Studies on aggregation-propensities and secondary structural transformations of proteins

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Abstract The insoluble and fibrillar aggregates of some proteins are thought to be the pathological cause of neurodegenerative diseases. The aggregation-propensities of different types of proteins were investigated by Thioflavine T fluorescence assay and atomic force microscopy imaging. Then, the structural transformations of the proteins from aqueous state to solid state were studied by circular dichroism spectroscopy. The results indicate that proteins of different secondary structure show variations in their aggregation-propensities, together with their various structural transformations from aqueous state to solid state. Our studies imply that the structural transformation of proteins from solution to solid state is closely associated with their aggregation-propensities, which will provide insight into the molecular mechanism of protein aggregation in neurodegenerative diseases.

Keywords Aggregation-propensity, Secondary structural transformation, ThT fluorescence, Atomic force microscopy imaging, Circular dichroism spectroscopy

CLC numbers Q51, Q336

1 Introduction

The insoluble and fibrillar aggregates of some proteins are thought to be the pathological cause of neurodegenerative diseases, and it is critical to reveal their secondary or tertiary structures for elucidating the molecular mechanism underlying the fibrillization of amyloidogenic proteins.[1-3] However, their intrinsic insolubility in aqueous buffer and inability to crystallize preclude using circular dichroism (CD) spectroscopy and solution nuclear magnetic resonance (NMR) to study their structures in solution, and X-ray diffraction to investigate their structures in crystals.[4] Fortunately, it has been found that the proteins on glass and mica surfaces can aggregate from aqueous buffer into noncrystalline thin films with good homogeneity after evaporation of solvent, as judged by transmission electron microscopy.[5] The change of proteins from aqueous state into noncrystalline thin-film state is reminiscent of their change from aqueous state into insoluble and noncrystalline fibrillar aggregates.

CD spectroscopy is a valuable technique for investigating the secondary structures of proteins in solution. The far-UV range of the amide CD spectra provides structural information for identifying α-helixes, β-sheets and random coils of various proteins.[6,7] Presently, solid-state CD spectroscopy is applicable to studying the molecular structure of amyloid-like protein aggregates, which are prepared as thin films deposited on transparent plates.[5,8-10]

We have studied five proteins with various secondary structures, including α-Syn$_{1-74}$, FF domain and 2WW domain of formin-binding protein 11 (FBP11), IgG-binding B1 domain of protein G (PGBD) and ubiquitin. α-synuclein (α-Syn) is a major component of Lewy bodies and Lewy neurites in the brains of

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patients with neurodegenerative diseases, such as Parkinson's disease, the Lewy body variant of Alzheimer's disease, dementia with Lewy bodies and multiple system atrophy.[11,12] α-Syn is readily aggregated into amyloid fibrils both *in vivo* and *in vitro*.[13-15] α-Syn$_{1-74}$ is the N-terminal 74-residue fragment of α-Syn with a natively unfolded structure. FF domain is a phosphopeptide-binding module, which might be involved in protein-protein interaction. FF domain is composed of three α- helixes arranged in an orthogonal bundle with a 3_{10} helix in the loop between the second and the third α-helixes.[16,17] 2WW domain has been found to interact with the proline-rich region of Huntingtin, which is encoded by the gene mutated in Huntington's disease.[18,19] Our NMR structure information obtained on the 2WW domain indicates that the domain is a compact module mainly composed of β-sheets (data not shown). PGBD is an α/β protein, which is an ideal model for the study of protein structural transformation due to its compactly folded structure and high stability.[20,21] Ubiquitin is a highly conserved α/β protein and plays a critical role in the ubiquitin-proteasome pathway.[22,23] The aggregation-propensities of five proteins are investigated by Thioflavine T (ThT) fluorescence assay and atomic force microscopy (AFM) imaging. In addition, the CD spectra of proteins in aqueous state and in solid state are compared for analysis of their secondary structural transformations.

2 Material and methods

2.1 Material

Ubiquitin (purity>95%) was purchased from Sigma. Genes encoding α-Syn$_{1-74}$, FF domain, 2WW domain and PGBD were subcloned into the pET3a vectors, and the respective proteins were expressed in *Escherichia coli* BL21 (DE3). α-Syn$_{1-74}$ and FF domain were purified through a CM sepharose fast flow cation-exchange column, while 2WW domain and PGBD were purified through a Q sepharose fast flow ion-exchange column. The fraction containing target proteins were further purified with a FPLC Superose-12 column (Amersham) equilibrated with 0.1 mol/L PBS buffer (0.1 mol/L phosphate, 0.1 mol/L NaCl, pH 7.0). Finally, the purities of proteins were identified by SDS-PAGE as above 95%.

