Amyloid-like Fibril Formation in an All β-Barrel Protein

PARTIALLY STRUCTURED INTERMEDIATE STATE(S) IS A PRECURSOR FOR FIBRIL FORMATION*

Acidic fibroblast growth factor from newt (Notophthalmus viridescens) is a ~15-kDa, all β-sheet protein devoid of disulfide bonds. In the present study, we investigate the effects of 2,2,2-trifluoroethanol (TFE) on the structure of newt acidic fibroblast growth factor (nFGF-1). The protein aggregates maximally in 10% (v/v) TFE. Congo red and thioflavin T binding experiments suggest that the aggregates induced by TFE have properties resembling the amyloid fibrils. Transmission electron microscopy and x-ray fiber diffraction data show that the fibrils (induced by TFE) are straight, unbranched, and have a cross-β structure with an average diameter of 10–15 Å. Preformed fibrils (induced by TFE) of nFGF-1 are observed to seed amyloid-like fibril formation in solutions containing the protein (nFGF-1) in the native β-barrel conformation. Fluorescence, far-UV CD, anilino-8-naphthalene sulfonate binding, multidimensional NMR, and Fourier transformed infrared spectroscopy data reveal that formation of a partially structured intermediate state(s) precedes the onset of the fibrillation process. The native β-barrel structure of nFGF-1 appears to be disrupted in the partially structured intermediate state(s). The protein in the partially structured intermediate state(s) is found to be “sticky” with a solvent-exposed non-polar surface(s). Amyloid fibril formation appears to occur due to coalescence of the protein in the partially structured intermediate state(s) through solvent-exposed non-polar surfaces and intermolecular β-sheet formation among the extended, linear β-strands in the protein.

Protein aggregation is a problem of importance not only in biotechnology but also in health-related industries (1, 2). Globular proteins in aqueous solution often tend to aggregate under a variety of conditions of concentration, temperature, pH, and ionic strength (3–6). The morphology of aggregates formed varies considerably and ranges from amorphous forms to highly structured fibrils (7–9). Structured fibrils formed in vitro closely resemble the highly organized amyloid fibrils found in association with a variety of human disorders, including Alzheimer’s disease, Creutzfeldt-Jakob disease, Huntington’s disease, and type II diabetes (10–19). Several studies show proteins that are apparently unrelated in sequence and, in their native conformation, aggregate into fibrils that have characteristic amyloid-like structural and histological features (16, 20–26). Recently, Bucciantini et al. (27) demonstrated that amyloid-like fibrils of SH3 domain (from bovine phosphatidylinositol 3-kinase) induced in vitro in 25% (v/v) 2,2,2-trifluoroethanol (TFE)1 (under appropriate conditions) are cytotoxic to fibroblast NIH3T3 cells. The amyloid fibrils generated ex vivo are observed to seed fibrillate in cultured NIH3T3 cells (23). Therefore, it is now increasingly believed that amyloid represents a generic form of polypeptide conformation, and all peptides/proteins have the potential to form amyloid-like fibrils under appropriate conditions (21–27).

The molecular mechanism underlying the amyloid fibril formation is poorly understood. Recent studies on proteins such as transthyretin (16, 25), lysozyme (26), α-synuclein (28), α-microglobulin (29), immunoglobulin light chain variable domain (30) suggest that amyloid fibril formation from the native state occurs via conformational changes leading to the formation of sticky amyloid-prone, partially structured intermediate(s). The partially structured intermediate state(s) is postulated to associate into oligomers and subsequently undergo structural rearrangement to form amyloid-like fibrils (8, 16). However, very little information exists on the conformational features of the “amyloid-prone” partially structured intermediate state(s).

In the present study, we investigate the TFE-induced conformational transitions in a β-barrel protein such as the newt acidic fibroblast growth factor (nFGF-1, Refs. 31 and 32). TFE is observed to induce amyloid-like fibrils in nFGF-1. The formation of amyloid-like fibrils is triggered by the accumulation of a partially structured intermediate state(s) in the TFE-induced unfolding pathway of nFGF-1.

