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2,2,2-Trifluoroethanol Induces Helical Conformation in an All β-Sheet Protein

G. Jayaraman, T. K. S. Kumar, A. I. Arunkumar, and C. Yu[1]

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

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The effect of 2,2,2-trifluoroethanol (TFE) on the structure of an all β-sheet protein, cardiotoxin analogue II (CTX II), from the Taiwan cobra (*Naja naja atra*) is studied. Using circular dichroism studies, it is found that higher concentrations of TFE induced a structural transition from β-sheet to α-helix, both in the native state (nCTX II) and in denatured but not disulfide reduced CTX II (dCTX II) samples. The β-sheet to α-helix conversion is shown to be cooperative. However, in denatured and reduced CTX II (rCTX II), TFE transforms a portion(s) of the protein backbone from a random coil to an α-helical conformation. Based on the solution structure of CTX II and the physical property of TFE, a possible mechanism for the observed backbone structural transitions induced by TFE is discussed. The results described in this paper question the significance of the structure of the "molten globule" intermediate(s) obtained in organic solvents such as TFE.

2,2,2-Trifluoroethanol (TFE) has been widely used as a structure inducing cosolvent [1, 2]. Numerous investigations, using peptides and proteins, have established a strong correlation between TFE-induced structure and the native protein structure [3–6]. In the peptide systems, TFE does not induce α-helix indiscriminately. Isolated fragments of proteins, having helical regions in the native state, exhibit higher helical content in TFE [7, 8]. Hence, TFE has been often used as a test for the intrinsic ability of peptides to adopt helical conformation in the native state. Although, TFE is assumed to stabilize helical structures in native proteins/peptides, this is not widely accepted since little is known about the effect of TFE on other secondary structural elements. Recently, Shiraki et al [9] examined the TFE-induced conformational transitions on the native structure of more than twenty proteins. The results of this study revealed that even predominantly β-sheet proteins such as β-lactoglobulin and concanavalin A show high helical propensity in TFE. Crystal structure of these two proteins showed that only a small percentage of their backbone exists in a helical conformation. Interestingly, secondary structure prediction analysis on these two predominantly β-sheet proteins revealed the propensity for significant helical content. Thus, it was argued that eventhough a protein does not possess helical segment(s) in its structure in water, TFE can successfully induce helical conformation even if the polypeptide backbone shows propensity to form helix. However, generalisation of the mode of action of TFE in inducing α-helix can only be made after studying the effect of TFE on a protein that does not have helical segments in its backbone, such as an all β-sheet protein(s). To-date, the helix inducing effect of TFE on all β-sheet proteins has not been studied. In this communication, we report the induction of α-helical conformation using TFE in an all β-sheet protein, cardiotoxin analogue II (CTX II) from Taiwan Cobra, *Naja naja atra* [10–12]. We also discuss the structural transitions occurring in the native CTX II (nCTX II), denatured but not disulfide reduced CTX II (dCTX II) and denatured and disulfide reduced CTX II (rCTX II), on the addition of increasing concentrations of TFE.

MATERIALS AND METHODS

Cardiotoxin analogue II (CTX II) was isolated and purified as per the procedure of Yang *et al* [13]. 2,2,2-Trifluoroethanol was purchased from Janssen Chemica, Belgium. dCTX II was prepared by dissolving appropriate amounts of CTX II in 6M

[1] Author to whom all correspondence should be addressed. Fax: 886 35 711082.

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guanidinium hydrochloride and incubating at room temperature for two hours. Similarly rCTX II sample was obtained by dissolving requisite amounts of CTX II in 6M guanidinium hydrochloride and 2% β-mercaptoethanol, overnight at room temperature.

Circular dichroism. All circular dichroism measurements were made on a Jasco J-720 spectropolarimeter using quartz cells of pathlengths 2 mm and 0.2 mm at 25°C. All protein solutions were made in deionised water. The concentrations of the cardiotoxin samples were estimated from the respective molar extinction coefficients at 280 nm. In case of rCTX II and dCTX II, all TFE titrations were carried out by a ten-fold dilution of the denaturants (to a final concentration of 0.6 M guanidinium hydrochloride with/without 0.2% β-mercaptoethanol) with appropriate concentrations of the TFE-water mixture. The percentage helicity was calculated from the ellipticity value at 222 nm as per the procedure reported by Chen *et al* [14]. The concentration of TFE is expressed as percentage volume to volume.

