Unfolding and Refolding of Cardiotoxin III Elucidated by Reversible Conversion of the Native and Scrambled Species†

Jui-Yoa Chang,*,‡ Thallampuranam Krishnaswamy S. Kumar,§ and Chin Yu*,§

Pharmaceuticals Research Laboratories, Novartis AG Basel CH-4002, Switzerland, and Department of Chemistry, National Tsing Hua University, Hsinchu, Taiwan

Received June 17, 1997; Revised Manuscript Received November 13, 1997

ABSTRACT: Cardiotoxin analogue III (CTX III) isolated from the venom of the Taiwan Cobra (*Naja naja atra*) is a small molecular weight, all β-sheet protein, cross-linked by four disulfide bridges. The unfolding and refolding mechanisms of CTX III have been examined by monitoring the reversible conversion of the native and scrambled species. It is found that, in the presence of a denaturant (urea/guanidinium hydrochloride) and a thiol catalyst, CTX III forms a mixture of scrambled species by shuffling its four native disulfide bonds. Complete unfolding of CTX III can be achieved using either 3.0−4.0 M guanidinium hydrochloride (GdmCl) or 5.0−6.0 M urea. It is observed that GdmCl is thermodynamically more potent but kinetically less efficient than urea in unfolding CTX III. The rate constants of unfolding of CTX III in 8 M urea are significantly greater than that obtained in 5.0 M GdmCl and 8.0 M GdmCl. Interestingly, upon removal of the denaturant, scrambled species of CTX III is found to refold spontaneously through dynamic reshuffling of the non-native disulfides to attain the native disulfide linkages. In addition, CTX III contains highly reactive lysines which are modified by trace amounts of cyanate contaminant which exists invariably even in high-grade urea solutions. The reactive lysines of CTX III are modified by cyanate both in the native and unfolded states of the protein. The modification is nonselective, and the modified product is found to consist of highly heterogeneous species. Surprisingly, these heterogeneous species of modified CTX III are observed to display stability and folding/unfolding properties indistinguishable from those of the native CTX III. The knowledge obtained from the present study, on the conditions to convert the scrambled species, could provide useful clues for a rational design for snake venom cardiotoxins with potential therapeutic applications.

The information required to drive a disordered polypeptide spontaneously to its native three-dimensional structure under physiological conditions is known to be encoded within its amino acid sequence (*1*). It was suggested that, within a limited time scale, such a folding process does not involve a random search of all possible conformational space (*2*). As a consequence, it has been generally understood that protein folding must proceed via preferred pathways and structured intermediates. Currently, there are two popular approaches to elucidate the early intermediates formed along the folding pathway. The pulse-labeling technique (*3, 4*) utilizes hydrogen−deuterium exchange to identify the backbone amides that become protected during the process of folding. Another approach to characterize the folding intermediates is the disulfide-quenching technique (*5, 6*). In this approach, the mechanism of the formation of native disulfides is followed by quenching the disulfide pairing during the process of folding using appropriate reagents. Monitoring protein folding coupled to disulfide bond formation has several advantages. It is generally believed that all disulfide bonds between cysteine residues are essentially equivalent, and any differences between those in proteins stem from the conformation of the protein (*7*). In this context, if intramolecular disulfide exchange is permitted, those consistent with the most stable conformation will be predominant (*8*). In addition, the advantage of trapping and characterization of folding intermediates using the disulfide-quenching technique helps to define the kinetic role(s) of the folding intermediates (*9*−*11*).

The disulfide folding pathway of bovine pancreatic trypsin inhibitor (BPTI) has been well characterized (*9, 12*). However, there has been intense debate regarding the nature and importance of the disulfide intermediates that form and accumulate during the folding pathway of BPTI (*9, 13*). Despite the disagreement(s), the folding pathway of BPTI is characterized by the formation of limited number of well-populated intermediates which possess one or two disulfide bonds. In addition, the intramolecular rearrangements of the two disulfide intermediates are proposed to be the rate-limiting step(s) in the folding of BPTI. Recently, the disulfide folding pathway of various single-domain proteins have been elucidated (*14*−*18*). The folding pathways of these proteins are characterized by the presence of fully

† This work was supported by the Taiwan National Science Council (NSC 86-2113-MOO7-001, NSC 86-2113-M007-003) and the Dr. C. S. Tsou Memorial Medical Research Advancement Foundation (VGHTH 86-0112) grants.

* Corresponding authors: Chin Yu at National Tsing Hua University or J.-Y. Chang at Novartis AG.

‡ Novartis AG.

§ National Tsing Hua University.

S0006-2960(97)01456-6 CCC: $15.00
Published on Web 04/28/1998

oxidized, non-native disulfide paired, scrambled intermediates in the initial stage of folding. The level of accumulation of such scrambled intermediates has been shown to vary depending on the redox potential of the folding buffer (*18*). The results of these studies have clearly demonstrated that scrambled species accumulated in the initial stage of folding are not off pathway "dead-end" products but rather are productive intermediates that could efficiently rearrange its disulfide bonds to yield the native protein (*16*−*19*).

Cardiotoxins are an interesting class of molecules with a wide array of biological properties, which include lysis of erythrocytes, selective killing of certain types of tumor cells, and specific inhibition of the activity of enzymes such as protein kinase C and Na^+-K^+ ATPase (for review, see refs *20* and *21*). Due to their anticoagulant activity snake venom, cardiotoxins have potential therapeutic applications (*22*). In addition, recently, cardiotoxins have been contemplated to form anion-selective ion channels (*23*). Owing to the broad spectrum of interesting biological properties exhibited by cardiotoxins, considerable research interest is focused on understanding the structure and folding aspects of snake venom cardiotoxins.

