BIOCHEMISTRY
including biophysical chemistry & molecular biology

Rapid Report

pubs.acs.org/biochemistry

The Newly Discovered Parkinson's Disease Associated Finnish Mutation (A53E) Attenuates α-Synuclein Aggregation and Membrane Binding

Dhiman Ghosh,[⊥] Shruti Sahay,[⊥] Priyatosh Ranjan, Shimul Salot, Ganesh M. Mohite, Pradeep K. Singh, Saumya Dwivedi, Edmund Carvalho, Rinti Banerjee, Ashutosh Kumar, and Samir K. Maji*

Department of Biosciences and Bioengineering, Indian Institute of Technology Bombay, Mumbai, India

Supporting Information

ABSTRACT: α-Synuclein (α-Syn) oligomerization and amyloid formation are associated with Parkinson's disease (PD) pathogenesis. Studying familial α-Syn mutants associated with early onset PD has therapeutic importance. Here we report the aggregation kinetics and other biophysical properties of a newly discovered PD associated Finnish mutation (A53E). Our *in vitro* study demonstrated that A53E attenuated α-Syn aggregation and amyloid formation without altering the major secondary structure and initial oligomerization tendency. Further, A53E showed reduced membrane binding affinity compared to A53T and WT. The present study would help to delineate the role of A53E mutation in early onset PD pathogenesis.

Aggregation and toxic oligomers formation by α-synuclein (α-Syn) is directly linked with Parkinson's disease (PD) pathogenesis.[1−3] Several *in vitro* studies have shown that the PD associated mutations alter the aggregation and oligomerization rates of α-Syn. It has been shown that among familial PD mutations, A53T, E46K, and H50Q accelerate α-Syn aggregation,[4−7] whereas A30P and G51D mutations delayed the aggregation kinetics and fibril formation *in vitro*.[8,9] Recently, a new familial PD mutation of α-Syn (A53E) associated with early onset PD and multiple system atrophy (MSA) was discovered in a Finnish family.[10] However, the effect of this mutation on α-Syn structure and aggregation is not known yet. We analyzed here for the first time the structure, aggregation, extracellular toxicity, and membrane binding properties of the newly discovered A53E mutant and compared these properties with WT α-Syn and its other two familial PD mutants, A53T and A30P.

For biophysical studies, all proteins were purified, and low molecular weight (LMW) preparation[2,6] was made at a concentration of 200 μM in 20 mM Gly-NaOH buffer, pH 7.4, 0.01% sodium azide. The aggregation reaction was initiated by incubating the LMW preparations at 37 °C with slight agitation. Aggregation kinetics and secondary structural changes were monitored by ThT binding assay and CD spectroscopy, respectively. ThT binding revealed a kinetic profile of nucleation dependent polymerization reaction consisting of three distinct phases (nucleation, elongation, and stationary phase) for all the proteins (Figures 1A and S1). Quantitative analysis of the ThT profile (methods section of the Supporting Information (SI)) showed the lag times for A53T, WT, A53E, and A30P are ∼42 h, ∼58 h, ∼135 h, and ∼195 h, respectively (Figure 1B), suggesting A53E substantially attenuated α-Syn aggregation. Since another PD mutation on the same site, A53T, accelerated the aggregation of α-Syn, it is possible that negatively charged side chain of Glu residue in A53E might affect the intermolecular interactions and β-sheet-rich fibril formation of α-Syn. We therefore studied a designed mutant, A53K, with an oppositely charged (positive) side chain of Lys.

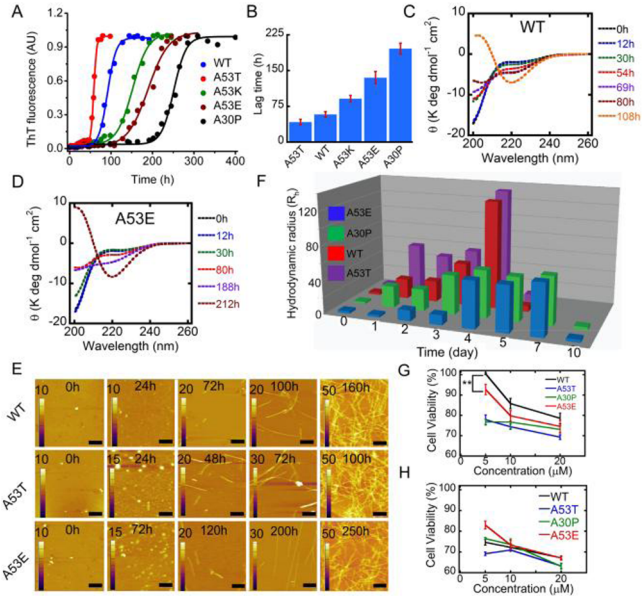

Figure 1. Fibrillation of α-Syn. (A) Aggregation kinetics of WT α-Syn and mutants measured by ThT binding assay (*Y*-axis represents normalized ThT fluorescence). (B) Bar diagram representation of lag time of their corresponding fibril formation. (C, D) CD spectroscopy showing secondary structural changes from random coil to β-sheet during fibrillation for WT and A53E, respectively. (E) Temporal morphological changes during aggregation of α-Syn monitored by AFM. (F) Time-dependent oligomerization of α-Syn monitored by DLS (standard error for each value is ∼2−10%). (G, H) Dose-dependent MTT reduction assay of α-Syn fibrils and oligomers at 72 h, respectively.

