Characterization of a partially structured state in an all-β-sheet protein

Thirunavukkarasu SIVARAMAN, Thallampuranam K. S. KUMAR, Gurunathan JAYARAMAN, Chien C. HAN and Chin YU*

Department of Chemistry, National Tsing Hua University, Hsinchu, Taiwan, Republic of China

Cardiotoxin analogue III (CTX III) is a low-molecular-mass all-β-sheet protein isolated from the Taiwan cobra (*Naja naja atra*) venom. A stable partially structured state similar to the 'molten globule' state has been identified for CTX III in a 3% (w/v) solution of 2,2,2-trichloroacetic acid at 298 K. This stable state has been structurally characterized using a variety of techniques such as CD, 1-anilinonaphthalene-8-sulphonate fluorescence binding, Fourier transform IR and two-dimensional NMR spectroscopy techniques. Direct assignment of the homonuclear two-dimensional NMR spectra of the protein in 3% trichloroacetic acid showed that drastic structural perturbation had not taken place in the protein and that the 'intermediate' state retained a significant portion of the native secondary-structural interactions. It is found that about 65% of the native β-sheet structural contacts are maintained in the partially structured state of CTX III in 3% trichloroacetic acid.

INTRODUCTION

It is generally accepted that the amino acid sequence of a protein determines its unique native structure [1]. To understand why proteins adopt particular three-dimensional structures, it is important to elucidate the hierarchy of interactions that stabilize the native state [2]. Identification and characterization of partially folded intermediates could help in dissecting the organizational levels in proteins [3–5]. One such stable partially structured intermediate is the 'molten globule' [6]. In the molten globule state, proteins have been shown to be compact with pronounced secondary structure but no rigid tertiary structure [7,8]. The intramolecular mobility of the side chains is greater in the molten globule state than in the native state [9]. There is growing evidence that these equilibrium molten globule intermediates may be on the kinetic pathway of protein refolding, and hence a detailed characterization of the structures of molten globule intermediates is critical to our understanding of protein folding [10,11]. The advent of several new methodologies has led to the identification and characterization of the molten globule state in a number of proteins, under a variety of denaturing conditions [12–15]. However, to date, it has not been possible to generalize convincingly about the concept of the molten globule as a universal equilibrium protein-folding intermediate(s) because of lack of substantial evidence of the occurrence of such intermediate states in the folding/unfolding pathway of proteins comprised entirely of β-sheet structure [16].

Cardiotoxin analogue III (CTX III), isolated from the venom of the Taiwan cobra (*Naja naja atra*), is a basic low-molecular-mass all-β-sheet protein, with four disulphide bridges [17–19]. Importantly, no portion of its backbone exists in a helical conformation but as double- and triple-stranded (Figure 1) antiparallel β-sheets [20].

It is well documented in the literature that the addition of acid to protein causes denaturation of the protein. Recently, molten globule states (often referred to as the A-state) have been characterized under acidic conditions in several proteins [22–24]. However, in all these proteins at least some portion of the backbone exists in a helical conformation. However, to our knowledge, molten globule states have not been identified previously along the acid-induced unfolding pathway of an all-β-sheet protein. In this paper, we describe a molten globule-like intermediate in an all-β-sheet protein, CTX III, in a 3% (w/v) solution of 2,2,2-trichloroacetic acid (TCA).

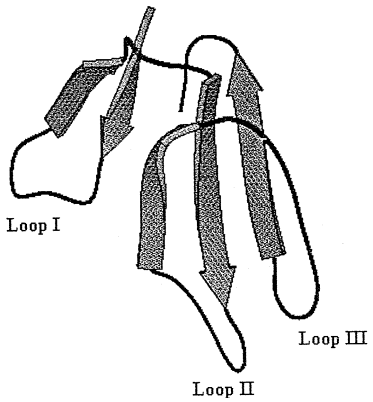

Figure 1 MOLSCRIPT [21] figure of CTX III showing the overall protein backbone fold

The ribbon arrows represent regions of the peptide backbone in β-sheet conformation.

MATERIALS AND METHODS

CTX III was purified by the procedure of Yang et al. [25]. TCA was purchased from E. Merck, Darmstadt, Germany. 1-Anilinonaphthalene-8-sulphonate (Mg^+ salt) (ANS) was from Sigma Chemical Co., St. Louis, MO, U.S.A. All other chemicals used were of high-quality analytical grade. Protein solutions were made in deionized water. All experiments, unless otherwise mentioned, were carried out at 25 ± 2 °C. The concentrations of TCA are reported as percentage (w/v).

Treatment with TCA

CTX III was treated with TCA by the method of Sagar and Pandit [26]. Protein solutions containing the appropriate concen-

Abbreviations used: CTX III, cardiotoxin analogue III; TCA, trichloroacetic acid; ANS, 1-anilinonaphthalene-8-sulphonate; FTIR spectroscopy, Fourier transform IR spectroscopy; DQF, double quantum filtered; MCD, main chain directed.

