The Disulfide Folding Pathway of Tick Anticoagulant Peptide (TAP), a Kunitz-Type Inhibitor Structurally Homologous to BPTI

Jui-Yoa Chang*

Pharmaceuticals Research Laboratories, Ciba-Geigy Ltd., Basel, CH-4002 Switzerland

Received March 21, 1996; Revised Manuscript Received May 21, 1996[⊗]

ABSTRACT: The pathway of oxidative folding of tick anticoagulant peptide (TAP, 60 amino acids and three disulfides) has been analyzed by characterization of the acid and iodoacetate trapped folding intermediates. The results reveal a high degree of heterogeneity of the one- and two-disulfide intermediates and the presence of three-disulfide scrambled species along the folding pathway. The picture of TAP folding differs significantly from the well-documented case of bovine pancreatic trypsin inhibitor (BPTI), despite the fact that both proteins share close structural homology in term of 3-D conformation and disulfide pattern.

Elucidation of the pathway of protein folding requires trapping, isolation and characterization of intermediates that adopt partially folded structures (Kim & Baldwin, 1990; Richards, 1991; Matthews, 1993; Evans & Radford, 1994). Two useful methods are currently available to tackle this problem. A newly developed technique of pulsed-label NMR (Roder et al., 1988; Udgaonkar & Baldwin, 1988; Jennings & Wright, 1993; Balbach et al., 1995) permits trapping and identification of amide groups that are engaged in the structured elements. This technique can in principle be applied to all types of proteins. However, intermediates trapped by this method are not amenable to chromatographic purification. An established alternative is to quench the disulfide pairing during the course of folding by acid or alkylating reagents and to follow the mechanism of the formation of native disulfides (Creighton, 1974; Creighton & Goldenberg, 1984; Scheraga et al., 1984; Weissmann & Kim, 1991). This technique is limited to the analysis of disulfide containing proteins, but a unique advantage is that species of trapped intermediates can be further purified for characterization. This method thus allows construction of a detailed folding pathway on the basis of the concentration, heterogeneity, and structure of disulfide isomers accumulated and trapped along the process of folding (Creighton, 1986, 1990).

The first case of well-documented disulfide folding pathway is bovine pancreatic trypsin inhibitor (BPTI),[1] a Kunitz-type protease inhibitor which comprises 58 amino acids and three disulfides. In the original model of BPTI folding (Creighton & Goldenberg, 1984; Creighton, 1990, 1992), eight well-populated one- and two-disulfide intermediates were identified. Five were shown to contain exclusively native disulfides, and those that adopted non-native disulfides have been suggested to be kinetically important intermediates (Creighton, 1992). In a subsequently revised version of BPTI model (Weissmann & Kim, 1991, 1992), it was further demonstrated that there existed five species of well-populated intermediates (two one-disulfide and three two-disulfide species) and all of them contain only native disulfide bonds. Despite this inconsistency, the model of BPTI folding maintains three principal conclusions: (a) the folding intermediates consist of only one- and two-disulfide species; (b) specific interactions that stabilize the native BPTI play a crucial role in guiding the folding early on and hence dictating the formation of limited numbers of well-populated intermediates that admit mainly native disulfides; and (c) the rate-limiting step of BPTI folding is the intramolecular rearrangements of two-disulfide intermediates to generate the immediate processor that rapidly form the third and final native disulfide.

The folding pathway of three other single-domain proteins, namely, hirudin (Chatrenet & Chang, 1993), potato carboxypeptidase inhibitor (PCI) (Chang et al., 1994), and human epidermal growth factor (EGF) (Chang et al., 1995a), all contain three disulfides as well and have the size similar to that of BPTI, has been recently analyzed. Their folding was shown to undergo an initial stage of nonspecific disulfide pairing that leads to the formation of scrambled three-disulfide species. This is followed by consolidation and refinement of scrambled species in the presence of thiol catalyst to attain the native structure. The mechanism of their folding shares two important characteristics that have not been described in the BPTI model (Creighton & Goldenberg, 1984; Creighton, 1990; Weissmann & Kim, 1991). One is the complexity of folding intermediates encountered at the stage of one- and two-disulfide species. In the cases of hirudin (Chatrenet & Chang, 1993) and PCI (Chang et al., 1994), the distinguishable fractions of one- and two-disulfide intermediates approach the number of possible isomers and there exists apparently no predominant species. In the case of EGF (Chang et al., 1995a), four well-populated one- and two-disulfide species were found among heterogeneous minor intermediates, but unlike the BPTI, only one of them contains native disulfides. The second distinct feature is the existence of three-disulfide scrambled species as folding intermediates. Scrambled proteins were originally observed in the case of ribonuclease A as folding was

* Corresponding address: K-121,104 Ciba-Geigy Ltd., Basel, CH-4002 Switzerland. Fax / Tel: +41 61 696 8313.

[⊗] Abstract published in *Advance ACS Abstracts,* August 1, 1996.

[1] Abbreviations: TAP, tick anticoagulant peptide; PCI, potato carboxypeptidase inhibitor; EGF, epidermal growth factor; BPTI, bovine pancreatic trypsin inhibitor; DTT, dithiothreitol; GdmCl, guanidine hydrochloride; GSH, reduced glutathione; GSSG, oxidized glutathione; Cys, cysteine; Cys−Cys, cystine.

S0006-2960(96)00691-5 CCC: $12.00

conducted in the presence 8 M urea (Anfinsen et al., 1961; Haber & Anfinsen, 1962). They have since been considered as misfolded structures of abortive folding. However, during the productive renaturation of hirudin, PCI and EGF, scrambled structures were found in abundance. The level of their accumulation along the folding pathway was shown to depend upon the redox potential and can be experimentally manipulated (Chang, 1994). Another surprising finding observed with hirudin and PCI is that the compositions of their one- and two-disulfide intermediates are hardly affected by the presence of denaturant, suggesting that non-covalent interactions play minimal role in dictating the relative concentrations of these intermediates.