Secondary structure prediction. The α-helix and β-sheet propensities in CTX II sequence was predicted using four different prediction methods. The prediction methods were that of Kotelchuck and Scheraga [15], Wu and Kabat [16], Chou and Fasman [17] and Ptitsyn and Finkelstein [18].

RESULTS AND DISCUSSION

CTX II is an all β-sheet small molecular weight (6.8 Kda) protein containing 60 amino acid residues with four disulfide bridges (Figure 1). It has no helical segment(s) in its backbone [19].

The circular dichroism (CD) spectra of nCTX II (Figure 2a) in the far UV region at neutral pH and 25°C shows a minimum centered around 213 nm, which is indicative of the backbone being in β-sheet conformation. Addition of TFE upto 60% (v/v) to nCTX II does not cause significant change in the ellipitcity value at 215 nm which implies that the backbone conformation is unaltered. However, further increase in TFE concentration, beyond 60%, induces helical conformation. This is evident from the appearance of the characteristic negative ellipticity bands centered at 208 nm and 222 nm. The far UV CD spectra of CTX II in 6M guanidinium hydrochloride, with (rCTX II) and without (dCTX II) β-mercaptoethanol are representative of random coil state(s) (Figure 2d). This aspect is also exemplified by the complete loss of the near UV CD signal (data not shown). We wanted to investigate if TFE could induce helix in a protein wherein all tertiary structural contacts (except the disulfide bridges) are disrupted. Hence we studied the effect(s) of TFE on dCTX II sample. Interestingly, upon addition of 20% TFE, dCTX II refolds into a β-sheet conformation (figure 2b). It can be noticed that, nCTX II also exists in a β-sheet conformation at 20%

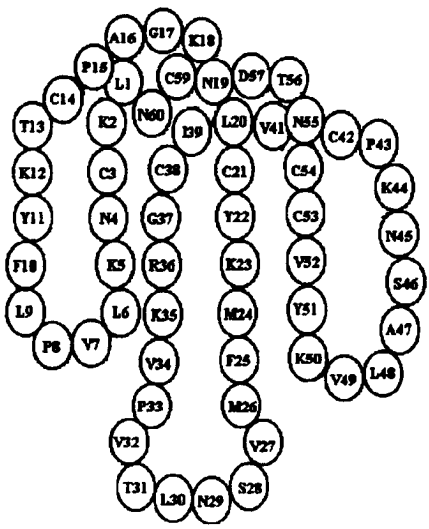

FIG. 1. The amino acid sequence of CTX II. The residues Asp40 and Arg58 are not shown for reasons of clarity. The four disulfide bridges in the protein are located between Cys3 and Cys21; Cys14 and Cys38; Cys42 and Cys53; and Cys54 and Cys59. Residues 2–4 and 11–13 constitute the anti-parallel double strand and residues 20–25, 35–39 and 50–54 form the anti-parallel triple stranded β-sheet.