Cardiotoxin III (CTX III) is isolated from the Taiwan Cobra (*Naja naja atra*). It is a highly basic (pI > 10), small molecular mass (6.8 kDa), all β-sheet protein cross-linked by four disulfide bridges (*20, 24, 25*). Solution structure of CTX III revealed that it is a "three finger" shaped protein with three loops emerging from a globular head (*26*). The head region of the molecule is fortified by the presence of four disulfide bonds formed between cysteine residues located at position 3-21, 14−38, 42−53, and 54−59 (*26*). The presence of these disulfide bridges are crucial to the maintenance of the three-dimensional structure of CTX III. Interestingly, the disulfide bonds in CTX III have been shown to inhibit the helix formation in the presence of helix-inducing cosolvents such as 2,2,2-trifluoroethanol (*27*). Recently, this stable partially structured intermediate state has been identified and characterized along the alcohol (*28*) and acid-induced unfolding pathways of this protein (CTX III).

The present study is aimed at elucidating the disulfide unfolding and refolding mechanisms of CTX III. However, unlike the conventional approach which involves reduction and reoxidation of disulfide bonds, the unfolding and refolding of CTX III were analyzed here by monitoring reversible conversion of the native and scrambled species through shuffling and reshuffling of the native and non-native disulfide bonds (*19*). In the present study, the effects of denaturants, such as urea and GdmCl, have been studied to understand the influence of noncovalent forces in the unfolding/refolding pathway(s) of CTX III.

EXPERIMENTAL PROCEDURES

Materials. CTX III was isolated from the Taiwan Cobra (*Naja naja atra*) using the method described (*29*). The protein was more than 95% pure as judged by HPLC and N-terminal sequence analysis. Reduced glutathione (GSH), cysteine (Cys), and 2-mercaptoethanol were purchased from Sigma. GdmCl (cat no. 112031) and urea (cat no. 108488) are products of Merck with minimum purity of 99.5%. Additional supplies of urea were obtained from Sigma, Bio-Rad (161-0731), and Amresco (Ohio), all with purity greater than 99.9%.

Unfolding of CTX III. The protein (0.5 mg/mL) was dissolved in the Tris-HCl buffer (0.1 M, pH 8.4) containing 0.25 mM of 2-mercaptoethanol and selected concentrations of the denaturant (urea or GdmCl). Unfolding was routinely carried out at 23 °C for 20 h. To monitor the kinetics and progress of unfolding, aliquots of the sample were removed in a time-course manner, quenched with 4% trifluoroacetic acid, and analyzed by HPLC. The unfolded sample was subsequently acidified with an equal volume of 4% trifluoroacetic acid and stored at −20 °C. To prepare the sample for refolding, the unfolded protein, which consists of a mixture of scrambled species, was passed through a PD-10 column (Pharmacia) equilibrated in 2% aqueous trifluoroacetic acid, lyophilized, and reconstituted in the folding buffer. The refolding was initiated by introduction of selected thiol catalyst.

Refolding of Scrambled CTX III. To initiate the folding, the unfolded protein (0.5 mg/mL) was redissolved in the same Tris-HCl buffer containing selected thiol catalyst (0.25 mM of 2-mercaptoethanol, 1 mM reduced glutathione, or 1 mM of cysteine). Folding was performed at 23 °C. Folding intermediates were trapped in a time-course manner by mixing aliquots of the sample with an equal volume of 4% trifluoroacetic acid in water. The rate constant of unfolding and refolding were estimated based on the relative recovery of the native and scrambled species obtained at various time points during unfolding/refolding of CTX III. Quantitative estimates of the recoveries of the various CTX III species were obtained from their HPLC peak areas. The rate constant of unfolding/refolding reactions were estimated by fitting the data to a single-exponential function ($y = A\exp^{-kt} + C$, wherein A is the amplitude of the phase, k is the apparent rate constant and C is the final amplitude) by the conventional Levenberg−Marquardt nonlinear least-squares method, yielding the rate constant(s) and phase amplitude(s) in the kinetic unfolding experiment.

Protein Analytical Methods. The disulfide content of scrambled proteins was determined by the dabsyl chloride precolumn derivatization method (*30*), which permits direct quantification of the disulfide bonds of proteins. Amino acid sequence analysis was performed 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. The apparatus has been described in detail elsewhere (*31*). The calibration was performed either externally or internally, by using standard proteins (Hypertensin, MW 1031.19; Synacthen, 2934.50, and Calcitonin, 3418.91).

RESULTS

Selection of Thiol Reagents for the Unfolding and Refolding of CTX III. The usefulness of cysteine, reduced glutathione, and 2-mercaptoethanol as thiol reagents to promote the unfolding and refolding of the native and scrambled proteins has been analyzed (*18*). During the process of unfolding, 2-mercaptoethanol was found to be a more suitable reagent because, in the presence of denaturant(s), cysteine and reduced glutathione form significant amounts of mixed disulfides with scrambled proteins (*18*).