Distinction between the mechanism of BPTI and hirudin bears implications of fundamental importance because they represent two very different models of protein folding. The BPTI folding is in line with the "framework model" (Ptitsyn, 1987; Kim & Baldwin, 1982; Baldwin, 1989; Karplus & Weaver, 1994) that stresses the importance of local interactions in reducing the conformational search and guiding the efficient protein folding through hierarchic condensation of native-like elements. The hirudin model, on the other hand, is more consistent with the "collapse model". The collapse model (Dill, 1990; Dill et al., 1995) depicts that protein folding undergoes an initial stage of rapid hydrophobic collapse followed by slower annealing in which specific interactions refine the structure rather than dominate the folding code.

This discrepancy may be attributed to the structural difference, particularly the dissimilarity of disulfide pattern, between BPTI and hirudin. It may also possibly reflect the vast diversity of mechanism that governs protein folding. In order to clarify this issue, we have chosen to analyze the folding pathway of tick anticoagulant peptide (TAP), a factor Xa specific inhibitor. TAP was first discovered at Merck (Waxman et al., 1990; Sandana et al., 1993). Its 3-D structure has been recently elucidated by NMR (Autuch et al., 1994; Lim-Wilby et al., 1995). TAP includes three disulfides and has a size (60 amino acids) nearly identical to that of BPTI. Most importantly, both TAP and BPTI belong to the Kunitz-type inhibitor and share close structural homology in term of disulfide pattern and three-dimensional conformation (see Figure 1).

EXPERIMENTAL PROCEDURES

Materials. Recombinant tick anticoagulant peptides (TAP) is produced by Ciba (CGP-55099). The purity of TAP was greater than 95% as judged by HPLC, mass analysis, and N-terminal sequence analysis. Reduced glutathione (GSH), oxidized glutathione (GSSG), cysteine (Cys), cystine (Cys−Cys), β-mercaptoethanol, and thermolysin (P-1512) were obtained from Sigma.

Control Folding Experiments. Those are foldings that are performed either in the Tris-HCl buffer alone (control −) or in the same buffer containing 0.25 mM of β-mercaptoethanol (control +). Results of control foldings serve as standard for measuring the efficiency of TAP folding in the presence of various redox agents. TAP (1.5 mg) was dissolved in 0.5 mL of Tris-HCl buffer (0.5 M, pH 8.5) containing 5 M of GdmCl and 30 mM of dithiothreitol.

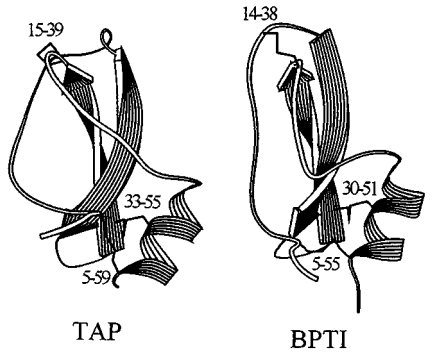

FIGURE 1: Schematic views of the native structures of TAP and BPTI. The three native disulfide bonds of TAP (Cys5−Cys59, Cys15−Cys39 and Cys33−Cys55) and BPTI (Cys5−Cys55, Cys14−Cys38 and Cys30−Cys51) are indicated.

Reduction and denaturation was carried out at 22 °C for 90 min. To initiate the folding, the sample was passed through a PD-10 column (Pharmacia) equilibrated in 0.1 M Tris-HCl buffer (pH 8.5). Desalting took about 1 min, and unfolded TAP was recovered in 1.1 mL, which was immediately diluted with the same Tris-HCl buffer to a final protein concentration of 1 mg/mL, both in the absence (control −) and presence (control +) of 0.25 mM β-mercaptoethanol. Folding intermediates were trapped in a time-course manner by mixing aliquots of the sample with equal volume of (a) 4% trifluoroacetic acid in water (reversible trapping) or (b) 0.4 M iodoacetic acid in the Tris-HCl buffer (0.5 M, pH 8.5) (irreversible trapping). In the case of iodoacetate trapping, carboxymethylation was performed at 22 °C for 30 min, followed by desalting using the PD-10 column. Trapped folding intermediates were separated by HPLC. To ensure that precipitation did not occur during the folding of TAP, two precautions were taken during this study: (a) The folding experiments were carried out at the protein concentration ranging from 0.3 to 3 mg/mL. The profile and complexity of the intermediates were found to be indistinguishable. (b) The total recovery of the intermediates (calculated by HPLC peak integration) was closely monitored after each step of sample manipulation (e.g., desalting) and compared between each time course trapped sample. A limit of deviation was found to be ±7−8%.

Carboxymethylation of Acid-Trapped Intermediates Isolated by HPLC. Acid-trapped intermediates were separated and isolated by HPLC. The samples were dried in a speedvac and immediately treated with 1 M iodoacetic acid in 0.1 mL of Tris-HCl buffer (0.5 M, pH 6.5) containing 40% (by volume) of dimethylformamide (Chang, 1993). The reaction was carried out for 20 min, and the carboxymethylated intermediates were removed from the excess reagent and salt by a NAP-5 column (Pharmacia).

Folding of TAP in the Presence of Redox Agents or Denaturants. The procedures of unfolding and refolding are as those described in the control folding experiments. Selected concentrations of redox agents or denaturants were introduced immediately after unfolded TAP was desalted through the PD-10 column. Folding intermediates were trapped reversibly or irreversibly as those described above.

