Denatured States of Tick Anticoagulant Peptide

COMPOSITIONAL ANALYSIS OF UNFOLDED SCRAMBLED ISOMERS*

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In the presence of denaturant and thiol catalyst, a disulfide-containing protein denatures and converts to a mixture of scrambled isomers, which can be purified and structurally characterized. Scrambled isomers adopt a different conformation and a varied extent of unfolding. Their relative concentration (composition) signals the state of unfolding of the denatured protein and is determined by the denaturing condition. In this report, tick anticoagulant peptide (TAP) (60 amino acids and 3 disulfides) has been denatured in the presence of urea, guanidine hydrochloride, guanidine thiocyanate, organic solvents, and at elevated temperature. The recoveries of scrambled TAP were analyzed. The results demonstrate that each denaturing condition generates a unique structure (composition of scrambled species) of denatured TAP. Among various species of scrambled TAP, the beads-form species contains the smallest disulfide loop and appears to represent the most extensively unfolded state. The yield of the beads-form species as a fraction of the total denatured TAP is invariably determined by the strength of the denaturing condition.

**Part of this work was completed at Novartis AG, Basel, Switzerland. This is publication number 196-IMM from the Institute of Molecular Medicine for the Prevention of Human Diseases, University of Texas-Houston Health Science Center. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This paper is dedicated to Dr. Hans J. Müllner-Eberhard.

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‡ The abbreviations used are: GdmCl, guanidine hydrochloride; X, scrambled; TAP, tick anticoagulant peptide; GdmSCN, guanidine thiocyanate; HPLC, high pressure liquid chromatography.
cretic trypsin inhibitor belong to the Kunitz-type inhibitor and share close structural homology in terms of disulfide patterns and three-dimensional conformation (25, 26).

EXPERIMENTAL PROCEDURES

Materials—TAP (CGP-55099) is a recombinant protein produced by Novartis. The protein is more than 95% pure as judged by HPLC and N-terminal sequence analysis. GdmCl, GdmSCN, urea, acetonitrile, ethanol, and 2-mercaptoethanol were products of Merck (Darmstadt, Germany) with purity of greater than 99%.

A Standard Protocol of Denaturation—The protein (0.5 mg/ml) was dissolved in the Tris-HCl buffer (0.1 M, pH 8.4) containing 0.25 mM 2-mercaptoethanol and selected concentrations of denaturants (urea, GdmCl, GdmSCN, or organic solvents) subjected to selected denaturing conditions (elevated temperature). When using denaturants, the reaction was typically performed at 23 °C for 20 h. In the case of heat denaturation, the reaction was carried out within 90 min (55 °C) and 45 min (69 °C). To monitor the kinetics and intermediates of unfolding, aliquots of the sample were removed at a defined interval, quenched with 4% trifluoroacetic acid, and analyzed by HPLC. The denatured samples were subsequently acidified with an equal volume of 4% trifluoroacetic acid and stored at −20 °C.

Criteria for the Completion of Denaturation and Construction of Denaturation Curves—The completion of denaturation, under a given denaturing condition, implies that conversion of the native species to the scrambled species has reached a state of equilibrium. This state of equilibrium also includes those among scrambled species. Two major criteria are applied here to ensure that denaturation has reached equilibrium. One is time course kinetics analysis, which indicates that conversion of the native TAP to a scrambled species has reached a plateau and the rate of scrambled to native species remains constant during prolonged incubation. These results have revealed that denaturation of TAP by denaturants (in the presence of 0.25 mM 2-mercaptoethanol) was completed generally within 4–6 h. Nonetheless, the reaction has been routinely allowed to proceed overnight (20 h). In the case of thermal denaturation, there are inherent difficulties in performing proteolytic analysis because of the heat-induced decomposition of disulfide bonds. Another method to verify the completion of denaturation is to reassociate (refold) fully reduced/denatured TAP under the same conditions that denature the native TAP. Both renaturation and denaturation are expected to reach the same state of equilibrium and generate end products that consist of the same ratio of scrambled to the native species (20).

The extent of denaturation at every given condition was simply calculated from the fraction of TAP (using HPLC peak area integration) that is converted to the scrambled species. These data were then used to construct the denaturation curves.

Structural Analysis of Scrambled TAP—Isolated fractions of scrambled TAP (~10 µg) were treated with 1 µg of thermolysin in 30 µl of N-ethylmorpholine/acetate buffer (50 mM, pH 6.4). Digestion was carried out at 23 °C for 16 h. Peptides were then isolated by HPLC and analyzed by amino acid sequencing and mass spectrometry to identify the disulfide-containing peptides.