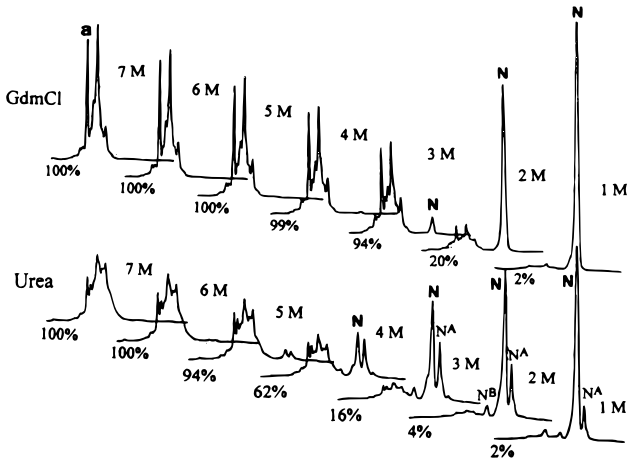

FIGURE 1: Unfolding of CTX III to the state of scrambled species using increasing concentrations of GdmCl (top panel) and urea (bottom panel). Unfolding was carried out in the presence of 2-mercaptoethanol (0.25 mM) and indicated concentrations of the denaturants for 16 h. The samples were acidified with 4% TFA and 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. A linear gradient of 20 to 56% solvent B in 30 min was used. Column was Vydac C-18 for peptides and proteins, 4.6 mm, 10 μm. Column temperature was 23 °C. **N** indicates the elution position of the native species. Fractions $\mathbf{N^A}$ and $\mathbf{N^B}$ contain native CTX III that are modified with 1 mol of cyanate, which appear only in the case of urea unfolding. Sequence analysis has shown that $\mathbf{N^A}$ and $\mathbf{N^B}$ each consists of heterogeneous species of single cyanate modified CTX III. All other fractions contain 4-disulfide scrambled species. One of the major fractions of scrambled CTX III generated in the presence of GdmCl is marked as "**a**".

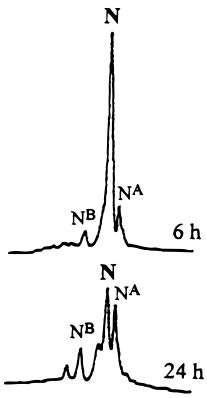

FIGURE 2: Modification of the native CTX III by the cyanate contaminant of urea. CTX III (0.2 mg/mL) was dissolved in Tris-HCl buffer (0.1 M, pH 8.4) containing 6 M urea. The sample was left at 23 °C for 6 and 24 h, acidified with 4% TFA, followed by HPLC analysis. Fractions $\mathbf{N^A}$ and $\mathbf{N^B}$ both contain species which exhibit a molecular mass of 43 Da higher than the native CTX III (6739). The 24 h sample is comprised of 30% of the native CTX-III and 70% of heterogeneous species modified with 1 (~35%), 2 (~25%) and 3 mol (~10%) of cyanate.

This aspect inevitably compromises the purity of scrambled species. Further optimization of the concentration of 2-mercaptoethanol was systematically investigated by allowing the native proteins to unfold overnight in solutions containing 6 M GdmCl and varying concentrations of 2-mercaptoethanol, using hirudin (*18*) and ribonuclease A as model proteins. The end-products were carboxymethylated, followed by amino acid composition analysis and molecular mass analysis. The results reveal that the concentration of 2-mercaptoethanol ranging 0.2−0.3 mM is optimal for efficient reshuffling of the disulfide bonds in CTX III during unfolding of the protein. At higher concentration of 2-mercaptoethanol, the disulfide bonds of scrambled proteins risk being partially reduced (J.-Y. Chang, unpublished data).

During the process of refolding of scrambled proteins, cysteine, reduced glutathione, and 2-mercaptoethanol were found to be equally useful. In many cases, cysteine and reduced glutathione were shown to be more effective than 2-mercaptoethanol in promoting the reshuffling of disulfide bonds (*18, 19*).

Unfolding of CTX III in the Presence of Thiol Catalyst and Denaturant. In the presence of a denaturant and a thiol catalyst, native CTX III reshuffles its native disulfide bonds to form a mixture of scrambled isomers. The extent of unfolding of CTX III in the presence of increasing concentrations of urea and GdmCl is shown in Figure 1. All species unfolded by GdmCl and urea contain negligible amount (<2−3%) of free cysteines. This was confirmed by amino acid composition analysis of the carboxymethylated samples. On the basis of composition of unfolded species, CTX III appears to adopt very different state of unfolding in the urea and GdmCl solutions. For instance, it was thought initially that the two fractions ($\mathbf{N^A}$ and $\mathbf{N^B}$, see the bottom panel of Figure 1) eluted near the native species represent partially unfolded structures that exist as transient intermediates only in the urea solution. It turned out that $\mathbf{N^A}$ and $\mathbf{N^B}$ indeed contain the native species (with four native disulfide bonds) that are chemically modified by 1 mol of cyanate. As it will be subsequently demonstrated, these cyanate modified CTX III not only possess disulfide stability comparable to that of the native CTX III, but also unfold and refold with efficiencies that are indistinguishable from that of the native species. Thus, in calculating the extent of unfolding (unfolding curves), $\mathbf{N^A}$ and $\mathbf{N^B}$ therefore are counted as the native species.

Control experiments have shown that cyanate modification occurs regardless of the origin of urea. The extent of modification is dependent upon the concentration of urea and the time of incubation (Figure 2). CTX III was modified by cyanate both in the native and denatured states, but the reaction rate of the scrambled form is about 3-fold slower than that of the native species. In the presence of 8 M urea, modification occurs almost exclusively at the scrambled form, since the rate constant of unfolding is 500-fold greater than that of cyanate modification of the native CTX III (Figure 3). Thus, the cyanate modification of unfolded CTX III partly accounts for the apparent difference of HPLC patterns of the unfolded states produced in urea and GdmCl (as observed in Figure 1). Under the unfolding conditions described here, more than 90% of the modified species are labeled with single cyanate. They are eluted within two major fractions ($\mathbf{N^A}$ and $\mathbf{N^B}$) next to the native species (Figure 2). Both $\mathbf{N^A}$ and $\mathbf{N^B}$ species contain the native disulfide pairings. Molecular weight analysis by MALDI mass spectrometry and sequence analysis by Edman's degradation showed that $\mathbf{N^A}$ and $\mathbf{N^B}$ consist of heterogeneous species of single cyanate modified CTX III. The modification distributes nonselectively among all the lysine residues of CTX III (data not shown). Although the data presented in this study does not permit us to provide a clear-cut explanation for the different elution positions for $\mathbf{N^A}$ and $\mathbf{N^B}$ species, it