2.2 Time course of aggregation process

200 μmol/L protein samples in 0.1 mol/L PBS buffer were sterilely filtered through 0.22 μm filters to remove any granular matter. Protein solutions were incubated in sterile Eppendorf tubes with continuous shaking at 37℃. As the incubation was progressing, aliquots of each protein were taken at various time points and stored at -20℃ for later assay by ThT (Aldrich) fluorescence. Each 20 μL of the incubated samples was added to 980 μL of 5 μmol/L ThT in 50 mmol/L glycine-NaOH buffer (pH 9.0), the ThT fluorescence shows a maximum in this buffer system. ThT specifically binds protein fibrils and the enhancement of ThT fluorescence was used to semiquantitatively estimate the relative rate of filament formation.[24] Fluorescence measurements were performed on a Hitachi F-4010 fluorophotometer. The emission intensities at 482 nm were recorded immediately after addition of the aliquot to the ThT solution with excitation at 446 nm.

2.3 Atomic force microscopy imaging

Each sample was prepared by depositing 5 μL of the solution on freshly cleaved mica (Alfa Aesar). After adsorption for 5 min, the mica surface was gently washed with deionized water to remove redundant buffer and proteins not firmly attached to the surface. Excess water was removed with condensed air. Images were obtained at a commercial AFM facility (nanoscope III, Digital Instruments) equipped with a 130 μm×130 μm scanner (J-scanner) by tapping mode imaging. The cantilevers with a nominal force constant of 20–100 N/m were used for the experiments. Each sample was observed in more than five regions to avoid experimental errors.

2.4 Circular dichroism measurements

All CD measurements were performed on a Jasco-715 spectropolarimeter at room temperature. The far-UV spectra of solution samples were recorded over a wavelength range of 250–190 nm using a cuvette of 1 mm pathlength at a scan speed of 10 nm/min and a time constant of 0.125 s. The concentration of each sample was 0.2 mg/mL in 0.1 mol/L

PBS buffer. Data were further processed by signal averaging, baseline subtraction and noise reduction. The near-UV spectra of solution samples were recorded over a wavelength range of 320–250 nm using a cuvette of 1-cm pathlength. The concentration of sample was 1 mg/mL in 0.1 mol/L PBS buffer. Other parameters adopted were the same as the far-UV spectra of solution samples. For solid-state CD measurements, 200 μL (1 mg/mL) protein in 0.1 mol/L PBS buffer was cast onto a 2-cm diameter cylindrical quartz glass for evaporating overnight (12–18 h) at room temperature until a dry thin film formed on the surface of the glass. The parameters for recording the solid sample were the same as the solution except for a time constant of 0.5 s. For a film, the concentration of proteins and film thickness (pathlength) are unclear, and it is difficult to calculate the molar ellipticities of proteins as in solution. As a result, the far-UV CD spectra of the proteins in dry films were presented as ellipticities (θ, mdeg). For the sake of comparison, all data for the solution CD measurements were also presented as relative ellipticities.

3 Results and discussion

The purities of α-Syn$_{1-74}$, FF domain, 2WW domain, PGBD and ubiquitin are identified by SDS-PAGE as above 95% (Fig.1), and the five proteins are pure enough for our experiments. Fig.2 shows the time course for the aggregation of α-Syn$_{1-74}$, FF domain, 2WW domain, PGBD and ubiquitin by ThT binding assay. Upon incubation, α-Syn$_{1-74}$ and FF domain aggregate quickly with an obvious increase in ThT fluorescence intensity and reach a stable phase within 1 day. The enhancement in ThT fluorescence of α-Syn$_{1-74}$ is much higher than that of FF domain, and a possible explanation is that the aggregates of α-Syn$_{1-74}$ have a stronger ThT binding ability than those of FF domain. However, 2WW domain, PGBD and ubiquitin have no such characteristic changes even after incubation for 6 days.

AFM is used to directly observe the morphology of protein fibrils after ThT fluorescence assay. As revealed by AFM imaging, only 6-day incubation sample of α-Syn$_{1-74}$ and FF domain formed long regular fibrils, corresponding to their ThT fluorescence enhancement (Fig.3A, B). As for three other proteins, no fibrils were visible after incubation for 6 days, which also conform to their ThT fluorescence data (data not shown). In agreement with ThT fluorescence assay, the results from AFM imaging show the presence of fibrils in α-Syn$_{1-74}$ and FF domain samples and the absence of fibrils in three others.