Protein Analytical Methods—The disulfide content of scrambled proteins was determined by the dabsyl chloride precolumn derivatization method (27), which permits direct quantification of the disulfide bonds of proteins. Amino acid sequence analysis was performed with a Hewlett-Packard G-1000A sequencer. The matrix-assisted laser desorption ionization mass spectrometer was a home-built time of flight instrument with a nitrogen laser of 337-nm wavelength and 3-ns pulse width. The calibration was performed either externally or internally by a nitrogen laser of 337-nm wavelength and 3-ns pulse width. The calibration was performed either externally or internally by a nitrogen laser of 337-nm wavelength and 3-ns pulse width. The calibration was performed either externally or internally by a nitrogen laser of 337-nm wavelength and 3-ns pulse width. The calibration was performed either externally or internally by a nitrogen laser of 337-nm wavelength and 3-ns pulse width. The calibration was performed either externally or internally by a nitrogen laser of 337-nm wavelength and 3-ns pulse width. The calibration was performed either externally or internally by a nitrogen laser of 337-nm wavelength and 3-ns pulse width.

Nomenclature of Scrambled Species—To simplify the description of scrambled species of TAP, they are designated by the following: X-TAP-(species assigned on HPLC), where X stands for scrambled. For instance, the potency of a denaturant (scrambled species), whereas unfolding describes the state of the denatured protein. For instance, the potency of a denaturant to denature TAP is determined by the fraction of TAP that is converted to scrambled species after the reaction has reached equilibrium. On the other hand, the potency of a denaturant to unfold TAP is determined by the composition of scrambled species and specifically by the recovery of the most extensively unfolded scrambled species (X-TAP-a). In addition, potency and efficiency have been used alternatively to compare GdmCl and urea. Potency of a denaturant is defined by the extent of protein denaturation (and unfolding) as the reaction has reached a state of equilibrium. Efficiency is related to the kinetics (rate constant) of unfolding.

RESULTS

The Composition and Disulfide Structures of X-TAP—Along the reversible pathways of unfolding and refolding of TAP (21, 28), 11 fractions of X-TAP have been detected as intermediates. Among them, there exist seven major fractions that constitute more than 90% of the total concentration of X-TAP. Four of them were structurally characterized (28). An additional three fractions, e, h, and i, have been isolated here. They were digested by thermolysin at pH 6.5. Peptides were then isolated by HPLC and characterized by amino acid sequencing and mass spectrometry. Each was shown to consist of a single species. Their disulfide structures, presented in Fig. 1, are the basis for the examination of denatured states of TAP.

Denaturation of TAP in the Presence of Urea, GdmCl, and GdmSCN—Denaturation of the native TAP was analyzed in the presence of increasing concentrations of urea, GdmCl, and GdmSCN. The resultant denaturation curves are shown in Fig. 2. GdmSCN is more potent than GdmCl and urea. Based on the concentration that is required to achieve the same extent of denaturation, GdmSCN is about 2.5-fold more potent than GdmCl, whereas urea and GdmCl display almost indistinguishable potency in denaturing native TAP. Indeed, at a low concentration of denaturant (3 M), urea is actually more potent than GdmCl.

The comparable potency of urea and GdmCl in denaturing native TAP provides a useful case for further analysis of the kinetics of denaturation. TAP can be almost fully denatured (>94%) by both urea and GdmCl at concentrations ranging from 6 to 8 M (Fig. 2). However, the kinetics of its denaturation are dependent upon the concentration of the denaturant. At 8 M urea, the rate constant of denaturation, which was measured to be 0.0137 min⁻¹, is about 1.5- to 3-fold greater than that performed at 7 and 6 M urea. With GdmCl, the rate constants differ only slightly: 0.0138 min⁻¹ at 8 M GdmCl; 0.0163 min⁻¹ at 7 M GdmCl; and 0.0115 min⁻¹ at 6 M GdmCl. Surprisingly, the rate constant of TAP denaturation at 7 M GdmCl is somewhat greater than that performed at 8 M GdmCl. This marginal difference is well reproducible.