Structural Analysis Well-Populated Scrambled TAP. Isolated fractions of scrambled TAP (~30 μg) were treated with 3 μg of thermolysin in 100 mL 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

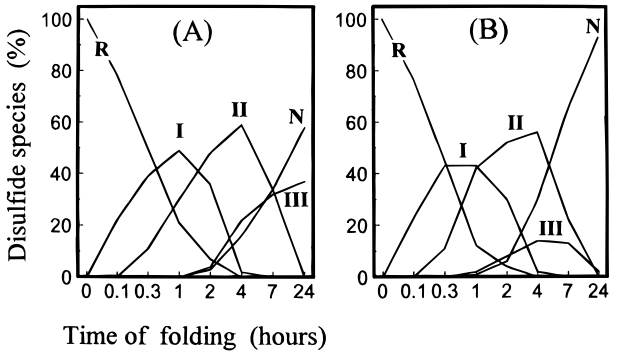

FIGURE 2: Quantitative analysis of the five classes of disulfide species of TAP along the pathway of the "control minus" (A) and "control plus" (B) folding. **I**, **II**, and **III** stand for one-disulfide, two-disulfide, and three-disulfide scrambled species, respectively. The distribution of disulfide species (**R, I**, **II**, and **III**) was deduced from the mass analysis (by MALDI mass spectrometry) and amino acid composition analysis of iodoacetate-trapped samples. Distinction between **III** and **N** was based on HPLC peak integration.

analyzed by amino acid sequencing and mass spectrometry in order to identify the disulfide containing peptides.

Amino Acid Analysis, Amino Acid Sequencing and MALDI Mass Spectrometry. Amino analysis was performed with the dabsyl chloride precolumn derivatization method which permits direct evaluation of the disulfide (cystine) content (Chang & Knecht, 1991). Amino acid sequencing was done with a Hewlett-Packard G-1000A sequencer. The MALDI mass spectrometer was a home-built time of flight (TOF) instrument with a nitrogen laser of 337 nm wavelength and 3 ns pulse width (Boernsen et al., 1990). The apparatus has been described in detail elsewhere. The calibration was performed either externally or internally, by using standard proteins (Hypertensin, MW 1031.19; Synacthen, MW 2934.50; and Calcitonin, MW 3418.91).

RESULTS AND DISCUSSION

Folding Intermediates of TAP are Highly Heterogeneous. Reduced and denatured TAP was first allowed to refold in the Tris-HCl buffer in the absence and presence of thiol catalyst (0.25 mM β-mercaptoethanol). These two folding experiments were designated as "control minus" (without β-mercaptoethanol) and "control plus" (with β-mercaptoethanol). The folding intermediates were trapped in a time-course manner either by acid quenching or by reaction with iodoacetic acid. Those intermediates were then characterized for their chromatographic behavior by HPLC, disulfide content by amino acid composition analysis and distributions of disulfide species by MALDI mass spectrometry (Figure 2). The HPLC patterns of acid-trapped intermediates are presented in Figure 3. Three groups of intermediates advanced spontaneously and sequentially along the folding pathway. Each consists of isomers existing in a state of equilibrium. The first group of intermediates which appeared after 5 and 20 min of folding contains mainly one-disulfide species (**I**). At least six or seven fractions of one-disulfide intermediates have been identified (see 20 min sample of Figure 3, fractions marked 1−6). Further analysis of carboxymethylated samples revealed that fractions 2 and 6 each contain at least 2 and 3 species, respectively. The second group which are predominant species of the 2 and 4 h trapped samples is composed of two-disulfide species (**II**). There exists a minimum of ten fractions of two-disulfide species. Most minor fractions overlap with one-disulfide species. The three major two-disulfide fractions are numbered from 7 to 9 (Figure 3). Again, these well-populated two-disulfide fractions all consist of more than 3−4 species (data not shown).

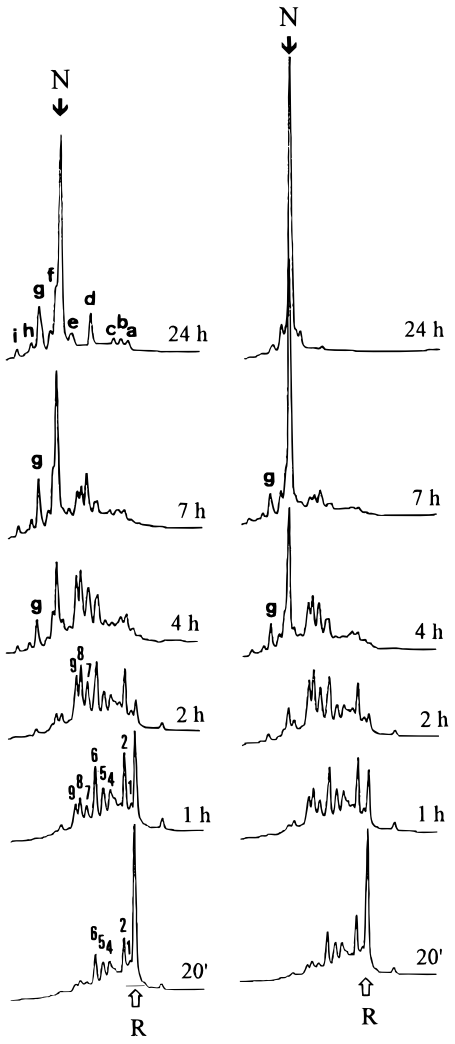

FIGURE 3: Acid-trapped intermediates of the "control minus" (left column) and "control plus" (right column) folding of TAP. Folding was performed in the Tris-HCl buffer (0.1 M, pH 8.4) in the absence (control minus) and presence (control plus) of β-mercaptoethanol (0.25 mM). The samples were analyzed by HPLC using the following conditions. Solvent A was water containing 0.1% trifluoroacetic acid. Solvent B was acetonitrile/water (9:1, by volume) containing 0.1% trifluoroacetic acid. The gradient was 28%−45% solvent B linear in 40 min. Column was Vydac C-18 for peptides and proteins, 4.6 mm × 10 mm. Column temperature was 23 °C. **R** and **N** indicate the elution positions of the fully reduced and the native species. Major fractions of one-disulfide and two-disulfide intermediates are numbered from 1 to 6 and from 7 to 9, respectively. Scrambled three-disulfide species are marked alphabetically (24 h sample, left column).

