

# Effects of the mutations Ala<sup>30</sup> to Pro and Ala<sup>53</sup> to Thr on the physical and morphological properties of $\alpha$ -synuclein protein implicated in Parkinson's disease

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**Abstract**  $\alpha$ -Synuclein ( $\alpha$ -syn) protein has been found in association with the pathological lesions of a number of neurodegenerative diseases. Recently, mutations in the  $\alpha$ -syn gene have been reported in families susceptible to an inherited form of Parkinson's disease. We report here that human wild-type  $\alpha$ -syn, PD-linked mutant  $\alpha$ -syn(Ala30Pro) and mutant  $\alpha$ -syn(Ala53Thr) proteins can self-aggregate and form amyloid-like filaments. The mutant  $\alpha$ -syn forms more  $\beta$ -sheet and mature filaments than the wild-type protein. These findings suggest that accumulation of  $\alpha$ -syn as insoluble deposits of amyloid may play a major role in the pathogenesis of these neurodegenerative diseases.

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**Key words:**  $\alpha$ -Synuclein; Parkinson's disease; Lewy body; Self-aggregation; Amyloid; Neurodegenerative disease

## 1. Introduction

Parkinson's disease (PD) and dementia with Lewy bodies (DLB) are pathologically characterised by the presence of Lewy bodies (LBs) and Lewy neurites (LNs) accompanied by dead and dying neurones (reviewed in [1]). Immunohistochemical studies of LBs and LNs and immunoelectron microscopy studies of LB filaments indicate that the presynaptic protein  $\alpha$ -synuclein ( $\alpha$ -syn) forms the major filamentous component of LBs and LNs [2–5]. It has also been shown that some of the LBs in PD display thioflavine-S reactivity, indicating the presence of amyloid-like filaments [6,7]. This suggests that abnormal deposition of  $\alpha$ -syn may be involved in the pathogenesis of PD and DLB. This proposal has been supported by the recent discovery of mutations in the  $\alpha$ -syn gene in families susceptible to inherited forms of PD [8,9].

One mutation, found in certain Italian and Greek families, results in an Ala to Thr substitution at position 53 of  $\alpha$ -syn [8] in a region predicted to adopt an  $\alpha$ -helical structure surrounded by  $\beta$ -sheets. The other mutation, Ala30 to Pro, has been detected in a family of German origin, unrelated to the families in which the Ala53Thr mutation was found [9]. These substitutions have been proposed to disrupt the  $\alpha$ -helical

structure in this region and extend the  $\beta$ -sheet, rendering the protein more prone to self-aggregation [8–11]. This may lead to the formation of abnormal deposits which could contribute to nerve degeneration in the brain, similar to the accumulation of the  $\beta$ -amyloid protein (A $\beta$ ) in Alzheimer's disease (AD), Prion protein in prion diseases and Huntingtin protein in Huntington's disease (HD) (reviewed in [12]).

Studies of neurodegenerative disorders such as AD [13–15], prion protein diseases [16–18], and HD [19] have highlighted the importance of the biophysical properties of mutant cellular proteins in initiating and propagating neuronal lesions found in these neurodegenerative disorders. We therefore investigated some of the biophysical properties of recombinant human wild-type  $\alpha$ -syn and the PD-linked mutants  $\alpha$ -syn(Ala30Pro) and  $\alpha$ -syn(Ala53Thr). We report here that mutant  $\alpha$ -syn proteins developed more  $\beta$ -sheet structure and formed more mature filaments than wild-type as revealed by SDS-PAGE, thioflavine-S staining, electron microscopy and circular dichroism studies.

## 2. Materials and methods

### 2.1. Preparation of $\alpha$ -syn proteins

Recombinant  $\alpha$ -syn proteins were expressed in *Escherichia coli* and purified as previously described [20].

### 2.2. Preparation of aggregated 'aged' solutions of $\alpha$ -syn proteins

$\alpha$ -Syn proteins were dissolved in PBS (phosphate-buffered saline pH 7.4) at a concentration of 110  $\mu$ M, and the resulting solutions were aged for 7 days at 37°C.

### 2.3. Electron microscopy (EM)

A 10  $\mu$ l sample was placed on a carbon-coated copper grid, and incubated for 5 min. The droplet was then displaced with 10  $\mu$ l of 0.5% (v/v) glutaraldehyde and incubated for an additional 5 min. The grid was then washed with five drops of water and wicked dry. Finally, the sample was stained with 10  $\mu$ l of 2% (w/v) uranyl acetate solution for 2 min. This solution was wicked off, the grid was air-dried, then viewed on a JEOL TEMCX 100 II electron microscope and photographed.

