

Events in the Kinetic Folding Pathway of a Small, All β -Sheet Protein*

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The folding of cardiotoxin analogue III (CTX III), a small (60 amino acids), all β -sheet protein from the venom of the Taiwan Cobra (*Naja naja atra*) is here investigated. The folding kinetics is monitored by using a variety of techniques such as NMR, fluorescence, and circular dichroism spectroscopy. The folding of the protein is complete within a time scale of 200 ms. The earliest detectable event in the folding pathway of CTX III is the formation of a hydrophobic cluster, which possess strong affinity to bind to nonpolar dye such as 1-anilino-8-naphthalene-sulfonic acid. Quenched-flow deuterium-hydrogen exchange experiments indicate that the segment spanning residues 51–55 along with Lys²³, Ile³⁹, Val⁴⁹, Tyr⁵¹ and Val⁵² could constitute the “hydrophobic cluster.” Folding kinetics of CTX III based on the amide-protection data reveals that the triple-stranded, antiparallel β -sheet segment, which is located in the central core of the molecule, appears to fold faster than the double-stranded β -sheet segment.

The protein folding pathway is the kinetic process in which disordered polypeptide chain proceeds to fold into its highly ordered native structure (1). Folding of a polypeptide chain into its unique three-dimensional structure is believed to be encoded in its amino acid sequence (2). The transformation of the unfolded chain to its native state obviously does not involve a random search of the conformational space, because such a process would require too much of time (3). Most proteins are known to fold within a time span of a few minutes if not seconds (4). Therefore, it is obligatory that the initial events in protein folding should limit the conformational space and thus specify the pathway for unfolded polypeptide chain to attain its native state. Hence, it is important to understand the structural interactions that come into play during the early events of folding (5, 6). These interactions, consequently, predetermine the three-dimensional structure of the protein.

Folding to the native conformation is a highly cooperative process for small, single domain proteins (7). Although, transient intermediates have been observed during the folding process, it is still not clear at what stage the cooperativity in the folding process arises. Characterization of early folding intermediates provides vital clues(s) to understand the cooperativity phenomenon observed in the folding of small proteins.

Recently, with the advent of powerful techniques such as the quenched-flow deuterium-hydrogen exchange in conjunction with two-dimensional NMR experiments, it has become feasible to characterize the transient intermediates that occur in the early stages of folding (8–10). The quenched-flow hydrogen-deuterium exchange technique is particularly powerful, because it permits many specific sites within a protein to be probed on the millisecond time scale (11). This technique has been successfully used to study the folding pathway of several proteins (8). Interestingly, most of these proteins are composed of helical conformation or possess both α -helical and β -sheet segments. Roder *et al.* (11), studying the early folding events in cytochrome C, a predominantly helical protein, found that helix formation occurs very quickly within the low millisecond time scale. The fast time scales of helix formation (in cytochrome C) are not surprising, because the hydrogen bonding interactions involved in helix formation are local. Since hydrogen bonding in β -sheet formation involves interactions between distant parts of the polypeptide chain, it is expected that the folding rates of β -sheet segments would be slower than α -helices. In this context, we investigate the early folding events in the folding pathway of a small, all β -sheet protein such as cardiotoxin analogue III (CTX III)¹ from the Taiwan cobra (*Naja naja atra*).

CTX III isolated from the venom of the Taiwan cobra (*N. naja atra*) is a 60-amino acid, highly basic, all β -sheet protein (12–14). The three-dimensional structure of CTX III shows that the protein is “three-finger” shaped with three loops projecting from a globular head (14, 15) as shown in Fig. 1. The protein is cross-linked by four disulfide bonds. The secondary structure of CTX III (14) includes five β -strands arranged to form double and triple-stranded antiparallel β -sheets (Fig. 1). In the native state, CTX III does not show any helical segments, and interestingly, secondary structure prediction analysis on the amino acid sequence of CTX III shows that no portion(s) of the protein has any propensity to adopt a helical conformation. Thus, CTX III is an ideal choice to understand the early folding events in an all β -sheet protein.

In the present study, the kinetic folding pathway of CTX III is examined using a variety of techniques such as stopped-flow circular dichroism, stopped-flow fluorescence, and quenched-flow deuterium-hydrogen exchange. The results obtained herein are consistent with the hydrophobic collapse model of protein folding.

EXPERIMENTAL PROCEDURES

Materials—CTX III was purified as per the method reported earlier (14). 1-Anilino-8-naphthalene sulfonic acid (ANS) was purchased from

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¹ The abbreviations used are: CTX III, cardiotoxin analogue III; ANS, 1-anilino-8-naphthalene sulfonic acid; GdnHCl, guanidine hydrochloride; TFE, 2,2,2-trifluoroethanol.

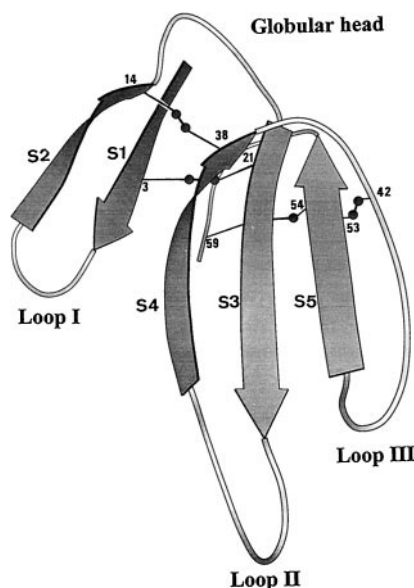


FIG. 1. MOLSCRIPT representation of the structure of CTX III showing the overall solution backbone folding. The ribbon arrows represent regions of the peptide backbone in the β -sheet conformation. S1–S5 represent the various β -strands (strand I to strand V) in the structure of CTX III. The disulfide bonds in the protein are located between residues 3 and 21, 14 and 38, 42 and 53, and 54 and 59.

Sigma. Guanidine hydrochloride (GdnHCl, ultrapure) was purchased from Merck, Germany. All other chemicals used were of high quality. Carboxymethylated CTX III was prepared as per the procedure reported by Goldberg *et al.* (16).

GdnHCl-induced Denaturation—Equilibrium unfolding was monitored by far-UV and near-UV CD as a function of GdnHCl concentration. CD measurements were made on a Jasco J720 spectropolarimeter. CD spectra were collected with the slit width set to 430 μ m, a response time of 1 s, and a scan speed of 20 nm/min. Each spectrum was an average of at least five scans. Secondary structure measurements were made at 213 nm with protein concentrations of 160 μ M in a 0.02-cm path length cuvette. Near-UV CD measurements at 270 nm were carried out using a protein concentration of 160 μ M with a cuvette with a path length of 0.1 cm. The sample temperature was maintained using a Neslab RTE-110 circulating water bath at 25 ± 0.2 °C.

Data Analysis—Equilibrium unfolding data obtained using GdnHCl as denaturant were converted to plots of F_U , the fraction of protein in the unfolded state, versus denaturant concentration using the equation,

$$F_U = (X_O - (X_F + m_F[D])) / ((X_U + m_U[D]) - (X_F + m_F[D])) \quad (\text{Eq. 1})$$

where X_O is the value of the spectroscopic property measured at denaturant concentration [D]. X_F and X_U represent the intercepts, and m_F and m_U the slopes of the folded and unfolded base lines of the data, respectively, and were obtained from linear least squares fits of the base lines.