The subsequent decrease of two-disulfide intermediates is accompanied by the buildup of three-disulfide TAP, which includes scrambled species (**III**) and the native structure (**N**) (see Figure 3, 4, 7, and 24 h samples). From this stage on, the recovery of native protein is dependent upon the presence of free thiols to catalyze the conversion of scrambled species to the native species (Figure 2). When folding was performed in the buffer alone ("control minus", see left column of Figure 3), free cysteines of two- and one-disulfide species apparently functioned as thiol catalyst during the early stage

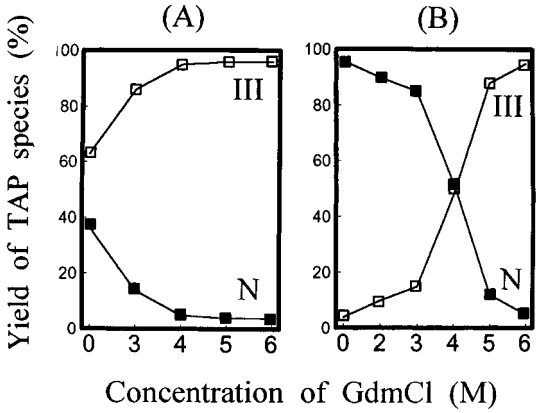

FIGURE 4: End products of TAP folding carried out in the presence of different concentrations of GdmCl. The folding was carried out overnight (16 h) in the Tris-HCl buffer containing 0−6 M GdmCl. The experiments were performed both in the absence (A) and presence (B) of β-mercaptoethanol (0.25 mM). The end products of folding contain invariably a mixture the native (**N**, ■) and scrambled species (**III**, □). The ratio of the native/scrambled TAP was determined from the HPLC peak integration.

of folding. As the folding progressed, free cysteines depleted gradually and scrambled species therefore become trapped and accumulated in an exponential manner. Under these conditions, about 45% of the protein was recovered as scrambled TAP. In the presence of β-mercaptoethanol, scrambled species appeared as transient intermediates and converted to the native structure quantitatively ("control plus", right column of Figure 3). At least 10 fractions of scrambled TAP have been observed (Figure 3, left column, marked alphabetically from a to i).

Folding of TAP in the Presence of Denaturant. Folding of TAP was further performed in the alkaline buffer containing varied concentrations of GdmCl (2−6 M), both in the presence and absence of thiol catalyst (0.25 mM of β-mercaptoethanol). These experiments were designed to evaluate the influence of denaturant on the mechanism of TAP folding. The results were compared with the two control folding experiments shown in Figure 3. After 16 h, all foldings were shown to end up with three-disulfide TAP, containing a mixture of varied ratio of the native species and scrambled species.

The presence of denaturant affects the recovery of the native TAP. In the group of experiments that include β-mercaptoethanol (Figure 4B), the recovery of the native TAP remains close to quantitative at low concentration of GdmCl (2−3 M). However, the yield drops precipitously as the concentration of GdmCl reaches 4−5 M, a critical range in which specific non-covalent interactions that guide the conversion of scrambled species to the native structure appear to be largely nullified. At 6 M GdmCl, the recovery of the native TAP was only 5%. These results are consequences of equilibrium between scrambled and the native TAP in the presence of thiol catalyst and denaturant. The equilibrium constant is a function of the strength of denaturant. Under a given concentration of GdmCl, the same state of equilibrium can be reached by driving the equilibration either from the end of the fully reduced species (folding) or the native species (unfolding). For instance, when fully reduced TAP was allowed to refold in the presence of 4 M GdmCl and 0.25 mM β-mercaptoethanol, only 51% ± 4% of the protein attains the native structure. Conversely, when

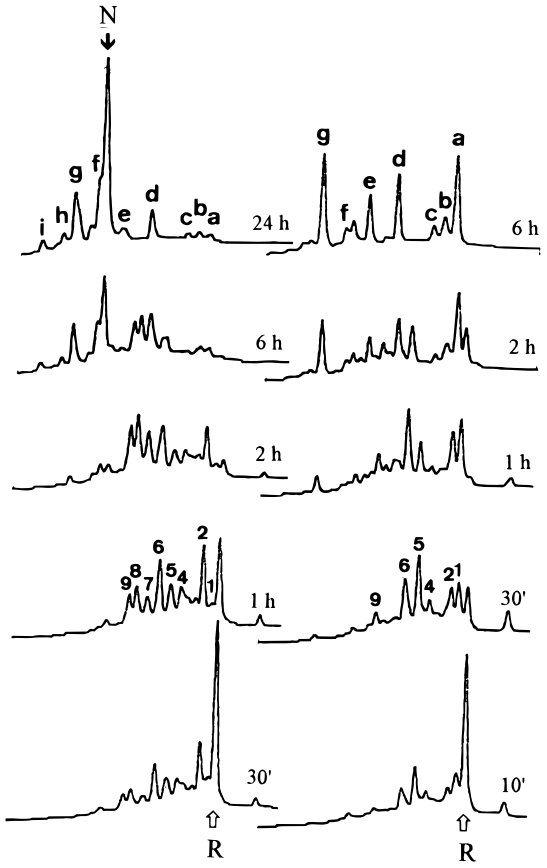

FIGURE 5: Effect of 6 M GdmCl on the compositions of folding intermediates of TAP (trapped by acid). Examples presented here are two folding experiments performed in the presence (right) and absence (left, control minus) of 6 M GdmCl. No thiol catalyst was included in these experiments. In both cases, the end products of folding (top chromatograms) consist of only three-disulfide species of TAP. The recovery of native TAP was 3% and 49%, respectively. The compositions of all three classes of folding intermediates were affected by the presence of denaturant.

the native TAP was placed under the same conditions, 49% ± 4% of the protein will unfold and reshuffle its native disulfides to settle as scrambled species (data not shown). In the group of experiments that exclude β-mercaptoethanol, the recovery of native TAP is significantly lower, from 40% of the control experiment (no denaturant) to less than 5% as the concentration of GdmCl reaches 4 M (Figure 4A).