### 2.4. Circular dichroism

Spectra were recorded at 21°C over a range 190–250 nm on a JASCO J720 spectropolarimeter. An aliquot of the protein solution (about 130  $\mu$ l) was placed in a quartz cell (0.2 mm pathlength). All spectra were corrected by subtracting the baseline of the appropriate solvent. Results are expressed as molar ellipticity,  $[\theta]$ , in units of deg cm<sup>2</sup> dmol<sup>−1</sup>.

## 3. Results and discussion

Interest in the  $\alpha$ -syn protein was spurred by reports of its

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**Abbreviations:** AD, Alzheimer's disease; A $\beta$ ,  $\beta$ -amyloid protein; DLB, dementia with Lewy bodies; HD, Huntington's disease; LB, Lewy body; LN, Lewy neurite; NAC, non-A $\beta$  component of AD amyloid; PD, Parkinson's disease

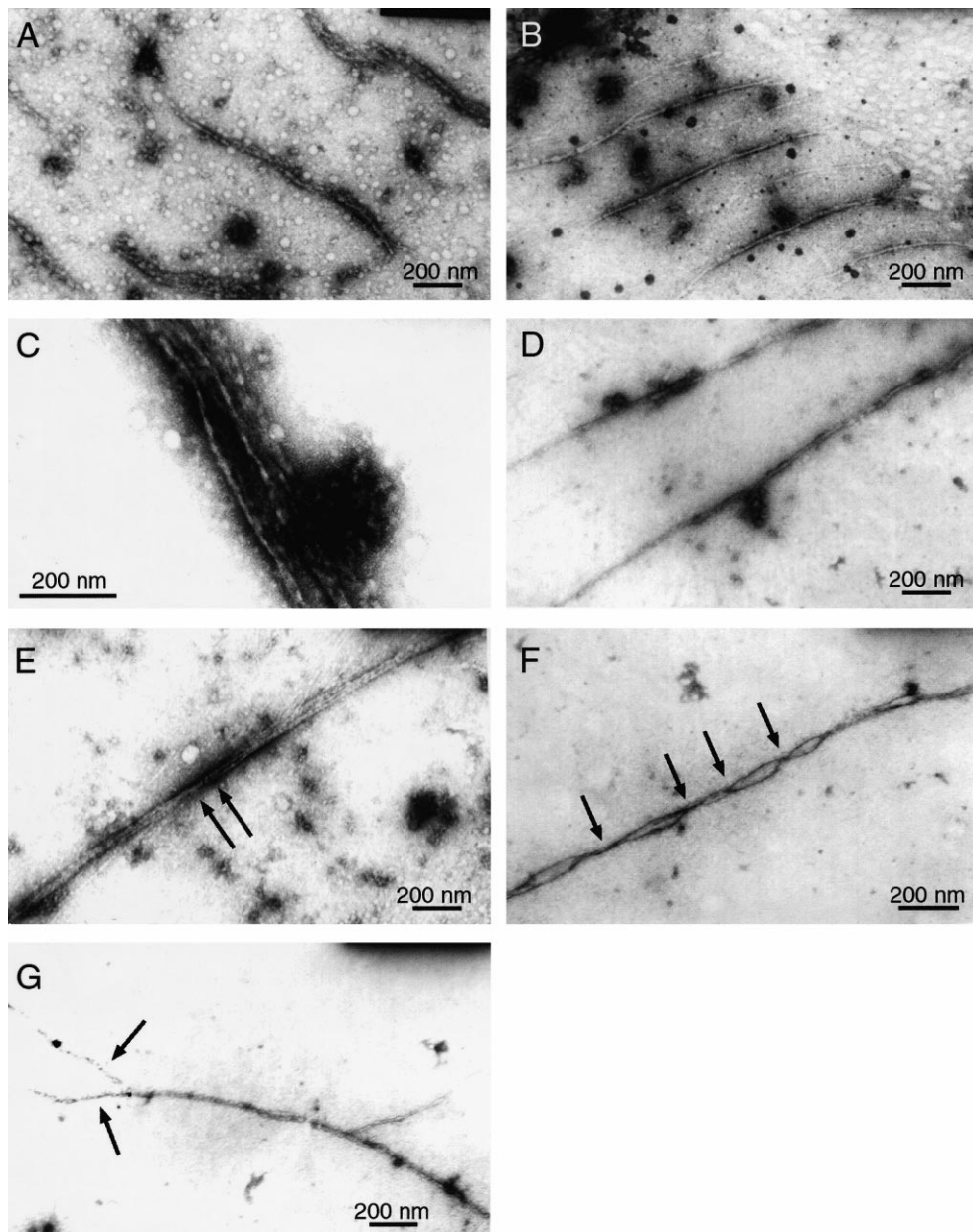


Fig. 1. Negatively stained electron micrographs (EM) of filaments obtained from aged  $\alpha$ -syn proteins. A:  $\alpha$ -syn. B,C:  $\alpha$ -syn(Ala30Pro). D–G:  $\alpha$ -syn(Ala53Thr). The EM showed different morphology of filaments including: short irregular 6–8 nm wide filaments (A); short straight 8–10 nm wide filaments (B); long straight bundles of 10–12 nm wide filaments (C); long straight individual 10–12 nm wide filaments (D); twisted (arrowed) filaments with varying width of 7–12 nm (E); two 6 nm wide filaments coiled around each other and joined at intervals (arrowed) to form a filament with a variable diameter (F); 25 nm wide branched filament with 6–10 nm extensions (arrowed) at the end (G). Scale bar, 200 nm.

