Pesticides directly accelerate the rate of α-synuclein fibril formation: a possible factor in Parkinson's disease

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Abstract Parkinson's disease involves intracellular deposits of α-synuclein in the form of Lewy bodies and Lewy neurites. The etiology of the disease is unknown, however, several epidemiological studies have implicated environmental factors, especially pesticides. Here we show that several pesticides, including rotenone, dieldrin and paraquat, induce a conformational change in α-synuclein and significantly accelerate the rate of formation of α-synuclein fibrils in vitro. We propose that the relatively hydrophobic pesticides preferentially bind to a partially folded intermediate conformation of α-synuclein, accounting for the observed conformational changes, and leading to association and subsequent fibrillation. These observations suggest one possible underlying molecular basis for Parkinson's disease. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Aggregation; Rotenone; Paraquat; Partially folded intermediate

1. Introduction

Parkinson's disease is the second most common neurodegenerative disease affecting as many as one million Americans, mostly over the age of 60. Recent investigations have shown that Parkinson's disease is a protein deposition disease involving intracellular deposits of α-synuclein in the form of Lewy bodies and Lewy neurites [1]. The etiology of Parkinson's disease is unknown, but recent work has shown that, except in extremely rare cases, there appears to be no direct genetic basis [2]. However, several epidemiological studies have implicated environmental factors, especially pesticides [3–8].

α-Synuclein, a relatively abundant brain protein of 140 amino acids and of unknown function, was first identified in association with synaptic vesicles [9]. α-Synuclein belongs to the class of proteins known as natively unfolded; i.e. the purified protein at neutral pH is substantially disordered [10,11]. Fibrils of α-synuclein have been reported in Lewy bodies from individuals with Lewy body diseases, as well as in vitro [12–17]. We have recently established that the fibrillation of α-synuclein involves a critical partially folded intermediate [11].

Here we show that certain pesticides can significantly stimulate the formation of α-synuclein fibrils. Since these agents also induce a conformational change in α-synuclein, it is likely that this partially folded conformation is a critical precursor to association and fibrillation. These observations suggest an underlying molecular basis for Parkinson's disease and related Lewy body diseases.

2. Materials and methods

2.1. α-Synuclein preparation

A new procedure for producing and purifying α-synuclein was developed, by fusing its gene to a chitin-binding domain (CBD)/intein system (IMPACT: New England Biolabs) and expressing the fusion protein in *Escherichia coli*. This system allowed simple purification of α-synuclein by binding of the CBD fusion protein with a chitin column, followed by addition of a thiol agent (dithiothreitol or cysteine) to induce the cleavage reaction of the intein and release the α-synuclein. The resultant protein, which was highly homogeneous by polyacrylamide gel electrophoresis, was indistinguishable from authentic α-synuclein based on its electrophoretic mobility and molecular mass determined by electrospray mass spectrometry.

2.2. Pesticides

Diethyldithiocarbamate (DDC), dieldrin, paraquat and rotenone were obtained from Sigma and used without further purification. The final concentrations in the incubation solutions were 100 μM.

2.3. Fibril formation

Solutions of 0.5 ml α-synuclein (0.5 mg/ml (35 μM)) at pH 7.5 in 10 mM phosphate buffer were stirred at 37°C in glass vials with micro stir-bars. Fibril formation was monitored with thioflavine T (TFT) fluorescence [18]: aliquots of 10 μl were removed from the incubated sample and added to 1.0 ml of 25 μM TFT. The presence of fibrils was confirmed by electron microscopy (EM) (negative staining with uranyl acetate) and atomic force microscopy.

2.4. Fourier transform infrared (FTIR) spectra

Attenuated total reflectance (ATR) data were collected on a Nicolet 800SX FTIR spectrometer equipped with an MCT detector. The IRE ($72\times10\times6$ mm, 45° germanium trapezoid) was held in a modified SPECAC out-of-compartment ATR apparatus. [19] Typically 1024 interferograms were co-added at 4 cm^{-1} resolution. Data analysis was performed with GRAMS32 (Galactic Industries). Secondary structure content was determined from curve fitting to spectra deconvoluted using second derivatives and Fourier self-deconvolution to identify component band positions. Hydrated thin film samples were prepared by drying 50 μl of 1 mg/ml α-synuclein solution on a ZnSe crystal with dry N_2. The infrared spectra were collected, followed by Fourier transformation using the spectrum of the clean crystal as a background. Water (liquid and vapor) components were subtracted from the protein spectrum.

