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Secondary Structure Formation Is the Earliest Structural Event in the Refolding of an All β-Sheet Protein

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The refolding kinetics of cobrotoxin (CBTX), a small-molecular-weight (~7 kDa) all β-sheet protein, has been monitored using a variety of biophysical techniques. The secondary structure formation and hydrophobic collapse occur as distinct events during the refolding of the protein. Complete secondary structure formation occurs prior to the clustering of the hydrophobic residues. The late stage(s) of the refolding pathway of CBTX is characterized by change(s) in the local environment and optical asymmetry of the indole ring of the sole tryptophan residue. The results obtained in the present study, to our knowledge, represent the first unambiguous experimental support for the framework model of protein folding.

Key Words: **all β-sheet protein; hydrophobic collapse; framework model.**

Understanding the earliest events in protein folding are crucial to learn the mechanism of the refolding process. In general, it is still not clear whether the driving force in protein folding is hydrogen bonding or hydrophobic interactions. Kauzmann contemplated that hydrophobic collapse could to be the dominant force governing protein folding because hydrogen bonding to the solvent water molecules would strongly favor the unfolded state (1). This is opposed to the views of Mirsky and Pauling, who proposed that protein folding is largely directed by the formation of hydrogen bonds (2). These contradicting viewpoints form the crux of many phenomenological models that have been proposed to explain how a protein can fold, averting a random search over its vast conformational space. Among them, two models are frequently used to describe the earliest enigmatic steps of protein folding. The secondary structure framework model postulates that certain elements of native secondary structure are formed very fast, and upon further refolding, the tertiary structure slowly develops (3). The hydrophobic collapse model proposes a non-specific compactization of the polypeptide chain into a structureless globule, which reduces the conformational search and consequently facilitates the formation of specific secondary and tertiary structure (4). In practice, it has proved difficult to ascertain whether initial hydrophobic collapse of the polypeptide chain precedes or succeeds secondary structure formation. Recently, Agashe *et al.* provided the first experimental evidence for the "hydrophobic collapse" model (5). Conversely, although there are numerous reports regarding the formation of native secondary structural elements in the very early stages of folding, an unambiguous conclusion regarding the sequence of structural events (hydrophobic clustering and secondary structure formation) could not be drawn, as spectroscopic changes reminiscent of hydrophobic collapse were found to occur on a similar time scale as that of the secondary structure development (6). In addition, the formation of secondary structure in the very early stages of folding as detected by optical techniques could not be completely authenticated by other direct methods of detection such as quenched-flow hydrogen-deuterium exchange (7). To-date, there is no clear-cut experimental evidence to support that hydrophobic collapse succeeds secondary structure formation. Herein, we investigate events in the refolding pathway of cobrotoxin (CBTX), a small (~8 kDa), all β-sheet protein from the Taiwan cobra (*Naja naja atra*) venom (8, 9). The refolding kinetics of CBTX provides the first example wherein complete secondary structure formation takes place prior to the hydrophobic collapse.

MATERIALS AND METHODS

Cobrotoxin was purified from the crude Taiwan cobra venom according to the procedure reported by Yang *et al.* (10). Guanidinium hydrochloride (GdnHCl, ultrapure) was purchased from Merck, Germany. Deuterated GdnHCl was prepared by repeated lyophilization of GdnHCl dissolved in D_2O. The authenticity of the sample prepared

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was verified using 1D ^{1}H NMR experiments. 1-Anilino-8-napthalene sulfonate (NH_4^+ salt) was purchased from Sigma Chemical Co., U.S.A. Glycine-d_5, D_2O and DCl were purchased from Cambridge Isotope Laboratories, U.S.A.

GdnHCl-induced unfolding. Experiments on cobrotoxin were performed using near and far UV circular dichroism (CD) spectroscopy. All CD spectra were collected on a Jasco J720 spectrometer with the silt width set to 430 μm, response time of one second and scan speed of 20 nm/min. Measurements in the far UV region (190–250 nm) were made at a protein concentration of 160 μm (in 10 mM acetate buffer, pH 3.4) using 0.02 cm pathlength quartz cell. The near UV (250–320 nm) measurements were done using a quartz cell of pathlength 0.1 cm. All data were corrected for background noise and represent an average of five scans. The sample temperature was controlled using a Neslab RTE temperature controller. Data analysis was performed using standard protocols (11).