For a two-state $F \rightleftharpoons U$ unfolding pathway, the free energy of unfolding by denaturant (ΔG_U) at concentration [D] is related to F_U by a transformation of the Gibbs-Helmholtz equation in which the equilibrium constant of unfolding in the transition zone, $k_{app} = F_U / (1 - F_U)$, $F_U = e^{-\Delta G_U/RT} / (1 + e^{-\Delta G_U/RT})$. It is assumed that the free energy of unfolding, ΔG_U , has a linear dependence on the concentration of the denaturant [D],

$$\Delta G_U = \Delta G(H_2O) + m_G[D] \quad (\text{Eq. 2})$$

wherein $\Delta G(H_2O)$ and m_G are the intercept and slope, respectively, of the plot of ΔG_U versus the concentration of the denaturant. m_G is the measure of the cooperativity of the unfolding

reaction and $\Delta G_U(H_2O)$ is the free energy difference between the folded and unfolded states in the absence of any denaturant.

Stopped-flow Fluorescence Measurements—All stopped-flow fluorescence measurements were performed at 5 ± 0.2 °C using a SF-61 stopped-flow spectrofluorimeter (Hi-Tech Scientific Co.). The concentration of ANS was varied between 50 and 250 μ M by appropriately mixing the stock solution of ANS (300 μ M) in the native buffer (10 mM sodium acetate buffer, pH 3.0). 1.6 mM of equilibrium-unfolded CTX III (in 6 M GdnHCl) was diluted 10-fold into 270 μ l of the ANS-containing native buffer. This procedure ensured that the concentrations of the protein (160 μ M) and GdnHCl (0.6 M) were unchanged during the refolding reaction in the presence of different concentrations of ANS. The binding of ANS was monitored by setting the excitation and emission wavelengths at 418 and 497 nm, respectively. The bandwidth used in all the measurements was 2 nm. The dead time of stopped-flow mixing device was 4.0 ms.

Stopped-flow CD Measurements—Stopped-flow CD measurements were carried out using a stopped-flow apparatus from Biologic attached to a Jasco J-720 spectropolarimeter. The refolding reaction was initiated by a 10-fold dilution of the GdnHCl-denatured CTX III sample. The path length of the optical cell used for all CD measurements was 1 mm. All data were acquired at 5 °C, and the change in ellipticity at appropriate wavelengths (213 and 270 nm) was monitored as a function of time. The kinetic data were expressed as a sum of exponential terms. Nonlinear least squares curve fitting was used to obtain the number of exponential phases with their amplitudes and apparent rate constants.

Quenched-flow Experiments—All experiments were carried out at 5 ± 0.1 °C using a RQF-63 rapid mixing quenched-flow apparatus (Hi-Tech Scientific Co.). Complete denaturation and exchange of the backbone amide protons with deuterium was achieved by dissolving CTX III (30 mg/ml) in 6 M deuterated guanidine hydrochloride in D_2O at pH 6.6 ± 0.2 . Deuterated guanidine hydrochloride was obtained through repeated cycles of dissolving ultrapure GdnHCl in D_2O followed by repeated lyophilization. Refolding of the denatured protein was initiated by 10-fold dilution with 50 mM glycine- d_5 (pH 3.0) in H_2O . At this pH, negligible labeling occurred. After variable refolding times ranging from 9.8 to 190.4 ms, the solution was diluted again to 10 times the initial protein volume with 0.2 M sodium borate (pH 9.6) to initiate labeling of the deuterated amides in CTX III with protons. After a lapse of 10 ms, the labeling pulse was stopped by a further 3.4-fold dilution of the initial protein volume with 1 M HCl in water. The final pH was about 3.2, at which the hydrogen/deuterium exchange (for the amides in native CTX III) was minimal. It should be mentioned that there were no signs of intermolecular aggregation of the protein during any of the refolding steps in the quenched-flow experiment. This was ensured from the steady state CD (in the far UV region) and 1H one-dimensional NMR spectra of the refolded protein sample. It should be mentioned that absorbance of the refolded protein sample(s) at 300 nm also did not indicate aggregation of the protein during refolding. After the final step of refolding, the protein samples were concentrated by ultrafiltration at 4 °C.

A magnitude COSY spectrum of each sample was recorded on a 600-MHz NMR spectrometer at 5 ± 0.1 °C. 256 increments over 1024 data points were collected. All of the NMR data were processed using UXNMR software on a Silicon Graphics workstation. The intensities of the intraresidue ($C^{\alpha}H$, NH) cross-peaks were normalized based on the aromatic cross-peak ($C^{\delta}H$, $C^{\epsilon}H$) intensities of Phe²⁵ and Tyr²². The proton occupancy of individual amides at the zero time point of refolding were

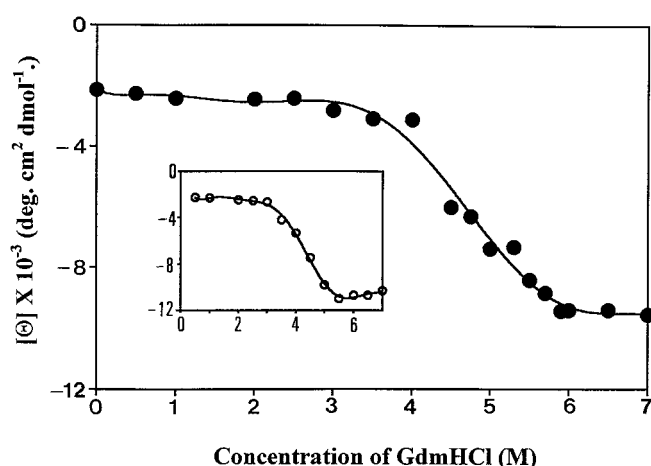


FIG. 2. **GdnHCl-induced denaturation of CTX III at pH 6.8.** Denaturation was monitored by measurement of mean residue ellipticity at 213 nm. The *inset* depicts the renaturation profile of CTX III denatured by 6 M GdnHCl using the 213-nm mean residue ellipticity.

obtained by a 10-fold dilution of the 6 M GdnHCl-denatured CTX III (in D_2O) with the pulse labeling borate buffer in H_2O at pH 9.6. It is important to mention that the first step of initiation of folding (without the exchange) at pH 3.6 using HCl-glycine buffer used at other refolding time points is eliminated for the zero time point proton occupancy estimation. The pulse labeling step using the borate buffer (pH 9.6) process ensures exchange of all deuterium atoms by protons and also the protein folds back to its native state. The intensities of the cross-peaks of the residues in the fingerprint region of the COSY spectrum at this time point (the zero time point) represent the initial proton occupancy. It is pertinent to mention that the relative percentage proton occupancy of individual residues at the zero time point (and in subsequent refolding times) was estimated by comparing with the amide proton intensities of the corresponding residues in the fingerprint region of the COSY spectrum of the protein dissolved in D_2O at pH 3.6. The intensities of the cross-peaks of the individual amide protons of the native protein dissolved in D_2O at pH 3.6 are assumed as 100%. The time courses of change in proton occupancies were fitted to a single exponential decay ($y = A \exp^{-kt} + C$, where A is the amplitude of the phase, k is the apparent rate constant, and C is the final amplitude) by the Levenberg-Marquardt nonlinear least squares method, yielding rate constants and phase amplitudes in the kinetic folding experiment. All data analysis were performed using Kaleidagraph software (Synergy Software).