3. Results

Solutions of recombinant α-synuclein at pH 7.5, 10 mM phosphate buffer, stirred at 37°C, formed fibrils over a period of days to weeks, depending on the protein concentration and

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ionic strength. However, the major determinant of the rate of fibrillation was the degree of agitation, the more vigorous the agitation, the faster the rate of fibril formation. In fact, with very vigorous agitation, the effects of variables such as ionic strength, protein concentration and various additives were greatly attenuated. As observed for other aggregating systems, the kinetics of α-synuclein aggregation exhibit an initial lag, followed by an exponential growth period, followed by a leveling off as fibril formation comes to a halt (Fig. 1). Under the standard assay conditions used (pH 7.5, 37°C, 10 mM phosphate buffer), the lag was >2 weeks for 35 μM α-synuclein. Fibril formation was monitored with TFT fluorescence [18], and the presence of fibrils was confirmed by EM (negative staining with uranyl acetate) and atomic force microscopy.

3.1. Pesticide-stimulated α-synuclein fibril formation

If, as suggested by epidemiological studies, pesticide exposure increases the risk of Parkinson's disease, there are several possible mechanisms that can be envisaged. The simplest of these is that the pesticide itself directly interacts with α-synuclein and leads to accelerated fibrillation. Thus we examined the effect of a number of commonly used pesticides on the kinetics of α-synuclein fibrillation. As shown in Fig. 1, the pesticides, representing different chemical classes, dramatically accelerated the rate of formation of fibrils when incubated with α-synuclein at pH 7.5, 37°C. DDC and dieldrin were the most effective, under the experimental conditions used, both in terms of enhanced kinetics and amounts of fibrils formed. Particularly noteworthy is the observation that rotenone had a substantial accelerating effect on α-synuclein fibrillation, since it has recently been reported that rotenone can induce the major features of Parkinson's disease in rats [20]. The experiments in Fig. 1 used low concentrations of α-synuclein and 100 μM pesticide. Accelerated fibrillation was also observed with higher concentrations of α-synuclein (150 μM) and with 15 μM pesticide (data not shown). Although the concentration of α-synuclein in dopaminergic neurons is not accurately known, it is likely to be at least 200 μM.

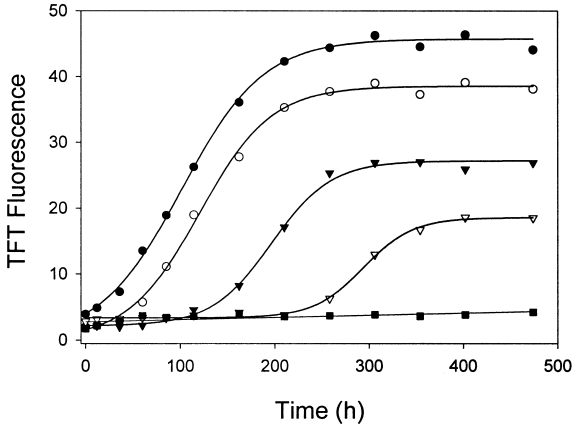


Fig. 1. Kinetics of α-synuclein fibril formation in the presence of pesticides. Solutions of α-synuclein (35 μM) were incubated with stirring at 37°C, in 10 mM phosphate buffer, pH 7.5, in the presence of the indicated pesticides (100 μM) as described in the text. Fibril formation was monitored by the increase in TFT fluorescence. Key: control, ■; DDC, ●; dieldrin, ○; paraquat, ▼; rotenone, ▽. The lag times (h) and rate constants for fibril growth (elongation) (h^{-1}) were as follows: DDC (9.9, 0.023), dieldrin (42.5, 0.026), rotenone (137.2, 0.035), and paraquat (241.2, 0.038).