Received: August 19, 2014
Revised: September 25, 2014
Published: September 30, 2014

   dx.doi.org/10.1021/bi5010365 | *Biochemistry* 2014, 53, 6419−6421

The mutation of A53K also delayed the aggregation kinetics of α-Syn, where the lag time was (~90 h) (Figure 1B, results section of SI). The data suggest that a charged residue at the 53 position may affect α-Syn folding and the fibrillation process. In contrast, a neutral side chain of Thr (A53T) may rather facilitate the hydrophobic interactions, and the -OH group of Thr may also participate in the H-bonding interactions thereby favoring fibril formation. Consistent with the ThT binding assay, the CD data revealed slower conformational transition of A53E from a random coil to β-sheet structure compared to WT and A53T (Figures 1C,D and S2).

Further, to study the temporal change of morphology during aggregation of WT, A53T, and A53E, time-dependent atomic force microscopy (AFM) analysis was performed. Immediately after LMW preparation, all three synucleins showed a mostly low ordered amorphous structure (Figures 1E and S3). However, with the progression of time, gradual transformation of an ordered structure with the formation of higher order oligomers was observed. After 72 h of incubation, A53T showed long fibrillar morphology, whereas WT took ~100 h for the same (Figures 1E and S3). In contrast to A53T and WT, A53E showed slower transformation of morphology and fibril growth. The small and thin protofibrils like structures appeared only after 120 h. After 200 h of incubation, A53E showed formation of long, thin fibrils that gradually converted into a dense network after ~250 h. Thus, our time-dependent AFM study revealed slower aggregation kinetics for A53E consistent with our ThT binding assay and CD spectroscopy data. Further, the AFM data suggest that A53E took more time for oligomer formation and its subsequent conversion to fibrils. Therefore, the population time of oligomers and small filaments is longer for A53E compared to WT. Recent studies suggested that the early formed oligomers could be the most probable toxic species responsible for neurodegeneration in PD.[2] We thus analyzed whether A53E mutation affects the early oligomerization properties of α-Syn. To test this, two different methods, size exclusion chromatography (SEC) and photo-induced chemical cross-linking of unmodified proteins (PICUP), were performed. In SEC profile, constant area under the monomeric peak (~15 mL) (Figure S5) ruled out the different oligomerization tendencies of these proteins immediate after dissolution. However, the larger area under the oligomeric peak (~7.5 mL) by A53T and WT compared to A53E could be due to higher light scattering at 280 nm by larger size oligomers formed by A53T and WT. Further, a PICUP study showed similar oligomer distributions consisting of monomers along with a low amount of dimers, trimers, and pentamers for all the mutants (Figure S6). The SEC and PICUP data thus suggest no significant difference in initial oligomerization properties of WT α-Syn and its mutants. To examine the possible differences in the oligomerization states of all synucleins during aggregation, time-dependent oligomer formation was monitored using dynamic light scattering (DLS). With the progression of time, we observed a difference in the oligomer size distributions across different α-Syn mutants (Figures 1F and S7). A53T, A30P, and WT formed oligomers of a bigger size after 24 h of incubation, whereas A53E did not undergo any increase in size. A53T and WT reached maximum oligomer size on day 4, which decreased subsequently on their fibril formation on day 5. In contrast, slower fibrillizing mutants A30P and A53E maintained a consistent oligomer size through days 4, 5, and 7 of incubation with a subsequent decrease in oligomer size by day 10 (Figure 1F). The data suggest that A30P and A53E accumulate oligomers for a longer duration of time. At the end of the assembly reaction, the fibrils formed (Figure S4) were used to test the cellular toxicity using MTT assay. The dose-dependent cellular toxicity assay suggests that at low fibril concentration (5 μM), all mutants were slightly more toxic than WT. However, at higher fibril concentration (20 μM), no significant difference in toxicity was observed (Figure 1G). Since oligomers are suggested to be more toxic[2] and our AFM data showed that A53E formed globular oligomers at 72 h of incubation, dose-dependent cellular toxicity was further studied at 72 h. Interestingly, we did not observe any significant differences in toxicity between WT and mutants (Figure 1H). A53E rather showed slightly less toxicity at a lower concentration compared to WT and other mutants, the reason for which is not clear.