RESULTS

Equilibrium Unfolding—Equilibrium unfolding as a function of GdnHCl concentration was monitored by far and near UV CD spectroscopy. CTX III is an all β -sheet protein lacking in tryptophan residues. Hence, the ellipticity changes at various concentrations of GdnHCl were examined at 213 nm (far UV region) and 270 nm (near UV region). It was ensured that the protein is completely unfolded at 6 M GdnHCl. The ellipticity changes can be fitted to a two-state model (native $\rightleftharpoons$ unfolded) for unfolding (Fig. 2). Fig. 2 shows the equilibrium denaturation curve obtained for CTX III at pH 6.8, 25 $^{\circ}C$, using GdnHCl as the denaturant. In both far UV (213 nm) and near UV (270 nm), CD gave nearly superimposable denaturation curves, and therefore the unfolding process of CTX III has been assumed to fit to a two-state model (data not shown). The free energy of unfolding in the absence of denaturant was obtained by linear extrapolation of $\Delta G(D)$ to zero denaturant concentration. The values obtained for $\Delta G_U(H_2O)$ from the GdnHCl de-

naturation profile monitored at 213 and 270 nm were similar within experimental error. The m_G value, which is a measure of the cooperativity of the unfolding reaction, was found to be 0.67 kcal $\cdot$ mol $^{-1}\cdot$ M $^{-1}$. The concentration of denaturant (C_m) at which the protein (CTX III) is half-unfolded (when $\Delta G_U = 0$) is estimated to be 4.75 M, by monitoring the changes in ellipticity at 213 nm. The renaturation of CTX III from the 6.0 M GdnHCl obtained by monitoring the ellipticity changes at 213 nm is depicted in the *inset* of Fig. 2. It can be deduced from the refolding curve that the unfolding CTX III by GdnHCl is perfectly reversible (Fig. 2, *inset*), and this aspect helps us to understand the molecular events in the folding pathway of the protein (CTX III).

Refolding Kinetics Monitored by Optical Stopped-flow Measurements—The refolding kinetics of CTX III was monitored by using stopped-flow fluorescence and stopped-flow CD techniques. The lack of tryptophan in the protein necessitated the use of ANS as an extrinsic probe to monitor the folding kinetics by stopped-flow fluorescence. When ANS is present during refolding, it is found to bind strongly to the protein within 8 ms (Fig. 3), which is close to the dead time of mixing in the stopped-flow instrument. As a consequence, the rise in ANS emission cannot be monitored accurately. As the folding of protein proceeded, the emission intensity of ANS increased in the burst phase and then decreased steadily in the slow phase (Fig. 3, *inset*). The decrease in the ANS fluorescence signal is triphasic with time constants of $\tau_1 = 50.1 \pm 7.0$ ms, $\tau_2 = 291.5 \pm 4.0$ ms, and $\tau_3 = 5.5 \pm 1.8$ s. The kinetic curve reveals that most of the changes in the ANS fluorescence are complete within 200 ms (Fig. 3).

Stopped-flow CD experiments were carried out to complement the fluorescence data by investigating the formation of secondary structure (far UV CD) and tertiary structure (near UV CD) during the folding process of CTX III. The kinetics of refolding measured from ellipticity change(s) at 270 nm arising from the tyrosine residues are shown in Fig. 4A. This figure shows that most of the changes are over in the first phase of refolding, which occurs within 100 ms. The 270-nm ellipticity changes have been fitted to a three-exponential decay function. An initial phase starting at 12.3 ms rapidly decays within 20 ms. To overcome the technical problems of curve fitting, we have ignored fitting the initial phase. The ellipticity changes occurring at higher refolding times have been fitted by shifting the zero time point to 20 ms. The remainder of the CD signal evolves within about 200 ms. The refolding kinetics of CTX III monitored in the far UV region at 213 nm (Fig. 4B) presents several interesting features. In the time range (20 ms) just above the dead time of the stopped-flow CD instrument, the ellipticity value at 213 nm decreased significantly (Fig. 4B). During this phase, the changes in the ellipticity at 270 nm (Fig. 4A) are minimal, are similar to the ellipticity values during this time period, and are similar to those obtained for completely unfolded CTX III at this wavelength (270 nm). After the burst phase decrease, the ellipticity value at 213 nm increased slowly to attain the value of the native protein. Such an "overshoot" behavior as shown in Fig. 4B has been reported in several other proteins (17) and has been attributed to the formation of nascent nonnative helix formation during the burst phase of folding of proteins (17, 18).

The Anomalous CD Overshoot Phenomenon—To examine if the overshoot in CTX III is due to the formation of nascent, nonnative helix formation, we carried out refolding experiments in a helicogenic solvent such as 2,2,2-trifluoroethanol (TFE). CTX III is an all β -sheet protein, and its three-dimensional solution structure reveals that no portion of the polypeptide chain of CTX III exists in helical conformation. Recently, it

FIG. 3. Stopped-flow kinetics of refolding of CTX III monitored by ANS fluorescence. The final protein concentration was 160 μ M. The fluorescence (γ_{ex} = 418 nm, γ_{em} = 497 nm) was monitored for 1.5 s. Nonlinear least squares fits of the data give values of $\tau_1 = 50.1 \pm 7.0$ ms, $A_1 = 28.7 \pm 3.0$; $\tau_2 = 291.5 \pm 40.0$ ms, $A_2 = 37.4 \pm 2.0$; $\tau_3 = 5.5 \pm 1.8$ s, and $A_3 = 29.7 \pm 7.5$. The *inset* clearly depicts the hydrophobic collapse that occurs in the burst phase of folding.