Stopped-flow CD measurements. Stopped-flow CD experiments were performed using SFM-3 Biologic stopped-flow apparatus attached to a Jasco J720 spectrometer. The cell pathlength was 2 mm and the dead time of the instrument was estimated to be about 5 ms. Refolding of the protein was initiated by a 10-fold dilution of the protein (cobrotoxin) unfolded in 3 M GdnHCl, with 10 mM acetate buffer, pH 3.4. Refolding was monitored at 5°C. The data presented is an average of 50 scans. The ellipticity changes at 214 and 284 nm were analyzed using the Microcal origin software and the data were fit to mono- and biexponential equations. Non-linear best square fitting of the ellipticity changes yielded the amplitude and rate constants of the different phases.

Stopped-flow fluorescence. All stopped-flow fluorescence measurements were performed at 5°C on a SF-61 stopped-flow specrofluorometer (Hi-tech, Scientific, UK). Refolding experiments were performed by mixing one part of the denatured protein (in 3 M GdnHCl) with 9 parts of the refolding buffer (10 mM acetate buffer, pH 3.4). ANS binding experiments were performed at various concentrations (between 50 and 250 μM) of the dye (ANS), by an appropriately mixing the stock solution of ANS in the refolding buffer (10 mM acetate buffer, pH 3.4). The binding of ANS was monitored by setting the excitation and emission wavelengths at 418 and 497 nm, respectively. The kinetic data were analyzed by a non-linear least squares fit to the equation

$$A_{(t)} = A_i \exp^{(-kt)} + A_x,$$

wherein $A_{(t)}$ is the amplitude change at time t, A_x is the amplitude at infinite time, A_i is the amplitude at zero time of phase i, and k is the rate constant of phase i.

Quenched-flow D-H exchange experiments. The quenched-flow D-H experiments were carried out using a RQF-63 rapid mixing quench apparatus (Hi-tech scientific, UK) at 5°C. The methodology adopted for the quenched-flow experiments was the same as that used in the study of the refolding kinetics of a snake venom cardiotoxin (12). Fully unfolded cobrotoxin was prepared by taking up lyophilized cobrotoxin at a concentration of 3 mg/ml in D_2O containing 3.0 M guanidinium deuterochloride (GdnDCl at pH 6.0 ± 0.2) and incubated at 50°C overnight to ensure complete exchange of all its amide protons. Refolding of the protein was initiated by a 10-fold dilution of unfolded cobrotoxin with 50 mM glycine-d_5 (pH 3.4) buffer in H_2O. At this pH, negligible exchange occurred. After the desired refolding times ranging from 9.8 to 500 ms, the solution was diluted again to 10 times the initial protein volume with 0.1 M borate buffer (pH 9.6) to initiate labeling of the deuterated amides in cobrotoxin with protons. The labeling was stopped after an interval of 10 ms, by a further 4.5 fold dilution of the initial protein volume with 0.62 M HCl. The final pH of the solution was maintained about 3.4.

Magnitude COSY spectrum of each sample (at various refolding times) was recorded at 5 ± 0.1°C. Using a Bruker DMX 600 NMR spectrometer. 256 increments over 1024 data points were collected. All NMR spectra were processed using the UXNMR software on a Silicon Graphics workstation. The time courses of change in proton occupancies were fitted to a single exponential fit ($Y = A.e^{-kt} + C$, 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.

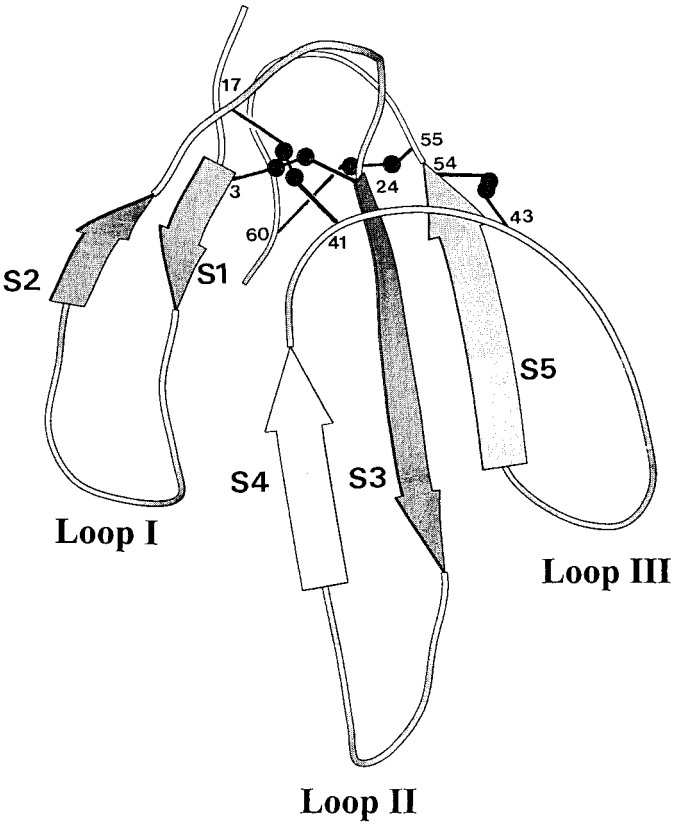

FIG. 1. MOLSCRIPT representation of the three-dimensional structure of cobrotoxin. The ribbon arrows represent the various β-strands in the protein.