association with the pathological lesions of a number of neurodegenerative diseases [2–5,21–23]. The recent discovery that mutations in the  $\alpha$ -syn gene give rise to families susceptible to an inherited form of PD [8,9] has aroused scientific interest in this protein, but the mechanism by which these mutations give rise to PD is not known. However, one possibility is that  $\alpha$ -syn containing these mutations aggregates more rapidly [8–11]. We therefore decided to examine the biophysical properties of  $\alpha$ -syn so that we could determine whether the PD-linked mutations had a major impact on these properties.

In order to examine the possible self-aggregation of  $\alpha$ -syn, we aged solutions of recombinant  $\alpha$ -syn for 7 days and we investigated whether any self-oligomerisation was taking

place. As expected, self-oligomerisation of wild-type and mutant  $\alpha$ -syn proteins was detected as revealed by SDS-PAGE (data not shown). Larger amounts of dimeric species and oligomers were formed in aged than in fresh solutions. Furthermore, aggregated  $\alpha$ -syn species were found in the stacking gel in aged samples, suggesting the formation of larger aggregates that did not enter the separating gel. Similar observations have been reported for  $\alpha$ -syn isolated from LBs [4]. During the preparation of this article, Hashimoto et al. [7] reported that wild-type  $\alpha$ -syn can self-aggregate from solution and form amyloid-like filaments at pH 6.9, but not at pH 7.4. We, in contrast, were able to detect aggregation of both wild-type and mutant  $\alpha$ -syn at pH 7.4 when we incubated the

