Studies of the aggregation of an amyloidogenic α-synuclein peptide fragment

J. Madine*, A.J. Doig*, A. Kitmitto† and D.A. Middleton*†

Faculty of Life Sciences, The University of Manchester, P.O. Box 88, Manchester M60 1QD, U.K. and †Faculty of Medical and Human Sciences, The University of Manchester, P.O. Box 88, Manchester M60 1QD, U.K.

Abstract

The deposition of α-syn (α-synuclein) fibrils in Lewy bodies is a characteristic feature of individuals with neurodegenerative disorders. A peptide comprising the central residues 71–82 of α-syn [α-syn(71–82)] is capable of forming β-sheet-rich, amyloid-like fibrils with similar morphologies to fibrils of the full-length protein, providing a useful model of pathogenic α-syn fibrils that is suitable for detailed structural analysis. We have studied the morphology and gross structural features of α-syn(71–82) fibrils formed under different conditions in order to obtain reliable conditions for producing fibrils for further structural investigations. The results indicate that the rate of aggregation and the morphology of the fibrils formed are sensitive to pH and temperature.

Aggregation of α-syn (α-synuclein)

Amyloid fibril formation is thought to be a central event in various neurodegenerative diseases. Many proteins have the ability to produce amyloid-like structures, including the presynaptic protein α-syn, which is found in Lewy bodies [1]. EM (electron microscopy) has been used to show that α-syn fibrils both in brain extracts and formed in vitro are rigid, unbranched, 10 nm wide assemblies characteristic of amyloid fibrils [2]. X-ray diffraction data has shown that the fibrils are composed of β-strands with spacings of 4.7 and 10 nm representing the regular repeats of a cross-β structure [3,4]. Fibrils of α-syn and other amyloidogenic proteins form by a nucleation-dependent mechanism, with an initial lag phase followed by a growth period [5].

The rate of fibrillization of α-syn increases at higher temperatures [6,7], possibly owing to an increase in the lag time required to produce the partially folded intermediate, which nucleates fibril growth. The rate of α-syn aggregation and the morphology of the fibrils formed are also influenced by pH. Hoyer et al. [8,9] observed that amorphous aggregates form at pH 4 and 5, whereas fibrillar aggregates are produced at pH 6 and 7. Other studies suggest that low pH may promote fibril formation by neutralizing negative charges within the C-terminus, which enhances hydrophobic interactions, promoting formation of the partially folded intermediate seen at increasing temperatures [7].

The central hydrophobic region of α-syn is believed to be responsible for the protein aggregation, since the deletion of residues V71TGVTAVAQKTV82 in this region abolishes fibril formation [10]. Furthermore, α-syn(71–82) (peptide comprising the central residues 71–82 of α-syn) forms amyloid fibrils of similar morphology to α-syn, suggesting that this region contributes to the fibrillogenic core of the full-length protein [10,11]. Structural studies on fibrils formed by α-syn(71–82) could provide insight into the aggregation process and architecture of full-length α-syn fibrils. Here, we have studied the ability of α-syn(71–82) to aggregate under different conditions of pH and temperature, in order to determine reliable conditions to produce fibrils for detailed structural analysis.

Measurement of α-syn(71–82) aggregation

A 900 µM aqueous solution of α-syn(71–82) was buffered to the required pH (10 mM phosphate for pH 7 and 10 and 10 mM sodium acetate for pH 4) and incubated with continuous agitation, at 37°C (pH 4, 7 and 10) or at 20°C (pH 7). Samples were taken for analysis immediately after preparation of the solution, when it was expected that the peptide was monomeric, and again after 6 weeks. Insoluble aggregates were removed by centrifugation at 16000 g and the percentage aggregation was analysed after alkaline hydrolysis using the 1’amino-reactive agent ninhydrin to assess the concentration of α-syn(71–82) in the supernatant. The analysis shows that pH 7 is the most effective in achieving aggregation (100% at 20 and 37°C), with pH 10 giving approx. 80% aggregation and pH 4 showing less than 20% aggregation (Figure 1). Temperature did not appear to affect the amount of aggregation, with 37 and 20°C both showing 100% aggregation at pH 7.