* To whom all correspondence should be addressed.

trations of TCA were prepared by the addition of the requisite amount of the stock solution of TCA (80 %, w/v) to aqueous CTX III solution. The TCA-treated protein solutions were incubated at 25 °C for 2 h. The precipitated protein samples were pelleted down rapidly by centrifugation at 704 ***g*** (3000 rev./min) for 20 min. Protein concentration in the supernatant was determined using the molar absorption coefficient of CTX III at 280 nm. Irrespective of the protein concentration used, it was found that it was possible to redissolve the precipitated protein. Degradation of the redissolved protein was assessed by recording a one-dimensional ^{1}H-NMR spectrum and comparing it with that of the untreated CTX III (native protein) samples.

CD

CD spectra were recorded on a Jasco spectropolarimeter, model J720, equipped with an interfaced personal computer. The instrument was calibrated with [^{2}H]ammonium-10-camphorsulphonate. The results are expressed as mean residue ellipticity, $[\theta]$, which is defined as $[\theta] = \theta_{obs}/60lc$, where θ_{obs} is the observed ellipticity in degrees, c is the concentration in dmol/l, l is the length of the light path in cm and constant 60 is the number of amino acid residues in the protein. All CD spectra were measured with appropriate concentrations using 0.2 mm and 2 mm pathlength cells.

ANS-binding studies

ANS-binding studies were carried out on a Hitachi F-3010 spectrofluorimeter. Appropriate volumes containing 400 μM ANS and 20 μM CTX III containing the requisite concentrations of TCA were mixed and the fluorescence spectra were recorded between 370 and 600 nm using an excitation wavelength of 350 nm. Appropriate background corrections were made in all the spectra.

Fourier transform IR (FITR) spectroscopy

All FTIR spectra were recorded on a Bomem DA8 FTIR spectrometer. Protein samples containing appropriate amounts of TCA were prepared (in water) to a final concentration of approx. 10 mg/ml. For each sample about 3–5 μl was placed between a pair of CaF_2 windows and mounted in a dismountable cell. For all samples, 100 interferrograms were collected and Fourier-transformed to generate a spectrum with resolution of 2 cm^{-1}. Second-derivative spectra were calculated with optimal band-pass filters.

^{1}H-NMR spectroscopy

^{1}H-NMR spectra were recorded at 25 °C on a Bruker DMX 600 spectrometer. All the spectra were obtained by dissolving appropriate amounts of lyophilized samples of CTX III in a mixture of 90 % water and 10 % ^2H_2O containing the requisite amount of plain TCA. One-dimensional NMR spectra were obtained with a spectral sweep width of 7508 Hz. NOESY spectra of CTX III in 3 % TCA were acquired using mixing periods of 100 and 200 ms. For NOESY experiments, presaturation was applied during the mixing period. The spectrum was acquired with 2048 complex data points in t_2 (detection time) and 512 data points in t_1 (evolution time) dimension. TOCSY spectra were recorded with a mixing period of 75 ms. All experiments were performed with quadrature detection in the phase-sensitive mode using time-proportional phase incrementation. TOCSY and NOESY data were apodized using a 90°-shifted sine-bell function in both dimensions; a 45°-shifted sine-bell function was applied to the double-quantum-filtered (DQF)-COSY data. Before Fourier transformation, the data were zero-filled to 4096 points. Spectral assignments were achieved using the TOCSY, DQF-COSY and NOESY spectra recorded for CTX III in 3 % TCA. The NOE connectivities for backbone protons were obtained using the main-chain-directed (MCD) approach [27].

RESULTS AND DISCUSSION

TCA is a well-known protein-precipitating agent. Its effect on CTX III was studied over an acid concentration range of 0–60 %. Figure 2 shows the percentage of protein in solution on addition of increasing concentrations of TCA. The protein precipitation curve is U-shaped. It can be seen that between 0 and 3 % TCA, there is no precipitation of the protein (Figure 2). Above 3 % TCA, the protein solution first turns turbid, and precipitation is almost complete at 15 % TCA. However, above 35 % TCA, there is very little precipitation and most of the protein is in solution. The effect of TCA can be classified into three stages on the basis of the percentage of the protein in solution. In stage 1, at or below 3 % TCA, most of the protein remains in solution (Figure 2). In stage 2, at intermediate TCA concentrations ranging from 5 to 35 %, the recovery of the protein is very much less, implying that most of the protein is precipitated. In the final stage, at TCA concentrations above 35 % the extent of precipitation dramatically decreases and almost all the protein (approx. 100 %) is in solution (Figure 2). Acid treatment is known to cause covalent damage to proteins. However, reverse-phase HPLC profiles and one-dimensional ^{1}H-NMR of CTX III treated with either 3 % or 45 % TCA revealed no evident covalent damage to CTX III (results not shown). Nevertheless, the possibility of deamidation cannot be completely ruled out. Although TCA is routinely used as a protein-precipitating agent, the mechanism is still not well understood. The precipitation of CTX III observed in the TCA concentration range 5–35 % could be due to 'salting out' of the protein. At the molecular level, the salting out phenomenon could be due to reversible association or irreversible aggregation of the protein molecules. We found that the protein precipitated at various concentrations of TCA is completely soluble in water, and the redissolved protein yields a similar one-dimensional ^{1}H-NMR spectrum to that of the native protein (results not shown). This indicates that the TCA-induced salting out effect leading to protein precipitation is due to

