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Influence of Backbone Conformation on Protein Aggregation

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Abstract: Effect(s) of organic solvents on an all β-sheet protein are investigated to understand the influence of backbone conformation on protein aggregation. Results obtained in the present study reveal that protein aggregation is accompanied by the formation of non-native β-sheet conformation. In contrast, induction of non-native helical segments in the protein is found to inhibit aggregation. The differential effects of the secondary structures on protein aggregation are proposed to stem from the disparity in the nature of the hydrogen bonds and packing of the side chains of hydrophobic residues in the β-sheet and α-helix conformation. In our opinion, the results of the present study provide useful hints to develop methods to alleviate the problems of both in vitro and in vivo protein aggregation.

Introduction

Understanding the molecular mechanism underlying protein aggregation is of prime importance not only in biotechnology but also in the health-related industries.[1] Protein aggregation is believed to be involved in several disorders, such as Alzheimer's disease, cystic fibrosis, and prion diseases.[2] Furthermore, formation of inclusion bodies induced by protein aggregation is often a major obstacle in large-scale heterologous expression of proteins.[3] To develop strategies for preventing protein aggregation the mechanism(s) and pathways by which proteins aggregate must be characterized. In this pursuit, in the present study, we investigate the influence of the backbone conformation of proteins in the induction of the aggregation reaction. The results obtained herein clearly show that aggregation is strongly linked to the nature of the secondary structure induced in the protein under the influence of a solvent. It is found that induction of non-native β-sheet conformation favors protein aggregation, but in contrast, generation of non-native helical segments in the protein is observed to inhibit the aggregation reaction.

Experimental Section

Materials. Recombinant *newt* acidic fibroblast growth factor (nFGF-1) was expressed in *E. coli* and purified according to the method described by Arunkumar et al.[4] Carbonic anhydrase was purchased from Sigma Chemical Co., USA. TFE and ethanol were procured from Janssen Chemica, Belgium. Acetonitrile was obtained from Merck, USA. All other chemicals used in this study are of high-quality analytical grade. All solutions were made in 10 mM phosphate buffer (pH 7.2) containing 100 mM NaCl (at 20 °C).

Turbidity Measurements. Turbidity measurements were performed on a Hitachi U-3310 spectrophotometer. All measurements were made after 3 h of incubation (at 20 °C) of the protein (nFGF-1) in appropriate concentrations of the organic cosolvents. The concentration of the protein used in the turbidity experiments was 100 μg/mL. The turbidity of the solutions was measured by absorbance at 350 nm. The path length of the sample cell used was 10 mm.

Circular Dichroism. All CD measurements were made on a Jasco J-720 spectropolarimeter. CD studies carried out at 100 μg/mL were performed using a 0.2 cm quartz cell in the wavelength range of 190−250 nm. A step size of 0.1 nm, an average time of 3 s, and an average of 10 scans were used to generate the data. The far-UV CD spectra were smoothed by using the noise reducing option in the software supplied by the vendor (JASCO).

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 1 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 using the conventional high-pressure method. The native protein sample was prepared by lyophilizing the protein and mixing the lyophilized powder with the KBr pellet. All spectra were recorded with a wavenumber resolution of 2 cm^{-1}. For each spectrum, 64−200 interferograms were collected and averaged, and a Happ-Genzel apodization function was applied before FT. All processing procedures were carried out so as to optimize the quality of the spectra in the amide I region, between 1600 and 1700 cm^{-1}.

Results and Discussion

Acidic fibroblast growth factor from newt (nFGF-1) is a 17 kDa, all β-sheet protein, bereft of disulfide bonds (Figure 1).[4] The secondary structural elements include 12 antiparallel

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Figure 1. Molscript representation of the backbone folding of nFGF-1. The secondary structural elements in the protein include 12 antiparallel β-strands arranged into a β-barrel structure.

β-strands arranged into a β-barrel motif (Figure 1). The structure of nFGF-1 in its native state lacks helical segments. Hence, nFGF-1 is an ideal protein to understand the action of helix inducing solvents.