Examination of time course trapped samples further revealed that the denaturant affects the composition of all three classes of folding intermediates. For one- and two-disulfide species, the differences are most evident by comparing the 1 h intermediates refolded in the absence of denaturant (Figure 5, left column) and the 30 min sample performed in the presence of 6 M GdmCl (Figure 5, right column). Fractions that become diminished in the presence of 6 M GdmCl (e.g., fractions 2 and 8) presumably contain species which adopt structures that are sensitive to the denaturant. The distinction of three-disulfide scrambled species is displayed by the 6 and 24 h samples shown in Figure 5. Among the ten fractions of scrambled TAP, three (a, d, and g) are favored in the presence of denaturant and three (d, f, and g) become well-populated in the absence of denaturant.

Another noticeable influence of the denaturant is exhibited by the approximately 3-fold acceleration of the intermediates flow (Figure 5). This effect may be explained by the stability

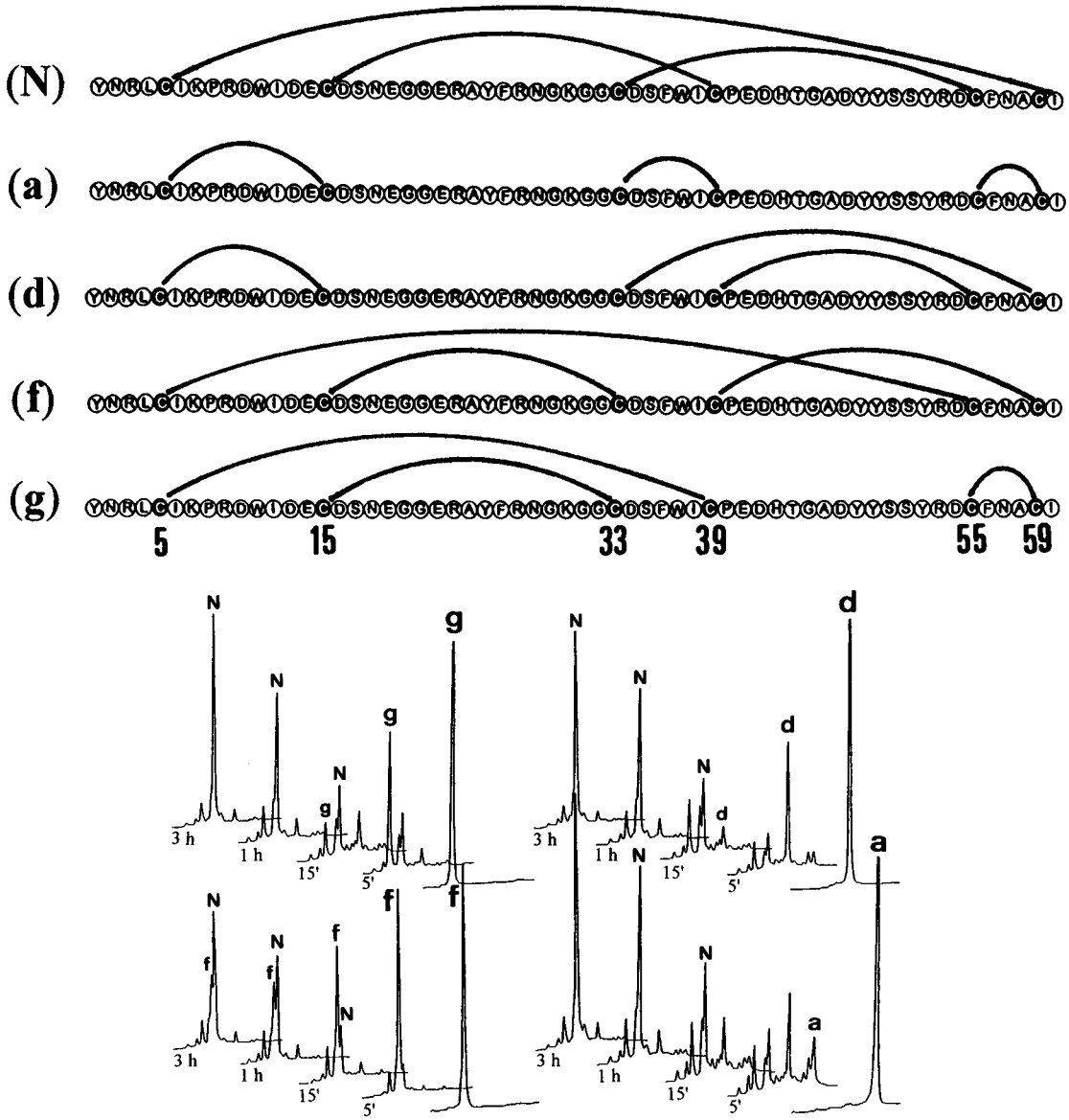

FIGURE 6: Disulfide structures of well-populated scrambled TAP and the pathway of their consolidation to form the native structure. The structures were derived from the sequence and mass analysis of thermolytic peptides of purified scrambled TAP. The consolidation was performed at 23 °C in the Tris-HCl buffer (0.1 M, pH 8.4) containing 1 mM of cysteine. The protein concentration was 1 mg/mL. Intermediates were trapped by mixing aliquots of the sample with 2 vol of 4% trifluoroacetic acid and analyzed by HPLC using conditions described in the legend of Figure 3.

difference of the intermediates. When folding of TAP was performed in the absence of denaturant, many species of one- and two-disulfide intermediates are apparently stabilized by non-covalent interactions (this is why they are denaturant sensitive). Such intermediates are inclined to be more complacent and less reactive to convert along the folding pathway. This conclusion is supported by the contrasting results obtained from hirudin and PCI. In these two cases (Chatrenet & Chang, 1993; Chang et al., 1994), the HPLC patterns of their one- and two-disulfide intermediates are hardly affected by the denaturant. Consequently, the flow rate of their folding intermediates remain indistinguishable, regardless of whether folding was carried out in the absence or presence of 6 M GdmCl.

