-disulfide bonds.

The extent of denaturation is measured by the fraction of native BPTI converted to scrambled isomers. Fig. 2 presents the denaturation curves of BPTI under increasing concentrations of urea, GdmCl and GdmSCN. The unexpected finding is that urea (8 M) and GdmCl (6 M), the two most commonly applied strong denaturing conditions, are unable to denature BPTI at all. Even at 8 M GdmCl, the denaturation of BPTI remains incomplete. Indeed, BPTI can be effectively dena-

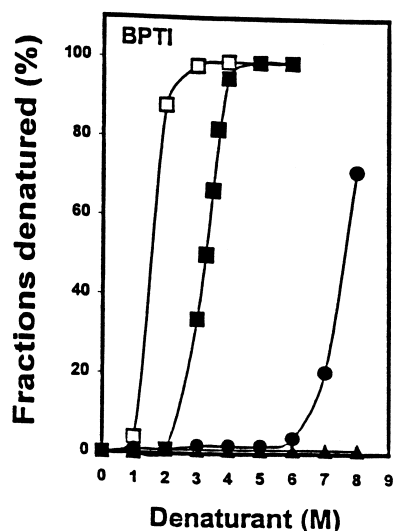


Fig. 2. The denaturation curves of BPTI and TAP. Denaturation is measured by the fraction of native BPTI (or TAP) converted to the scrambled isomers. The denaturants are GdmSCN (■), GdmCl (●), urea (▲) and GdmSCN at 55°C (□). Denaturation was carried out at 23°C for 24 h in Tris-HCl buffer (0.1 M, pH 8.4) containing 2-mercaptoethanol (0.25 mM) and increasing concentrations of the indicated denaturant.

tured only with GdmSCN. Denaturation of BPTI occurs at 3–4 M of GdmSCN (Fig. 1). At an elevated temperature (55°C), rapid denaturation of BPTI takes place at 1–2 M of GdmSCN.

3.2. The disulfide structure of denatured BPTI

Denatured BPTI was shown by HPLC analysis to comprise at least eight fractions of fully oxidized scrambled isomers (Fig. 1). The most predominant fraction is designated X-BPTI-b (where X stands for scrambled). This fraction was isolated for structural analysis. It was digested with thermolysin. Peptides were then separated by HPLC (Fig. 3) and analyzed by Edman sequencing and MALDI mass spectrometry in order to identify peptides that contain disulfide bonds. The results, summarized in Table 1, unambiguously demonstrate that X-BPTI-b contains Cys5-Cys14, Cys30-Cys38 and Cys51-Cys55 and adopts the *beads-form* disulfide pairing (Fig. 4).

The structure of denatured BPTI, as signalled by the composition of scrambled isomers, varies under increasing concentrations of GdmSCN (Fig. 1). Specifically, the recovery of X-BPTI-b as a fraction of the total denatured BPTI rises from 34 to 56% as the concentration of GdmSCN increases

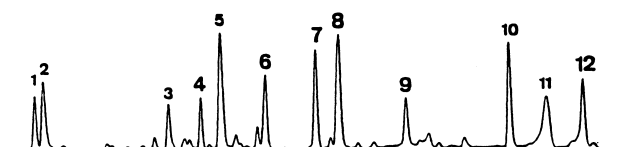


Fig. 3. Peptide maps of X-BPTI-b derived from the thermolysin digestion. All major fractions were isolated and analyzed by Edman sequencing and MALDI mass spectrometry. The results are summarized in Table 1. Peptides were separated using the HPLC conditions described in the legend of Fig. 1, except for using a different gradient consisting of 0–60% B, linear, over 90 min.

from 3 to 6 M (Fig. 5). Among the 14 possible scrambled isomers of BPTI, X-BPTI-b contains the smallest combined disulfide loops and apparently represents the most extensively unfolded structure. The increasing yield of X-BPTI-b clearly indicates a progressive expansion of the BPTI polypeptide chain induced by the rising concentration of GdmSCN.

BPTI is not alone in displaying these properties. The predominance of the *beads-form* isomer under strong denaturing conditions has been similarly observed with the structures of denatured insulin-like growth factor (IGF-1) [11] and tick anticoagulant peptide (TAP) [10]. TAP is a factor Xa specific inhibitor structurally homologous to BPTI. In the case of TAP, the recovery of the *beads-form* isomer as a fraction of the total denatured TAP grows from 23 to 63% as the concentration of GdmSCN increases from 2 to 6 M. The data are included in Fig. 5 for comparison.

3.3. The unusual conformational stability of BPTI

The stability of BPTI stands out among numerous disulfide containing proteins that have been characterized by the disulfide scrambling technique. Their relative stability, defined by the concentration of denaturants required to denature 50% of the protein, is shown in Table 2. BPTI is the only case that exhibits total resistance against 8 M urea and 6 M GdmCl. Based on the denaturation curves of GdmSCN, BPTI is about 2-, 3- and 4-fold more stable than IGF-1, TAP and RNase A, respectively. However, comparison of their relative stability is dependent upon the nature of the denaturant. The order of stability among IGF-1, TAP and RNase A differs in the case of urea denaturation. Also, as a denaturant, GdmCl is generally 2–3-fold more potent than urea [5]. This is true for BPTI as well as for IGF-1 [11], and RNase A [30]. But in the case of TAP [10], urea is about as effective as GdmCl. These discrepancies are intriguing and most likely attributable to the differential mode of action between GdmCl and urea [3,5,31,32] – a long standing issue that remains to be fully understood.