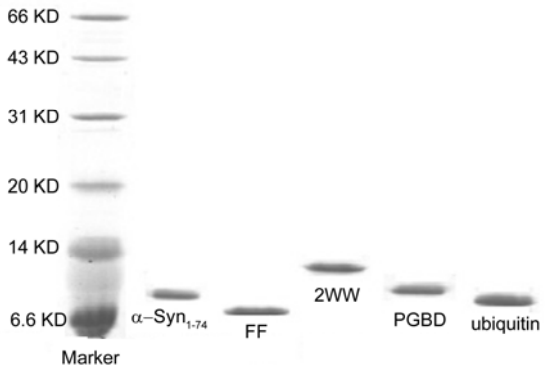

Fig.1 Purity of proteins was identified by 15% SDS-PAGE and the polyacrylamide gel was stained with Coomassie Blue.

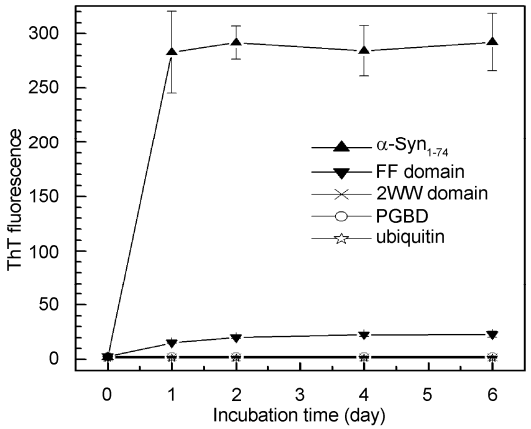

Fig.2 Comparison of α-Syn$_{1-74}$ (up triangles), FF domain (down triangles), 2WW domain (crosses), PGBD (circles) and ubiquitin (stars) for their abilities to aggregate into fibrils. Data are presented as the means ± standard error.

After investigating the aggregation-propensities of the five proteins, we further study their secondary structural transformation from the aqueous state to noncrystalline solid state to find out the underlying molecular mechanism. The solution CD spectrum of α-Syn$_{1-74}$ presents a strong negative peak at 197 nm, which is indicative of a random coil structure. The solid-state CD spectrum of α-Syn$_{1-74}$ shows a strong negative peak at 217 nm and a positive peak at 193 nm, implying a typical β-sheet dominant structure

(Fig.4). During the change from aqueous state into solid state, the secondary structure of α-Syn$_{1-74}$ transforms from random coil into typical β-sheet. The result conforms to the report that the fibrillar aggregates of α-Syn mainly comprises β-sheets as revealed by FTIR spectroscopy and low-resolution X-ray diffraction.[14,25]

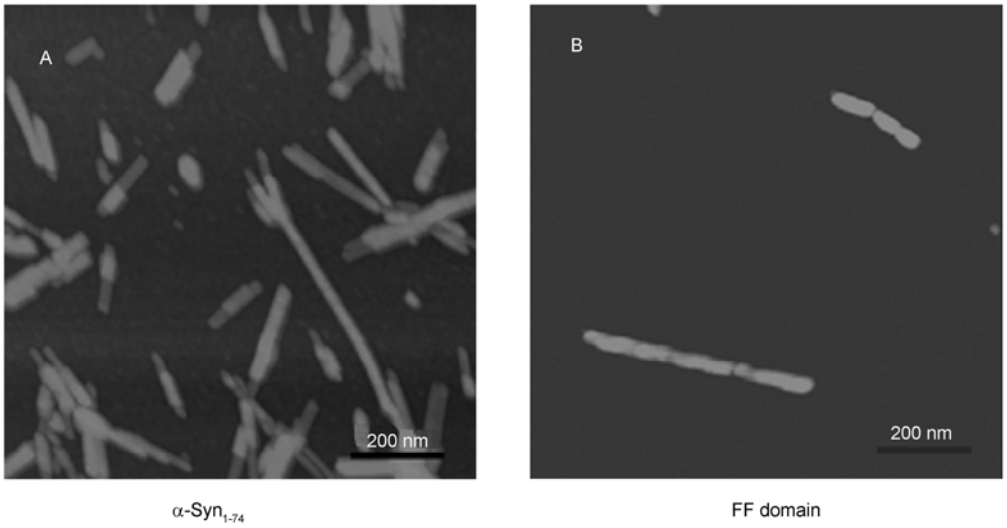

Fig.3 AFM images of (A) α-Syn$_{1-74}$ and (B) FF domain. The photographs were obtained from the samples incubated with 6-day continuous shaking. The two are topographical height images of 1 μm^2 in area and the scale bar denotes 200 nm. The brightness represents the heights of areas.