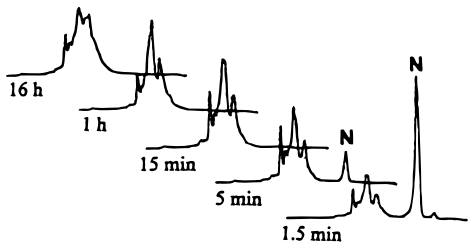

FIGURE 3: Time course unfolding of CTX III in the presence of 8 M urea. CTX III (0.2 mg/mL) was dissolved in Tris-HCl buffer (0.1 M, pH 8.4) containing urea (8 M) and 2-mercaptoethanol (0.25 mM). Unfolding was carried out at 23 °C and time-course unfolded samples were trapped with 4% TFA, followed by HPLC analysis. Slow chemical modification of scrambled species by the cyanate in the urea solution(s) accounts for the change in the pattern observed between the 15 min and 16 h samples.

is probable that the cyanate-induced carbamylation of lysine residues spread in various locations in the three-dimensional structure of CTX III (*26*) subtly alters the local microenvironment (hydrophobicity) resulting in difference(s) in the retention times of $\mathbf{N^A}$ and $\mathbf{N^B}$ species upon reversed-phase HPLC. It is known that minor chemical modifications of side-chain groups in proteins, such as deamidination also causes significant differences in the retention times in reversed-phase HPLC (*32*).

GdmCl is more potent than urea. CTX III unfolds abruptly at 2−3 M of GdmCl and 4−5 M of urea, respectively. The compositions of scrambled CTX III generated by GdmCl and urea are similar but not identical. Comparison of their difference needs to exclude the complication of cyanate modification. The true difference is best illustrated by comparing the 5 min, 8 M urea unfolded scrambled species (Figure 3) with the 4 M GdmCl produced scrambled CTX-III (Figure 1). There are 104 possible scrambled isomers of CTX III. It is not known how many of them populate in the unfolded state of CTX III. However, in four different cases of 3-disulfides containing proteins, an average of 80% of all possible scrambled isomers have been detected (*17*, *19*, *33*).

Kinetics of CTX-III Unfolding. Conditions wherein it is possible to denature CTX III quantitatively were further compared for their kinetics to unfold the native CTX III. Denaturing conditions include 6−8 M of urea and 4−8 M of GdmCl (see Figure 4, panels A and B). In the solution containing 8 M urea, the unfolding of CTX III completed within 10 min. The rate constant of unfolding at 8 M urea is about 2.8- and 10-fold greater than those performed in 7 and 6 M urea, respectively (Figure 4, panel C). By contrast, 5 M GdmCl was found to be kinetically more effective than 6−8 M GdmCl. The rate constant of CTX III unfolding at 5 M GdmCl was nearly 3.5-fold greater than that carried out at 8 M GdmCl (Figure 4C). The results also reproducibly demonstrate that 8 M urea is more effective than the optimized concentration of GdmCl (5 M) in unfolding CTX III.

Reduction of the Native and Scrambled CTX III in the Absence of Denaturant. When the native CTX III was treated with strong reducing agent (DTT) in the absence of denaturant, reduction of its four disulfide bonds proceeded via an all-or-none mechanism without appreciable accumulation of 3-, 2- or 1-disulfide species (Figure 5, left panel). This is observed with all time-course trapped intermediates

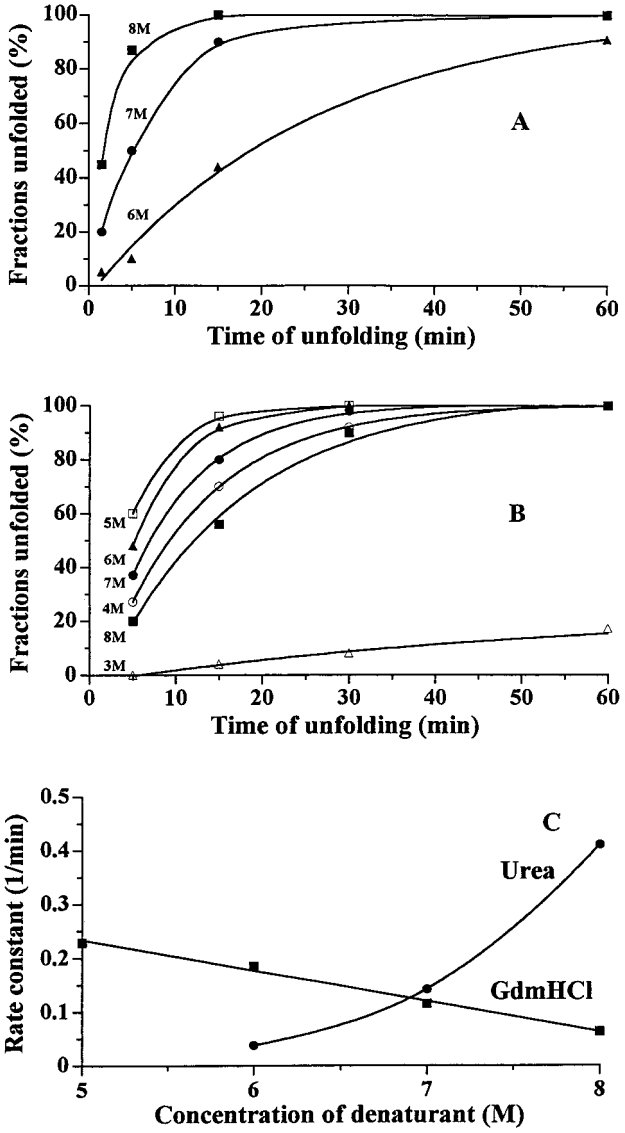

FIGURE 4: Kinetics of unfolding of CTX III at different concentrations of the denaturants. (A) Unfolding in urea, (B) unfolding in GdmCl, and (C) depicts the change(s) in the rate constant of the unfolding process of CTX III under various denaturant conditions. The unfolding conditions are the same as described in the legend of Figure 3.

at DTT concentration ranging from 2 to 50 mM. Reduction of the native CTX III in 50 mM DTT was completed within 30 min. In contrast, the disulfide bonds of scrambled CTX III could be effectively reduced with very low concentration of the reducing agent. For instance, at 0.5 mM of DTT, complete reduction of scrambled CTX III was achieved within 3 min (Figure 6). However, under these conditions, the native species remained practically intact.

