Trehalose inhibits fibrillation of A53T mutant alpha-synuclein and disaggregates existing fibrils

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A B S T R A C T
The aggregation of alpha-synuclein (AS) is pivotally implicated in the development of Parkinson’s disease (PD), inhibiting this process might be effective in treating PD. Here, by using circular dichroism spectroscopy, thioflavin T fluorescence, and atomic force microscopy, we found that trehalose at low concentration disaggregates preformed A53T AS protofibrils and fibrils into small aggregates or even random coil structure, while trehalose at high concentration slows down the structural transition into β-sheet structure and completely prevents the formation of mature A53T AS fibrils. Further work in vivo will be needed to evaluate its potential as a novel strategy for treating PD.

Introduction
Neurodegenerative disorders, such as Alzheimer's disease (AD) and Parkinson's disease (PD), are characterized by abnormal aggregation of certain protein in human brain. PD is the most common neurodegenerative movement disorder and featured by the formation of intraneuronal Lewy bodies in pathology. Alpha-synuclein (AS), a 140-residue protein that is largely unfolded in its physiological conformation but fibrillar as the major component in Lewy bodies in PD, is believed to be pivotally implicated in the pathogenesis of PD. Its soluble and oligomeric species are the precursor of AS fibrils and may be neurotoxic for dopaminergic neurons [1,2]. Therefore, preventing AS aggregation and reducing its neurotoxicity might hold the promise as a novel treatment for PD, a strategy which has been similarly investigated in other neurodegenerative disorders [3–6].

Trehalose contains many active –OH groups in its structure (Fig. 1), and has attracted special attention in this application. It was considered as a bioprotectant against stresses including desiccation and heat in the past [7], recently, it was found to be effective in inhibiting protein aggregation in vitro/vivo model with Alzheimer’s disease, Huntington’s disease and prion disease [5,8–10]. The striking similarity between the fibrillation of AS and beta-amyloid (Aβ)3 enables us to hypothesize that trehalose might be able to disrupt AS aggregation and potentially contribute to PD therapy. Thus, we investigated the impact of trehalose on the aggregation of A53T AS which is more prone to aggregate [11], and demonstrated its ability of inhibiting or reversing the process by using Circular Dichroism (CD) spectroscopy, Thioflavin T (ThT) fluorescence detection, and Atomic Force Microscopy (AFM).

Materials and methods
Genetic truncation and peptide synthesis
The DNA sequence encoding the full-length human AS was amplified by PCR, and cloned into the pET-15b expression vector (Novagen, San Diego, CA) using Ndel and Xhol restriction sites. The forward PCR primer was 5’-GCCGCATATGGATGTATTCATGGG-3’ and the reverse primer was 5’-ACTCCTCGAGTTAGCGTTTCG

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Abbreviations used: Aβ, beta-amyloid; AD, Alzheimer’s disease; AS, alpha-synuclein; PD, Parkinson’s disease; CD, circular dichroism; ThT, Thioflavin T; AFM, atomic force microscopy.
GTTCGTAG-3’. A point mutation of the AS substituted by threonine for alanine (A53T) was introduced by PCR. Constructs were confirmed by sequencing and then introduced into Escherichia coli Rosetta (DE3) cells (Novagen, San Diego, CA) to produce 6X His-tagged A53T AS. Protein production was induced by shaking the E. coli Rosetta cells with 0.8 mM IPTG at 37 °C for 4 h, and the recombinant proteins were then purified with a His-select column (Sigma, St. Louis, MO) according to the manufacturer’s protocol and dialyzed against 1X PBS overnight. Purity of recombinant A53T AS was verified by SDS–PAGE and Western blot analysis. Histidine tags were not eliminated since they rarely affect the characteristics of the protein [12,13].

Preparation of AS aggregations

The aggregates were produced by incubating A53T AS in 20 mM phosphate buffer (pH 7.5) at 37 °C for 7 days in a 0.5 ml Eppendorf tube without shaking. According to previous reports investigating the effect of trehalose on other proteins, 10 or 100 mM trehalose (Sigma, St. Louis, MO) was co-incubated with 2.0 mg/ml A53T AS in this study [9]. Ten microliters of the incubated solution was extracted at every given time to assess the A53T AS aggregation by means of CD spectroscopy, ThT fluorescence and AFM.

CD spectroscopy

CD spectrum of A53T AS was recorded using a Jasco spectropolarimeter with a quartz cell of 1 mm path length. Ten microliters of the incubated samples was extracted and diluted into 140 μl phosphate buffer (20 mM, pH 7.5) for CD measurement. The CD spectra were measured at the range from 190 to 250 nm. A blank control (20 mM phosphate buffer, pH 7.5) was subtracted from the studied spectra. All CD spectra were deconvoluted using Secondary Structure Estimation software of the Spectra Manager program developed by JASCO Corporation, Japan.