TFE-Induced Aggregation. Organic cosolvents such as 2,2,2-trifluoroethanol (TFE) have been shown to promote amyloid-type aggregates in many proteins.[5] In this context, we examined the effect(s) of various organic solvents on nFGF-1. The aggregation induced in the protein by the organic solvents was measured usng turbidity measurements at 350 nm. The turbidity changes occurring upon addition of TFE could be artibitarily classified into three phases. In the first phase, which extends up to 5% v/v TFE, no significant change could be discerned in the absorbance intensity at 350 nm, implying that aggregation is not induced in the protein in this range of alcohol concentration (Figure 2A). The second phase extending from 10 to 50% TFE represents the zone of aggregation (Figure 2A). In this phase, the protein solutions treated with TFE are found to turn turbid. Maximun aggregation of the protein could be noticed to occur at a TFE concentration of 10% v/v. Interestingly, in the third phase, which occurs beyond 50% v/v TFE, the protein solution remains clear with low 350 nm absorbance values (Figure 2A). Sedimentation equilibrium experiments on nFGF-1 in 60% v/v TFE revealed that the protein exists as a soluble monomer under these conditions (data not shown). It is pertinent to mention that the overall trend in the aggregation profile remains independent of the protein concentration. These results clearly suggest that the aggregation of nFGF-1 could be modulated by the concentration of the fluorinated alcohol.

Protein Aggregation Is Correlated to Induced Backbone Conformation. TFE is commonly used as a structure inducing cosolvent.[6] To understand the influence of the backbone conformation on the protein aggregation reaction, we performed far-UV CD experiments on nFGF-1 at various concentrations of TFE. The far-UV CD spectrum of nFGF-1 in its native state is typical of a type II β-barrel protein with a positive ellipticity

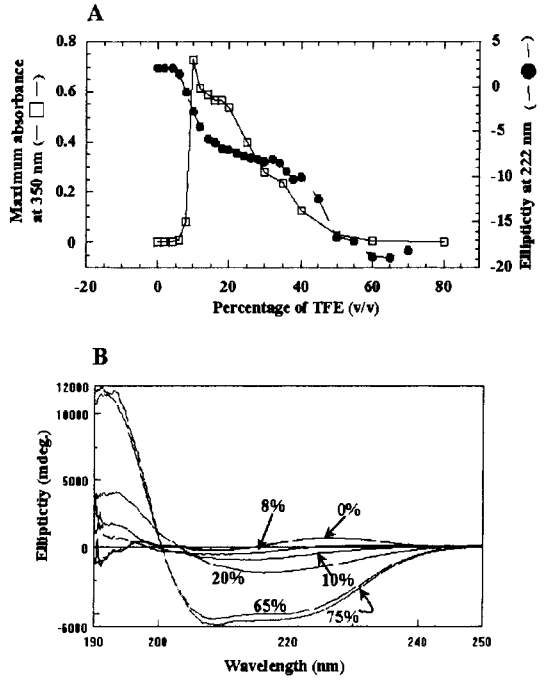

Figure 2. Absorbance (350 nm) and ellipticity (222 nm) changes at various percentage concentrations of TFE (panel A). Panel-B depicts the far-UV CD spectra of nFGF-1 at various percentage (v/v) concentrations of TFE. It could be deduced that the increase in the ellipticity intensity at 222 nm (signifying the formation of non-native helical segments) is compounded with the decrease in the turbidity of the protein solution.

band centered at 228 nm and a negative extrema at around 205 nm (Figure 2, panel B). The 228 nm CD band is believed to primarily represent the arrangement of the β-strands in the β-barrel architecture of the protein.[7] No discernible changes could be realized in the CD band (at 228 nm) in the spectra obtained in TFE in the concentration range of 0−5% v/v. However, in the second phase wherein aggregation is induced in the protein (at TFE concentrations between 10 and 50% v/v), the far-UV CD spectra show a negative ellipticity band centered at about 218 nm, reminiscent of proteins containing extended β-sheets (Figure 2, panel B). The loss of the positive CD band at 228 nm signifies the disruption of the native β-barrel architecture in the protein. The intensity of the 218 nm CD band is found to steadily increase with the increase in the TFE concentration up to 10% v/v (Figure 2, panel B). At higher concentrations of TFE (>50% v/v) wherein no or insignificant aggregation of the protein is observed, the far-UV CD spectra depict negative CD bands centered at 208 and 222 nm, suggestive of induction of helix conformation (Figure 2, panel B). As the three-dimensional structure of nFGF-1 lacks helical segments, the helical conformation observed in high concentrations of TFE (>50%(v/v)) appears to be essentially non-native in origin. The results presented thus far clearly suggest that protein aggregation is strongly linked to the nature of non-native secondary structural elements induced. Formation of non-native β-sheet conformation appears to favor aggregation and in contrast, induction of non-native helical segments is observed to stabilize the protein in a soluble monomeric state. The