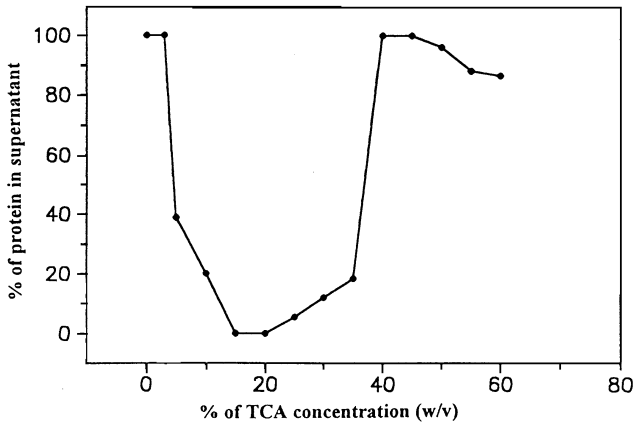

Figure 2 Extent of precipitation of CTX III at various concentrations of TCA

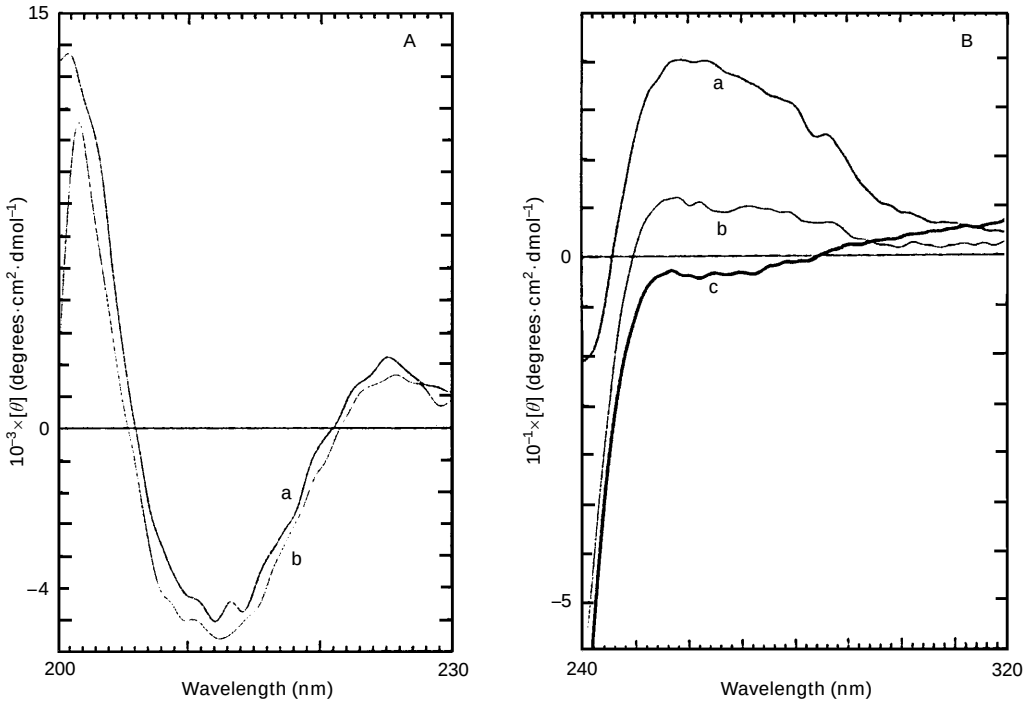

Figure 3 Far- (A) and near- (B) UV CD spectra of CTX III

a, Native CTX III; b, 3% TCA-treated CTX III; c, 45% TCA-treated CTX III.

reversible association and not to the irreversible aggregation of protein molecules. The 'salting in' effect observed at TCA concentrations above 35% could be due to complete unfolding of the protein. It is interesting to note that the other acids tested, such as HCl, acetic acid and monochloroacetic acid, did not exhibit protein-precipitation behaviour like that of TCA (results not shown).

The differential action of TCA (in terms of protein precipitation) at various concentrations gave us the first clue that it could be inducing structural transitions in the protein. Hence we decided to investigate the TCA-induced structural transitions in the protein (CTX III). It was found that the protein solutions turn turbid if the TCA concentration is raised above 3%. Hence all spectroscopic experiments on the protein (except UV spectroscopic measurements) were carried out at 3% TCA.