Further experiments were performed to compare the relative stability (disulfide bonds) of various intermediates of CTX III formed during refolding. A mixture of CTX III consisting of the native species (**N**), scrambled species (collectively underlined as in Figure 5), and cyanate-modified native CTX III ($\mathbf{N^A}$ and $\mathbf{N^B}$) was used as the starting material (see Figure 5, right panel). Reduction was carried out using different concentrations of DTT (0.2−15 mM), and each condition was analyzed in a time-course manner in order to examine the kinetics of reduction of different species. These experiments demonstrate that the native CTX III and cyanate

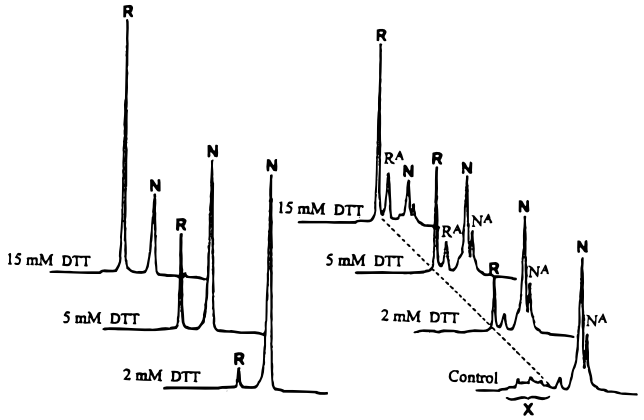

FIGURE 5: (Left) Reduction of the native CTX III in the absence of denaturant. CTX III (0.2 mg/mL) was reduced in the Tris-HCl buffer (0.1 M, pH 8.4) containing 2 mM, 5 mM, and 15 mM of DTT, respectively. The reactions were performed at 23 °C for 30 min and quenched with 4% TFA. Only the native species and the fully reduced species were detected in those samples. (Right) Analysis of the relative stability of various CTX III species. The control sample consists of the native species (**N**), the cyanate modified species ($\mathbf{N^A}$) and scrambled species (collectively underlined as **X**). This control sample was prepared by treating the native CTX III with 3 M urea and 0.25 mM 2-mercaptoethanol at 23 °C for 16 h. Urea and 2-mercaptoethanol were removed from the protein and the protein was then reduced in Tris-HCl buffer (0.1 M, pH 8.4) using 2, 5, and 15 mM of DTT for 30 min. **R** and $\mathbf{R^A}$ are fully reduced species of **N** and $\mathbf{N^A}$, respectively. The minor fraction of cyanate modified species ($\mathbf{N^B}$) is not marked here. $\mathbf{R^B}$, $\mathbf{R^A}$, and $\mathbf{N^B}$ are eluted at the same position.

modified CTX III ($\mathbf{N^A}$ and $\mathbf{N^B}$) possess nearly identical stability against DTT reduction. This is exemplified by their constant ratio throughout the process of reduction as shown in Figure 5.

Refolding of Scrambled CTX III. Scrambled CTX III refold spontaneously to form the native structure in alkaline buffer containing appropriate thiol catalyst. The process of refolding involves reshuffling of the non-native disulfide bonds and regrouping of the composition of scrambled isomers. Despite the heterogeneity of scrambled CTX III, a progressive transformation of the pattern of scrambled species is clearly visible along the pathway of refolding (Figure 6). For instance, one of the most predominant fraction of GdmCl unfolded CTX III (marked as "a") disappeared rapidly during the very early stage of folding. If the cases of hirudin and tick anticoagulant peptide are any indication (*17, 19*), this predominant fraction of scrambled CTX III may contain species which adopts the four-beads form of disulfide structure. The four-beads form isomer contains disulfides that are formed by four pairs of neighboring cysteines. Among the 104 scrambled isomers, it possesses the smallest combined size of disulfide loops and presumably represents the most extensively unfolded species.

Two sets of scrambled CTX III were analyzed. The GdmCl unfolded CTX III was able to refold to form the native species quantitatively (top panel of Figure 6). On the other hand, urea unfolded CTX III refolds and converts to the native species (**N**) as well as cyanate modified native species ($\mathbf{N^A}$ and $\mathbf{N^B}$). These results thus demonstrate that cyanate modification does not influence the efficiency of folding of scrambled CTX III.

The kinetics of refolding is strongly dependent upon, the redox potential of the thiol reagent. Using 2-mercaptoethanol