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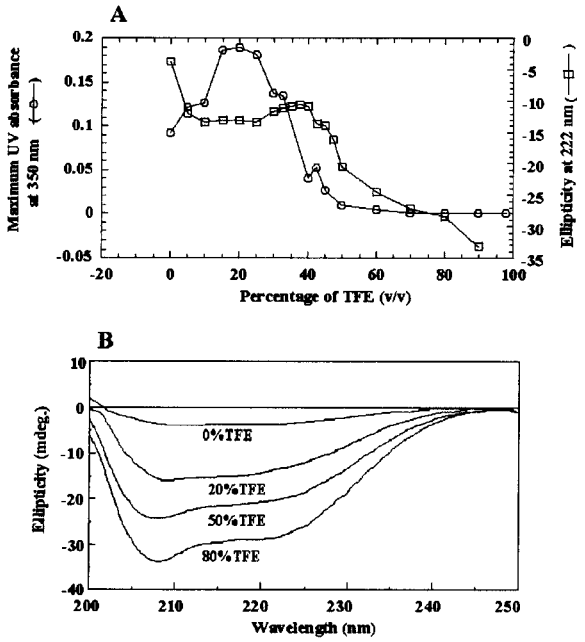

Figure 3. Changes in the 350 nm absorbance and the 222 nm ellipticity versus the percentage (v/v) concentration of TFE (panel A). Far-UV CD spectra of carbonic anhydrasse (CAB) at various percentage concentrations (v/v) of TFE are depicted in panel B.

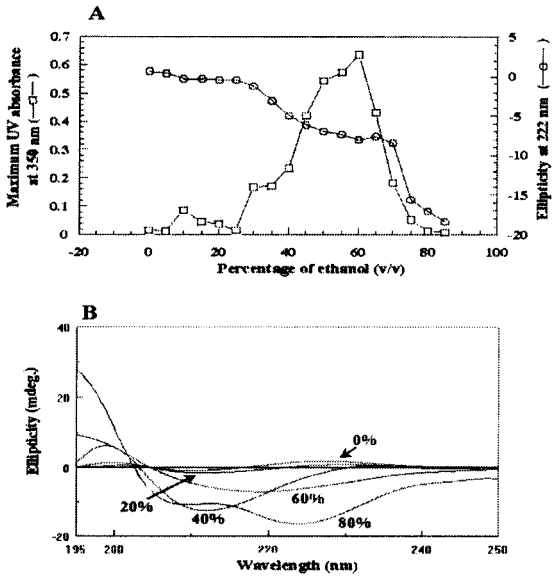

Figure 4. A plot of the changes in the 350 nm absorbance and the 222 nm ellipticity changes versus the percentage (v/v) concentration of ethanol is shown in panel A. It could be observed that non-native helix formation inhibits protein aggregation. The far-UV CD curves of nFGF-1 obtained in various percentage concentrations (v/v) of ethanol are shown in panel B.

generality of the observed phenomenon was tested by investigating the effects of TFE on another all β-sheet sheet protein such as the human carbonic anhydrase (CAB). Even in CAB, the TFE-induced structural transitions associated with the promotion or prevention of protein aggregation were found to be similar qualitatively to those observed in nFGF-1 (Figure 3). These results clearly suggest that protein aggregation is linked to the nature of non-native secondary structural elements induced in the protein.