ThT fluorescence assay

The fibrillation of A53T AS was evaluated by the ThT fluorescence with a fluorescence spectrophotometer (FLS-920, Edinburgh Instruments). Briefly, 10 μl of the incubated sample was added into 1 ml ThT solution (10 μM ThT in 20 mM phosphate buffer, pH 7.5). The fluorescence of the fibrillar A53T AS in a quartz cuvette with the light-path of 1 cm was induced at the excitation wavelength of 450 nm and recorded at the emission wavelength of 482 nm. The values of ThT fluorescence of the A53T AS were referred to the free ThT fluorescence and repeated for three batches of samples. All ThT data were fit with a sigmoidal model using Sigmoidal Fit Origin 7.5 software developed by OriginLab Corporation, USA.

AFM image

Two microliters of A53T AS samples was freshly collected and swiftly diluted to 5 μg/ml solution with 20 mM phosphate buffer. Ten microliters of the diluted samples was mounted onto the freshly cleaved mica for AFM observation. After being dried at room temperature for 5 min, the sample on the mica surface was gently rinsed with deionized water to remove the phosphate salt, and then dried in vacuum overnight. The images of the A53T AS were acquired under atmosphere by using the silicon probes in a tapping mode packaged in multi-mode SPM Nanoscope IV system (Veeco Metrology Group, USA). The aggregate dimensions of AS were analyzed by Depth model packaged in nanoscope software developed by Veeco Metrology Group, USA. Each curve height was obtained by averaging at least four AFM images for one sample.

Results

Impact of trehalose on the secondary structure of A53T AS

Structural alteration of A53T AS upon the trehalose treatment was examined with CD spectroscopy. Dose and time effects are shown in Fig. 2A–C. CD spectra were deconvoluted and the amount of random coil was compared in Fig. 2A–C. In the absence of trehalose, 2.0 mg/ml A53T AS was highly unfolded in solution with the most negative ellipticity noted at 198 nm in the initial phase (Fig. 2A), suggesting a dominant random coil conformation of A53T AS in the solution. With the passing of the incubation time, the minimum ellipticity at 198 nm decreased gradually, while the intensity of the negative peak at 219 nm increased slightly (Fig. 2B)[14,15], indicating that AS experienced a significant structural transition from random coil to beta-sheet structure (Fig. 2D-a)[6,16]. In the presence of 10 mM trehalose (Fig. 2B), the negative peak at 219 nm slightly increased during the incubation from day 0 to 4 as the initial random coil conformer attenuated simultaneously (Fig. 2D-b). Intriguingly, the altered structure of A53T AS was shown to experience a dramatic structural transition upon the 10 mM trehalose treatment during the incubation from day 4 to 7, with an increased minimum ellipticity at around 198 nm and an decreased ellipticity at around 219 nm (Fig. 2B), indicating that the secondary structure of A53T AS was influenced apparently by the 10 mM trehalose during the late incubation. As seen in Fig. 2D-b, during the late period of incubation in the presence of 10 mM trehalose, the content of A53T AS in the form of random coil increased sharply, suggesting a fast transition of secondary structure to random coil. On the other hand, in the presence of 100 mM trehalose, there was a typical evolution of the secondary structure during the whole period of incubation from day 0 to 7 (Fig. 2C), which is similar to the tendency observed during the incubation of A53T AS without trehalose (Fig. 2A and D-c), and completely different from that in the presence of 10 mM trehalose during the late period of the incubation.

Fibrillation dynamics and aggregate morphologies of A53T AS

ThT binds β-sheets, especially the extended ones present in amyloid fibrils[17,18]. In our experiments, ThT fluorescence intensity of A53T AS without trehalose (Fig. 3A) initially showed a low plateau (lag phase) during the first day, increased exponentially during day 1–3, and reached saturation after that. Under AFM, A53T AS without trehalose showed a characteristic fibrillar morphology [19–21]. In our research (Fig. 4), we observed small cake-like AS oligomers with the height of about 1.8 nm from day 0 to 1. Those oligomers elongated and thickened into “protofibrils” in the height of about 4 nm by day 2. The aggregates continued to lengthen and thicken into 6.5 nm protofibrils or fibrils at day 4, and some fibrils even started to be bifurcated like a “crotch”. By day 6, the bifurcated fibrils twisted and cross-linked into fibrils
in the height of 12 nm, and eventually formed fibrillar plaques by day 7. Taking the CD results (Fig. 2A) into account, we conclude that A53T AS in the absence of trehalose firstly assembles into partially folded oligomers during the lag phase, then grows into β-sheet-rich protofilaments or protofibrils with an increase of ThT fluorescence intensity during the exponential phase, eventually assembles into the entwined fibrils and fibrillar plaques with a final plateau of ThT fluorescence intensity due to the exhaustion of soluble monomers and the formation of oligomeric intermediates [22].