MATERIALS AND METHODS

Heparin-Sepharose was purchased from Amersham Biosciences. Labeled 15N,15C, D2O, and TFE-d3 were purchased from Cambridge Isotope Laboratories. 1-Anilino-8-naphthalene sulfonate (magnesium salt), thioflavin T (ThT), and Congo red were purchased from Sigma. All other chemicals used were of high quality analytical grade. Unless otherwise mentioned, all solutions were made in 100 mM phosphate buffer (pH 7.0) containing 100 mM sodium chloride. All experiments were performed at 20 °C.

Protein Expression and Purification—Recombinant nFGF-1 was prepared from transformed Escherichia coli BL21(DE3)pLysS cells. The nFGF-1 DNA construct consisting of 486 base pairs was inserted between the NdeI and BamHI restriction sites. The expressed protein was

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1 The abbreviations used are: TFE, 2,2,2-trifluoroethanol; nFGF-1, newt acidic fibroblast growth factor; ThT, thioflavin T; ANS, 1-anilino-8-naphthalene sulfonate; HSQC, heteronuclear single quantum coherence; FT-IR, Fourier transform infrared spectroscopy.

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For the seeding experiments, various aliquots 100–500 μl of sealing solution (TFE-induced preformed fibrils) of the protein was added to freshly prepared solution (TFE induced preformed fibrils) of the protein. The sealing experiments were carried out with appropriate controls. The first control involved the incubation of an identical solution of nFGF-1 in the absence of the aliquot of the sealing solution, and the second control involved the dilution of an approximate aliquot of the sealing solution into phosphate buffer. The sealing (aggregation) was monitored by the increase in the absorbance at 350 nm. In addition the sealing solution was analyzed by transmission electron microscopy to obtain definite evidence for the existence of fibrils and analyzing their morphology.

 stead State Fluorescence—Fluorescence experiments were performed on a Hitachi F2500 spectrofluorimeter at 2.5- or 10-nm resolution. Intrinsic fluorescence measurements were made at a protein concentration of 100 μg/ml using an excitation wavelength of 280 nm. 1-Anilino-8-naphthalene sulfonate (ANS) binding experiments were carried out on nFGF-1 at various concentrations of TFE using an excitation wavelength of 390 nm. The emission was monitored between 420 and 600 nm. The concentrations of the dye (ANS) and the protein (nFGF-1) used were 200 μM and 100 μg/ml, respectively.

NMR Experiments—All NMR experiments were performed on Bruker Avance-600 NMR spectrometer at 20 °C. A 5-mm inverse probe with a self-shielded z-gradient was used to obtain all gradient-enhanced 1H, 15N heteronuclear single quantum coherence (HSQC) spectra. 15N decoupling was accomplished using the globally optimized alternating-phase rectangular pulse sequence. 2048 complex data points were collected in the [H,15N] HSQC experiments. 512 complex data points were collected in the 15N dimension. The HSQC spectra were recorded at 32 scans at all concentrations of TFE. The concentration of the protein sample was 0.5 mg/ml in 95% H2O and 5% D2O (containing 100 mM phosphate and 100 mM sodium chloride). 15N chemical shifts were referenced using the consensus ratio of 0.01013291189. All spectra were processed on a Silicon Graphics work station using XWINNMR and AURELIA software.

Fourier Transform Infrared Spectroscopy (FT-IR)—The samples for the FT-IR spectral measurements were prepared as follows. Protein aggregates formed in 15% (v/v) TFE (at a protein concentration of 10 mg/ml) were centrifuged at 10,000 rpm using a desktop centrifuge for 20 min, and the supernatant was removed carefully. The precipitant was dried overnight in a vacuum desiccator. The dry powder of the aggregates was enclosed in a KBr tablet by a conventional high pressure method. FT-IR spectra of nFGF-1 at 0% (v/v) and 70% (v/v) TFE were acquired by dissolving the protein in 99% D2O containing appropriate concentrations of TFE-d6. All spectra were recorded with a wave number resolution of 2 cm⁻¹. For each spectrum, 64–200 interferograms were collected and averaged, and a Happ-Genzel apodization function was applied before Fourier transformation. All processing procedures were carried out so as to optimize the quality of the spectrum in the amide I region, between 1600 and 1700 cm⁻¹.