Ethanol mimics the structural changes induced by TFE. It is important to understand if the correlations observed between backbone conformation of the protein and the aggregation reaction are unique to TFE. In this context, we monitored the effect(s) of ethanol on nFGF-1. The aggregation profile of nFGF-1 in various concentrations of ethanol (monitored by turbidometric measurements at 350 nm) shows a similar trend as observed in TFE. In the ethanol concentration range of 0 to 25% (v/v), no or very small changes occur in the absorbance intensity at 350 nm (Figure 4, panel A). The protein is found to aggregate in the alcohol concentration range of 40 to 75% v/v (Figure 4, panel A). At higher concentrations of alcohol (>75% v/v), the protein solutions remain clear with little or no aggregation. As observed in TFE, in the second phase of the aggregation profile (in 40 to 75% v/v ethanol), the far-UV CD spectra of the protein are representative of extended β-sheet structure with a negative ellipticity maxima at 218 nm (Figure 4, panel B). The far-UV CD spectra of nFGF-1 collected in ethanol concentrations greater than 75% v/v (wherein the protein remains in the soluble form) show helix characteristic ellipticity bands at 208 and 222 nm (Figure 4, panel B). Thus, these results clearly show that the influence of the backbone conformation on the aggregation reaction is a general phenomenon and not merely restricted to TFE. The differences in the helix inducing potencies of TFE and ethanol probably account for the observed disparity in their ability to inhibit aggregation. Hirota et al.,[8] comparing the effects of various alcohols in inducing the helical structure in mellitin, discovered that fluorinated alcohols such as TFE and 3,3,3,3′,3′,3′-hexafluoro-2-propanol (HFIP) have higher inducing potency than the nonfluorinated alcohols. The enhanced helix inducing tendency of the fluorinated alcohols was shown to arise from their ability to cluster and consequently provide a local nonpolar environment, wherein hydrophobic interactions are weakened and hydrogen bonds are strengthened.

Protein Aggregation Cannot Be Alleviated without the Induction of Non-Native Helix Conformation. Acetonitrile (ACN) is a mild solvent, which in some cases has been reported to induce β-sheet conformation in proteins.[9] It has been successfully employed to study the α-helix to β-sheet conformational transition(s) in peptides derived in Alzheimer's Aβ peptides.[10] Hence, ACN is an apt solvent to verify the relationship between the induction of non-native helical conformation and the inhibition of protein aggregation. In contrast to TFE and ethanol, the aggregation profile of the protein in ACN shows only two phases. The absorbance intensity at 350 nm does not change appreciably in the initial phase of the ACN titration (0−60% v/v, Figure 5, panel A). However, the protein is found to aggregate steadily beyond an acetonitrile concentration of 60% v/v. Unlike in TFE and ethanol, the protein solution remains turbid even at high concentrations of ACN. The far-UV CD spectra of nFGF-1 at concentrations of ACN lesser than 40% v/v show spectral features resembling that of the protein in its native state (Figure 5, panel B). However, beyond 45% v/v ACN, wherein the protein shows a strong propensity to aggregate, the far-UV CD spectra show a negative extrema

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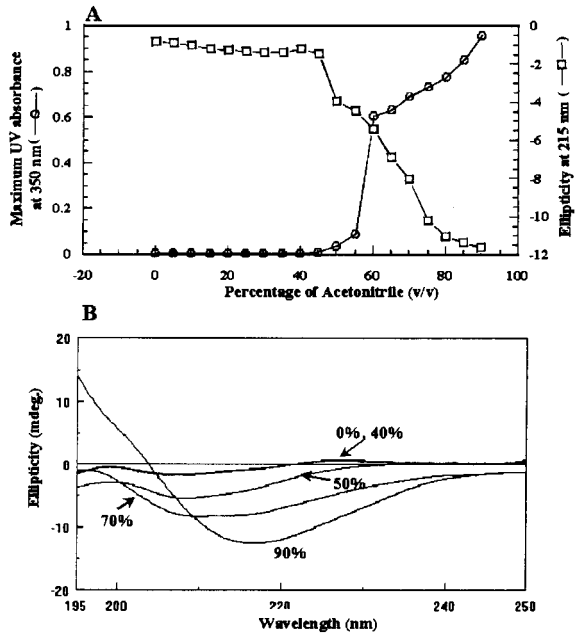