In the presence of 10 mM trehalose (Fig. 3B), the ThT fluorescence intensity of A53T AS increased during the first three days. Subsequently, the ThT fluorescence intensity reduced sharply, which was consistent with the phenomenon observed under AFM. During the incubation from day 0 to 3 (Fig. 5), there was no significant difference in the morphology of the A53T AS aggregates in the presence of 10 mM trehalose compared with that in A53T AS alone (Fig. 4). However, during the late period of trehalose treatment from day 4 to 5 (Fig. 5), those A53T AS protofibrils or fibrils disassembled into doublestrand rosaries and less ordered aggregates. By day 7, those assemblies dissolved into small aggregates or even disappeared (Fig. 5). The evolution of the aggregate’s morphology in the presence of 10 mM trehalose agrees well with the formation of more random coil in the CD spectra (Fig. 2B and D-b) and low fluorescence intensity in ThT fluorescence (Fig. 3B) during the late incubation.

In the presence of 100 mM trehalose, the slope of the exponential phase lessened (Fig. 3C) compared with that in A53T AS alone, while the fluorescence intensity increased continuously from day 3

![Fig. 2. Trehalose-induced conformational alterations in A53T AS. Far UV circular dichroism spectra of AS were measured in the absence (A) or presence of 10 mM (B) or 100 mM (C) trehalose. Spectra showed here were collected after incubation for 0 day (solid line in black), 3 days (short dash in blue), and 6 days (dash dot in red) and were average of three independent experiments. The random coil contents of A53T AS as a function of trehalose concentrations and incubation time were compared after deconvolution of the CD spectra(D), without (a) (C) or with 10 mM (b) (●) or 100 mM (c) (△) trehalose. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)](image1)

![Fig. 3. Inhibition of A53T AS fibrillation by trehalose. The fibrillation kinetics of A53T AS, in the absence (A) (○) or presence of 10 mM (B) (●) or 100 mM trehalose, (C) (△), was detected by ThT fluorescence emission at 485 nm; error bars = SD, n = 3. Solid lines represent sigmoid fitting results.](image2)
to 5, and then reached a higher plateau. Under AFM, there was no fibrillar aggregates but some small oligomers and protofilaments during the incubation from day 0 to 7 (Fig. 6). It is in line with our corresponding CD results (Fig. 2C) in which a little bit more soluble $\beta$-sheet structure appeared during the late incubation. Taken together, trehalose at high concentration could slow down the formation of $\beta$-sheet aggregates during the early period of incubation, stabilize and increase the partially-folded oligomers or $\beta$-sheet-rich protofilaments which increase the ThT fluorescence intensity, and prevent the formation of the mature A53T AS fibrils during the late period of incubation.

**Discussion**

In summary of the results from CD, ThT fluorescence and AFM, the vitro experiments reported here provide a unique insight into the role of trehalose in affecting the dynamics and morphology of A53T AS aggregates. In the absence of trehalose, though random coil structures are predominant initially, the reduction of this structure (Fig. 2) after incubation could lead to an increase in the overall hydrophobicity of A53T AS and the presence of partially folded structures with beta-structure [6], which accelerates the fibrillation of A53T AS and the formation of the aggregate [23].
The aggregation and fibrillation kinetics of A53T AS alone follows a typical sigmoidal model of nucleated polymerization, during which the initial lag phase corresponds to the formation of critical nuclei, the subsequent exponential growth phase to the fibril elongation, and the final plateau to the exhaustion of soluble monomers and the formation of oligomeric intermediates [24–27]. In this work, the fibrillation process of A53T AS is a typical nucleation-dependent aggregation procedure similarly observed in other studies. During the 'nucleation' step occurring in the first day of incubation, the A53T AS monomers assembled into partially-folded oligomers which act as nucleus or seeds. Once the oligomers reached a sufficient amount, they grew into protofibrils or fibrils during 'elongation' step and then into the entwined or cross-linked larger fibrils in a thermodynamically favorable manner. In the presence of 10 mM trehalose, the 'nucleation' step of A53T AS was similar to that without trehalose. However, the preformed fibrils disassembled into small aggregates or even soluble random coil structure during the 'elongation' step. Moreover, in the presence of 100 mM trehalose, the 'elongation' step of A53T AS was effectively suspended as diagramed in Fig. 7.