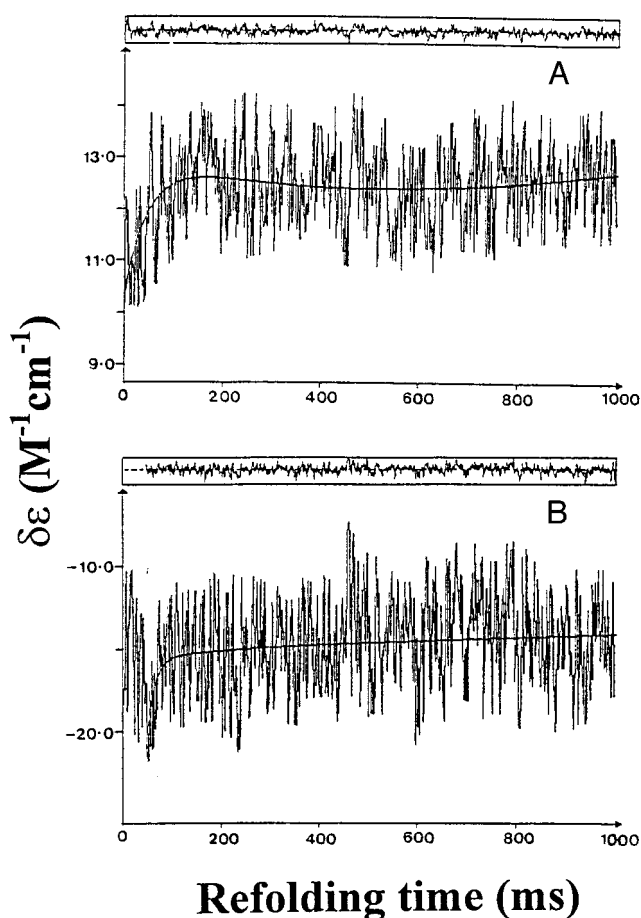
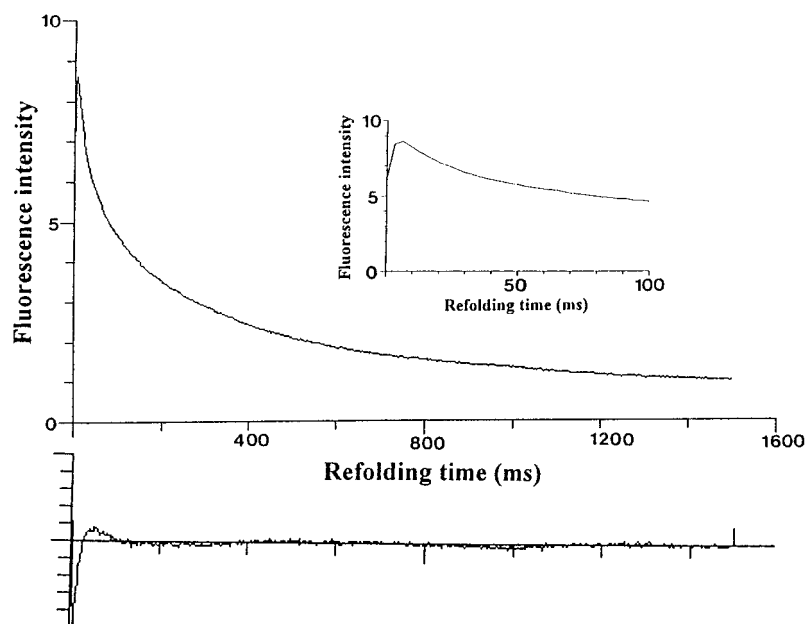


FIG. 4. Stopped-flow kinetics of refolding of CTX III monitored by circular dichroism at 270 nm (A) and 213 nm (B). Nonlinear least squares fits of the data obtained at 270 nm yield values of $\tau_1 = 48.7 \pm 0.8$ ms, $A_1 = -29.0 \pm 1$; $\tau_2 = 592 \pm 234$ ms, $A_2 = 33 \pm 7.5$; $\tau_3 = 5.0 \pm 0.12$ s, and $A_3 = -37.0 \pm 7.5$.

has been demonstrated that TFE nonspecifically induces helix conformation in CTX III (13) at higher concentrations ($\geq 90\%$, v/v). It can be argued that if the CD overshoot phenomenon (as observed in CTX III) were due to the formation of nonnative helix, then refolding CTX III in TFE-water mixtures at concen-

trations less than 90% (v/v) would be expected to cause the intensity of the CD overshoot to increase due to the stabilization of the nascent, "non-native" helix. From the curves depicting the changes in the 222-nm ellipticity upon refolding CTX III in the buffer alone and in the buffer containing various TFE concentrations (Fig. 5), it is found that the CD overshoot signal in the burst phase of refolding does not show the expected increase in the ellipticity at 222 nm when the refolding of the protein is carried out separately in the refolding buffer containing 10 and 50% (v/v) concentrations of TFE (Fig. 5). Based on these results, we believe that the CD overshoot signal observed in CTX III may not be linked to the formation of transient, nonnative helical conformation. Chaffotte *et al.* (20), investigating the overshoot phenomenon in the early stages of refolding of hen egg white lysozyme reported that the "burst" of ellipticity (in the far UV CD region) that occurs in the dead time of the experiments does not reflect significant changes in the secondary structure of the protein. They believe that the CD overshoot essentially reflects changes in the state of side chains, with an important contribution arising from disulfide bonds (21). Interestingly, Varley *et al.* (22), studying the kinetics of folding of interleukin-1 β , which is an all β -sheet protein but lacks disulfide bridges, did not observe the overshoot phenomenon. Similarly, in proteins such as cytochrome C, ubiquitin, and staphylococcal nuclease, which are also characterized by the lack of disulfide bridges, the anomalous far UV CD ellipticity overshoot phenomenon is not observed (20). It appears that there exists a strong correlation between the overshoot of CD ellipticity in the burst phase of refolding and the presence of disulfide bonds in the native state of the protein. In conclusion, the results presented thus far clearly indicate that the CD overshoot phenomenon is in some way related to the attainment of asymmetry of the disulfide bonds during the process of protein folding. To understand the contribution of the disulfide bonds to far UV CD overshoot phenomenon observed during the refolding of CTX III, we studied the "refolding" of S-carboxymethylated CTX III. There were no significant change(s) in the ellipticity (at 213 nm) values upon refolding. Importantly, the overshoot in the 213-nm ellipticity is not observed when the S-carboxymethylated CTX III sample dissolved in 6 M GdnHCl is diluted (refolded) 10 times with the refolding buffer. Thus, the results of these experiments unambiguously demonstrate that the far UV CD overshoot phenom-

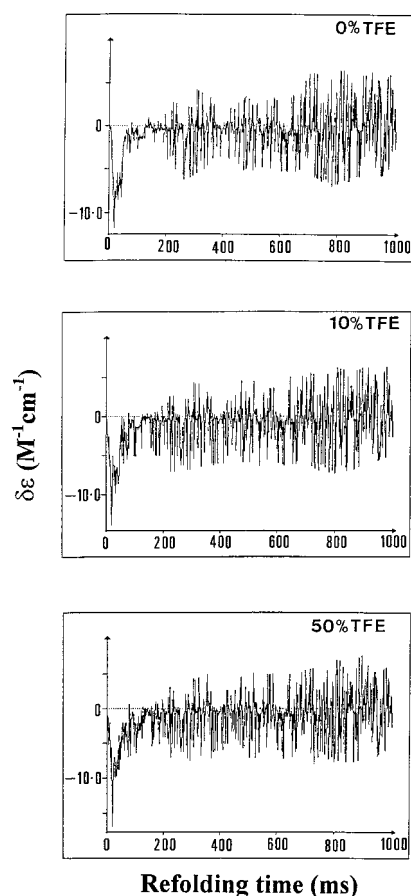


FIG. 5. Refolding of CTX III monitored by stopped-flow circular dichroism at 222 nm in various concentrations of TFE. Refolding of CTX III in the presence of even 50% TFE (v/v) did not result in any significant increase in the intensity of the CD signal (at 222 nm) during the burst phase of refolding.

enon observed during the refolding of CTX III is significantly linked with the asymmetrization of the disulfide bridge(s) in the protein. It is of interest to note that the CD overshoot phenomenon is not observed when CTX III treated with 2 M GdnHCl (at this concentration of GdnHCl, the structure of CTX III is unperturbed) is refolded with the refolding buffer, indicating that the overshoot in the CD signal is connected with the reorganization of tertiary structural interactions during the refolding of CTX III (data not shown). The 270-nm tyrosine ellipticity evolves completely only after 50 ms (Fig. 4A), and hence the possibility of the far UV CD overshoot being associated with the attainment of the asymmetric environment of the optically active aromatic residues is remote. However, at the present juncture, we cannot totally exclude this possibility. In addition, our results show that, at least in the refolding of CTX III, no transient, nonnative helix conformation(s) is formed during the early stages of protein folding.