Disulfide Structures of Well-Populated Scrambled TAP. There are 74 possible disulfide isomers (15 one-disulfide, 45 two-disulfide, and 14 scrambled three-disulfide) that may serve as folding intermediates of TAP. Our data have shown that a substantial portion of them do exist along the folding pathway. However, scrambled three-disulfide species represent the only class of intermediates that can be purified to homogeneity by HPLC. Four species of well-populated scrambled TAP (a, d, f, and g), which constitute more than 70% of the total scrambled TAP, were isolated. Their disulfide structures were deduced from sequence and mass spectometric analysis of thermolysin digested peptides. The structures are summarized in Figure 6 (top panel).

One unique feature is that none of these four scrambled TAP contain a native disulfide. Species a which is well-populated only in the presence of denaturant adopts the "three-bead" structure. The same type of structure was found with the most predominant scrambled species of hirudin (Chang et al., 1995b) and EGF (Chang et al., 1995a) as well. The only difference is that the three bead species of hirudin and EGF are predominant both in the presence and absence of denaturant. Species f, which contains the second largest disulfide loop (Cys5−Cys55), is the only well-populated scrambled TAP that is highly sensitive to the denaturant. Its

share of scrambled structures reduced from 30% in the absence of denaturant to less than 3% in the presence of 6 M GdmCl. This indicates that species f is stabilized by specific non-covalent interactions. Whether these interactions are native-like still remain to be elucidated. The fact that species f contains no native disulfide does not rule out the possibility that it may adopt native-like structures. However, exchange of Cys15−Cys39 (native) to Cys15−Cys33 (scrambled) requires the reversed orientation of one major stretch of β-strand (Figure 1). It is difficult to imagine that such change will not have a profound effect on the overall conformation of TAP. Further analysis of this species by NMR may provide interesting information.

Consolidation of Scrambled TAP. Well-populated species of three-disulfide intermediates were compared for their ability to convert to the native structure. They were dissolved in the folding buffer and a thiol catalyst (1 mM Cys) was introduced to initiate the reshuffling of their non-native disulfides. Along the process of consolidation, a purified scrambled TAP simultaneously equilibrates with other scrambled isomers as well as converts to the native species (Figure 6). Eventually, all scrambled species convert to the native structure. The results reveal that a scrambled TAP appears to equilibrate faster with those that already share a common disulfide. This phenimenon is not unexpected since such conversions involve reshuffling of only two pairs of disulfide. For instance, species f converts to species g about 3-fold faster than to species d. Both f and g share Cys15−Cys33. Another example is the rapid conversion of a to d. In this case, both contain Cys5−Cys15. Species a, d, and g display comparable efficiency in generating the native TAP. But species f is about 50% less productive. The sluggish activity is evidently attributed to the unique stability of species f. This property is consistent with those observed in the case of hirudin, in which denaturant sensitive species of scrambled hirudins are less productive as well (Chang, 1995).

Efficiency of Oxidative Folding of TAP is Promoted by a Two-Stage Mechanism. Folding of disulfide-containing proteins is known to be mediated by redox agents, such as reduced and oxidized glutathione (GSH/GSSG) (Sexena & Wetlaufer, 1970; Creighton, 1984). Their influences on the folding behavior of ribonuclease A and lysozyme were investigated in details (Creighton, 1984 1984; Lyles & Gilbert, 1991; Rothwarf & Scheraga, 1993). We have recently demonstrated that GSH and GSSG mediate two distinct stages of hirudin folding (Chang, 1994). While GSSG enhances the rate of disulfide formation, GSH serves primarily to catalyze the disulfide reshuffling of non-native disulfides. Furthermore, it was shown that Cys/Cys−Cys is more potent than GSH/GSSG.

The efficacy of these two systems of redox agents on promoting the folding of TAP has been systematically analyzed here. The effect of Cys (or GSH) was found to resemble that of β-mercaptoethanol. In the presence of Cys (1 mM) alone, scrambled three-disulfide species appear as transient intermediates and folding of TAP can be achieved quantitatively within 3 h (column 1 of Figure 7). Cys−Cys and GSSG play a different role. They enhance the rate of disulfide formation and accelerate the flow from the fully reduced species (R) to the three-disulfide species. As a consequence, scrambled three-disulfide species accumulated rapidly when folding of TAP was performed in the buffer

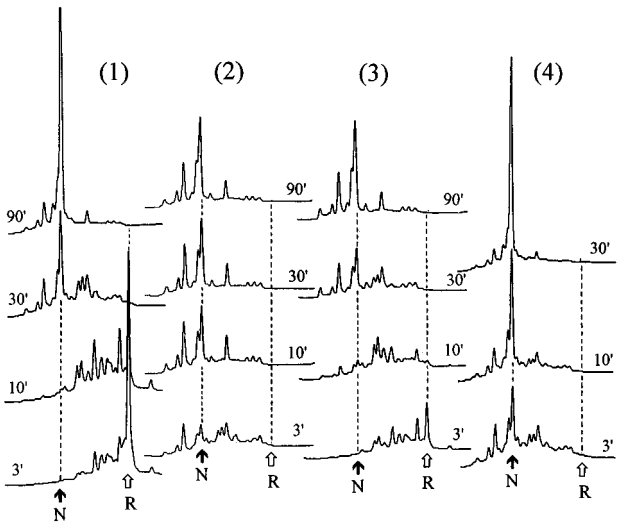

FIGURE 7: Folding of TAP is promoted by redox agents in a two-stage mechanism. Reduced TAP was allowed to refold in the Tris-HCl buffer (0.1 M, pH 8.4) containing (1) Cys (2 mM), (2) Cys−Cys (0.5 mM), (3) GSSG (0.5 mM) and (4) Cys/Cys−Cys (4 mM/2 mM). In the presence of Cys−Cys or GSSG alone, the folding intermediates accumulated rapidly as scrambled species. To facilitate the comparison of the progression of folding intermediates, the windows between **R** and **N** are marked by broken lines. The folding intermediates were trapped by acid.