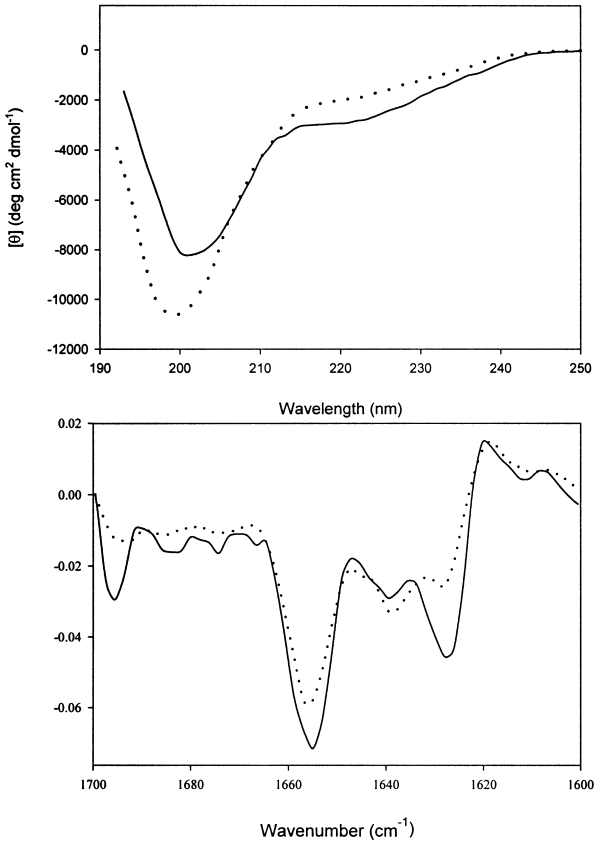


Fig. 2. Pesticide-induced conformational changes in α-synuclein. Top panel: far-UV CD spectra of 35 μM α-synuclein in 10 mM phosphate buffer, pH 7.5; dotted line is the control (no added pesticide), solid line is in the presence of 100 μM DDC. Bottom panel: second derivative ATR FTIR spectra of the amide I region of α-synuclein in the absence (dotted) and presence of 100 μM rotenone after 1 h incubation at 37°C, pH 7.5.

3.2. Pesticide-induced conformational changes in α-synuclein

In order to investigate the underlying molecular mechanism for the accelerated fibril formation we looked for conformational changes induced by the pesticides. As noted, α-synuclein is a natively unfolded protein, meaning that in the purified state at pH 7.5 it exhibits biophysical properties expected for an unfolded protein, rather than a tightly packed, native globular molecule [11]. The presence of the pesticides that increased the rate of fibrillation induced a time-dependent increase in the amount of secondary structure in α-synuclein, as monitored by circular dichroism (CD) (Fig. 2). Comparison of the time-dependent changes in CD and fibril formation revealed that the conformational changes are slow, but precede association of α-synuclein, as monitored by light scattering and fibril formation. At low ionic strength the spectrum was indicative of a substantially unfolded protein. FTIR analysis confirmed that the conformational change involves an increase in β-structure (Fig. 2).

3.3. α-Synuclein aggregation involves a branched pathway leading to both amorphous and fibrillar aggregates

An interesting feature of the data shown in Fig. 1 is that the final TFT signal is different for different pesticides. The most likely explanation is that there are fewer fibrils present in those systems with smaller final TFT signals. This might arise if there were competing pathways for α-synuclein aggregation.

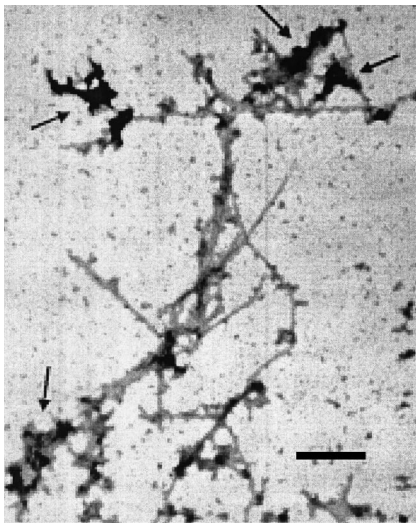


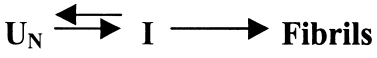
Fig. 3. Aggregation of α-synuclein leads to both fibrils and amorphous (arrows) deposits. Negatively stained (uranyl acetate) electron micrograph.

This was confirmed by electron micrograph images of the aggregated material, which showed both fibrils and amorphous deposits, Fig. 3, and filtration experiments, which showed no soluble α-synuclein present at the end of the reaction.