Kinetics of Amide Proton Protection—Since the amide protons in CTX III have been assigned in the two-dimensional COSY NMR spectrum (14, 15), it is possible to unambiguously follow the kinetic protection for 43 separate amide residues involved in secondary and tertiary structural interactions (Fig. 6). These amide protons are distributed throughout the molecule. All the residues were adequately described by a single-exponential decay (Fig. 7). The time constants τ (the inverse of rate constant, k) of refolding of the various residues are listed in Table I. If we arbitrarily subdivide the time constants in Table I into two classes, fast ($\tau < 20$ ms) and slow ($\tau > 40$ ms), then the residues in the fast class appear to be primarily

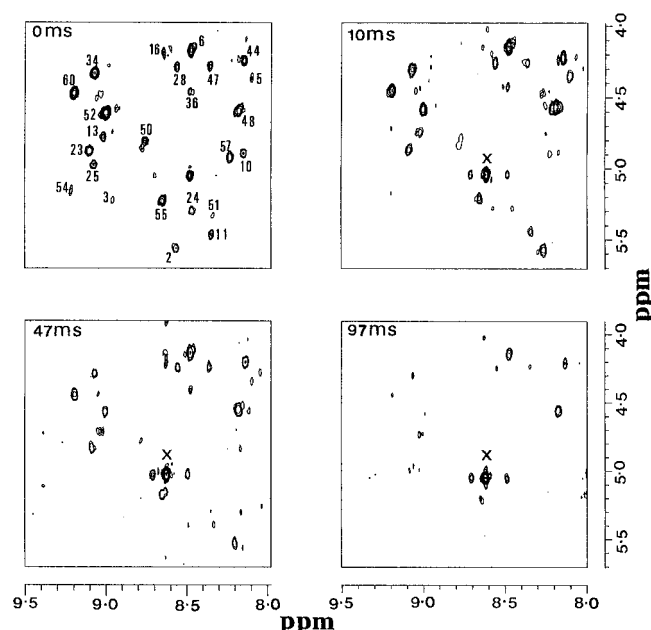


FIG. 6. Magnitude COSY spectra of CTX III samples prepared by quenched-flow hydrogen exchange methods at various pulse labeling time periods. The labeled NH-C^αH cross peaks represent residues that have undergone the amide deuterium-proton exchange at various refolding time periods. The cross-peak labeled as X originates from the protonated contaminant(s) in glycine- d_5 used in the refolding buffer.

associated with the hydrophobic core of the molecule, whereas those in the slow class are mostly located on the periphery of the folded protein structure. Comparison of the time constants of the various residues listed in Table I suggests that there could be initial rapid hydrophobic collapse occurring during the refolding of the protein. Interestingly, the time constants of Lys²³, Ile³⁹, Val⁴⁹, Tyr⁵¹, Val⁵², Cys⁵³, Asp⁵⁷, and Arg⁵⁸ are less than 20 ms (Table I). In the native state of the protein, these residues are distributed in both the structured and unstructured regions of the CTX III molecule. The small refolding time constant of Lys² ($\tau = 21.0$ ms) is worth mentioning. The solution structure of CTX III (12, 15) reveals that the amide proton of Lys² is hydrogen bonded to the carbonyl group contributed by Arg⁵⁸. This interaction tethers the N- and C-terminal ends of the CTX III molecule together. Comparing the time constants of folding of Lys² with those of the residues involved in the initial "hydrophobic collapse," it appears that the clustering of hydrophobic residues in the early stages of folding (< 20 ms) helps the N- and C-terminal ends of the molecule come to close proximity. This aspect favors a hydrogen bond formation between Lys² NH and Arg⁵⁸ CO, which could promote the formation of the native tertiary structural interactions in the subsequent stages of folding. In the native state of the molecule, the amide proton of Val⁴⁹ is involved in hydrogen bonding with the carbonyl group of Thr⁴⁶, forming a type 1 β -turn (12, 15). The significantly small folding time constant for Val⁴⁹ ($\tau = 5.5$ ms) could be attributed to the very early formation of the type 1 β -turn in the backbone of the polypeptide chain between Thr⁴⁶ and Val⁴⁹ (Table I).

It can be seen in Fig. 7 that the residues that occupy the relatively unstructured portions of the molecule (such as Leu⁶, Lys¹⁸, Thr²⁹, and Val⁴¹) show high refolding time. In addition, the rate of the deuterium-hydrogen exchange is slower in these residues. This indicates that these residues belong to the slow folding category. The local structures (wherein some of these slow folding residues are thought to be located) in the native protein generally tend to form at longer refolding times (8). In