CD spectra of native CTX III in water showed a broad positive signal from 290 to 260 nm (Figure 3B). The near-UV CD signal in proteins is generally believed to stem from the aromatic residues in the protein the aromatic rings of which have restricted motions because of non-covalent interactions with neighbouring residues in the three-dimensional structure of the protein. On unfolding (denaturation), these non-covalent interactions are lost, which results in the disappearance of the near-UV CD signal of the protein. In the case of CTX III, the near-UV CD signal centred around 270 nm is primarily due to two tyrosine residues, Tyr-22 and Tyr-51 [20]. The solution structure of CTX III shows that these two tyrosine residues are buried inside the protein and have been shown to be important in the structural maintenance of CTX III [20]. Thus changes in the near-UV CD signal in CTX III should clearly reflect changes in the tertiary-structural interactions in the protein. The negative ellipticity peak centred at 215 nm indicates that the secondary structure of the protein is predominantly β-sheet. However, the CD spectrum

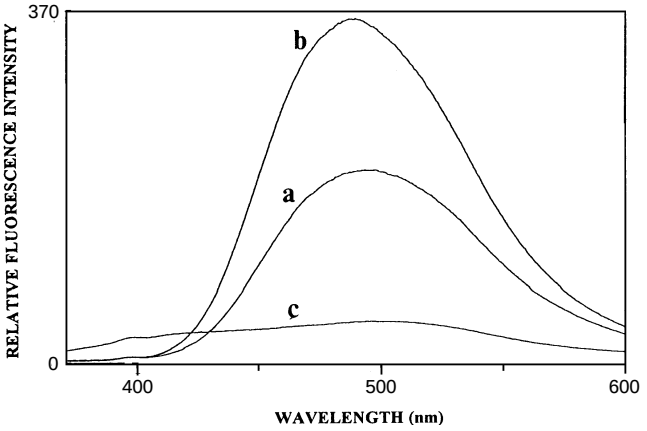

Figure 4 Emission spectra of ANS bound to CTX III in water (curve a), CTX III in 3% (w/v) TCA (curve b) and CTX III in 45% (w/v) TCA (curve c)

of the protein in the near-UV region at 3% TCA shows a dramatic fall in the tertiary-structural signal, signifying considerable loss of the tertiary-structural interactions in the protein (Figure 3B). In contrast, the CD spectrum of the protein in 3% TCA in the far-UV region shows only a minor change, implying that the native secondary-structural elements remain unperturbed (Figure 3A). The characteristics of pronounced secondary structure with little or no tertiary structure (on treatment with 3% TCA) are similar to those ascribed to proteins in the molten globule state(s). It is pertinent to mention here that there were no significant change(s) (compared with the native protein) in the near- and far-UV CD spectra of CTX III up to 2.5% TCA

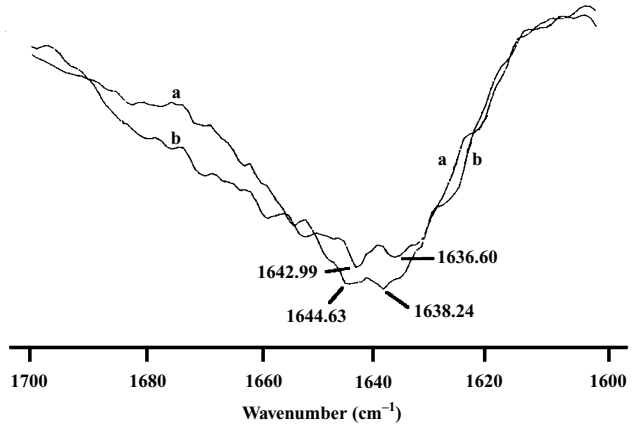

Figure 5 Second-derivative FTIR spectra in the amide I region of native CTX III in water (curve a) and CTX III in 3% TCA/water (curve b)

(results not shown). Finally, the complete loss of the near-UV CD signal of the protein in 45 % TCA indicates that the protein has completely lost its tertiary-structural interactions (Figure 3B). We could not record the far-UV region CD spectrum of the protein in 45 % TCA because of optical interference by TCA at high concentrations.

With the use of CD as the principal technique, molten-globule-like intermediates have been reported in the acid unfolding pathways of several proteins [28–30]. These acid-induced intermediate state(s) are often referred to as the A-state(s). However, Scholtz and Baldwin [31], studying the perchlorate-induced denaturation of RNase A, had cautioned that CD spectra of protein/peptides in high salt/acid concentrations could yield spurious CD spectra in the far-UV region suggestive of secondary-structure formation. Thus interpretations based on such results could lead to erroneous conclusions. To overcome this problem and to confirm the formation of a molten-globule-like state in 3 % TCA, we also employed other spectroscopic techniques.