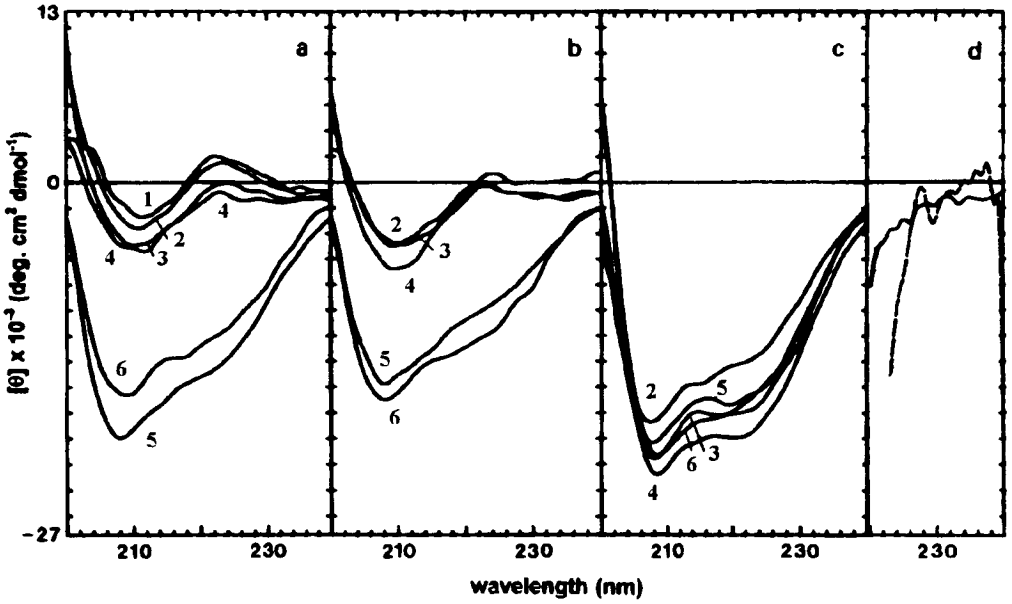

FIG. 2. Far UV CD spectra of (a) nCTX II, (b) dCTX II, (c) rCTX II; curves 1–6 represents spectra in 0%, 20%, 40%, 60%, 80% and 90% TFE (v/v) at 25°C, respectively. (d) The spectra of guanidinium hydrochloride (6M) denatured CTX II with (dashed line) and without (solid line) 0.2% β-mercapto ethanol in water.

TFE concentration (Figure 2a). Even upto 60% TFE concentration, dCTX II remains in a β-sheet conformation. However, increasing concentrations of TFE beyond 70% results in a cooperative β-sheet to α-helix transformation of the backbone (in dCTX II). Surprisingly, the maximum percentage of helix (∼30%) induced upon titration with TFE is less in the case of dCTX II as compared to nCTX II (∼40%). This may be due to the residual guanidinium hydrochloride (0.6M) present in dCTX II samples at all concentrations of TFE. The CD spectra of rCTX II (Figure 2c), upon addition of increasing concentrations of TFE upto 90%, presents a contrasting picture to the one observed for nCTX II, the 208 nm and 222 nm CD bands characterising the α-helix appear even upon addition of 20% TFE. Upto 40% TFE, the intensity of these helix characteristic CD bands steadily increases with the increase in TFE concentration. The percentage of helix induced almost remains unchanged between 40% and 60% TFE concentrations. A further marginal rise in helical content is observed beyond 60% TFE. A maximum of 62% helix is induced in the protein (rCTX II) in 70% TFE (Figure 3). This corresponds to about thirty-seven residues in CTX II. It is however, not clear whether the induced helix is due to the transformation of residues comprising the β-sheet or of the residues hitherto not involved in the secondary structure formation, in the native state (nCTX II) of the protein. From the results obtained, it is clear that the backbone structural transitions undergone by nCTX II, dCTX II and rCTX II, under the influence of increasing concentrations of TFE are different. In nCTX II, the protein backbone transforms from β-sheet to α-helix. In dCTX II, the backbone transition is from a random coil to β-sheet to α-helix. In rCTX II the transformation is directly from a random coil to a α-helix. Goto and coworkers [9, 20] have recently reported that TFE can only induce helix in these portion(s) of the sequence of a protein which are either helical in the native state of the protein or have a propensity to form a helix. It is pertinent to mention here that all the four secondary structure prediction methods employed herein showed that no portion(s) of the backbone of CTX II has a propensity to exist in a helical conformation. In this background of results, we believe that the induction of helix in an all β-sheet protein such as CTX II is not only novel but also significant and interesting.