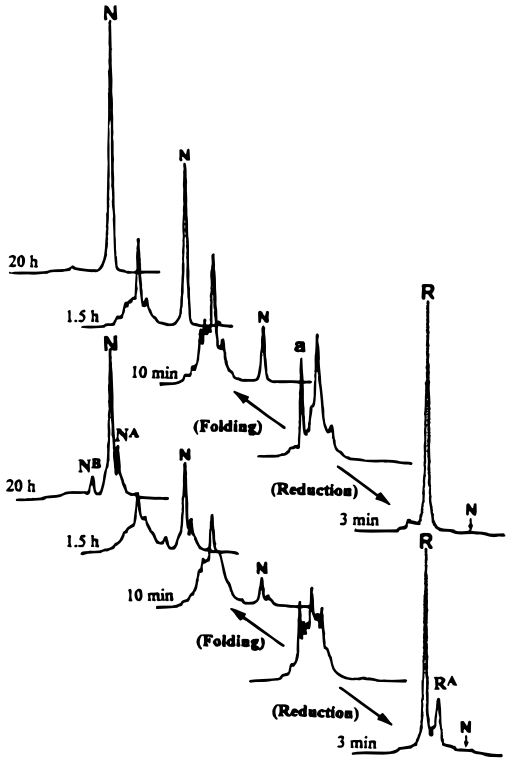

FIGURE 6: Folding and reduction (reductive unfolding) of scrambled CTX III. Two sets of scrambled CTX III, denatured by 6 M GdmCl (top panel) and 6 M urea (bottom panel), were analyzed. The folding was performed in Tris-HCl buffer (0.1 M, pH 8.4) containing 2-mercaptoethanol (0.25 mM) as thiol reagent. Reduction was carried out in the same buffer with DTT (0.5 mM). The protein concentration was 0.2 mg/mL. The intermediates of both folding and reduction (unfolding) were trapped with 4% TFA in a time course manner. Reduction of scrambled CTX III proceeds through undergoes heterogeneous intermediates (not shown) and completes within 3 min. **N** indicates the native species. $\mathbf{N^A}$ and $\mathbf{N^B}$ are cyanate modified CTX III with native disulfide bonds. **R** and $\mathbf{R^A}$ are fully reduced species of **N** and $\mathbf{N^A}$, respectively.

(0.25 mM) as catalyst, the rate constant of refolding of scrambled CTX-III was found to be 0.0052 ± 0.0004 min^{-1}. Additional thiol catalysts have been investigated as well. Cysteine (1 mM) is indistinguishable from GSH (1 mM) and both are about 2-fold more effective than 2-mercaptoethanol (0.25 mM) in promoting the folding of scrambled CTX III.

DISCUSSION

In studying the folding pathway of disulfide containing proteins, the protein has to be unfolded first. The most commonly adopted method is to treat the protein with strong denaturant and a reducing agent (*15, 34*). The fully reduced and denatured protein is then allowed to refold and reoxidize, and the folding pathway is identified by trapping and analyzing the partially reoxidized intermediates (e.g., 1- and 2-disulfide species) (*6, 12, 35*). An alternative is to denature the protein in the absence of reducing agent. In this case, all native disulfide bonds remain intact during the reversible process of unfolding and refolding (*36, 37*). Both methods have been widely applied to elucidate the folding mechanism of proteins that are stabilized by disulfide bridges (*12, 14, 18, 19, 38*−*40*). A third approach is permiting unfolding and refolding to proceed via shuffling and reshuffling of the native and non-native disulfide bonds. In the presence of a denaturant and a thiol catalyst, the native protein unfolds by

shuffling its native disulfides and converts to a mixture of scrambled species (*19*). All scrambled species contain the intact number of disulfide bonds but are comprised of at least two non-native disulfide linkages. Upon the removal of denaturant, scrambled proteins reshuffle their non-native disulfides and refold spontaneously back to the native state (*17, 19*). The process of disulfide reshuffling that leads to the formation of the native structure represents a unique picture of protein folding that is distinguished from those observable by conventional folding techniques. This novel method is applied here to analyze the unfolding and refolding mechanism of CTX III. A major limitation in the case of CTX III which contains four disulfide bonds, is the number of scrambled species. In CTX III, there exist 104 possible non-native scrambled isomers. Even if only a fraction of them populate in the unfolded state of CTX-III, their separation and structural characterization would still be a daunting task. The HPLC chromatogram clearly indicates the presence of highly heterogeneous species of scrambled CTX III. Nonetheless, the results have revealed a number of crucial properties of CTX III, and some of them have not been fully exploited.

One unusual property of CTX III is that it contains highly reactive lysines that are readily modified by the trace amounts of cyanate contaminant present in the urea solution (*41*). This side reaction occurs in CTX III both in the native and the denatured (scrambled) states. Cyanate modification of CTX III was observed with high purity grade of urea obtained from several different suppliers. Those same batches of urea, however, had no effect on four other proteins which were investigated in our laboratory, suggesting that the lysines of CTX III are uniquely reactive (*42*). Furthermore, cyanate modification is nonspecific and distributes randomly among all the lysine residues in CTX III. In light of this heterogeneity, it is particularly interesting to notice that those heterogeneous species of modified CTX III display properties of stability and folding which are indistinguishable from that of the native CTX III.

Another unique property of CTX III is that the native species exhibits a longer retention time than any other denatured species on reversed-phase HPLC. This phenomenon is remarkable because, for most proteins, the native structure is typically more hydrophilic than unfolded (denatured) structures and usually eluted faster on the HPLC with mechanism of separation based primarily on hydrophobic interaction(s). The abnormal behavior of CTX III could be attributed to the presence of lysyl residues. Basic amino acids, such as lysine and arginine, despite their hydrophilicity, are known to exhibit prolonged retention time(s) due to their interaction with the silica based support of reversed-phased chromatography. CTX III is a basic protein with high content of lysine. In the native structure of CTX III, most lysines are found to locate on the surface of the protein (*26*) and their interaction with the silica based support may account for this anomalous property of CTX III.