Figure 5. Panel A shows the changes in the 350 nm absorbance and the 215 nm ellipticity changes occurring at various concentrations of acetonitrile. Panel B depicts the far-UV CD profile of nFGF-1 at various percentage concentrations (v/v) of acetonitrile. It could be noticed that the aggregation of the protein is directly linked to the non-native β-sheet formation.

centered at around 218 nm, signifying the formation of extented β-sheet conformation (Figure 5, panel B). The intensity of the 218 nm negative ellipticity band is observed to increase steadily with increasing concentrations of ACN, suggestive of a progressive induction of the non-native β-sheet elements in the protein (Figure 5, panel B). Far-UV CD curves representative of helix conformation could not be observed at any acetonitrile concentration. It appears that failure of ACN to prevent aggregation could be related to its inability to induce non-native helical conformation in the protein.

Possible Mode(s) of Action. We are tempted to tentatively rationalize the structural effects underlying the induction and inhibition of aggregation of nFGF-1 in TFE. It is reasonably well established that aqueous−organic mixtures exert mild denaturant effects by disrupting the tertiary and quaternary structures of proteins.[11] The effects of alcohols on proteins are considered to primarily arise from the low polarity of the solvent which weakens the hydrophobic interactions that stabilize the compact native structures of proteins.[12] In this context, the first structural transition induced by various organic solvents (TFE, ethanol, and ACN) appears to be the disorganization of the hydrophobic contacts stabilizing the native β-barrel architecture, leading to the formation of a sticky structural intermediate characterized by extended β-strands (Figure 6). The disorganization of the native β-barrel architecture of nFGF-1 is probably reflected in the loss of the positive ellipticity band at 228 nm and concomitant appearance of the negative CD band at 218 nm signifying the formation of non-native extended β-sheet

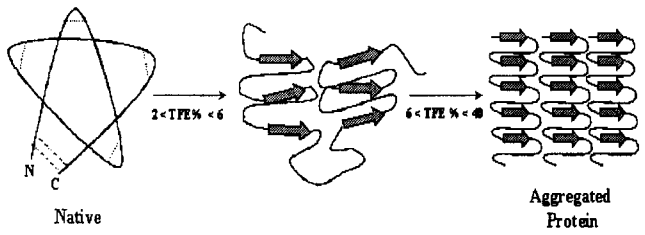

Figure 6. Schematic representation of the possible structural changes induced by TFE in nFGF-1. As the initial step, TFE appears to disrupt the native β-barrel structure of nFGF-1 to generate extended β-sheets. These β-sheets subsequently appear to coalesce together by forming an interstrand β-sheet among the partially denatured, monomeric units of the protein. However, at higher concentrations of TFE, formation of intrastrand, non-native helix segments appears to inhibit protein aggregation.