It has been shown previously that ANS, a fluorescent hydrophobic probe, has a stronger affinity for the molten globule intermediate than for the protein in the native or fully unfolded state [6]. We therefore carried out ANS-binding studies with TCA-treated and untreated CTX III samples. The wavelength of maximum emission (λ_{max}) of ANS in the presence of untreated CTX III was 498 nm (Figure 4, curve a). In the presence of CTX III treated with 3 % TCA, the λ_{max} of ANS is blue-shifted to 484 nm (Figure 4, curve b). The emission intensity of ANS is almost twice that of the untreated native protein. This blue shift in the ANS emission maximum, accompanied by the steep increase in its emission intensity, suggests increased accessibility of hydrophobic sites for ANS binding in the protein treated with 3 % TCA. Such a feature is characteristic of the molten globule state. Interestingly, in human retinol-binding protein, the emis-

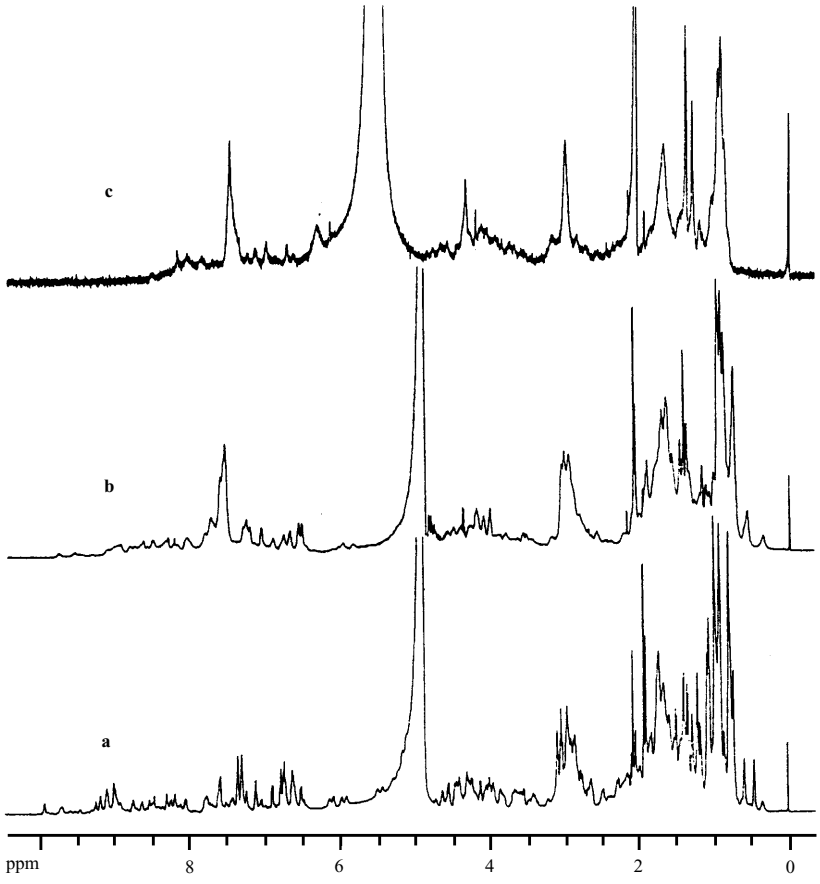

Figure 6 One-dimensional 1H-NMR spectra of CTX III at 25 °C in (a) native state (90% H_2O/10% ^2H_2O) (b) 3% (w/v) TCA/H_2O and (c) 45% (w/v) TCA/H_2O