Unfolding experiments demonstrate that GdmCl is about 2-fold more potent than urea in unfolding CTX III, as have been found in many different proteins (*41, 43*). However, the results of the kinetics of unfolding is puzzling. First, there exists an optimized concentration of GdmCl. Five molar GdmCl is the most effective concentration to unfold the native CTX III. Kinetically, 5 M GdmCl is about 3.5 times more effective than 8 M GdmCl. CTX III is not alone in displaying this property. An optimized concentration of GdmCl was also required in the case of denaturation of tick anticoagulant peptide (Chang, unpublished data). Second, urea (8 M) is actually found to be more effective than 5 M GdmCl in unfolding CTX-III, despite the fact that urea is about 2-fold less potent (equilibrium constant) than GdmCl as the denaturant (Figure 1). The underlying mechanism of this discrepancy is still unclear. GdmCl and urea have been routinely used as protein denaturants, and significant differences have been pointed out in the extent of unfolding of protein(s) achieved in these denaturants (*44, 45*). GdmCl and other charged denaturants have been shown to have some stabilizing effects on proteins due to charge−charge interactions (*46*−*48*). Hagihara et al. (*48*) have recently demonstrated that GdmCl at low concentrations refolds acid-unfolded apomyoglobin and cytochrome *c*, stabilizing the molten globule state. These authors envisage that GdmCl serves as sources of anions (chloride anions), which bind to the positively charged groups of the protein resulting in the screening of the electrostatic repulsion's that leads to unfolding. Goto et al. (*50*) and Palleros et al. (*51*), studying the effect(s) of various anions in proteins, unequivocally demonstrated that the freely ionizing acids could be potential sources of anions, which in-turn can substantially stabilize proteins by predominantly interacting with positively charged centers in proteins. In a related study, Goto and Aimoto (*52*) using an amphiphilic peptide showed that the peptide in the presence of NaCl adopts a monomeric conformation in which two amphiphilic α-helices interact with each other intramolecularly through hydrophobic residues. The results of this study imply that binding of the anions to the positively charged groups leads to increased hydrophobic interactions among nonpolar residues in peptides/proteins, promoting stabilization. It is quite possible that a similar phenomenon occurs during the unfolding of CTX III in GdmCl. Snake venom cardiotoxins, in general, are highly basic proteins (pI > 10.5) and have been shown to contain dense cationic clusters comprising of positively charged residues on the surface of the molecule (*53*). It is quite possible that the chloride anions contributed by GdmCl could partially stabilize the protein structure by binding to the positively charged clusters in the protein resulting in the delay in the unfolding process. This aspect could account for the slower rates of unfolding of CTX III in GdmCl. It is pertinent to mention here that the unfolding experiments with tick anticoagulant peptide (another basic protein) showed that rates of unfolding in GdmCl are slower as compared to that in urea (unpublished results).

Native CTX-III, upon reduction with DTT in the absence of a denaturant, reduction of the four disulfide bonds undergoes an all-or-none mechanism without significant accumulation of partially reduced species as intermediates. This mechanism is consistent with those observed in the cases of hirudin, tick anticoagulant peptide, and ribonuclease A (*32*). The disulfide stability of CTX III (against reduction) is also comparable to that of tick anticoagulant peptide.

Finally, folding experiments of scrambled CTX III reveal a dynamic reshuffling of the non-native disulfides and reorganization of scrambled isomers along the process of folding. Unfortunately, the complexity of scrambled species

and the absence of their structural information have limited further interpretation of these data. More knowledge about the folding of scrambled CTX III may be uncovered through mass spectrometry (*54*, *55*) and NMR (*3*, *4*) analysis. The application of electrospray mass spectrometry will be particularly useful in the case of CTX III. The abundance of lysyl residues in CTX III should provide valuable probes for the analysis of charge distribution and the state of protein's conformation (*56*) along the folding pathway of scrambled CTX III.

Recently, there have been attempts to express snake venom cardiotoxins in bacterial host systems (*57*−*59*). However, invariably, in all the cases, the expressed protein (CTX III) was trapped as inclusion bodies, rendering it difficult to obtain high yields of the protein. It is known that proteins trapped as inclusion bodies exist in partially structured states with mismatched disulfide bridges with structural features resembling the scrambled disulfide intermediates (*60*−*62*). We believe that the results of the present study on the experimental conditions optimal for realization of the native state from the scrambled species could provide useful leads to recover the cardiotoxins in their native forms from the Inclusion bodies. In addition, understanding the disulfide folding pathway of CTX III would provide clues to refold other homologous and pharmacologically important constituents of snake venoms such as the neurotoxins.