RESULTS AND DISCUSSION

Cobrotoxin is three-finger shaped, all β-sheet protein cross-linked by four disulfide bonds (Fig. 1). The secondary structural elements in the protein include five β-strands arranged into anti-parallel double and triple stranded β-sheet domains (13). The protein lacks helical conformation.

Equilibrium unfolding of CBTX is a two-state transition with no detectable intermediate(s) (data not shown). Formation of β-sheet structure was monitored by the changes in the 214 nm ellipticity using stopped-flow circular dichroism spectroscopy (Fig. 2). More than 80% of the total expected amplitude change is realized within about 30 ms after refolding is initiated. The changes in the 214 nm ellipticity are complete within the first 75 ms of refolding of the protein from

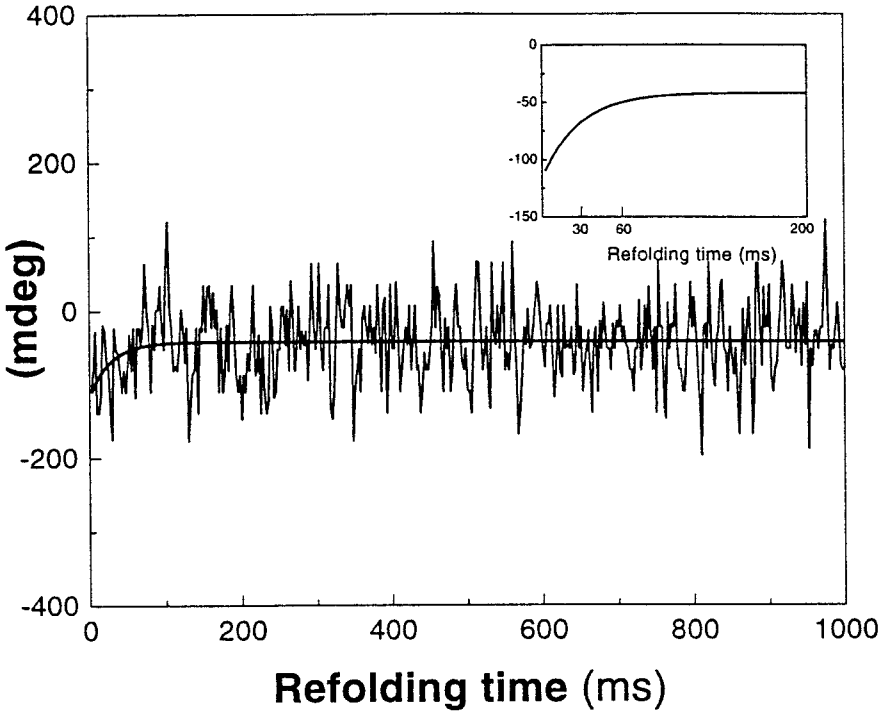

FIG. 2. Stopped-flow kinetics of the refolding of cobrotoxin monitored by the ellipticity changes at 214 nm. The rate constant estimated from the observed changes at 214 nm is 27.5 ± 2.5 ms.

the unfolded state(s) in 3 M guanidinium hydrochloride. The average time constant of residues involved in the backbone hydrogen bonding is estimated to be 26.2 ms (Fig. 2, inset). These results authenticate that stable secondary structure formation occurs within 75 ms of refolding.

Quenched-flow deuterium-hydrogen (D/H) exchange in conjunction with two-dimensional NMR experiments is a powerful tool to characterize the transient intermediates that occur in the early stages of folding (14, 15). Using this technique, it is possible to probe many specific sites within a protein on the millisecond time scale. We used the quenched-flow D/H technique to complement the results obtained by monitoring the ellipticity changes at 214 nm, using the stopped-flow CD. Figure 3 depicts the magnitude COSY spectra of cobrotoxin at two different pulse labeling times. The fingerprint region (NH-$C^{\alpha}H$) of the magnitude COSY