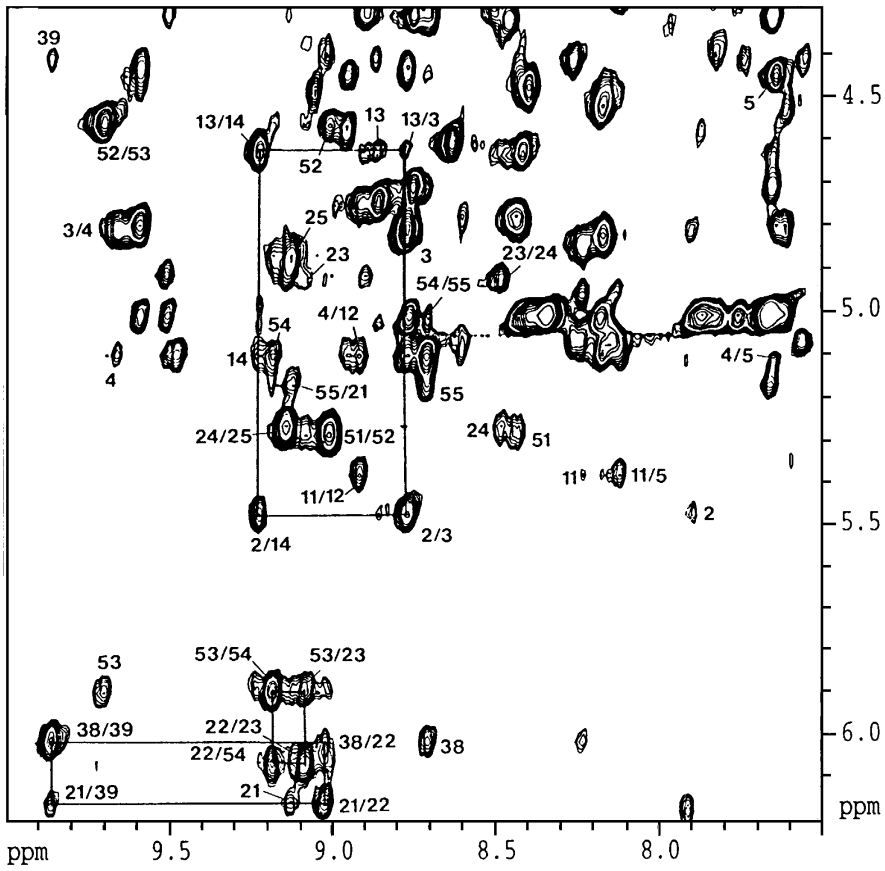

Figure 7 NOESY spectrum (200 ms mixing period) of CTX III in 3% (w/v) TCA depicting the NOEs in the fingerprint ($C^{\alpha}H$,NH) region

The sequential interstrand cross-peaks ($C^{\alpha}H$,NH) are labelled and the intraresidual cross-peaks are indicated according to the position of the corresponding amino acid residue(s), in the protein sequence.

sion intensity of ANS when bound to the acid-induced molten globule is five times that observed when ANS is bound to the native state of the protein [32]. It can be seen in Figure 4 (curve c) that, in 45% TCA, CTX III shows an emission intensity that is much lower ($I_{max} = 95$) than that of native CTX III in water. This indicates that in 45% TCA, the protein is completely unfolded, resulting in decreased affinity of the protein for ANS. The results of ANS-binding studies support the contention that CTX III exists in a molten-globule-like state in 3% TCA.

We performed FTIR experiments to support further our CD and ANS-binding experiments on the identification of a partially structured state of CTX III in 3% TCA. The amide I region (1600–1700 cm^{-1}) of the FTIR spectrum contains contributions from the C=O stretching vibration of the amide group (about 80%) with a minor contribution from the C–N stretching vibration [33]. The exact frequency of the amide I absorptions are predicted to be influenced by the strength of any hydrogen bonds involving amide C=O and N–H groups. Since each secondary-structural motif is associated with a characteristic hydrogen-bonding pattern between the amide C=O and N-H groups, it is to be expected that each type of secondary structure will give rise to characteristic amide I absorption [34,35]. The second-derivative spectrum of CTX III in water shows a strong amide I maximum at 1638 cm^{-1}, indicating that the secondary structure is predominantly β-sheet (Figure 5). The native spectrum also shows another band at 1644 cm^{-1}. This represents the random-coil conformation in the protein. CTX III treated with 3% TCA exhibits a similar IR spectrum to that of the native protein with the amide I band centred at 1636 cm^{-1}, indicating that the native secondary-structural interactions (β-sheet) are mostly intact. The shift of the amide I band in the spectrum of CTX III in 3% TCA could be mainly due to the change in pH on addition of TCA. There is an increase in the intensity of the amide band (at 1643 cm^{-1}) representing the random-coil conformation in the spectrum of CTX III in 3% TCA, indicating an increase in the random-coil conformation (Figure 5). The second-derivative IR spectrum of CTX III treated with 45% TCA looked very strange (for reasons unknown) and no meaningful inference could be made (results not shown). From the FTIR results it is clear that CTX III exists in a molten-globule-like state in 3% TCA.

It has been reported previously that acid-induced molten globule states show a higher tendency to aggregate (reversibly or irreversibly). In principle, the residual structure obtained in molten globule states could also be due to aggregation. The presence of aggregation can also be detected by FTIR. An increase in the intensity of the amide I band around 1615–1620 cm^{-1} often indicates the formation of a structure due to intermolecular hydrogen-bonding. The absence of this feature from our data indicates that the residual structure is not due to aggregation (Figure 5).