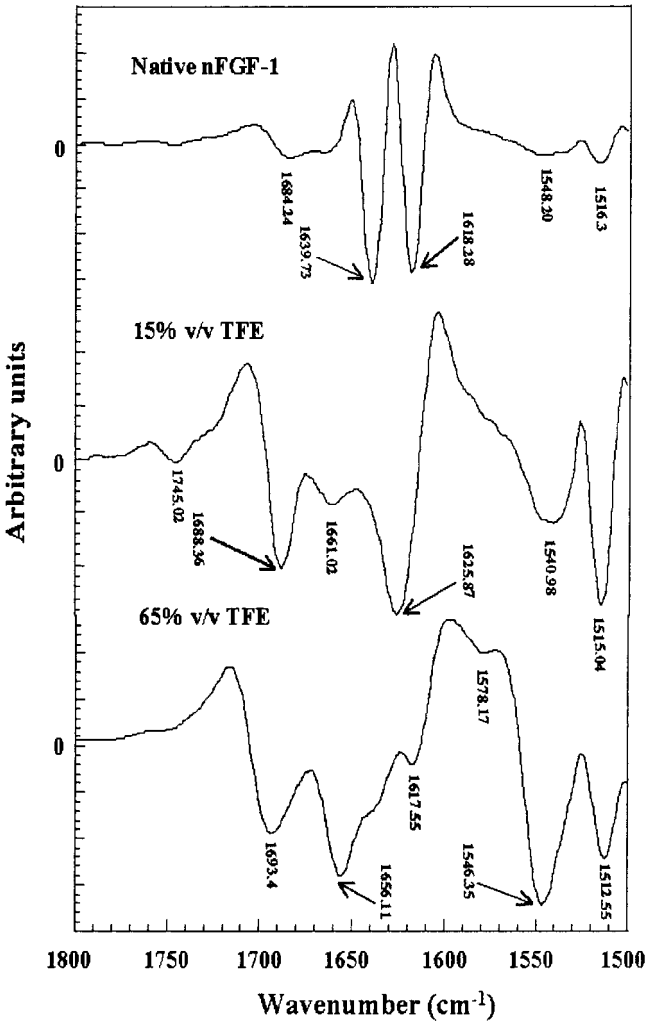

Figure 7. FTIR spectra of nFGF-1 in its native and 15% TFE states. The arrowheads indicate the exact wavenumber of the peak(s) in the spectra.

structure (Figure 2, panel B). The loss of the native β-barrel structure of nFGF-1 is also evident from the FTIR spectra collected in 15% v/v TFE, wherein the 1618 and 1639 cm^{-1} amide 1 doublet bands characterizing the β-barrel structure disappear (Figure 7). The potency to disorganize the native structure of the protein appears to be inversely related to the solvent polarity.[13] Therefore, acetonitrile being the most polar of the solvents (used in this study) exhibits the weakest tendency

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to disrupt the native structure of the protein. The aggregation observed in the protein in TFE (in the concentration range of 10 to 50% v/v) is probably triggered by the coalescence of the "sticky" extended β-sheet elements (in the protein) to form insoluble, intermolecular β-sheets (Figure 6). Formation of intermolecular β-sheets is corroborated by the presence of the 1688 cm^{-1} (Figure 7) band in the FTIR spectra of the TFE-induced protein aggregate. In addition to the intermolecular hydrogen bonds which favor aggregation, the enhanced solvent exposure of the hydrophobic side chains in the protein in the non-native β-sheet conformation also appears to provide a conducive environment for the condensation of the polypeptide chains to form higher order aggregates.[12,13] In general, peptides which have conformational propensity to form β-sheet(s) have poor solubility (in aqueous solutions) due to their tendency to aggregate through nonspecific interactions among solvent-exposed hydrophobic surface(s) or through intermolecular β-sheet formation.[14]

The inhibition of protein aggregation observed in higher concentrations of the water−alcohol mixture appears to be due to weakening of the hydrophobic interactions stabilizing the intermolecular β-sheet structure in the protein aggregate. The loss of hydrophobic contacts appears to favor the formation of intramolecular hydrogen bonds (over intermolecular hydrogen bonds) leading to the formation of helix conformation.[12,13] The induction of helix conformation in water−organic solvent mixture could be rationalized in terms of transfer free energy (ΔG) of protein groups from water to organic solvent.[8,15] The ΔG values of transfer of hydrophobic groups from water to alcohol are negative, and those of polar groups are positive. As a consequence, in cosolvents such as alcohols, the nonpolar groups in the protein tend to be solvent exposed while the polar amide groups tend to be buried, resulting in the formation of helix conformation. In contrast, the inability of acetonitrile to induce helix conformation in the protein could be attributed to its high polarity (as compared to the alcohols) which provides a favorable environment for the formation of intermolecular β-sheets (rather than intramolecular hydrogen bonds resulting in the formation of helix conformation).[12,13] Helix formation, in general, increases packing of the hydrophobic residues and consequently decreases their solvent exposure.[16] These structural features disfavor protein aggregation. It should be mentioned that more direct experimental evidence is needed to validate our proposal.

The results of the present study unambiguously suggest that protein aggregation is intricately related to the backbone conformation of the protein. In addition, these results provide useful information for the evolution of new strategies to prevent both in vitro and in vivo protein aggregation. The β-sheet to α-helix structural transition induced by TFE could be used as a useful model to understand the conformational switch of the prion protein from the soluble α-helical form to the β-sheet conformation leading to the amyloid formation.

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