At present, there are three hypotheses explaining the mechanism by which trehalose stabilizes protein folding: water-layer with preferential exclusion, water-replacement, and mechanical-entrapment [26]. The first hypothesis may be a partially reasonable explanation regarding its protein stabilizing effect based on our findings. In the presence of high concentration, trehalose molecules cluster with each other surrounding A53T AS molecules, weakening the inter-peptide hydrophobic interactions, thus increasing its stability. There are eight active –OH groups in the trehalose molecule (Fig. 1), each of which can interact with N or O atoms in the amino residues of the A53T AS protein to form the direct H-bonds between trehalose and peptides. In the meantime, indirect H-bonds formed via water molecule shells exist as well according to the water layer hypothesis [26]. The direct or indirect interaction between trehalose and A53T AS became stronger than that of the inter-H-bond within A53T AS, facilitating the formation of peptide-water-trehalose copolymer and preventing the formation of the inter-H-bond of A53T AS [28]. It is consistent with our observation that trehalose at high concentration allow AS to assemble into the partially folded oligomers and protofibrils, stabilize the aggregates morphology [25], and strongly inhibit the formation of the mature AS fibrils. In the presence of a low concentration of trehalose in our experiments, the interaction of the inter-H-bond within A53T AS monomers is dominant at the early period of incubation, and trehalose is not potent enough to inhibit the self-assembly of A53T AS monomers into partially-folded oligomers or beta-sheet-rich profibrils. This process is similar to that in the incubation of A53T AS in the absence of trehalose. In the late stage of incubation, however, with the exhaustion of A53T AS monomers and the diminished formation of the inter-H-bond within A53T AS monomers, trehalose enters the assembled A53T AS and breaks the preformed inter-H-bond within A53T AS, therefore, it loosens the ordered structure and leads to the process of disassembling into soluble small aggregates or even random coil structure.

Recently, some compounds such as rifampicin [29], curcumin [4] have been extensively investigated as potential therapies for PD and other neurodegenerative disorders. In the current work, trehalose showed the ability to inhibit the aggregation of A53T AS, thus may also be beneficial for treating PD. However, better understanding of the bio-toxicity of various species of aggregated AS will be crucial to develop trehalose as a novel therapeutic strategy. Previously it is believed that AS fibril, rather than other AS species, plays a causative role in PD by disrupting the membrane [30], but AS protofibrils or oligomers are reported to be more neurotoxic than fibrils [1,31–33], especially the ‘doughnut-shaped’ oligomers [34]. Therefore, future research should be done to distinguish the relative neurotoxicity of particular AS species in vivo and assess the effect of trehalose in PD animal models. Interestingly, trehalose was reported to have autophagy-enhancing [35] and anti-inflammatory [36] effects, which are pertinent to the pathology in PD. Furthermore, trehalose can slow down aging in Caenorhabditis elegans [37], a process closely associated with PD pathogenesis, it also reduces the formation of polyglutamine aggregates and correct motor dysfunction in a transgenic mouse model of Huntington’s disease [5], another neurodegenerative disorder.
disorder with abnormal protein deposits in brain, and provides neuroprotection in a mouse model of tauopathy with Parkinsonism [38]. Hopefully, trehalose might be useful for treating PD in vivo, which is being under way and awaits further verification.

**Conclusion**

To conclude, based on the CD spectroscopy, ThT fluorescence and AFM images of A53T AS, the conformation of A53T AS could change from random coil to β-sheet and undergo an obvious fibrillation process following a nucleation-dependent aggregation mechanism when the A53T AS was incubated for a period of time in absence of trehalose. In the fibrillation process, the A53T AS monomers assembled into soluble β-sheet oligomers or protofilaments in the ‘nucleation’ step, and then assembled into protofibrils and/or the entwined and cross-linked large fibrils during the ‘elongation’ step. When trehalose was present at low concentration in the A53T AS solution, it inhibited preformed protofibrils or fibrils from assembling into the twisted proto-cross-linked large fibrils, and even disassembled the preexisting fibrils into small aggregates or random coil conformers. Furthermore, when trehalose was present at high concentration in the A53T AS solution, it inhibited the formation of intra- or inter–H-bond in A53T AS with active –OH, efficiently preventing the self-assembling of A53T AS into the protofibrils and fibrils.

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**References**


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**Fig. 7. A hypothetical fibrillation process of A53T AS in the presence of trehalose.**

![Diagram of A hypothetical fibrillation process of A53T AS in the presence of trehalose.](image-url)