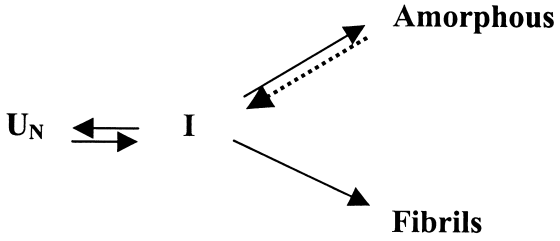
4. Discussion

Our results demonstrate two important points: certain pesticides induce a conformational change in α-synuclein, and these same compounds also accelerate the rate of α-synuclein fibril formation. We propose that the underlying basis of both key observations is that certain pesticides directly interact with α-synuclein to bring about a conformational change to a partially folded state with a high propensity to aggregate. We have recently shown that the natively unfolded state of α-synuclein arises from the large net negative charge at neutral pH and the low intrinsic hydrophobicity, and that factors that decrease the net charge or increase the relative hydrophobicity lead to population of a partially folded intermediate, which correlates with fibrillation [11,21]. We believe that relatively non-polar pesticides may preferentially bind to the partially folded intermediate conformation (I, in Scheme 1) and hence shift the equilibrium from the natively unfolded state (U_N) to the intermediate [11].

The intermediate then associates, leading eventually to fibrils. Precedence for such a ligand-binding-induced shift in equilibrium from an unfolded state to favor a partially folded state has previously been reported [21–23]. The driving force appears to be the preferential affinity of the intermediate for the ligand due to the greater exposure of contiguous hydrophobic regions on the surface of the intermediate relative to the natively unfolded state. In the intermediate, interactions of hydrophobic side-chains result in large hydrophobic patches,



Scheme 1.



Scheme 2.

increasing the potential affinity of the protein for non-polar pesticides, as well as increasing self-association. This self-association leads to formation of the critical nucleus on the pathway to fibrils. The pesticides that stimulated α-synuclein fibril formation have in common the properties that they are both relatively hydrophobic and relatively water-soluble. Based on our observation that 8-anilinonaphthalene-1-sulfonic acid also stimulates the aggregation of α-synuclein (data not shown), we predict that many compounds that are relatively hydrophobic and water-soluble may induce α-synuclein fibril formation.

Interestingly, α-synuclein appears to aggregate not only as ordered fibrils, but also as amorphous deposits. We attribute the decreased TFT signal in the presence of some of the pesticides to competition between fibrillation and amorphous aggregation, as evidenced by the presence of amorphous deposits in EM images. This means that there is an underlying branched pathway (Scheme 2) in which the partially folded intermediate partitions between pathways to either amorphous or fibrillar aggregates. Scheme 2 is undoubtedly an oversimplification: it is possible that there are two forms of the intermediate present, one leading to amorphous deposits, the other to fibrils. Individual pesticides interact selectively with the intermediate species on the respective pathways, leading to relatively more or less fibrils.

The results suggest that a variety of environmental factors can accelerate α-synuclein aggregation by increasing the concentration of the critical partially folded intermediate conformation that leads to aggregation. For pesticides the effect is due to their preferential binding to the partially folded intermediate conformation. This is an alternative mechanism to that proposed by Betarbet et al. [20], who suggest that a rotenone-induced defect in mitochondrial complex I is responsible for the observed nigrostriatal dopaminergic degeneration in rotenone-treated rats. Our observations of a direct stimulation by pesticides on α-synuclein fibrillation are very relevant to Parkinson's disease because they suggest that in the presence of pesticides, herbicides or other small soluble hydrophobic molecules (including some products of oxidative stress), the concentrations of α-synuclein required to cause rapid formation of fibrils are significantly reduced. The presence of pesticides in the brain has been demonstrated [24,25]. It remains to be determined how quickly α-synuclein fibrils will form with the concentrations of α-synuclein and pesticides found in neurons. The concentration of both α-synuclein and pesticide in the dopaminergic neurons is unknown at present.

Thus direct interactions between α-synuclein and environmental agents could play a role in the pathogenesis of nigrostriatal degeneration and thus in the etiology of sporadic Parkinson's disease. The critical cytotoxic agent remains

unknown, and could be an intermediate on the aggregation pathway, the amorphous or fibril deposits, or an indirect effect, such as disruption of proteasome function due to the aggregated α-synuclein.

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