RESULTS AND DISCUSSION

nFGF-1 is a ~15-kDa, all β-sheet protein with no disulfide bonds (31). The protein lacks helical segments, and the secondary structural elements include 12 β-strands arranged into a β-barrel architecture (Fig. 1).

Effect(s) of TFE on the Backbone Conformation—The original objective of this study was to investigate the specificity/non-specificity of the helix-inducing effect(s) of 2,2,2-trifluoroethanol. In this context, we monitored the secondary structural changes in nFGF-1 at various concentrations of TFE using far-UV circular dichroism (CD) spectroscopy. The far-UV CD spectrum of nFGF-1 (200–250 nm) shows two prominent ellipticity bands (a positive ellipticity band at 228 nm and an intense minimum at around 205 nm) characteristic of type II β-barrel proteins (Fig. 2, inset). The 228-nm CD band is believed to primarily represent the arrangement of the β-strands in the β-barrel architecture of the protein. No significant change(s) occurs in the intensity of the 228-nm CD band at a TFE concentration lower than 5% (v/v) (Fig. 2). However, at a TFE concentration of 8% (v/v), the far-UV CD spectra of the protein show dramatic changes (Fig. 2). The positive CD band centered at 228 nm is lost, implying the disruption of the native β-barrel architecture. The loss of the positive CD band at 228
nm is paralleled by the appearance of a negative CD band at around 218 nm in 20% TFE (v/v) (Fig. 2), suggesting the formation of extended β-sheet conformation. The intensity of the negative ellipticity band at 218 nm is observed to steadily increase with the increase in the TFE concentration from 10 (v/v) to 50% (v/v) (Fig. 2). At higher concentrations of TFE (>50% (v/v)), the far-UV CD spectra of the protein show two negative ellipticity bands at 208 and 222 nm, indicating the induction of helical conformation (Fig. 2). Because nFGF-1 in its native conformation lacks helical segments, the induced helix conformation (at higher concentrations of TFE (>50% v/v)) appears to be essentially non-native in origin. The percentage of non-native helix estimated from the far-UV CD spectrum of the protein 70% (v/v) is about 20%. The majority (>60%) of the backbone of the protein (33, 34) (in 70% (v/v) TFE) is estimated to be unstructured. The results discussed above clearly suggest that the conformational transitions induced by TFE in nFGF-1 occur in two stages. In the first stage, the β-barrel conformation is disorganized, resulting in the formation of extended β-sheet conformation. In the second stage, portions of the backbone of the protein form non-native helix conformation.

Influence of Backbone Conformation on Protein Aggregation—The protein samples treated with TFE in the concentration range of 10% (v/v) to 45% (v/v) and incubated at room temperature (~25 °C) for more than 3 h are observed to turn turbid. Hence, the aggregation of the protein (nFGF-1) is examined systematically by monitoring the changes in the 350-nm absorbance (scattering) at various concentrations of TFE (after 3 h of incubation of the protein at 25 °C in various concentrations of TFE). Interestingly, the aggregation profile shows that the protein tends to aggregate maximally between 8 and 45% (v/v) TFE, wherein the protein is observed to exist in an extended β-sheet conformation (Fig. 3). The extent of aggregates formed appears to increase with the increase in the formation of the extended β-sheet conformation in the protein. In addition, the aggregates formed (at TFE concentrations between 10% to 50% (v/v) TFE) are long and thread-like. Beyond this range of TFE concentration (>50% (v/v) TFE), the protein solution turns clear (with low 350-nm absorbance values) even after 48 h of incubation at 25 °C (Fig. 3). These results suggest that the aggregation of nFGF-1 is related to the nature of non-native secondary structural elements induced in the protein (by TFE). Formation of non-native extended β-sheet conformation appears to promote aggregation, and induction of non-native helix conformation correlates with the inhibition of protein aggregation. Similar observations were made by Dobson and co-workers, based on exhaustive point mutations in the muscle acylphosphatase protein (35, 36). A clear-cut kinetic partitioning between aggregation and folding of proteins was observed. Protein aggregation was strongly correlated to the β-sheet propensity of the regions of the protein (muscle acylphosphatase) in which the point mutations were located (35). In addition, mutations that stabilize helix conformation were shown to decrease the aggregation process, and those that destabilized the helix increased the aggregation rate (35).