To investigate the possible effect on the conformational properties due to A53E substitution, heteronuclear single quantum coherence (HSQC) spectroscopy was performed with WT, A53T, and A53E. An overlay of ^{1}H, ^{15}N-HSQC spectra for WT (black), A53T (red), and A53E (green) has been shown in Figures 2A and S8A. The data showed almost similar spectra of WT, A53T, and A53E with relatively narrow dispersions in the proton dimension for all proteins, characteristics of unfolded

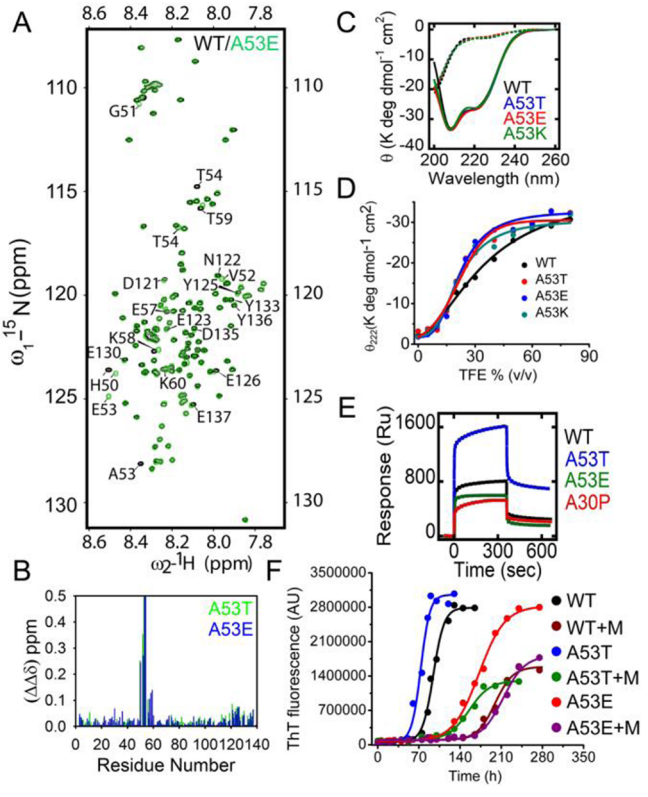

Figure 2. Structure and membrane binding studies of α-Syn. (A) Overlaid heteronuclear single-quantum coherence (HSQC) spectra of WT and A53E. (B) Chemical shift perturbations of A53T and A53E as compared to WT. (C) CD spectra of WT and its mutants in the presence (solid lines) and absence (broken lines) of 0.5% SDS. (D) θ_{222} values of the corresponding CD spectra for WT and its mutants in the presence of various TFE concentrations. (E) Relative membrane binding assessed by response unit (Ru) of all proteins at 100 μM concentration monitored by SPR. (F) Aggregation kinetics of WT, A53T, and A53E in the presence (+M) and absence of membrane (1:1 mass ratio) monitored by ThT fluorescence.

dx.doi.org/10.1021/bi5010365 | *Biochemistry* 2014, 53, 6419−6421

structure. The chemical shift differences however suggest perturbation of chemical shifts for residues surrounding the A53E mutation site (Figure 2B). This may be due to the alteration of local chemical environment by A53E substitution. Significant chemical shift changes were also observed for the residues at the extreme C-terminus of α-Syn. In contrast to chemical shift perturbation data, the secondary structural propensity however did not show any major alteration due to A53E or A53T mutation (Figure S8B). It is reported that α-Syn interacts with membrane and shows helical conformation in a membrane mimetic environment.[11] It has been proposed that A53E mutation may destabilize the second helix of the α-Syn.[10] To study this, the secondary structure of A53E was analyzed in two helix favoring conditions (0.5% SDS and various TFE concentrations). All proteins adopted helical conformation to a similar extent (similar θ_{222} value) both in 0.5% SDS and 80% TFE (Figure 2C,D). Further, TFE titration data indicated a gradual increase in helicity for all proteins with increasing concentration of TFE (for details refer SI, Figure S9). These data clearly indicated that A53E mutation did not alter major secondary structure either in physiological buffer conditions or in a membrane-mimicking condition.