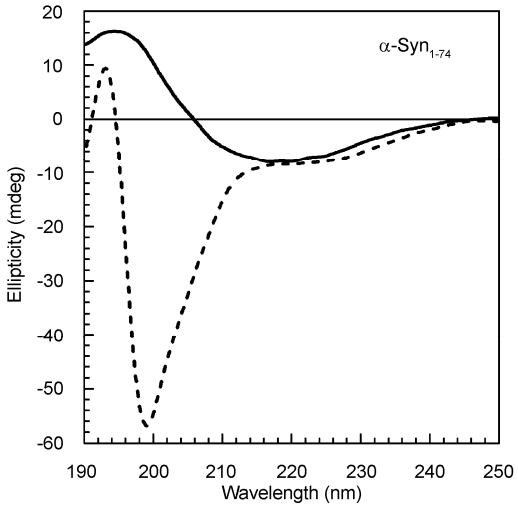

Fig.4 Far-UV CD spectra of α-Syn$_{1-74}$ in solid state (solid line) and in aqueous state (dashed line).

FF domain shows a typical double-peak spectrum in solution indicating a high α-helical content, which is consistent with the result of NMR analysis.[17] Compared with its solution spectrum that shows strong negative peaks at 208 nm and 222 nm, FF domain in solid state exhibits a strong negative peak at 224 nm and a small negative shoulder at around 210 nm. The peak enhancement at 224 nm and redshift suggest that FF domain in the solid state transforms into a structure containing β-sheets and probably aggregates into an amorphous amyloid form (Fig.5).

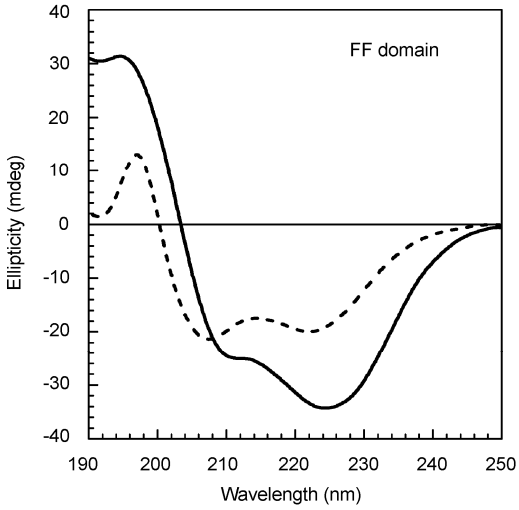

Fig.5 Far-UV CD spectra of FF domain in solid state (solid line) and in aqueous state (dashed line).

Unlike the other four proteins, 2WW domain contains seven tyrosine and four tryptophan residues, whose aromatic side chains lead to a strong peak in the near-UV CD spectra (Fig.6 A). Furthermore, the aromatic side chains absorb light in the far-UV region, causing interferences with the amide CD spectra of 2WW domain in solution. As a result, 2WW domain gives a spectrum different from that of typical

β-sheets in solution. In the solid state, the negative peak of 2WW domain becomes flattened, and the positive peak at 230 nm shifts a little (Fig.6 B). The broad negative peak is probably associated with the weak CD signal of 2WW domain in thin film. It seems that 2WW domain in solid state retains its β-sheet-rich structure.

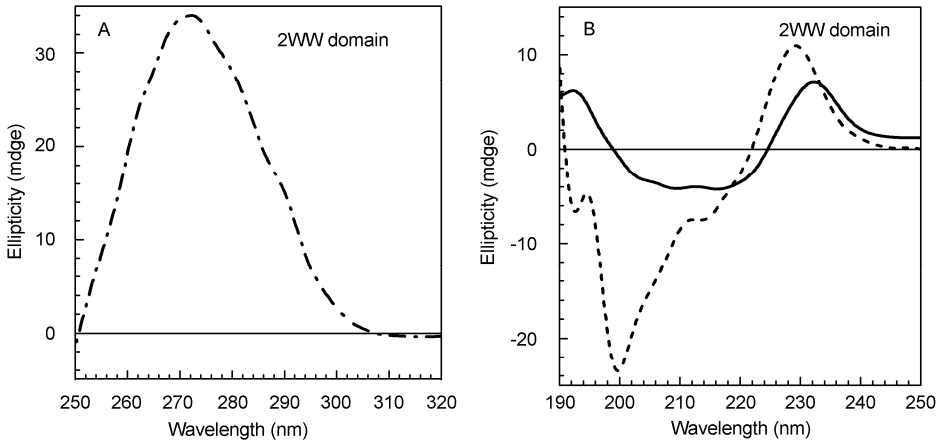

Fig.6 CD spectra of 2WW domain. (A) Near-UV CD spectra of 2WW domain in aqueous state (dash-dot line). (B) Far-UV CD spectra of 2WW domain in solid state (solid line) and in aqueous state (dashed line).