Examination of the composition of denatured TAP reveals...
the differential potency and distinct mode of actions of urea and GdmCl (Fig. 3). Using GdmCl and GdmSCN as the agents, denatured TAP consists of four well populated scrambled species, \( a, d, g \), and \( e \). In contrast, only two of them, \( d \) and \( g \), predominate in the urea solution. In comparing these two sets of denatured structures (urea versus GdmCl), one notices that the composition of urea-denatured TAP resembles that of the folding intermediates of GdmCl-denatured TAP (Fig. 4). When X-TAP produced by 6 M GdmCl was allowed to refold, X-TAP-\( a \) and X-TAP-\( e \) decreased rapidly. This suggests that TAP adopts a more advanced state of unfolding in the GdmCl and GdmSCN solutions and that X-TAP-\( a \) and X-TAP-\( e \) are highly unfolded species that become well populated only under these conditions. Furthermore, the pattern of X-TAP does not always remain constant under the same denaturant. The relative yield of X-TAP-\( a \)/X-TAP-\( g \) increases from 0.16 to 0.4 as the concentration of urea doubles from 4 to 8 M. At the same time, the concentration of X-TAP-\( a \) as a fraction of the total X-TAP increases almost linearly from 9 to 16.5% (Fig. 5). The increase of X-TAP-\( a \) is even more dramatic in the cases of GdmCl and GdmSCN denaturation (Fig. 5). The results thus indicate that the recovery of X-TAP-\( a \) is related to the strength of denaturing conditions and can be used to plot the unfolding curves of TAP (Fig. 5). These unfolding curves are distinguished from the denaturation curves shown in Fig. 2.

Denaturation of TAP in Organic Solvents—Organic solvents promote denaturation of proteins presumably through association with the hydrophobic residues of the protein, thereby disrupting hydrophobic bonding. As for urea and GdmCl, the potency of organic solvents in denaturing proteins depends on their concentration. Therefore, denaturation of TAP was also characterized in the presence of increasing concentrations of acetonitrile and ethanol. The denaturation curves obtained with both organic solvents are included in Fig. 2. The denaturation potency of acetonitrile is about 2–3-fold that of ethanol. Although the maximum extent of TAP denaturation in the presence of a high concentration of acetonitrile (9.6 M, 50% by volume) reaches 90%, it is only close to 60% in the buffer containing 8.6 M (50% by volume) ethanol. Beyond 50% content of acetonitrile or ethanol (by volume) in the buffer, TAP will increasingly precipitate.

Several important features can be concluded from the composition of X-TAP generated by organic solvents (Fig. 6). 1) The denatured TAP comprises only two well populated species of scrambled isomers, X-TAP-\( d \) and X-TAP-\( g \). 2) The recovery of X-TAP-\( a \), similar to the cases of urea and GdmCl unfolding, is dependent upon the concentration of the organic solvent. The content of X-TAP-\( a \) as a percentage of the total X-TAP increases from 2 to 14% as the concentration of acetonitrile rises.
from 4 to 8 M (Fig. 6). 3) In the case of ethanol or at low concentration of acetonitrile, small but significant amounts of X-TAP-a and X-TAP-i are detected (Fig. 6). These two species contain disulfide loops that are twice the size of that of X-TAP-d, X-TAP-g (Fig. 1). X-TAP-h and X-TAP-i accumulate during the pathway of refolding of GdmCl-denatured TAP (Fig. 4). Apparently, they adopt structures more compact than that of X-TAP-d, X-TAP-g, or X-TAP-a. 4) The denaturation curve and the composition of X-TAP generated by acetonitrile resemble those produced by urea.

Heat Denaturation of TAP—A time course denaturation of the native TAP at 55 °C is shown in the left panel of Fig. 6. The extent of TAP denaturation reaches a plateau after about 1 h. Prolonged incubation leads to the decomposition of scrambled species, as judged by their HPLC patterns. The rate of denaturation increases by 3-fold as the temperature rises from 55 to 69 °C (Fig. 6). The process of thermal denaturation is reversible. As the sample was removed from the heating block and placed at room temperature, X-TAP renatured spontaneously to form the native structure (Fig. 4).

The composition of heat-induced X-TAP is different from those generated by denaturants. It comprises all seven identified X-TAPs. Aside from the presence of X-TAP-h and X-TAP-i, the recovery of X-TAP-a is surprisingly high. For the samples that are denatured at 55 and 69 °C, the content of X-TAP-a accounts for 17 and 21% of the total X-TAP, respectively. These are 60 and 90% higher than the content of X-TAP-a found in the sample denatured by 7.7 M acetonitrile. Another characteristic of heat-induced X-TAP is the existence of approximately 4% X-TAP-e, which populates well only in the presence of GdmCl and GdmSCN but is barely detectable in solutions containing urea or acetonitrile.