Structure and morphology

The structure of α-syn(71–82) within the fibril samples was analysed using CD (Figure 2A) and the morphologies of the fibrils formed were assessed by transmission EM using...
negative staining methods (Figure 2B). CD analysis of the peptide solutions confirmed that α-syn(71–82) is initially unfolded at the outset of incubation. The peptide converts into a new species with higher β-sheet content upon incubation at 37 °C (pH 7) or 37 °C (pH 10) for 6 weeks, consistent with aggregation into amyloid fibrils (Figure 2A). The lower intensity of the 37 °C (pH 7) spectrum may be due to aggregates dropping out of the path of the spectrometer beam. In contrast, the peptide incubated at 37 °C (pH 4) remained unfolded like the monomer, suggesting that the structural rearrangements necessary for amyloid formation do not occur at this pH (Figure 2A, d). The CD spectrum of the peptide sample after incubation at 20 °C (pH 7) was consistent with a structural change in the peptide accompanying aggregation, but the outcome of the structural transition was not clear from the spectrum.

Insoluble aggregates of α-syn(71–82) formed after 6 weeks of incubation were analysed by EM after negative staining of the samples. Examination of the preparation at pH 4 did not find any defined fibrillar structure in contrast with samples at pH 10 and pH 7 and 37 °C. The morphology of the insoluble deposits formed in these preparations was reminiscent of amyloid fibrils, being narrow and elongated and approx. 10 nm in width. The fibrils formed at pH 10 were observed to have lengths ranging from 50 to 300 nm (Figure 2B, i) with those formed at pH 7 (Figure 2B, ii) found to be generally longer with lengths between 300 and 500 nm. The species produced at pH 7 at 20 °C was observed to be generally shorter and less regular than typical amyloid fibrils, with dimensions of approx. 10–100 nm (Figure 2B, iii).

Conclusions
This work has studied the formation, structure and morphologies of α-syn(71–82) peptide samples incubated under different pH and temperature conditions. It is shown that incubation at pH 4 reduces the rate of aggregation and prevents the formation of fibrils for a 6 week period. This is presumably due to neutralization of the C-terminal carboxy group at low pH, since no other group in the peptide has a pKa in the range 4–7. This implies that a bond from the C-terminal carboxy group is crucial for stabilizing amyloid formation. This could be a salt bridge, either to Lys80 or

Figure 1 | Analysis of the extent of aggregation of α-syn(71–82) after 6 weeks incubation under different aggregation conditions, as assessed by the concentration of peptide remaining in solution

Figure 2 | Experiments to examine the structural and morphological changes in α-syn(71–82) after incubation for 6 weeks, under varying conditions of pH and temperature

CD spectra of the peptide samples (A) show a structural rearrangement from an initial unfolded conformation (a) towards structures with β-sheet content upon incubation at 37 °C at pH 7 (b) and pH 10 (c) but remains unfolded at pH 4 (d). Following incubation at 20 °C (pH 7), the CD spectrum of the peptide was consistent with a transition from the initial unfolded conformation (e). (B) Electron micrographs of negatively stained preparations showed morphological differences between the samples incubated at (i) pH 10, 37 °C; (ii) pH 7, 37 °C, and (iii) pH 7, 20 °C. Scale bars, 100 nm.
the positively charged N-terminus of the peptide. Following incubation at pH 7 and 10, the peptide undergoes substantial aggregation into amyloid-like fibrils with similar morphology to those produced by α-syn [3]. A decrease in temperature appears to result in shorter structures than typical amyloid fibrils, accompanied by a transition of the peptide from its monomeric, unfolded conformation into a structure with less β-sheet content. Together these results confirm that this region of α-syn is able to aggregate into amyloid fibrils, and that the rate of aggregation and fibril morphology is sensitive to pH and temperature. The rates of formation and morphology of the fibrils may be related to differences in the structures of the constituent peptide molecules under the various incubation conditions.

This work was supported financially with a studentship and equipment grant from the Alzheimer’s Research Trust.

References


Received 4 July 2005