In solution, the spectrum of PGBD presents a positive peak at 195 nm and a broad negative peak between 210 nm and 220 nm, indicating that it comprises both α-helix and β-sheet structures as revealed by NMR.[20] In the solid state, PGBD gives a spectrum of indistinguishable shape from the one in solution (Fig.7 A). In Fig.7 B, ubiquitin shows a typical α/β structure with two negative peaks at 208 nm and at around 222 nm in solution. In the solid state, ubiquitin gives a spectrum similar to the one in solution, except for a slight reduction of the peak at 208 nm as compared with that at 222 nm. The possible explanation is that ubiquitin undergoes subtle rearrangements from solution to the solid state and appears more compact with some flexible residues adopting an ordered structure.

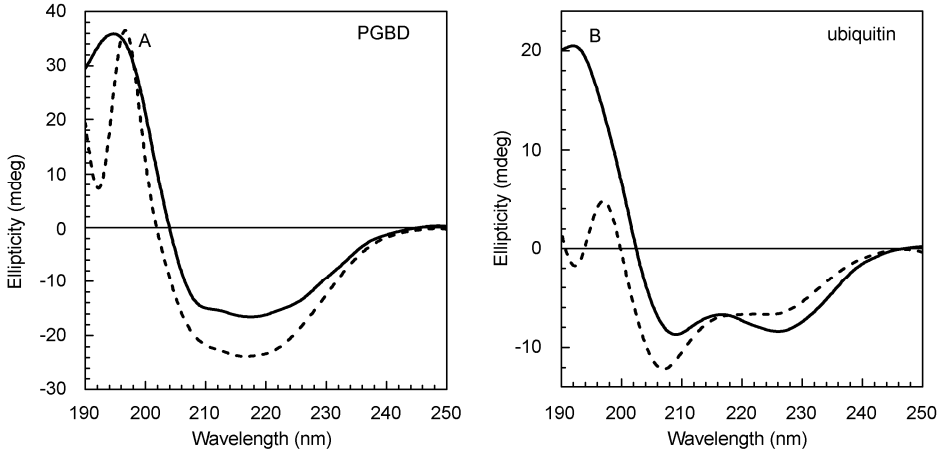

Fig.7 Far-UV CD spectra of (A) PGBD and (B) ubiquitin in solid state (solid line) and in aqueous state (dashed line).

Different types of proteins show variations in their aggregation-propensities as well as their secondary structural transformation profiles from aqueous state to solid state as revealed by CD spectra. The natively unfolded α-Syn$_{1\text{-}74}$ undergoes a structural transformation into β-sheets in the solid state in concomitance with its ability to aggregate into fibrils. The α-helical FF domain transforms into a new structure with the formation of some β-sheets in the solid state, and it is also readily to form fibrils upon incubation. On the other hand, the other three proteins keep their secondary structures in solid state similar to those in

aqueous state, and they are not prone to aggregate into fibrils after incubation. The β-sheet-rich 2WW domain in solid state retains its secondary structure in aqueous state. PGBD and ubiquitin, mainly composed of α-helixes and β-sheets, show no or slight structural rearrangement during change from solution to solid state due to their compact structures. The above experiments were performed *in vitro* under imitated physiological conditions (0.1 mol/L PBS, pH 7.0, 37℃) to provide theoretical basis for the spontaneous aggregation of proteins *in vivo*. It has been found that the secondary structural transformations and aggregation-propensities of proteins are related to the ionic strength and pH of the adopted buffer system to some extent. When the ionic strength and pH of the buffer system seriously deviate from the physiological conditions, the significant change of protein structures may happen and it will alter the aggregation-propensities of proteins accordingly.[26-28] Our preliminary studies under physiological conditions imply that the secondary structural transformation of proteins from solution to solid state is closely associated with their aggregation-propensities, which will provide insight into the molecular mechanism of protein aggregation in neurodegenerative diseases.

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