Furthermore, a systematic study has been performed to investigate the combined effect of denaturant and temperature. In every case, the addition of high temperature has resulted in a dramatic increase of the yield of X-TAP-a to the extent that a sequential denaturation by 6 M GdmSCN (7 h at 23 °C) and elevated temperature (69 °C, 15 min) denatured more than 99.5% of the native TAP with 85% of the denatured species adopting the disulfide structure of X-TAP-a.

Inhibition of TAP Denaturation by Protein Stabilizers—Protein stabilizers are compounds (salts, sugars, amino acids, etc.) used to preserve the native conformation of proteins in solution (29). Their presence is known to shift the equilibrium constant of N (native)/D (denatured) in favor of the native structure. Two commonly used protein stabilizers, NaCl and lysine, were examined here. They were compared specifically for their ability to inhibit the denaturation of TAP by urea (8 M), GdmCl (6 M), acetonitrile (7.7 M), and high temperature (55 or 69 °C).

The results show that NaCl and lysine are able to effectively inhibit all denaturing conditions, with the exception of GdmCl (Fig. 7). The extent of inhibition is, in general, inversely related to the potency of denaturing conditions. For instance, in the presence of 1 M NaCl, the extent of TAP denaturation by 8 M urea and 7.7 M acetonitrile is reduced by 42 and 81%, respectively, whereas the denaturation by 6 M GdmCl remains practically unaffected (Fig. 7). Indeed, even at 4 M GdmCl that
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The concentrations of X-TAP-i (100 amino acids) > X-TAP-f (88 amino acids) > X-TAP-e (56 amino acids) > X-TAP-g (56 amino acids) > X-TAP-d (52 amino acids) > X-TAP-a (20 amino acids). Those with large disulfide loops (X-TAP-i, -h, and -f) presumably assume a more compact conformation and thermodynamically might be stabilized by additional noncovalent interactions (either native or non-native like). This proposition is supported by two key observations. 1) The concentrations of X-TAP-i, -h, and -f become significant only under mild denaturing conditions (Figs. 6). 2) X-TAP-i, b, and f are the three major species that accumulate along the pathway of the refolding of GdmCl-denatured TAP (Fig. 4). In contrast, X-TAP-a, which contains the smallest disulfide loops, represents the most extensively unfolded form of scrambled TAP. When GdmCl is removed from the sample, the concentration of X-TAP-a decreases precipitously along the pathway of refolding (Fig. 4). Most strikingly, the recovery of X-TAP-a, as a fraction of the total X-TAP, is invariably determined by the strength and concentration of the denaturant. This has been observed with all denaturants investigated here (Fig. 5). Thus, recovery of X-TAP-a signals the extent of unfolding of the denatured TAP. This work therefore provides a useful method for measuring the extent of unfolding of the denatured protein. However, it is important to point out that the property observed with TAP may not necessarily apply to other disulfide-containing proteins. Proper spacing of cysteines in the sequence of a protein will probably be a major prerequisite.

Different Denaturants Generate Different Structures of Denatured TAP—Another important finding of this study is the demonstration that different denaturants each produce a unique pattern of the structure of denatured TAP (see Figs. 3 and 6). The differences reflect both their relative potency to denature the native TAP, as well as their relative ability to unfold the denatured species. The results are best illustrated by the comparison of urea and GdmCl. 1) Both GdmCl and urea denature the native TAP not only with similar potency (Fig. 2) but also with nearly indistinguishable kinetics. This property is somewhat unexpected because of the general experience that GdmCl is more potent than urea (7). Indeed, some disulfide-containing proteins, such as hirudin and epidermal growth factor, can be practically denatured only with GdmCl. For hirudin, GdmCl is 9-fold more potent than urea (data not shown). In the widely investigated case of ribonuclease A, the potency of GdmCl as a denaturant exceeds that of urea by 2.5–3-fold. These results, therefore, demonstrate that the relative potency of GdmCl and urea in denaturing the native structures can vary substantially from protein to protein. 2) Despite their comparable potency to denature native TAP, GdmCl is definitely more potent than urea in unfolding TAP. This is illustrated by the distinctive compositions of X-TAP generated by both denaturants (Fig. 3) and by the significantly higher recovery of X-TAP-a with GdmCl denaturation (Fig. 5). 3) Urea and GdmCl also differ in their interactions with protein stabilizers (co-solvents). Whereas NaCl (1 M) can drastically inhibit the denaturation of TAP by 8 M urea, it does not interfere with the TAP denaturation by 6 M GdmCl at all. These results thus indicate that NaCl is able to counteract the destabilizing effect of urea but not that of GdmCl.