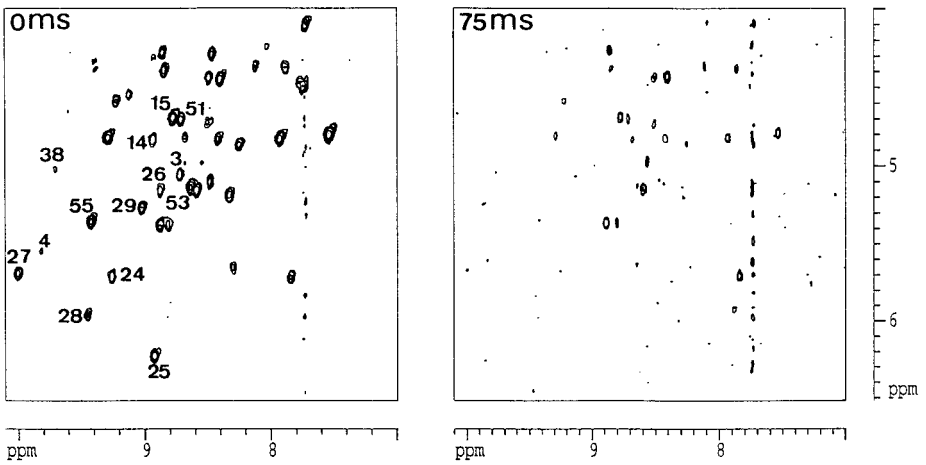

FIG. 3. Magnitude COSY spectra of CBTX samples prepared by quenched-flow hydrogen exchange methods, at two different pulse refolding time periods. The NH-$^{\alpha}$CH cross peaks observed in the spectra after 75 ms of refolding represent the residues in the unstructured portions of the CBTX molecule. The labeled cross-peaks (at 0 ms) represent the residues in the secondary structure.

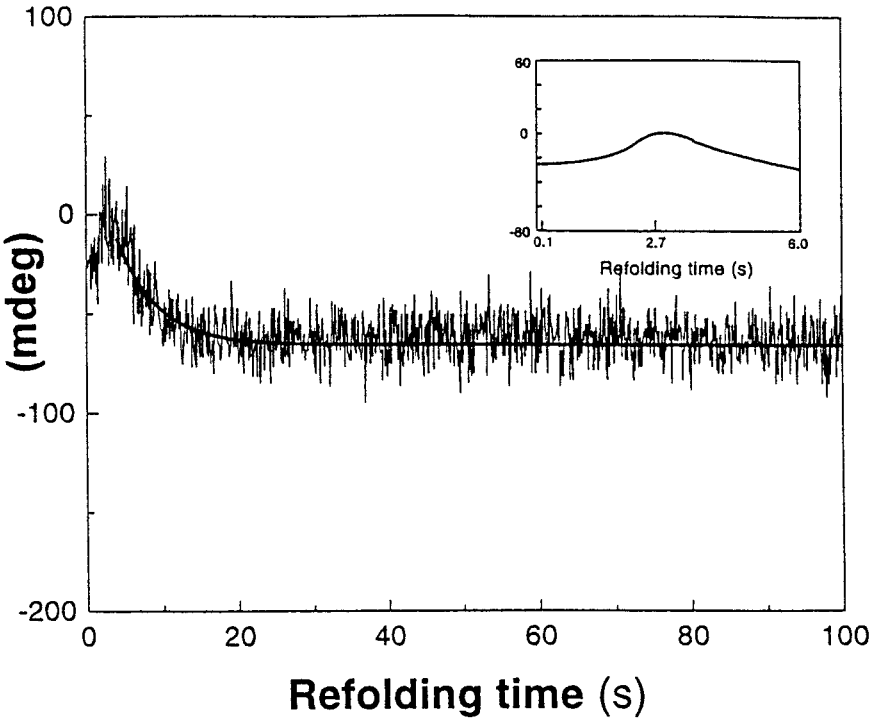

FIG. 4. Refolding kinetics of cobrotoxin monitored by the ellipticity changes at 284 nm. To overcome technical problems of curve fitting, we ignored fitting the initial phase (up to 2.7 s) of the CD curve. The remainder portion of the CD curve could be fitted to a single exponential yielding a rate constant of 5.17 ± 0.9 s.

spectrum (Fig. 3, spectrum at 0 ms refolding time) of cobrotoxin in the native state shows 24 well-resolved cross-peaks representing the secondary and tertiary structural interactions in the protein. Interestingly, these amide protons are distributed uniformly in the protein molecule. Monitoring the refolding of cobrotoxin by quenched-flow D/H exchange technique at various pulse labeling times, reveals that changes in the amide proton(s) protection are mostly complete within 75 ms of refolding (Fig. 3). This aspect could be discerned from the magnitude COSY spectrum obtained at a refolding time of 75 ms which shows that most of the cross-peaks representing the amide protons involved in secondary structural interactions are missing or present at low intensity (Fig. 3).