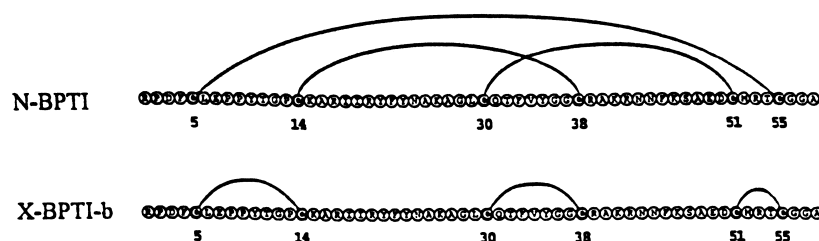


Fig. 4. Disulfide structures of the native BPTI and the predominant species of scrambled BPTI (X-BPTI-b). The disulfide pairing of native BPTI was elucidated by Kassell and Laskowski [14]. The structure of scrambled BPTI was concluded from the data given in Table 1.

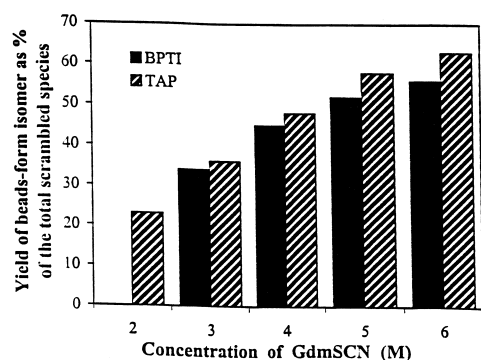


Fig. 5. Recoveries of X-BPTI-b as a fraction of the total denatured BPTI under increasing concentrations of GdmSCN. The denaturing conditions are described in the text. GdmSCN has a maximum solubility of around 6–6.5 M in Tris–HCl buffer. The data should be allowed a standard deviation of $\pm 5\%$. The content of the *beads-form* isomer of the GdmSCN denatured TAP is included here for comparison. TAP is a protease inhibitor structurally homologous to BPTI. The results of TAP is taken from reference [10].

The outstanding conformational stability of BPTI has important implications for its unique folding mechanism. Aside from the data demonstrated here, numerous 1- and 2-disulfide isomers of BPTI were shown to be capable of taking on stable, native-like structures. A highly stable 2-disulfide intermediate of BPTI containing [Cys5–Cys55; Cys30–Cys51] has been characterized along the pathway of reductive unfolding [25]. It is formed directly by reduction of the native protein. Nuclear magnetic resonance analysis has shown that 1-disulfide isomers engaging [Cys5–Cys55] and [Cys30–Cys51], and additional 2-disulfide isomers containing [Cys30–Cys51; Cys14–Cys38] and [Cys5–Cys55; Cys14–Cys38] can all adopt native-like conformations [33–36]. Along the pathway of oxidative folding of BPTI, the existence of those stable structural elements governs the formation of predominant intermediates that admit mainly native disulfide bonds and precludes the formation of scrambled 3-disulfide species. However, it is not clear whether the compartment of stable structural elements, as that observed in the case of BPTI, represents a general property of small disulfide containing proteins. The disulfide folding pathways of several single domain proteins

Table 2

The concentration of denaturant required to denature 50% of the protein^a

Protein	GdmSCN (M)	GdmCl (M)	Urea (M)
PCI ^b	0.7	1.45	> 8 ^h
RNase A ^c	0.75	2.25	5.75
TAP ^d	1.0	4.2	4.0
IGF-1 ^e	1.5	3.2	5.5
Hirudin ^f	2	5	> 8 ^h
BPTI ^g	3.25	7.5	> 8 ^h

^aDetermined by the method of disulfide scrambling between the native and scrambled isomers. The data should be allowed a standard deviation of $\pm 5\%$.

^bChang and Aviles, unpublished data.

^cReference [30].

^dReference [10].

^eReference [11].

^fBulychev and Chang, unpublished data.

^gThe current study.

^hAt 8 M urea, 45% of PCI, 16% of hirudin and less than 2% of BPTI were denatured.

have been recently characterized in our laboratory [13,37,38]. The results reveal a wide diversity of folding mechanisms. Hirudin and potato carboxypeptidase inhibitor (PCI) are two distinct examples [37,38]. Their folding pathways are characterized by (1) a high degree of heterogeneity of 1- and 2-disulfide intermediates and (2) the presence of fully oxidized scrambled isomers as folding intermediates that has not been observed in the case of BPTI. Both hirudin and PCI differ from BPTI not only by their relative conformational stability but, most importantly, also by their distinctive fashion of stabilizing the three native disulfide bonds. Unlike BPTI, reduction of hirudin and PCI in the absence of denaturant (reductive unfolding) undergoes an all-or-none mechanism without accumulation of 2- and 1-disulfide unfolding intermediates [39,40].

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