TFE-induced Aggregates Possess Amyloid Fibril-like Properties—The aggregates of nFGF-1 formed in TFE (in 8–40% (v/v) TFE) have a fibrillar texture, suggesting that they could possess properties characteristic of amyloid fibrils. This possibility was examined by characterizing the tinctorial and ultrastructural properties of the TFE-induced aggregates.

Congo red is a hydrophobic dye routinely used to identify amyloid fibril formation by proteins (37). The absorption maximum of the dye is known to undergo a red shift upon binding to ordered repetitive β-sheet structures in the amyloid fibrils (38). Although the TFE-induced aggregation is maximum at
10% (v/v), Congo red binding is observed maximally at 15% (v/v) TFE (Fig. 4A). The dye binding is not only accompanied by a significant increase in the absorbance intensity at 490 nm but also by a prominent red shift in the absorbance maximum (490 to 520 nm) in the absorption maximum, implying the formation of amyloid-like fibrils. Panel B depicts the changes in the emission intensity of thioflavin T upon binding to nFGF-1 in various concentrations of TFE. The emission intensity of the dye is maximum in 15% (v/v) TFE, suggesting maximum accumulation of the amyloid type of fibrils under these conditions. The inset (in panel B) shows the emission spectra of thioflavin T in various concentrations of TFE.

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10% (v/v), Congo red binding is observed maximally at 15% (v/v) TFE (Fig. 4A). The dye binding is not only accompanied by a significant increase in the absorbance intensity at 490 nm but also by a prominent red shift in the absorbance maximum (490 to 520 nm, Fig. 4A, inset). Beyond 15% (v/v) TFE the intensity of the dye at 490 nm decreases steadily with the increase in concentration of the fluoro alcohol. These results suggest that the fibrils formed maximally at 15% (v/v) TFE have amyloid-like characteristics.

The nature of the aggregates formed were also probed using ThT. ThT is a fluorescent dye used as a diagnostic to identify amyloid fibril formation (39). The emission intensity of ThT is known to increase significantly upon binding to the linear array of β-strands in the amyloid fibrils. There is no appreciable change(s) in the fluorescence intensity of the dye at TFE concentrations lower than 10% (v/v) (Fig. 4B). However, an 8-fold increase in the fluorescence intensity at 485 nm is observed when the dye binds to the protein in 15% TFE (Fig. 4B). These results provide further evidence suggesting that amyloid-like fibrils of nFGF-1 accumulate maximally in 15% (v/v) TFE.

The ultrastructure of the aggregates (of nFGF-1) formed in 15% (v/v) TFE was examined by transmission electron microscopy and x-ray fiber diffraction. Electron micrographs reveal that the aggregates formed are straight and unbranched, with an average diameter of about 10–15 nm (Fig. 5A) and is similar to the width of the fibrils formed from other amyloidogenic proteins (39). X-ray fiber diffraction analysis of the nFGF-1 aggregates shows a dominant reflection at 4.7 Å on the meridian and associated equatorial reflection at 10.3 Å, typical of those expected for fibrils with a cross-β structure (Fig. 5B). The meridional reflection (at 4.7 Å), which primarily arises from the spacing between the β-sheet structure, is sharp and intense (Fig. 5B). The equatorial reflection (at 10.3 Å), which is generally attributed to the intersheet spacing, is relatively diffused (Fig. 5B). Thus, the results of the electron microscopy and x-ray fiber diffraction experiments authenticate that the aggregates formed in 15% (v/v) TFE possess features resembling that of the amyloid fibrils.