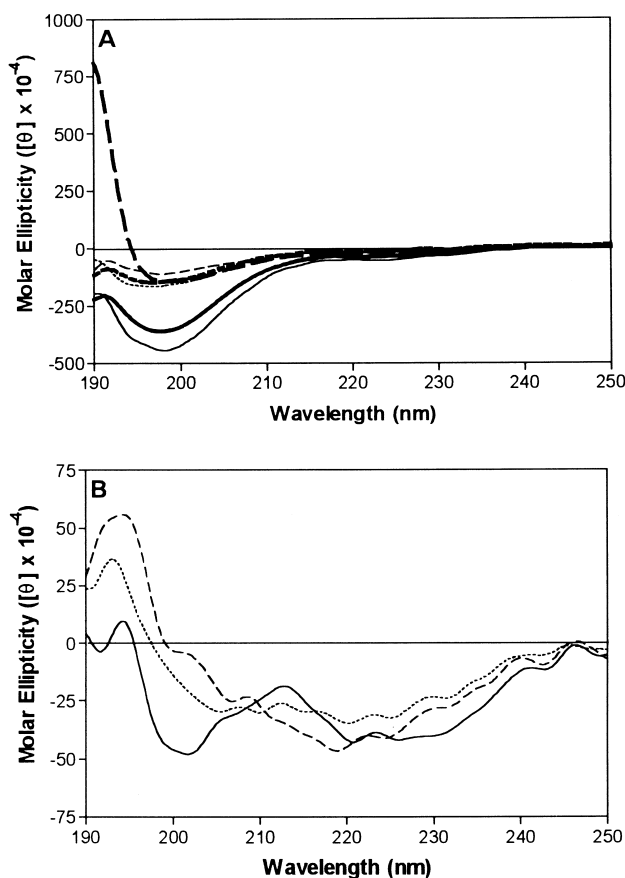


Fig. 2. CD spectra. A: Effect of ageing on the CD spectra of solutions of  $\alpha$ -syn,  $\alpha$ -syn(Ala53Thr) and  $\alpha$ -syn(Ala30Pro). CD spectra of solutions of  $\alpha$ -syn proteins (110  $\mu$ M) in PBS were obtained immediately upon preparation and after incubation at 37°C for 7 days. CD spectra were obtained after dilution to a final concentration of 10  $\mu$ M in PBS:  $\alpha$ -syn at time 0 (light solid line);  $\alpha$ -syn after 7 days (heavy solid line);  $\alpha$ -syn(Ala53Thr) at time 0 (light dashed line);  $\alpha$ -syn(Ala53Thr) after 7 days (heavy dashed line);  $\alpha$ -syn(Ala30Pro) at time 0 (light dotted line);  $\alpha$ -syn(Ala30Pro) after 7 days (heavy dotted line). B: CD spectra of solutions of  $\alpha$ -syn proteins in acetonitrile/PBS (1:1). CD spectra were obtained after dilution of a fresh stock solutions of  $\alpha$ -syn proteins (110  $\mu$ M) in PBS with appropriate concentration of acetonitrile/PBS to give a final concentration of acetonitrile 50% and 10  $\mu$ M of the proteins:  $\alpha$ -syn (solid line);  $\alpha$ -syn(Ala53Thr) (dashed line);  $\alpha$ -syn(Ala30Pro) (dotted line).

proteins at a higher concentration and for a longer period of time than reported in [7].

The formation of  $\alpha$ -syn filaments in a crossed  $\beta$ -pleated sheet conformation was assessed by thioflavine-S staining and by EM. Aggregates were formed from both aged wild-type and mutant  $\alpha$ -syn proteins and were thioflavine-S positive (data not shown), indicative of the presence of aggregates with  $\beta$ -pleated sheet conformation characteristic of amyloid-like filaments [24]. In contrast, fresh samples were thioflavine-S negative, indicating the absence of any aggregates. We used EM to examine uranyl acetate-stained filament preparations, made from fresh and aged samples formed under similar conditions as used for the aggregation assay (Fig. 1). This revealed filaments of variable length and of 6–25 nm in diameter from aged samples of  $\alpha$ -syn proteins, similar in size to those found in LBs and LNs ([3] and references therein). Filaments of diameter 6–8 nm wide are referred to as protofilaments while those of diameter 10 nm and above are referred to as

mature filaments. Clumps of amorphous material were also present in some preparations. We did not detect any filaments in the fresh samples. We observed differences in the morphology of the filaments formed by wild-type and mutant  $\alpha$ -syn under similar conditions. The wild-type  $\alpha$ -syn forms mainly short, irregular 6–8 nm wide unbranched protofilaments (Fig. 1A) together with a very few long single straight 10 nm wide mature filaments (data not shown). The filaments were sometimes associated with small clumps of amorphous material. In the case of the  $\alpha$ -syn mutants, some small amorphous clumps were also seen, but the samples mostly consisted of single, isolated filaments. Representative examples of the latter are shown in Fig. 1B–G, with filaments of diameter between 6 and 25 nm and of variable length of 50–600 nm. The aggregates of mutant  $\alpha$ -syn(Ala30Pro) formed various morphologies, including protofilaments, similar to those seen in the wild-type (Fig. 1A), as well as short 8–10 nm wide single filaments (Fig. 1B), but the majority of long mature filaments were seen either individually (data not shown) or as bundles of 10–12 nm wide filaments (Fig. 1C). In the case of  $\alpha$ -syn(Ala53Thr), similar filaments were observed (Fig. 1D) as well as twisted single filaments with a width varying between 7 and 12 nm (Fig. 1E) and also coiling of 6 nm wide protofilaments to produce cable-like structures joined at intervals (Fig. 1F). We have also seen branched mature filaments with slender 6–10 nm wide extensions at the end of a 25 nm wide filament (Fig. 1G). Goedert and co-workers have proposed a model in which the  $\alpha$ -syn molecules in LBs and LNs assembled first to form protofilaments, two of which could associate to produce a variable twisted mature filament [3]. Indeed, we have observed such a morphology (Fig. 1G) in a sample of aggregated  $\alpha$ -syn(Ala53Thr). This model is also supported by our finding that wild-type  $\alpha$ -syn formed mainly protofilaments (Fig. 1A) compared to the  $\alpha$ -syn mutants which formed mainly mature filaments under similar conditions (Fig. 1B–G).