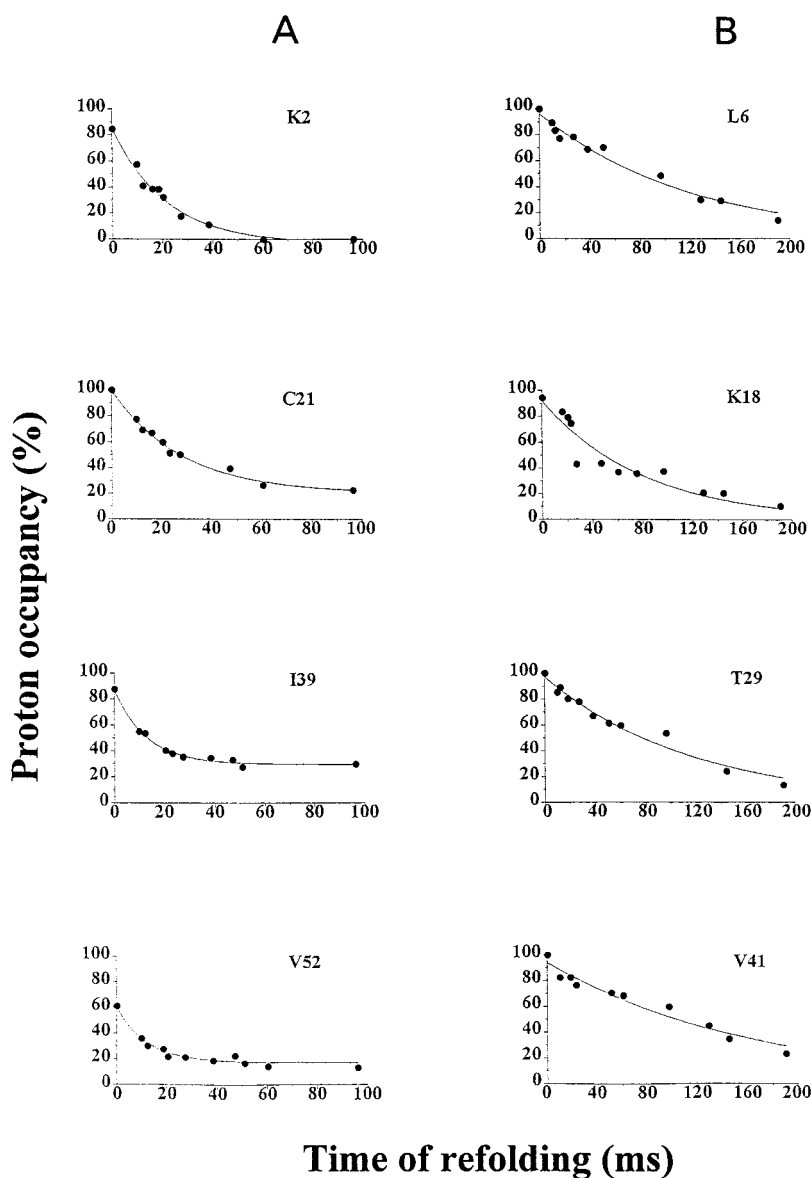


FIG. 7. Comparison of the observed time courses of amide proton occupancy of a selected residues as a function of the length of the folding time. All residues were fit to a first order exponential decay (for further details, see "Experimental Procedures"). Panel A depicts the percentage of proton occupancy of residues located in the secondary structure region. Lys² (K2) belongs to the double-stranded β -sheet segment. Cys²¹ (C21), Ile³⁹ (I39), and Val⁵² (V52) belong to the triple-stranded β -sheet segment. The percentage of proton occupancy for residues located in the unstructured regions (Leu⁶ (L6), Lys¹⁸ (K18), Thr²⁹ (T29), and Val⁴¹ (V41)) are represented in panel B. Since the changes in the proton occupancy are not completed within the refolding time of 190.4 ms for the residues given in panel B, a clear time-dependent asymptote (C) could not be obtained for the curves representing these residues. Hence, we used the exponential function, $y = Ae^{-kt}$ (by eliminating the asymptote (C = 0)) to fit time course changes in the amide proton occupancy of these residues (given in panel B)).

contrast to the slow folding residues, the residues involved in the secondary structure formation in the native state of the protein show proton occupancy in the range of 10–20% after 100-ms refolding.

Comparison of the kinetics of the amides in the various β -strands comprising the double and triple-stranded β -sheets in CTX III (Table II) shows that there is a clear pattern in the rate(s) of formation of the five β -strands. The average folding time constants of the β -strands (Table II) in the protein are as follows: strand I, $\tau = 34.8$ ms; strand II, $\tau = 35.1$ ms; strand III, $\tau = 23.6$ ms; strand IV, $\tau = 18.3$ ms; and strand V, $\tau = 17.2$ ms. Formation of strand II and strand I comprising the double-stranded β -sheet segment appears to occur almost simultaneously. The average time constant of residues in the double-stranded β -sheet is 35.0 ms (Table II). The triple-stranded β -sheet segment appears to fold faster than the double-stranded β -sheet segment. The average time constant of residues comprising the triple-stranded β -sheet segment is 19.7 ms. Among the β -strands (strands III, IV, and V) constituting the triple strand, residues in strand V fold rapidly with an average time constant of 17.2 ms (Table II). Strand IV ($\tau \approx 18.3$ ms) appears to fold faster than strand III (23.6 ms). In summary, it appears that the formation of the triple-stranded

β -sheet domain occurs before the double-stranded β -sheet segments, as shown in Fig. 8. It should be mentioned that the difference(s) in the values of the time constants of folding of residues in the structured and unstructured regions of the protein molecule is not very distinct (Table I). However, considering the small size of the protein and the extensive cross-linking of the molecule by the occurrence of four disulfide bridges, the observed narrow difference(s) in the time constants of refolding between the residues involved in the secondary structure of the molecule and those of residues in the unstructured portion(s) of the CTX molecule is quite significant.

DISCUSSION

CTX III belongs to the class of three-finger proteins. The solution structure of the protein shows that three loops emerge from the globular head (14, 15). The secondary structure of the protein is exclusively β -sheet (hence all β -sheet protein) with no portion of the polypeptide chain in helical conformation (Fig. 1). The antiparallel double- and triple-stranded β -sheet segments in CTX III are stabilized by the four disulfide bridges. It is a highly stable protein, and thermal denaturation studies have demonstrated that CTX III melts only at temperatures

TABLE I
Protection of amide hydrogens during the refolding of CTX III

Residue	Amplitude	Time constant	Structural context
	%	ms	
Lys ²	88.80 ± 04.3	21.00 ± 02.2	Strand I
Lys ⁵	64.16 ± 07.3	33.00 ± 09.9	
Leu ⁶	96.33 ± 02.6	120.48 ± 08.7	Loop I
Val ⁷	76.71 ± 05.4	52.36 ± 10.5	
Phe ¹⁰	49.47 ± 05.2	36.90 ± 10.2	Strand II
Tyr ¹¹	61.90 ± 08.3	35.21 ± 11.7	
Lys ¹²	54.18 ± 05.3	33.67 ± 07.9	
Lys ¹⁸	91.83 ± 06.5	80.78 ± 12.4	Globular head
Cys ²¹	79.72 ± 03.3	27.62 ± 02.8	Strand III
Tyr ²²	87.65 ± 06.0	30.77 ± 05.1	
Lys ²³	42.38 ± 03.2	13.51 ± 02.0	
Ala ²⁸	67.07 ± 04.1	22.52 ± 03.2	Loop II
Thr ²⁹	97.17 ± 02.8	116.28 ± 09.5	
Lys ³¹	71.82 ± 07.4	55.90 ± 13.1	
Val ³²	98.88 ± 02.4	163.93 ± 17.5	
Val ³⁴	59.59 ± 21.4	50.50 ± 41.3	Strand IV
Lys ³⁵	54.07 ± 05.8	36.36 ± 10.0	
Arg ³⁶	64.02 ± 04.7	32.15 ± 06.2	
Cys ³⁸	60.15 ± 05.0	35.59 ± 08.0	
Ile ³⁹	58.19 ± 02.1	12.44 ± 00.9	
Val ⁴¹	94.00 ± 03.1	161.29 ± 15.6	Globular head and loop III
Lys ⁴⁴	84.20 ± 06.8	49.51 ± 11.3	
Leu ⁴⁸	94.16 ± 24.9	129.87 ± 69.1	
Val ⁴⁹	51.27 ± 03.9	05.50 ± 01.2	
Tyr ⁵¹	39.62 ± 05.0	12.73 ± 03.9	Strand V
Val ⁵²	44.48 ± 03.0	11.78 ± 01.6	
Cys ⁵³	37.50 ± 03.4	13.60 ± 02.7	
Asn ⁵⁵	78.70 ± 06.9	21.27 ± 05.0	
Asp ⁵⁷	56.71 ± 01.3	09.80 ± 00.5	Globular head
Arg ⁵⁸	38.67 ± 04.6	12.95 ± 03.0	
Cys ⁵⁹	64.04 ± 03.0	172.40 ± 29.7	
Asn ⁶⁰	63.92 ± 03.8	51.81 ± 09.4	