Seeding Fibril Formation with Preformed Fibrils—Seeding is a common phenomenon in aggregation and gelation processes, as it is in crystallization (40). It has been reported that aliquots of pre-formed fibrils formed by AB peptide when it is added to clear solutions substantially accelerated the rates of formation of amyloid-like fibrils (8). Recently, Bucciantini et al. (27) demonstrate that pre-formed amyloid fibrils (formed in vitro) of proteins including SH3 domain from phosphatidylinositol 3-kinase and the N-terminal domain of the E. coli HypF protein, which are not associated with amyloidoses, are highly cytotoxic and induced amyloid-like fibrils in cultured NIH3T3 fibroblast cells (27). In this context, we investigated the seeding potency of the amyloid-like fibrils of nFGF-1 induced in TFE. The potency of the pre-formed fibrils of nFGF-1 (induced in
15% TFE (v/v) to seed fibril formation was examined by monitoring the time-dependent changes(s) in the 350-nm absorbance upon the addition of aliquots of pre-formed fibrils into solutions (1 mg/ml) of native nFGF-1. It could be observed that the rate of induction of aggregation depends on the amount of the pre-formed fibrils added. However, the total aggregation induced appears to be independent of the concentration of the pre-formed fibrils used for seeding. Panel A shows the thioflavin T binding affinity of the induced aggregates. The control experiment (open triangles) with ThT added to nFGF-1 (in 10 mM phosphate buffer (pH 7.2) containing 100 mM NaCl (in the absence of the pre-formed fibrils)) shows no time-dependent increase in the emission intensity at 485 nm. These results suggest that the induced aggregates possess properties of the amyloid fibrils.

We performed ThT binding experiments to verify if the aggregates seeded by the pre-formed fibrils have amyloid-like characteristics. A significant time-dependent increase in the fluorescence intensity of the dye (ThT) at 485 nm could be observed upon incubation of the native protein with pre-formed fibrils (Fig. 6B). These results suggest that the induced aggregates (upon seeding) indeed possess properties resembling the amyloid fibrils. In addition, transmission electron micrographs revealed that the induced aggregates are fibrillar with an average diameter of about 15 nm. In summary, the results of the seeding experiments clearly demonstrate that fibril growth (in nFGF-1) is dominated by a nucleation mechanism similar to that observed in crystal growth and gelation processes. Such in vitro seeding has also been observed in studies of human lysozyme and its amyloidogenic variants (41). Recently, fibrils generated under in vitro conditions in hen egg white lysozyme (which is not associated with amyloid diseases) have been shown to seed fibril formation of protein in the native conformation (42). Similarly, in vitro studies with the AB peptide associated with Alzheimer’s diseases suggest that origin of the rapid onset of amyloid diseases and infectivity of prion-related diseases occurs by a nucleation (seeding) mechanism (8).

**Formation of a Partially Structured Intermediate(s)—**

2 S. Srisailam, T. K. S. Kumar, D. Rajalingam, and C. Yu, unpublished results.
Recently, there is increased interest in understanding the molecular events leading to the formation of amyloid fibrils. Based on experimental evidence available in different proteins, it is now increasingly believed that amyloid fibril formation involves the ordered self-assembly of partially folded species that are crucial soluble precursors of fibrils (22–26, 35–37). In this context, we monitored the conformational changes in the protein before amyloid-like fibril formation.

The fluorescence spectrum of nFGF-1 shows an emission maximum at around 308 nm (Fig. 7, inset). The fluorescence of the lone tryptophan residue located at position 121 is significantly quenched in the native state of the protein (43–46). This quenching effect is attributed to the presence of imidazole and pyrrole groups in the vicinity of the indole ring of the tryptophan (Trp-121) residue (31). However, the quenching effect is relieved upon unfolding of the protein, yielding a fluorescence