Having found that  $\alpha$ -syn proteins can self-aggregate and form filaments in a crossed  $\beta$ -pleated sheet conformation as revealed by SDS-PAGE, EM and thioflavine-S staining, circular dichroism (CD) spectroscopy was used to study the conformational preferences of the  $\alpha$ -syn proteins. CD spectra have been used to distinguish between proteins or peptides displaying predominantly  $\alpha$ -helical,  $\beta$ -sheet or random coil conformations. The first are characterised by a maximum at 192 nm and minima at 208 and 222 nm and the second by a maximum at 195 nm and a single minimum at 218 nm. In both cases, the maximum has greater intensity than the minimum. Random conformations are characterised by a minimum at 197 nm. CD spectra were recorded from fresh and aged solutions of  $\alpha$ -syn proteins. The spectrum of the fresh  $\alpha$ -syn(Ala53Thr), with a minimum at 196 nm, changed over 7 days in PBS to a spectrum with a maximum at 190 nm and a minimum at 198 nm (Fig. 2A). We interpret this change in the spectrum as indicating a transition from predominantly random conformation to a mixture of  $\beta$ -sheet and random conformations. Similar observations have been reported for comparison studies between fragments of wild-type A $\beta$  and A $\beta$  containing the Flemish mutation [13]. In contrast, when a similar experiment was carried out on wild-type  $\alpha$ -syn and  $\alpha$ -syn(Ala30Pro) under similar conditions, the spectrum of both proteins showed little change upon ageing of the solutions for 7 days in PBS, with only a slight decrease in intensity in the minimum at 197 nm which is characteristic of random con-

formations (Fig. 2A). As discussed above, examination by SDS-PAGE and EM showed signs of self-aggregation and filament formation in all the  $\alpha$ -syn proteins examined. These aggregates had a structure in crossed  $\beta$ -pleated sheet conformation as revealed by thioflavine-S staining (data not shown). Thus the fact that we did not directly observe a transition to  $\beta$ -sheet during ageing of wild-type  $\alpha$ -syn and the mutant  $\alpha$ -syn(Ala30Pro) using CD may be due to the particular kinetics of aggregation for these proteins (reviewed in [25]). Similar findings during aggregation of A $\beta$  fragments [13] as well as a bacterial protein OsmB [26] were interpreted by the authors as suggesting that the aggregate does not contribute to the CD signal because the concentration of soluble aggregates is low. The use of the membrane-mimicking solvent aqueous acetonitrile, which is known to stabilise  $\beta$ -sheet [13,27–30], emphasises the structural differences between wild-type and mutant  $\alpha$ -syn proteins. Freshly prepared solutions in acetonitrile/PBS mixtures (1:1) of  $\alpha$ -syn mutants exhibit  $\beta$ -sheet conformation featuring a minimum at around 218 nm and a maximum about 195 nm (Fig. 2B). The  $\beta$ -sheet content of mutant  $\alpha$ -syn(Ala53Thr) is even higher than that of the mutant  $\alpha$ -syn(Ala30Pro) as suggested by the intensity of the CD curves. In contrast, wild-type  $\alpha$ -syn under similar conditions gave a spectrum with minima around 225 nm and 202 nm and with a maximum at 194 nm (Fig. 2B). We interpreted this spectrum as a mixture of random and  $\beta$ -sheet conformations.

Here we have presented data showing that PD-linked mutant  $\alpha$ -syn proteins developed more  $\beta$ -sheet structure and formed more mature filaments than wild-type  $\alpha$ -syn. These results suggest that mutant  $\alpha$ -syn could form filaments and deposit in LBs and LNs faster than the wild-type protein. Our results may therefore explain how patients carrying these mutations in the  $\alpha$ -syn gene could develop PD early in life. Deposition of aggregates of certain neuronal proteins to form neurotoxic filaments is a popular concept in neurodegenerative diseases such as AD [31], prion diseases [32] and HD [19]. Our results are in keeping with the emerging theme of conformation-dependent toxicity and extend this theme to include PD and DLB.

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