Previous studies suggested that α-Syn interaction with the membrane play a significant role in its native function as well as its malfunctioning in PD pathogenesis.[12] We used the surface plasmon resonance (SPR) technique to study the negatively charged membrane (PE/PC) and α-Syn interaction. Figures 2E and S10 show an increment of response unit (Ru) for all proteins upon binding to the membrane surface in a concentration-dependent manner. The relative dissociation constants (K_d) calculated from the Ru values were ∼71 μM, ∼57 μM, ∼80 μM, and ∼18 μM for A53E, WT, A30P, and A53T, respectively, suggesting A53E has lower affinity toward membrane compared to WT. The charge repulsion between negatively charged Glu53 and negatively charged vesicle may attenuate the binding between them. The higher membrane binding of A53T mutant could be attributed to the interaction between OH group of Thr side chain and lipid molecules in the vesicles. The data suggest that optimum membrane binding may be required for α-Syn function, and altered membrane binding affinity as shown here for familial PD associated mutants (A53E, A30P, and A53T) may contribute to PD pathogenesis. In light of previous reports of α-Syn aggregation being significantly affected by the presence of membrane,[13] we further studied aggregation of α-Syn and its two mutants A53T and A53E in the presence of membrane (PE/PC) with a mass ratio of 1:1. The presence of membrane delayed the aggregation kinetics of all the proteins. However, A53T in the presence of membrane still showed faster aggregation kinetics, whereas WT and A53E aggregated almost at a similar rate (Figure 2F). Our data suggest that lipid environment might also play a significant role in modulating aggregation of α-Syn and its mutants *in vivo*. As previously shown for familial PD mutations A30P and G51D,[4,9] our current study thus suggests that A53E attenuates α-Syn aggregation in contrast to the familial PD mutations, A53T, E46K, and H50Q.[4,6,7] Due to the different effects of PD associated mutations on the aggregation rate of α-Syn, the correlation of α-Syn aggregation with disease pathogenesis is not straightforward. Moreover, several factors may contribute to α-Syn mediated toxicity in PD including membrane interaction and soluble oligomer formation of α-Syn. In this context, A30P has been shown to oligomerize rapidly despite having delayed fibrillation kinetics.[4] G51D mutation however has been shown to delay oligomerization as well as fibrillation kinetics of α-Syn[9] as shown here for A53E, which may enhance the duration of the α-Syn oligomers population. Moreover, the impaired membrane binding of A53E mutant as shown here (similar to A30P and G51D) may also hinder vesicle mediated axonal transport leading to their accumulation in the neuronal cell body resulting in aggregation and disease pathogenesis.[14] Our current study thus reveals the alterations caused by A53E mutation on the structure, aggregation, and membrane binding capability of α-Syn, which will have significant value to understand the role of A53E mutation in early onset PD pathogenesis.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Detailed description of material methods and supplementary figures. This material is available free of charge via the Internet athttp://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: 91-22-2576-7774. E-mail: samirmaji@iitb.ac.in.

Author Contributions

⊥D.G. and S.S. contributed equally.

Funding

This work was supported by grants from DBT (BT/PR14344Med/30/501/2010;BT/PR13359/BRB/10/752/2009, ICMR (5/20/9(Bio)/2011-NCD-I) Govt. of India.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors acknowledge the national NMR facility (TIFR), AFM, and SPR facility (IRCC, IIT Bombay), Prof. G. Krishnamoorthy (TIFR) and Dr. Veenita Shah for DLS and SPR experiments, respectively. S.S., D.G., and A.K. are thankful to SPMF (CSIR), UGC and Ramalingaswami fellowship, Govt. of India, respectively.

■ REFERENCES

(1) Spillantini, M. G., et al. (1997) *Nature 388*, 839−840.
(2) Winner, B., et al. (2011) *Proc. Natl. Acad. Sci. U.S.A. 108*, 4194−4199.
(3) Cookson, M. R. (2005) *Annu. Rev. Biochem. 74*, 29−52.
(4) Conway, K. A., Harper, J. D., and Lansbury, P. T. (1998) *Nat. Med. 4*, 1318−1320.
(5) Greenbaum, E. A., et al. (2005) *J. Biol. Chem. 280*, 7800−7807.
(6) Ghosh, D., et al. (2013) *Biochemistry 52*, 6925−6927.
(7) Choi, W., et al. (2004) *FEBS Lett. 576*, 363−368.
(8) Conway, K. A., et al. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 571−576.
(9) Fares, M. B., et al. (2014) *Hum. Mol. Genet. 23*, 4491−4509.
(10) Pasanen, P., et al. (2014) *Neurobiol. Aging 35*, 2180 e2181−2185.
(11) Eliezer, D., et al. (2001) *J. Mol. Biol. 307*, 1061−1073.
(12) Auluck, P. K., et al. (2010) *Annu. Rev. Cell. Dev. Biol. 26*, 211−233.
(13) Zhu, M., and Fink, A. L. (2003) *J. Biol. Chem. 278*, 16873−16877.
(14) Jensen, P. H., et al. (1998) *J. Biol. Chem. 273*, 26292−26294.