The $^1$H,$^15$N HSQC spectra of nFGF-1 in various concentrations of TFE (panel A). Panel B depicts the $^1$H,$^15$N chemical shift perturbation in nFGF-1 in 8% (v/v) TFE. The protein undergoes gross conformational changes in 8% (v/v) TFE. Amide protons of residues Lys-26, Lys-142, Ala-143, Leu-145, Leu-147, Leu-149, and Asp-154 (indicated at the top of the respective bars in panel B) connecting the N- and C-terminal ends of the protein (through hydrogen bonds) also show significant chemical shift perturbation. The $^1$H,$^15$N chemical shift perturbation data shown in panel B represents the weighted average (of $^1$N and $^1$H (31) chemical shift differences of residues in the protein in 0% (v/v) TFE and 8% (v/v) TFE. The drastic decrease in the dispersion of the cross-peaks in the HSQC spectrum of the protein in 70% (v/v) TFE indicates that most portions of the protein exist in a disordered state(s). Only small portions of the sequence of nFGF-1 appear to assume non-native helical conformation in 70% (v/v) TFE.

FIG. 9. $^1$H,$^15$N HSQC spectra of nFGF-1 in various concentrations of TFE (panel A). Panel B depicts the $^1$H,$^15$N chemical shift perturbation in nFGF-1 in 8% (v/v) TFE. The protein undergoes gross conformational changes in 8% (v/v) TFE. Amide protons of residues Lys-26, Lys-142, Ala-143, Leu-145, Leu-147, Leu-149, and Asp-154 (indicated at the top of the respective bars in panel B) connecting the N- and C-terminal ends of the protein (through hydrogen bonds) also show significant chemical shift perturbation. The $^1$H,$^15$N chemical shift perturbation data shown in panel B represents the weighted average (of $^1$N and $^1$H (31) chemical shift differences of residues in the protein in 0% (v/v) TFE and 8% (v/v) TFE. The drastic decrease in the dispersion of the cross-peaks in the HSQC spectrum of the protein in 70% (v/v) TFE indicates that most portions of the protein exist in a disordered state(s). Only small portions of the sequence of nFGF-1 appear to assume non-native helical conformation in 70% (v/v) TFE.
spectra with an emission maxima at around 350 nm (Fig. 7, inset). Hence, the ratio of 350- to 308-nm fluorescence reliably describes the conformational transitions occurring during the unfolding of the protein (46). The fluorescence spectra of nFGF-1 does not appreciably change at lower concentrations of TFE (<8% (v/v) TFE). However, in the TFE concentration range of 8 to 15% (v/v) TFE, the ratio of the 350- to 308-nm fluorescence drastically increases, suggesting substantial loss of tertiary structural interactions in the protein (Fig. 7). The TFE-induced unfolding effects on the protein are maximum at 15% (v/v) TFE. Thus, analyzing the intrinsic fluorescence data in conjunction with that obtained using far-UV CD (indicating β-sheet segments in the protein) suggests that nFGF-1 exists in a partially structured state(s), with extended β-sheets and loosely packed side chains. Interestingly, at higher concentrations of TFE (>50 (v/v) TFE), the fluorescence spectra show a significant decrease in the 350-nm emission, suggesting a change in the microenvironment of the tryptophan residue upon induction of non-native helical conformation in the protein.

ANS is a popular hydrophobic dye that is known to bind to solvent-exposed hydrophobic surfaces in a manner characteristic of the formation of partially structured state(s) (47). The dye generally exhibits weak binding affinity to the native and unfolded state(s) of protein (46). The binding affinity of ANS to the protein does not significantly change below 8% (v/v) TFE (Fig. 8). However, beyond this TFE concentration (>8% (v/v) TFE), the fluorescence intensity of the dye at 485 nm increases drastically, reaching a maximum value at 15% (v/v) TFE. The intensity of the dye upon binding to the protein in 15% (v/v) TFE is about 20 times that observed when it (ANS) is bound to the native state of the protein (Fig. 8). In addition, the dye upon binding to the protein in 15% (v/v) TFE shows a prominent blue shift of about 42 nm (from 520 to 475 nm) in the wavelength of maximum emission (Fig. 8). These spectral characteristics clearly indicate that a partially structured state(s) with a solvent-exposed non-polar surface(s) accumulates (maximally at 15% (v/v) TFE) before the formation of amyloid-like fibrils. Beyond 15% (v/v) TFE, the fluorescence intensity of ANS at 485 nm decreases progressively (with the increase in the concentration of the fluoro alcohol (TFE)), indicating a depletion in the population of the partially structured intermediate (exhibiting high binding affinity to ANS) state(s) (Fig. 8). It appears that the partially structured intermediate state(s), which is accumulated maximally at 15% (v/v) TFE, is sticky and has a high tendency to aggregate through solvent-exposed hydrophobic surface(s) and intermolecular β-sheet formation (to be discussed below). The resultant aggregates possibly rearrange to form organized amyloid-like fibrils.