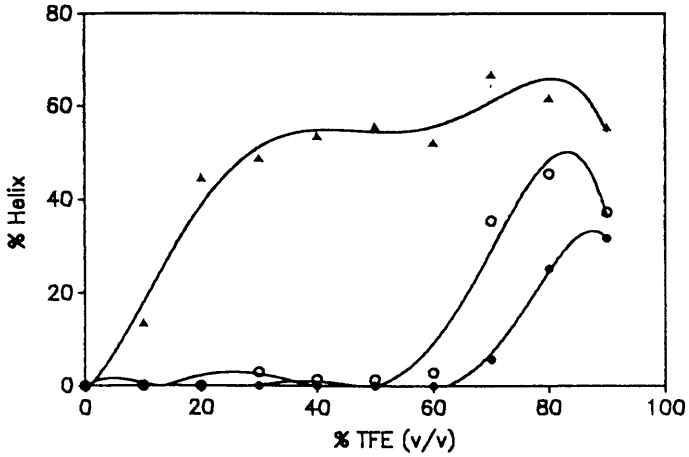

FIG. 3. Percentage of α-helix induced at various concentrations of TFE in nCTX II (o), dCTX II (●), and rCTX II (▲).

The mechanism of induction of helix in proteins by TFE is still a subject of intense debate. Several properties of TFE have been suggested to be responsible for the observed structural stabilization. The dielectric constant of the TFE is one-third that of water and hence it should closely approximate the interior of proteins and also should strengthen interactions between charged groups. Nelson and Kallenbach [21] demonstrated that the dominant TFE effect is due to its weaker basicity than water. Consequently, TFE would decrease the hydrogen bonding of amide protons to the solvent and hence strengthens intramolecular hydrogen bonding. The net result of this effect is the induction of the α-helix [21]. As TFE is less polar solvent, intrastrand hydrogen bonding within the polypeptide is favoured over interstrand hydrogen bonding. Hence TFE stabilises an α-helix and not β-sheet. In addition, the low polarity of TFE interupts hydrophobic interactions and therefore act as a potential denaturant of tertiary and quaternary structure [22, 23].

The TFE-induced structural transitions observed in nCTX II, dCTX II and rCTX II can be rationalised based on the native structure of CTX II and the physical property of TFE. CTX II consists of five β-strands emerging from the globular head [19]. These five strands align to yield an antiparallel double stranded β-sheet and a triple stranded β-sheet (Figure 1). The five β-strands are held in position by a mesh of four disulfide bridges in the globular head portion of the molecule. Since, the disulfide bonds in nCTX II are intact, the β-strands are forced closer to each other facilitating the formation of stable β-sheets. It requires 70% TFE to break the interstrand hydrogen bonds holding the β-sheet. The breakage of the interstrand hydrogen bonds promotes the intrastrand hydrogen bonding in the backbone, resulting in the formation of α-helix. This explains the β-sheet to α-helix transformation in nCTX II beyond 70% TFE (Figure 2a). In contrast, the disulfide bonds are cleaved in rCTX II (Figure 2c) and hence the β-strands drift away from one another. Under these conditions, the interstrand hydrogen bonding responsible for the formation of β-sheet is not favoured. In addition, as the hydrogen bonding of amide protons to the solvent is decreased due to the weaker basicity of TFE, intramolecular-intrastrand hydrogen bonding is established within the backbone of the protein aiding the formation of α-helix even at low TFE concentrations ($\geq$ 20%). This logic can explain the random coil to α-helix transition in rCTX II. Interestingly, in dCTX II, the formation of the β-sheet from the random coil precedes the formation of the helix (Figure 2b). As the disulfide bonds are intact in dCTX II, refolding by the dilution of the denaturant with lower concentrations of TFE brings the β-strands together facilitating the formation of the β-sheet. At higher concentrations, greater than 70%, TFE disrupts the formation of the β-sheet and converts the backbone into a helix. Thus, based on these results, it can be argued that TFE can induce helical conformation in peptide/proteins, nonspecifically.

To our knowledge, this is the first report, wherein induction of α-helix by TFE is shown in an all β-sheet protein. The findings of this study, in our opinion, has significant implications in the area of protein folding. The non-specific induction of α-helix by TFE as reported in the present study not only questions the validity of the non-hierarchial model [24] of protein folding but also raises doubts regarding the significance of non-native structures in the protein folding intermediates obtained in TFE.

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