TABLE II
Average time constant of folding of the various β -strands in CTX III

Structural context	Residues considered for averaging	Average time constant
		ms
Strand I	Lys ² , Lys ⁵	34.84 ± 10.7
Strand II	Phe ¹⁰ , Tyr ¹¹ , Lys ¹²	35.11 ± 07.3
Strand III	Cys ²¹ , Tyr ²² , Lys ²³	23.61 ± 02.9
Strand IV	Lys ³⁵ , Arg ³⁶ , Cys ³⁸ , Ile ³⁹	18.28 ± 03.6
Strand V	Tyr ⁵¹ , Val ⁵² , Cys ⁵³ , Asn ⁵⁵	17.19 ± 02.7

greater than 90 °C (23). In addition to the disulfide bridges, the protein is strongly stabilized by a core of hydrophobic residues. It is widely believed that the hydrophobic core bestows extraordinary structural stability to the protein (12). Recently, interesting studies on the protein folding aspects of snake venom cardiotoxins have been reported. Partially structured intermediate states have been identified along the alcohol- (13) and acid-induced (24) unfolding pathways of CTX III. In pursuit of our ultimate goal of understanding the folding/unfolding pathway(s) of CTX III, we have embarked on examining the kinetic events in the folding pathway(s) of this protein.

The Hydrophobic Collapse Detected by ANS Fluorescence—ANS has been proven to be a sensitive probe for monitoring the folding of globular proteins (25). The intense ANS fluorescence signals observed in the burst phase of folding are believed to be primarily due to the binding of ANS to nonpolar surfaces, presumably in the hydrophobic core, which in folding intermediates may be solvent-accessible. Such binding is therefore commonly used to measure the compactness of the bound intermediates (26). As described earlier, the burst phase of refolding of CTX III is characterized by the presence of highly

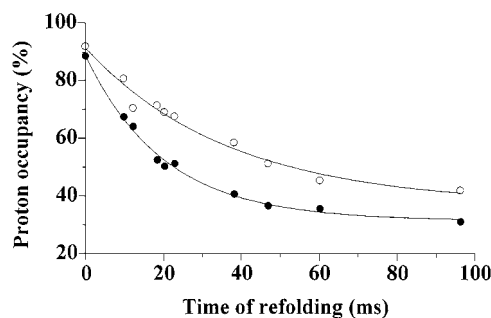


FIG. 8. Time courses for the protection of amides from exchange of the residues involved double- and triple-stranded β -sheets during the refolding of CTX III. The curves drawn represent the average of single exponential fits to the time course for individual amides in the double and triple-stranded β -sheet segments. Nonlinear least square fits of the data give values of 19.7 ms (filled circles) and 35.0 ms (open circles) for the average time constants of folding of the triple- and double-stranded β -sheet domains, respectively. Time courses of five and nine residues were averaged to trace the kinetic curves for the double- and triple-stranded β -sheets, respectively.

intense ANS fluorescence (Fig. 3). We believe that the strong ANS fluorescence signal observed during the burst phase is due to the binding of the nonpolar dye to the hydrophobic clusters in the very early stages of folding of CTX III. Interestingly, monitoring the 270-nm ellipticity, which signifies the asymmetric environment of the tyrosine residues in the protein, shows that CTX III regains less than 10% of the final ellipticity in native protein (at 270 nm) during the burst phase of folding (Fig. 4A). These results reveal that the specific tertiary structural interactions pertaining to the aromatic chromophores (tyrosines in this case) are not formed during the burst phase of folding. It is important to mention that the near UV CD signal at 270 nm for CTX III primarily stems from the tyrosine residue located at position 22. This residue contributes significantly to the stabilization of the native state of the protein. In this context, it would be of interest to note that Agashe *et al.* (26), examining the folding pathway of barstar, demonstrated that the protein collapses (during the burst phase of refolding) to a compact globule with a solvent-accessible hydrophobic core with no optically active secondary or tertiary structure.

Folding Events Detected by Amide Proton Pulse Exchange—It would be of interest to trace the chronological hierarchy of the formation of the various secondary structural segments in the refolding pathway of CTX III. The fifth β -strand (strand V) spanning residues 50–55 is largely hydrophobic. This β -strand is a component of the triple-stranded β -sheet segment in CTX III. It appears that this portion of protein along with other hydrophobic residues located at the C terminus forms the hydrophobic cluster that occurs in the burst phase of the folding pathway. Based on the small folding time constant (Table I), the residues such as Lys²³, Ile³⁹, Val⁴⁹, and Asp⁵⁷ along with the residues comprising strand V are believed to collapse into a hydrophobic cluster. The folding time constants of these residues is significantly small ($\tau < 20$ ms). The small folding time constant of Lys² ($\tau = 21.0$ ms) is interesting (Table I and Fig. 7). As stated earlier under “Results,” a hydrogen bond between the amide proton of Lys² and the carbonyl group of Arg⁵⁸ brings the N- and C-terminal ends of the backbone of the CTX III molecule spatially close. It appears that the formation of the “hydrophobic cluster” in the burst phase (< 20 ms) facilitates the formation of hydrogen bonding between Lys² NH and Arg⁵⁸ CO. The formation of this crucial hydrogen bond appears to initiate the subsequent events of folding in CTX III. Interestingly, the hydrogen bonding partners (carbonyl groups) for the amide groups of the residues in strand V are located in strand III of triple-stranded β -sheet. However, the average

folding time constant for residues in strand III is 23.6 ms and is greater than that of the residues in strand V (17.2 ms, Table II). This implies that strand V could be formed before strand III. In the absence of the interstrand hydrogen bonding with strand III, the amide deuterium in strand V is protected due to the formation of the hydrophobic cluster. Formation of such a nonpolar cluster probably secludes these residues in strand V from the solvent water, resulting in the observed slow exchange of amide deuterium with the solvent protons (27). On the whole, it is clear that the triple-stranded β -sheet segment forms early during the refolding of CTX III. Based on the average time constants of residues in the double- and triple-stranded β -sheet segments, it appears that the triple-stranded β -sheet ($\tau = 19.7$ ms) is formed before the double-stranded ($\tau = 35.0$ ms) β -sheet segment (Table II and Fig. 8). Partially structured intermediates have been identified and structurally characterized along the alcohol- (23) and acid-induced (24) unfolding pathways of CTX III. Interestingly, it is found that the triple-stranded β -sheet segment is persistently found in the intermediate states. To this extent, the results obtained in the kinetics folding pathway of CTX III are consistent with those obtained from equilibrium unfolding studies.