containing only Cys−Cys or GSSG (columns 2 and 3, Figure 7). The rate of this enhancement can be quantitatively evaluated by increasing the concentration of Cys−Cys and GSSG. In the presence of 0.5 mM Cys−Cys, the rate of disulfide formation increased 100-fold as compared to the folding carried out in the buffer alone (control minus) and the intermediates appeared after 10 min of folding were exclusively scrambled species (column 2, Figure 7). At higher concentration of Cys−Cys (2 mM), three-disulfide TAP formed quantitatively within 3 min. GSSG is about 10-fold less potent than Cys−Cys in promoting the disulfide formation of TAP (compare columns 2 and 3 of Figure 7), which is consistent with that observed in the case of hirudin. Under these conditions, however, about 60% of TAP was trapped as scrambled species. Their conversion to the native structure become a rate limiting step due to the absence of free thiols.

These results suggest that efficient folding of TAP requires an optimized combination of reduced and oxidized thiol agents. In the presence of 4 mmol of Cys and 2 mmol of Cys−Cys, complete folding of TAP can be achieved within 30 min (column 4, Figure 7).

Comparison of the Folding Mechanisms of TAP and Hirudin. The folding mechanism of TAP, in many aspects, is fundamentally indistinguishable from that of hirudin (Chatrenet & Chang, 1993), PCI (Chang et al., 1994), and EGF (Chang et al., 1995a). Those include the complexity of folding intermediates, the mode of their flow along the pathway and the mechanism of acceleration of folding in a two stage manner by redox agents. The most striking similarity is exhibited by the mechanism of the accumulation of three-disulfide scrambled intermediates. When folding was carried out in the alkaline buffer alone, about 50% of the protein reproducibly become trapped as scrambled species, unable to convert to the native structure due to the absence of thiol catalyst. This phenomenon was consistently

observed in the examples of hirudin, PCI, and EGF, as well as TAP.

However, there is one important difference concerning the property of folding intermediates that needs to be elaborated. Sensitivity of intermediates toward denaturant varies among these four proteins. In the cases of hirudin and PCI, only the composition of three-disulfide intermediates are affected by denaturant. For EGF (Chang et al., 1995a), both two- and three-disulfide intermediates are sensitive to the denaturant. In the case of TAP, all three classes of intermediates contain species that are susceptible to the denaturant. Species that are sensitive to the denaturant must adopt structures that are stabilized by non-covalent interactions. Their characterization by NMR should allow us to gain an insight into the nature of protein folding intermediates. Those structures do not necessarily have to be native-like. Even if they are, they most likely have to be broken and remade as folding progresses (Weissmann & Kim, 1995). Furthermore, the contribution of those stabilizing structures in terms of free energy (ΔG) is likely to be marginal, particularly at the levels of one- and two-disulfide species. This statement is supported by experimental results which show that the rate of reduction of scrambled TAP, which undergoes one- and two-disulfide intermediates, remains similar regardless of whether reduction is carried out in the absence or presence of 6 M GdmCl (data not shown).

Characterization of stable, well-populated intermediates has been a subject of major interest in the analysis of protein folding pathway (Kim & Baldwin, 1990). In a model that envisages hierarchical assembly of local secondary structures, these intermediates have been generally considered to adopt native-like structures and play an essential role in the pathway of productive folding (Creighton & Goldenberg, 1984; Oas & Kim, 1988; Creighton, 1990; Dadlez & Kim, 1995). The data obtained from TAP and hirudin indicate that existence of such stable intermediates does not necessarily facilitate the process of folding. On the contrary, they may play the role of impeding the flow of intermediates. The presence of stable scrambled species f (Figure 6). for instance, probably accounts for the rate-limiting step observed during the final stage of TAP folding.

Comparison of the Folding Mechanisms of TAP and BPTI. There are a number of similarity and crucial differences between the folding mechanism of TAP and that concluded from BPTI (Creighton & Goldenberg, 1984; Creighton, 1990, 1992; Weissman & Kim, 1991). (a) The one- and two-disulfide intermediates of TAP and BPTI each exist in a state of dynamic equilibrium. In the case of TAP, the state of equilibrium of one-disulfide species was observed as soon as the intermediates become detectable by HPLC and they remain in constant equilibrium throughout the folding process as transient intermediates. Two- and three-disulfide intermediates behave in the same fashion. Similar mechanism has also been observed with hirudin, PCI and EGF. Indeed, the results of equilibration experiments of isolated one- and two-disulfide BPTI, performed by Weissman and Kim (1992b) and Dadlez and Kim (1995), suggest that the property of BPTI folding intermediates may not be different at all from that of TAP and hirudin. (b) The folding intermediates of TAP are far more heterogeneous than what has been described in the case of BPTI. There are at least seven fractions of one-disulfide intermediates and ten fractions of two-disulfide intermediates. Most major fractions (e.g., 2, 6, 7, 8, and 9, Figure 1) further comprise multiple species, and unlike BPTI, there exist apparently no predominant one- or two-disulfide species of TAP. (c) The most important distinction, however, is the presence of scrambled three-disulfide TAP as folding intermediates, which have not been observed with BPTI. Similar to scrambled TAP and hirudin, there are species of folding intermediates of BPTI that become trapped as well along the course of folding. But these trapped intermediates of BPTI have been characterized as species that contain two native disulfides and two cysteines that are either buried (Weissmann & Kim, 1991, 1992a) or lack the flexibility (Creighton, 1990, 1992) to form the third native disulfide efficiently. It is, however, relevant to mention that scrambled species were indeed observed during the folding of BPTI (Creighton, 1975, 1977) and proBPTI (Weissmann & Kim, 1992a). The HPLC profile of scrambled proBPTI also bears a striking resemblance to that of scrambled hirudin and EGF. Considering that both TAP and BPTI share such close structural homology (Autuch et al., 1994), existence of these discrepancies cannot be more intriguing.