NMR spectroscopy facilitates the study at the level of individual amino acids residues during folding/unfolding of proteins (48). Heteronuclear correlation experiments have been shown to be very sensitive because of high magnetization transfer between directly bond nuclei (48). This aspect enables the use of $^1$H,$^{15}$N HSQC technique to investigate the conformational changes induced by TFE at high resolution. In general, $^1$H,$^{15}$N HSQC spectrum serves as a fingerprint of the conformational state of a protein (48). The HSQC spectrum of nFGF-1 in its native conformation (0% TFE) is well dispersed, and all the expected 126 $^1$H,$^{15}$N cross-peaks in the spectrum could be unambiguously assigned (31). No discernable changes could be observed in the HSQC spectrum of the protein acquired below a TFE concentration of 5% (v/v) (Fig. 9). We could not assess the structural features of the partially structured intermediate state(s) accumulated at 15% (v/v) TFE because the protein (even at 0.1 mM concentration) aggregates seriously after sample preparation (in 15% (v/v) TFE). Under these circumstances, the structural characteristics of the partially structured intermediate state(s) were predicted from the $^1$H,$^{15}$N chemical shift perturbation observed in the HSQC spectrum at 8% (v/v) TFE. $^1$H,$^{15}$N HSQC spectrum of the protein in 8% (v/v) TFE shows that many cross-peaks (in the spectrum) undergo significant chemical shift perturbation (Fig. 9B). These spectral features are indicative of gross conformational changes leading to the protein. Many cross-peaks correspond...
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Mechanism of Amyloid-like Fibril Formation—We are tempted to propose a model for the TFE-induced amyloid-like fibrils in nFGF-1. It is well known that alcohols such as TFE exert mild denaturant effects by partially disorganizing the tertiary and quaternary structures of proteins (49, 50). The denaturant effects of TFE on proteins primarily stem from their low polarity, which weakens the hydrophobic interactions that stabilize the compact native structures of proteins (51). In this background, the first conformational transition induced by TFE (at concentrations greater than 10% (v/v)) appears to be the disorganization of the hydrophobic contacts stabilizing the native β-barrel structure leading to the formation of a sticky partially structured intermediate state(s) (Fig. 10). The presence of solvent-exposed, non-polar surface(s) in the partially structured state(s) (populated in 15% (v/v) TFE) is evident from the high binding affinity of the protein in the intermediate state(s) to ANS (Fig. 8). Similarly, the disorganization of the native β-barrel architecture is probably reflected in the significant chemical shift perturbation of most of the cross-peaks in the 1H,15N HSQC spectrum in 8% (v/v) TFE and 15% (v/v) TFE. The 1H,15N HSQC spectra of the protein in 70% (v/v) TFE reveals that the chemical shift dispersion of the cross-peaks is vastly diminished, implying that large portions of the protein molecules are in a disordered conformation (Fig. 9A). The far-UV CD and NMR data (obtained in 70% (v/v) TFE) analyzed in conjunction suggests that the protein possibly exists primarily as a random coil with only small portions of the polypeptide backbone existing in non-native helix conformation. It should be mentioned that in the absence of detailed triple resonance data, the chemical shift indices of the resonances (1H,15N) in 70% (v/v) TFE cannot be estimated. This aspect precludes the assignment of non-native helical segment formed in the protein in 70% (v/v) TFE.

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