Urea and GdmCl are the two commonly used denaturants in protein chemistry (3, 7, 10, 13). Although the chemical mechanism is not fully understood, it is known that GdmCl and urea act by disrupting noncovalent interactions that stabilize the native protein (2). The existing evidence suggests that they cause water to become a better solvent for nonpolar amino acids and thus weaken the hydrophobic interaction, a dominant force of protein folding and stability (30). GdmCl, being a salt, also suppresses electrostatic interactions among charged groups of proteins. Pace and co-workers (31) have further shown that the unfolded states of proteins in urea and GdmCl solutions differ significantly in the extent of their interaction with denaturants. Liepinsh and Otting (32) recently demonstrated that urea binds preferentially to the pockets and grooves on the surfaces of proteins. The data presented here further indicate that urea and GdmCl act with two distinct modes of mechanism. These differences most likely reflect a differential capacity in neutralizing various noncovalent forces (hydrogen bonding, ion pairing, van der Waals, and hydrophobic interactions) that stabilize native proteins.

Thermal Denaturation—Thermal unfolding has not been widely applied in the experiments of reversible denaturation of proteins because of the risk of disrupting covalent structures that often leads to the irreversible denaturation of proteins (33). For disulfide-containing proteins, it has been shown that the combination of high temperature and alkaline pH can induce base-catalyzed β-elimination of disulfide bonds (34). This side reaction, which may occur even at mild alkaline pH of 8–9, causes native proteins to form a mixture of highly heterogeneous polymers that are intra- and intermolecularly cross-linked by lanthionine and lysinoalanine (35). In the case of TAP, denaturation at 55 and 69 °C is preferably carried out within 20 and 90 min, respectively, to avoid possible destruction of disulfide bonds. Conditions selected here allow the reversible renaturation of heat-denatured TAP.

The molecular composition of heat-denatured X-TAP is distinguished from that produced by various denaturants. The most intriguing aspect about the structure of heat-denatured TAP is the simultaneous presence of extensively unfolded isomers (X-TAP-a and X-TAP-e) and compact isomers (X-TAP-h and X-TAP-i). In solutions containing strong denaturants (GdmSCN and GdmCl), the denatured TAP attains a more advanced stage of unfolding. Therefore, X-TAP-a and X-TAP-e populate well, but X-TAP-h and X-TAP-i are absent (Fig. 3). In solutions with mild denaturants (e.g., 6.9 M ethanol), X-TAP-h and X-TAP-i appear, and the concentrations of X-TAP-a and X-TAP-e decrease accordingly. Coexistence of these four X-TAPs as found in heat-denatured TAP is somehow unique. Again, these data imply the complexity of noncovalent interactions that stabilize native TAP and indicate that the mechanism of thermal denaturation clearly differs from that of denaturants.

A Note of Caution—The conclusion of this study is based on the premise that the relative distribution of scrambled isomers
of TAP reflects merely the relative degree of unfolding of the conformational ensemble present in the selected denaturing condition. It is important to mention that the chemistry of the disulfide interchange is thermodynamically linked to the conformational ensemble, and this thermodynamic linkage could potentially alter the preferred conformational ensemble that exists in the absence of a disulfide interchange.

It is also relevant to point out that disulfide bonding signals only a small fraction of the total conformational states of denatured proteins. A given disulfide isomer, such as X-TAP-g, may still consist of many conformational isomers. Nonetheless, the ability to fractionate unfolded disulfide isomers, as demonstrated here with the case of TAP, provides a very useful tool to differentiate the state of denatured proteins and permits quantitative analysis of the denaturation curve and unfolding curve separately.

Acknowledgments—I appreciate the critical and useful comments of Dr. Dietmar G. Braun on this manuscript. I am also grateful to the insightful comment of the reviewer on the fundamental premise that this study is based upon. Part of the reviewer’s comment is included in the last part of the “Discussion” (“A Note of Caution”). I thank Dr. T Hawthorne and Dr. W. Maerki of Novartis for supplying the recombinant TAP.

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