Two different spectroscopic probes were employed to monitor the formation of tertiary structural interactions during the refolding of CBTX. The change(s) in ellipticity at 284 nm (Fig. 4) is largely due to the regainment of asymmetric environment of the sole tryptophan residue during refolding (12). Interestingly, the ellipticity monitored at 284 nm practically remains unchanged even up to 100 ms (Fig. 4), suggesting that little or no tertiary structural contacts develop in the protein during the time frame wherein complete secondary structural interactions are formed. In fact, the ellipticity at 284 nm begins to show discernible change(s) only after 1.5 s (Fig. 4). The changes in the near UV ellipticity are interesting (Fig. 4). Initially, the 284 nm ellipticity values are distinctly less negative. This trend is reversed after about 2.7 s of refolding and gradually the protein attains the ellipticity values expected for the protein in its native state. Although, this type of "undershoot" in the near UV CD signal is unprecedented in the literature, we opine that this phenomenon is linked to change(s) in the optical handedness of the sole tryptophan ring during the refolding process.

The hydrophobic dye 8-anilino napthalene-1-sulfonate (ANS) is routinely used as a specific tool to identify intermediate state(s) in protein folding (16). ANS binds to solvent accessible, non-polar surfaces in the protein folding/unfolding intermediate state(s). The binding of this dye is a measure of the exposure of the hydrophobic clusters and compactness of the bound intermediate(s). Less than 5% of the final amplitude of the ANS fluorescence is regained in the first 75 ms of refolding (Fig. 5). This aspect clearly implies that no or very meager extent of tertiary structure develops during this time scale wherein the secondary structural contacts in the protein are found to be completely established. The ANS fluorescence shows a steep increase to a maximum value at about 200 ms (Fig. 5, inset). Such a drastic increase in the fluorescence signal is believed to be due to binding of the non-polar dye to solvent-exposed hydrophobic clusters. Beyond 200

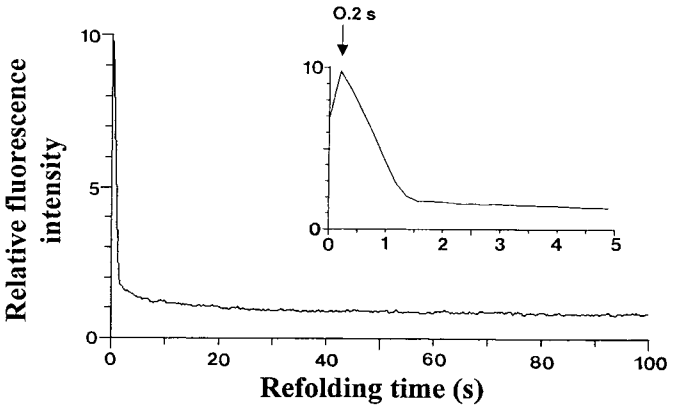

FIG. 5. Changes in the ANS fluorescence, during the refolding of CBTX. The inset figure represents the changes in the ANS fluorescence in the first 1 s of refolding of the protein. Changes in the ANS fluorescence after 200 ms best fitted to a two exponential function yielding rate constant values $k_1 = 3.53 \pm 0.55$ s and $k_2 = 21.92 \pm 3.12$ s.

ms, the ANS fluorescence shows a steady decrease as the hydrophobic core in the protein become inaccessible to the solvent (Fig. 5, inset).

It is rather surprising to find that folding of a small protein such as cobrotoxin proceeds through the occurrence of distinct structural events. Chronologically, the protein appears to rapidly (within 75 ms) form an open structure with most of the native secondary structural elements but lacking most of the tertiary structural contacts. Once the native secondary structural interactions are established, a pronounced clustering of the hydrophobic side chains (at ~200 ms) with strong binding affinity to ANS could be evidenced. Interestingly, at this time point (~200 ms), the protein seems to present characteristics of a 'molten globule' like state. The final stage of folding in CBTX appears to involve change(s) in the local environment and optical asymmetry of the indole ring of the sole tryptophan. In conclusion, the refolding pathway of CBTX is an unique example wherein secondary structure formation and hydrophobic collapse occur as well segregated events. The results obtained herein are in our opinion, the first unambiguous experimental support for the framework model of folding. The study, we believe, would also help to shed the general notion that folding of small proteins do not involve occurrence of distinct intermediate(s).

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