The protein in the molten globule state essentially has con-

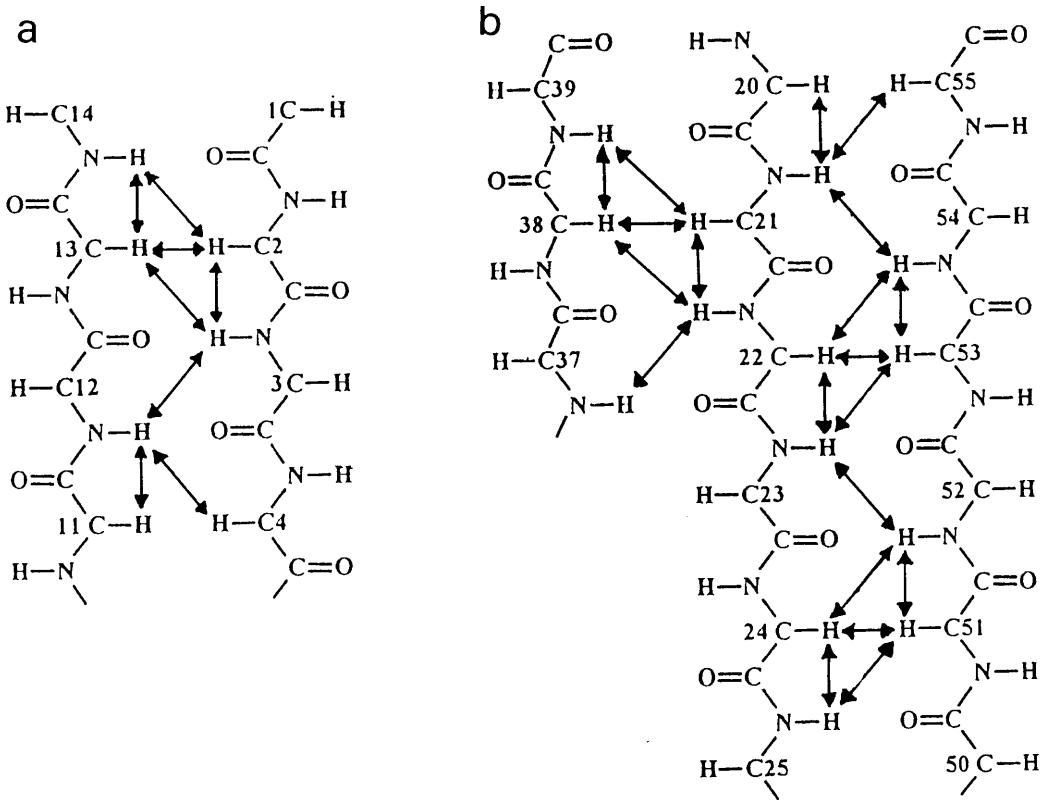

Figure 8 Schematic representation of the through-space NOE interactions in CTX III in 3% (w/v) TCA of (a) eight NOEs in the double-stranded region and (b) 20 NOEs in the triple-stranded region

Proton pairs that correspond to the cross-peak in the NOESY spectrum are connected by double-headed arrows. In total, 28 NOEs are observed for CTX III in the β-sheet region.

siderable secondary structure and greater flexibility for the side chains because of limited tertiary-structural contracts [6]. Therefore, in the molten globule state, the protein should yield NMR spectra showing a degree of chemical-shift averaging intermediate between the native and unfolded states. For native CTX III (Figure 6), the NMR linewidths are fairly narrow and show good dispersion and this reflects the highly specific inter-residue interactions within the compact folded state. These features can be quite clearly seen in the aromatic region of the spectrum (where the chemical shifts range from approximately 6.4 to 7.5 p.p.m.). In 3% TCA, the spectrum shows few resonances close to those in the unfolded state (Figure 6). Compared with the native state, the spectra of the protein in both 3% TCA (molten-globule-like state) and 45% TCA (unfolded state) show that the chemical-shift dispersion in the aromatic region of the spectrum was considerably reduced (Figure 6). This is due to the free motion of the aromatic rings, resulting from the loss of tertiary-structural contacts involving the aromatic residues in the molten-globule-like state (in 3% TCA) and unfolded state (45% TCA). Line broadening of resonances in ^{1}H NMR is generally associated with either chemical-exchange processes or restricted rotational motion as the result of aggregation of the protein molecules [6,8]. Since the TCA unfolding process is completely reversible, the latter possibility can be excluded in the present case. Line broadening is particularly evident in the aromatic region (6.4–7.5 p.p.m.) and in some of the peaks corresponding to the side-chain aliphatic protons (1–3 p.p.m.). This observation is consistent with the definition of a molten globule in which the side chains are believed to be more flexible (than in the native state) because of loss of some native tertiary-structural interactions in the molten globule state. The one-dimensional NMR spectrum of CTX III with 3% TCA (Figure 6, curve b) exhibits some features resembling that of the protein (CTX III) in the native state (Figure 6, curve a). For example, some of the reasonances below 1 p.p.m. are intact in both spectra. The resonances in this region of the NMR spectrum originate from the side-chain protons of Ile-39. From these results it appears that structural features of CTX III in 3% TCA are intermediate between the native (acid untreated) and the unfolded (with 45% TCA) states. Thus one-dimensional ^{1}H-NMR spectral results corroborate those obtained in CD, fluorescence and FTIR experiments, providing additional evidence for the existence of CTX III in a molten-globule-like state in 3% TCA.