A Global View of the Folding Events in CTX III—The kinetic events in the refolding pathway of CTX III as monitored by stopped-flow fluorescence clearly indicate the presence of a transient intermediate, which is characterized by the intense ANS fluorescence signal in the burst phase of folding. Intermediates with such spectral (intense ANS signal) characteristics have been detected in the burst phase of refolding of several proteins. The high affinity for ANS for these intermediates is thought to stem from a hydrophobic clustering of residues. Interestingly, the refolding kinetics followed by quenched-flow deuterium-hydrogen exchange of the amide protons reveals that the residues that possess refolding time constants less than 20 ms (fast folding residues) are apolar residues concentrated in the triple-stranded β -sheet segment and C-terminal loop. It appears that clustering of the hydrophobic residues in this portion of the CTX III molecules creates local structural geometry that is conducive for strong ANS binding observed in the burst phase of folding. The far UV CD overshoot phenomenon observed in the burst phase (8.0 ms) of refolding of CTX III is found to be associated with the attainment of asymmetry of the disulfide bond(s) in the protein. The head region of the CTX III molecule, wherein most of the apolar residues involved in the burst phase hydrophobic collapse are located, is extensively cross-linked by disulfide bridges. It is possible that the regaining of asymmetry of the disulfide bridge(s) in the protein could aid in the clustering of the hydrophobic residues (observed in the burst phase of folding) in the head region of the molecule. It appears that the “collapsed” intermediate observed in the burst phase of folding lacks most of the native tertiary structural interactions. This is exemplified by the fact that only about 10% of the total expected ellipticity at 270 nm is evolved in the burst phase of refolding (Fig. 4A). It is well known that the near UV CD signal (at 270 nm) in CTX III is mostly due to Tyr²², and it appears that this residue does not attain its asymmetric environment during the burst phase of refolding of the protein. This inference is supported by the high folding time constant (31 ms) obtained for Tyr²² using the quenched-flow deuterium-hydrogen experiments.

A common 50-ms phase is observed during the refolding of CTX III using the stopped-flow CD and fluorescence experiments. This phase is predicted to represent the formation of most of the native secondary and tertiary structural interactions in the protein. This formation is signified by the fact that most of the residues (monitored by the quenched-flow deuterium-

hydrogen exchange experiments) involved in the secondary structural interactions show folding time constants in the range of 30–50 ms. The slower phases observed probably represent the formation of local tertiary structural interactions and *cis-trans* proline isomerization.

Comparison with Folding Kinetics of Interleukin-1 β —The folding pathway of interleukin-1 β , another all β -sheet protein, has recently been studied using a variety of spectroscopic techniques including quenched-flow deuterium-hydrogen exchange experiments (22). It is quite informative to compare these results with those obtained in the present study on CTX III. In interleukin-1 β , the complete folding of the protein occurs on a time scale greater than 25 s (22). The slow folding rate of interleukin-1 β is in complete contrast to that observed for CTX III, wherein the folding is complete within 200 ms. The difference in the folding rates could be due to the difference in the molecular mass of two proteins. CTX III is 6.5 kDa, whereas interleukin-1 β is about 85 kDa. CTX III is cross-linked by four disulfide bridges located between residues 3 and 21, 14 and 38, 42 and 53, and 54 and 59. These disulfide bonds have been shown to be crucial for the maintaining the structural integrity of CTX III. The reduction of the disulfide bridges invariably results in the complete unfolding of the protein (CTX III). The solution structure of CTX III shows that the disulfide bonds in the protein stabilize the β -sheet element in the protein. The residues that are contemplated to be involved in the initial hydrophobic collapse are mostly located in the head portion of the molecule. This region is extensively cross-linked by disulfide bridges, and this cross-linking, we believe, could facilitate the clustering of hydrophobic residues observed in the early stages of folding of CTX III. The absence of disulfide bridges in interleukin-1 β , on the other hand, could be responsible for its slower refolding rate. Interestingly, just as in CTX III, the folding pathway of interleukin-1 β is characterized by the formation of intermediate state(s) (in the burst phase of folding) that exhibit high affinity for binding to ANS (22). One important difference between the folding kinetics of CTX III and interleukin-1 β is the rate at which the β -sheet conformational elements are formed. The formation of stable native secondary structure in interleukin-1 β , as measured by quenched-flow deuterium-hydrogen exchange experiments begins only after 1 s (22). Thus, the comparison of these two all β -sheet proteins shows that rates of protein folding do not only depend on the type of secondary structural conformation in the protein(s) but also strongly depend on other local or tertiary structural interactions of the protein in context.

Significance of the Hydrophobic Collapse—It is important to understand the significance of the hydrophobic collapse that occurs in the burst phase of refolding of several proteins including CTX III. Gutin *et al.* (28), examining the folding of proteins using a simple lattice model, showed that, depending on the interaction among residues, two different regions of folding are possible. A two-stage kinetics is observed when strong attraction among residues dominates in the folding process. The first stage is fast hydrophobic collapse into a compact nonnative globule, and the second stage is a slow search for the native conformation among these compact globules. They find that folding into the native state from the compact intermediate is an all-or-none transition. Gutin *et al.* (28) observed that native structure formation and compaction proceeded simultaneously from the random coil as an all-or-none transition when there was repulsion among residues. In real proteins, the interaction (attraction/repulsion) strongly depends on the physical conditions (concentration of denaturants, pH, and temperature) used during folding. In addition, the disulfide bonds and hydrophobic residues in the protein

tend to increase the average attraction among the residues and consequently favor the compaction of the molecule during refolding. CTX III is a highly hydrophobic molecule extensively cross-linked by four disulfide bridges. These features of the protein probably enable the polypeptide chain to undergo compaction during refolding prior to the assumption of the native conformation.

Camacho and Thirumalai (29) recently proposed that folding proceeds by a stagewise decrease of the number of available conformations. They suggest that at the first stage of folding, the polypeptide chain undergoes overall compaction and subsequently falls into one of the compact energy-minimized conformation. The native conformation is realized later by a search over a small number of energy-minimized compact conformation(s). However, several kinetics studies of random systems (30) have indicated that these minimum energy misfolded ("glassy") states represent deep traps and that escaping is extremely slow. Thus, it is believed that compaction itself may not increase the folding rate because it does not decrease the energy barriers en route to the native conformation (29, 30). Compaction is contemplated to slow down protein folding by decreasing the number of conformations available and, therefore, prohibits some conformations that form pathways with lower barriers (28).

Does Hydrophobic Collapse Precede the Formation of Secondary Structure?—It is worthwhile to address the question of whether hydrophobic collapse precedes secondary structure formation or *vice versa*. Contrasting reports exist in the literature on this question. Studies with isolated peptide fragments reveal that the secondary structure is only marginally stable in the absence of stabilizing tertiary structural interactions (31), which indicates that the formation of collapsed state(s) requires prior formation of secondary structure. On the contrary, Agashe *et al.* (26) demonstrated that the hydrophobic collapsed state exists in barstar, which lacks secondary structural interactions, suggesting that collapsed states in kinetic or equilibrium intermediates can exist even in the absence of secondary structure.

The results of the present study on CTX III indicate that the hydrophobic cluster is formed in the very early stages of the folding process. The formation of this hydrophobic cluster is primarily due to the coalescence of nonpolar residues located in strand V of the triple-stranded β -sheet segment. However, based on the results obtained in the present study, it is difficult to predict the hydrophobic clustering of residues. Since the residues comprising the hydrophobic cluster also form a part of the secondary structural element (strand V), the clustering hints at the possibility of the event of the hydrophobic collapse and the formation of secondary structure occurring simultaneously. However, there are obvious limitations to this conclusion, because there are residues that are involved in the secondary structure formation but fold on a slower time scale than

residues involved in the hydrophobic collapse. We believe a detailed submillisecond kinetic study is required to obtain a definitive answer to this question (32). Recently, CTX III has been cloned and expressed in high yields (19), and detailed folding studies with appropriate mutants could provide useful clues to understand the structural interactions that come into play during the early events of folding of CTX III.

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