ACKNOWLEDGMENT

The author thanks Dr. T. Hawthorne and Dr. W. Maerki for supplying the recombinant TAP and for supporting this study. The kindness of Dr. J. Priestle for preparing Figure 1 is highly appreciated.

REFERENCES

Anfinsen, C. B., Haber, E., Sela, M., & White, F. H., Jr. (1961). *Proc. Natl. Acad. Sci. U.S.A. 47*, 1309−1515.
Autuch, W., Güntert, P., Billeter, M., Hawthorne, T., Grossenbacher, H., & Wüthrich, K. (1994) *FEBS Lett. 352*, 251−257.
Balbach, J., Forge, V., van Nuland, N. A. J., Winder, S. L. Hore, P. J., & Dobson, C. M. (1995) *Nat. Struct. Biol. 2*, 865−870.
Baldwin, R. L. (1989) *Trends Biochem. Sci. 14*, 292−294.
Boernsen, K. O., Schaer, M., & Widmer, M. (1990) *Chimia 44*, 412−416.
Chang, J.-Y. (1993) *J. Biol. Chem. 268*, 4043−4049.
Chang, J.-Y. (1994) *Biochem. J. 300*, 643−650.
Chang, J.-Y. (1995) *J. Biol. Chem. 270*, 25661−25666.
Chang, J.-Y., & Knecht, R. (1991) *Anal. Biochem. 197*, 52−58.
Chang, J.-Y., Canals, F., Schindler, P., Querol, E., & Aviles, F. X. (1994) *J. Biol. Chem. 269*, 22087−22094.
Chang, J.-Y., Schindler, P., Ramseier, U., & Lai, P.-H. (1995a) *J. Biol. Chem. 270*, 9207−9216.
Chang, J.-Y., Schindler, P., & Chatrenet, B. (1995b) *J. Biol. Chem. 270*, 11992−11997.
Chatrenet, B., & Chang, J.-Y. (1993) *J. Biol. Chem. 268*, 20988−20996.
Creighton, T. E. (1974) *J. Mol. Biol. 87*, 563−577.
Creighton, T. E. (1975) *J. Mol. Biol. 95*, 167−199.
Creighton, T. E. (1977) *J. Mol. Biol. 113*, 295−312.
Creighton, T. E. (1984) *Methods Enzymol. 107*, 305−329.
Creighton, T. E. (1986) *Methods Enzymol. 131*, 83−106.
Creighton, T. E. (1990) *Biochem. J. 270*, 1−16.
Creighton, T. E. (1992) *Science 256*, 111−112.
Creighton, T. E., & Goldengerg, D. P. (1984) *J. Mol. Biol. 179*, 497−526.
Dadlez, M., & Kim, P. S. (1995) *Nat. Struct. Biol. 2*, 674−679.
Dill, K. A. (1990) *Biochemistry 29*, 7133−7155.
Dill, K. A., Bromberg, S., Yue, K., Fiebig, K. M., Yee, D. P., Thomas, P. D., & Chan, H. S. (1995) *Protein Sci. 4*, 561−602.
Evans, P. A., & Radford, S. E. (1994) *Curr. Opin. Struct. Biol. 4*, 100−106.
Haber, E., & Anfinsen, C. B. (1962) *J. Biol. Chem. 237*, 1839−1843.
Jennings, P. A., & Wright, P. E. (1993) *Science 262*, 892−896.
Karplus, M., & Weaver, D. L. (1994) *Protein Sci. 3*, 650−668.

Kim, P. S., & Baldwin, R. L. (1982) *Annu. Rev. Biochem. 51*, 459−489.
Kim, P. S., & Baldwin, R. L. (1990) *Annu. Rev. Biochem. 59*, 631−660.
Lim-Wilby, M. S. L., Hallenga, K., De Maeyer, M., Lasters, I., Vlasuk, G. P., & Brunck, T. K. (1995) *Protein Sci. 4*, 178−186.
Lyles, M. M., & Gilbert, H. F. (1991) *Biochemistry 30*, 613−619.
Matthews, C. R. (1993) *Annu. Rev. Biochem. 62*, 653−683.
Oas, T. G., & Kim, P. S. (1988) *Nature 336*, 42−48.
Ptitsyn, O. B. (1987) *J. Protein Chem. 6*, 273−293.
Richards, F. M. (1991) *Sci. Am.* 34−41.
Roder, H., Elöve, G., & Englander, S. W. (1988) *Nature 335*, 700−704.
Rothwarf, D. M., & Scheraga, H. A. (1993) *Biochemistry 32*, 2690−2697.
Sandana, M., Sandana, V., Rodkey, J., Wood, T., Ng, A., Vlasuk, G. P., & Waxman, L. (1993) *J. Biol. Chem. 268*, 20988−20996.
Sexena V. P., & Wetlaufer, D. B. (1970) *Biochemistry 9*, 5015−5023.
Scheraga, H. A., Konishi, Y., & Ooi, T. (1984) *Adv. Biophys. 18*, 21−41.
Udgaonkar, J. B., & Baldwin, R. L. (1988) *Nature 335*, 694−699.
Waxman, L., Smith, D. E., Arcuri, K. E., & Vlasuk, G. P. (1990) *Science 248*, 593−596.
Weissman, J. S., & Kim, P. S. (1991) *Science 253*, 1386−1393.
Weissman, J. S., & Kim, P. S. (1992a) *Cell 71*, 841−851.
Weissman, J. S., & Kim, P. S. (1992b) *Proc. Natl. Acad. Sci. U.S.A. 89*, 9900−9904.
Weissman, J. S., & Kim, P. S. (1995) *Nat. Struct. Biol. 2*, 1123−1130.

BI9606915