The solution structure of CTX III shows that the protein is an all-β-sheet protein with five β-strands, spanning residues 1–5, 10–14, 20–26, 34–39 and 50–55. These five β-strands together make up the antiparallel double- and triple-stranded β-sheet segments in the protein [20]. Using conventional two-dimensional NMR techniques, we attempted to characterize the molten-globule-like intermediate of CTX III in 3% TCA. Interestingly, the NOESY spectrum of CTX III in 3% TCA was fairly well dispersed (Figure 7). It showed a significant number of NOEs, which may arise from intra- as well as inter-residue through-space interactions (Figure 7). However, assignment of all spin systems in the molten-globule-like state was not feasible because of the extensive peak overlap in some regions of the spectrum. The overlap was especially large in the aromatic region. However, several spin systems could be immediately identified by com-

parison of the spectrum of the protein in the native state [20] with that of the protein in the molten-globule-like state in 3 % TCA. This served as the starting point for determining the location of adjacent spin systems through a combination of DQF-COSY, TOCSY and NOESY experiments using the MCD approach [27]. Although the chemical shifts of many of the amide proton resonances had changed in 3 % TCA compared with those in the native state in water, there were a few residues with amide proton resonance chemical shifts that did not undergo marked changes in 3 % TCA. The chemical shifts of the resonances corresponding to the amide protons of Cys-21, Tyr-22, Asn-4 and Cys-54 remained almost completely unaltered (Figure 7). These amino acid residues are all involved in the secondary-structure formation in the native state of the protein. Therefore these amide proton resonances were effectively used in tracing the residual backbone structure in 3 % TCA using the MCD (Figure 8) approach. Analysis of the NOESY spectrum of CTX III in 3 % TCA using the MCD approach revealed that there are eight NOEs in the double-stranded antiparallel β-sheet region (comprised of residues 2–4 and 11–14, Figure 8a) and 20 NOEs in the triple-stranded β-sheet region representing residues 20–25, 37–39 and 51–55 (Figure 8b). At a gross level, it appears that about 65 % of the native secondary-structural contacts are intact in the molten-globule-like intermediate of CTX III in 3 % TCA. The secondary-structural contacts that are missing in the protein treated with 3 % TCA are those at the β-sheet segments located in the triple-stranded β-sheet region. Previously, partially structured intermediates have been characterized using two-dimensional NMR in several proteins [37–39]. However, this is first instance of an acid-induced partially structured intermediate being characterized by two-dimensional NMR in an all-β-sheet protein.

It is of interest to analyse how a stable partially structured state could be realized in a small all-β-sheet protein such as CTX III. CTX III is a highly stable protein. The stability of the protein stems from the four disulphide bridges which fortify the head region of the molecule [20]. The disulphide bonds force the three loops to come close to each other. Thus the five β-strands in the protein are ideally placed to form the antiparallel double- and triple-stranded β-sheet segments in the protein. It appears that, at low acid concentrations, only the weak tertiary-structure interactions involving some of the aromatic and aliphatic residues in the protein are disrupted. From the two-dimensional NMR data, it is clear that the β-sheet segments that are disrupted in the molten-globule-like state are those that are located at the end portions of the loops, which are not stabilized by disulphide bonds. Thus the four disulphide bonds in CTX III appear to be crucial for the protection of the β-sheet secondary-structural elements in the molten-globule-like state in 3 % TCA. Interestingly, the characterization of the molten-globule-like state in a small protein such as CTX III disproves the theory that the occurrence of molten globule states is restricted to high-molecular-mass proteins.

Characterization of the molten globule state is important as it provides a clue to the forces that come into play during protein folding. Several proteins have been shown to exist in a molten globule state under a variety of denaturing conditions [24,40–43]. Partially structured intermediate states have been shown to occur along the acid-induced folding/unfolding pathway of several proteins [6,22,28,42]. These acid-induced partially structured intermediates are often referred to as A-states [6]. The A-state is reported to share most of the structural features characteristic of the molten globule states [6,42]. The partially structured state obtained in CTX III in 3 % TCA has structural features that closely resemble those of the molten globule state. As the TCA-induced unfolding of CTX III is completely reversible, the molten-globule-like state of CTX III in 3 % TCA could represent a meaningful protein folding intermediate. Thus far, molten globule states have been identified only along the folding/unfolding pathways of proteins in which significant portions of the backbone exist in a helical conformation [44]. Recently, human retinol binding protein has been shown to exist in the molten globule state under acidic conditions [38]. Although it is a predominantly β-sheet protein, more than 10 % of its backbone is known to adopt helical conformation in the native protein. It has not yet been possible to generalize the concept of molten globule as a universal equilibrium protein-folding intermediate(s). The lacuna is due to the lack of enough evidence of the occurrence of molten-globule-like intermediates in the folding pathway of proteins consisting entirely of β-sheet structure (all-β-sheet protein). Thus the identification of a molten-globule-like state in the TCA-induced acid unfolding pathway of CTX III (an all-β-sheet protein) demonstrated in the present study could pave the way for the generalization of the molten globule state as a universal protein-folding intermediate.

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