REFERENCES

1. Anfinsen, C. B. (1973) *Science 181*, 223−230.
2. Levinthal, C. (1968) *J. Chim. Phys. 65*, 44−45.
3. Udgaonkar, J. B., and Baldwin, R. L. (1988) *Nature 335*, 694−699.
4. Roder, H., Elöve, G., Englander, S. W. (1988) *Nature 335*, 700−704.
5. Creighton, T. E. (1986) *Methods Enzymol. 131*, 83−106.
6. Creighton, T. E. (1990) *Biochem. J. 270*, 1−16.
7. Givol, D., Delorenzo, F., Goldberger, K. F., and Anfinsen, C. B. (1965) *Proc. Natl. Acad. Sci. U.S.A. 53*, 676−684.
8. Creighton, T. E. (1992) *Science 256*, 111−112.
9. Creighton, T. E., Darby, N. J., and Kemmink, J. (1996) *FASEB J. 10*, 110−118.
10. Goldenberg, D. P. (1992) *Trend. Biochem. Sci. 17*, 257−261.
11. Weissman J. S., and Kim, P. S. (1995) *Nature Struct. Biol. 2*, 1123−1130.
12. Weissman, J. S., and Kim, P. S. (1991) *253*, 1386−1393.
13. Weissman J. S., and Kim, P. S. (1992) *Science. 256*, 112−114.
14. Chatrenet, B., and Chang, J.-Y. (1993) *J. Biol. Chem. 268*, 20988−20996.
15. Chang, J.-Y., Canals, F., Schindler, P., Querol, E., and Aviles, F. X. (1994) *J. Biol. Chem. 269*, 22087−22094.
16. Chang, J.-Y., Schindler, P., Ramseier, U., and Lai, P. H. (1995) *J. Biol. Chem. 270*, 9207−9216.
17. Chang, J.-Y. (1995) *J. Biol. Chem. 270*, 25661−25666.
18. Chang, J.-Y. (1994) *Biochem. J. 300*, 643−650.
19. Chang, J.-Y. (1996) *Biochemistry. 36*, 11702−11709.
20. Kumar, T. K. S., Lee, C. S., and Yu, C. (1996) *Adv. Exptl. Med. Biol. 391*, 115−129.
21. Kumar, T. K. S., Jayaraman, G., Lee, C. S., Arunkumar, A. I., Sivaraman, T., Samuel, D., and Yu, C. (1997) *J. Biomol. Struct. Dyn. 15*, 431−463.
22. Kini, R. M., and Evans, H. J. (1989) *Biochemistry 28*, 2403−2423.
23. Sun, Y. J., Wu, W. G., Chaing, C. M., Hsin, A. Y., and Hsiao, C. D. (1997) *Biochemistry 28*, 2403−2413.
24. Bhaskaran, R., Yu, C., and Yang, C. C. (1994) *J. Protein Chem. 13*, 503−504.
25. Yu, C., Bhaskaran, R., and Yang, C. C. (1994) *J. Toxin Toxicol. Rev. 13*, 291−315.
26. Bhaskaran, R., Huang, C. C., Chang, D. K., and Yu, C. (1994) *J. Mol. Biol. 235*, 1291−1301.
27. Kumar, T. K. S., Jayaraman, G., Lee, C. S., Sivaraman, T., Lin, W. Y., and Yu, C. (1995) *Biochem. Biophys. Res. Commun. 207*, 536−543.
28. Sivaraman, T., Kumar, T. K. S., Jayaraman, G., Han, C. C. and Yu, C. (1997) *Biochem. J. 321*, 457−464.
29. Yang, C. C., King, K., and Sun, T. P. (1981) *Toxicon 19*, 135−138.
30. Chang, J.-Y., and Knecht, R. (1991) *Anal. Biochem. 197*, 52−58.
31. Boernsen, K. O., Schaer, M., and Widmer, M. (1990) *Chimia 44*, 412−416.
32. Wearne, S. J., and Creighton, T. E. (1989) *Proteins 5*, 8−12.
33. Chang, J.-Y. (1997) *J. Biol. Chem. 272*, 69−75.
34. Jaenicke, R., and Rudolph, R. (1990) in *Protein Structure* (Creighton, Ed.) pp 191−224.
35. Scheraga, H. A., Konishi, Y., and Ooi, T. (1984) *Adv. Biophys. 18*, 21−41.
36. Radford, S. E., Dobson, C. M., and Evans, P. A. (1992) *Nature 358*, 302−307.
37. Houry, W. A., Rothwarf, D. M., and Scheraga, H. A. (1995) *Nat. Struct. Biol. 2*, 495−503.
38. Anfinsen, C. B., Haber, E., Sela, M., and White, F. H., Jr. (1961) *Proc. Natl. Acad. Sci. U.S.A. 47*, 1309−1515.
39. Haber, E., and Anfinsen, C. B. (1962) *J. Biol. Chem. 237*, 1839−1843.
40. Creighton, T. E. (1979) *J. Mol. Biol. 129*, 411−431.
41. Pace, N. C. (1986) *Methods Enzymol. 131*, 266−282.
42. Gatineau, E., Takechi, M., Bouet, F., Mansuelle, P., Rochat, H., Harvey, A. L., Montenay-Garestier, Th., and Menez, A. (1990) *Biochemistry 29*, 6480−6489.
43. Greene, R. F., and Pace, N. C. (1974) *J. Biol. Chem. 249*, 5488−5495.
44. Smith, L. J., Fiebig, K. M., Schwalbe, H., and Dobson, C. M. (1996) *Folding Des. 1*, 95−106.
45. Schwalbe, H., Fiebig, K. M., Buck, M., Jones, J. A., Grimshow, S. B., Spencer, A., Glaser, S. J., Smith, L. J., and Dobson, C. M. (1997) *Biochemistry 36*, 8977−8991.
46. Tsong, T. Y. (1975) *Biochemistry 14*, 1542−1547.
47. Ahmad, F., and Bigelow. J. (1979) *J. Mol. Biol. 131*, 607−617.
48. Hagihara, Y., Aimoto, S., Fink, A. L., and Goto, Y. (1993) *J. Mol. Biol. 231*, 180−184.
49. Rao, N. M., and Nagaraj, R. S. (1991) *J. Biol. Chem. 266*, 5018−5024.
50. Goto, Y., Takahashi, N., and Fink, A. L. (1990) *Biochemistry 29*, 3480−3488.
51. Goto, Y., and Aimoto, S. (1992) *J. Mol. Biol. 218*, 387−396.
52. Palleros, P. R., Shi, L., Reid, K. L., and Fink, A. L. (1993) *Biochemistry. 32*, 4314−4321.
53. Jang, J. Y., Kumar, T. K. S., Jayaraman, G., Yang, P. W., and Yu, C. (1997) *Biochemistry 36*, 14635−14641.
54. Miraz, U. A., Cohen, S. L., and Chait, B. T. (1993) *Anal. Chem. 65*, 1−6.
55. Miranker, A., Robinson, C. V., Radford, C. V., and Dobson, C. M. (1996) *FASEB J. 10*, 93−101.
56. Chowdhury, S. K., Katta, V., and Chait, B. (1990) *J. Am. Chem. Soc. 112*, 9012−9013.
57. Kumar, T. K. S., Yang, P. W., Lin, S. H., Wu, C. Y., Lei, B., Lo, S. J., Tu, S. C., and Yu, C. (1996) *Biochem. Biophys. Res. Commun. 219*, 450−456.
58. Chi, L. M., Vyas, A. A., Rule, G. S., and Wu, W. G. (1995) *Toxicon 32*, 1679−1683.
59. Chang, L. S., Wu, P. L., and Lin, J. (1996) *Biochem. Biophys. Res. Commun. 217*, 116−118.
60. Marston, F. A. O. (1986) *Biochem. J. 240*, 1−14.
61. Speed, M. A., Wang, D. I. C., and King, J. (1996) *Nat. Struct. Biol. 14*, 1283−1287.
62. Speed, M. A., Wang, D. I. C., and King, J. (1996) *Protein Sci. 6*, 99−100.

BI9714565