Accelerated α-synuclein fibrillation in crowded milieu

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Abstract Parkinson's disease is the second most common age-related neurodegenerative disease, resulting from loss of dopaminergic neurons in the substantia nigra. The aggregation and fibrillation of α-synuclein has been implicated as a causative factor in the disease, and the process of fibril formation has been intensively studied in vitro with dilute protein solutions. However, the intracellular environment of proteins is crowded with other macromolecules, whose concentration can reach 400 g/l. To address this discrepancy, the effect of molecular crowding on α-synuclein fibrillation has being studied. The addition of high concentrations of different polymers (proteins, polysaccharides and polyethylene glycols) dramatically accelerated α-synuclein fibrillation in vitro. The magnitude of the accelerating effect depended on the nature of the polymer, its length and concentration. Our results suggest that the major factor responsible for the accelerated fibrillation under crowded conditions is the excluded volume. **© 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: α-Synuclein; Fibril; Natively unfolded; Molecular crowding; Parkinson's disease

1. Introduction

Proteins have evolved to function within cells, where the concentration of macromolecules, including proteins, nucleic acids, and carbohydrates, and small solutes can be as high as 400 g/l [1]. These intracellular solutes can occupy about half of the total cellular volume [1–4]. Such media are referred to as 'crowded' rather than 'concentrated', as, in general, no individual macromolecular species is present at high concentration, and a special term, 'molecular crowding', has been introduced to describe the effect of high solute concentrations on chemical reactions [5,6].

Obviously, the volume occupied by solutes is unavailable to other molecules because two molecules cannot be in the same place at the same time. The thermodynamic consequences of this unavailable volume are called excluded volume effects [1,7]. Volume exclusion in biological fluids may have large effects on both stability of biological macromolecules [8–11] and macromolecular equilibria, including protein–protein interactions [6,12]. Moreover, molecular crowding may lead to significant alterations of the rates of chemical reactions, protein folding and macromolecular association [1,4–7,13]. Finally, it has been suggested that volume exclusion in physiological media could modulate the rate and extent of amyloid formation in vivo [6,14]. The validity of this hypothesis has been confirmed recently for the in vitro fibrillation of human apolipoprotein C-II [15].

α-Synuclein is a small (14 kDa), highly conserved presynaptic protein that is abundant in various regions of the brain [16–18]. Structurally, purified α-synuclein belongs to the family of natively unfolded proteins [19–21], which are characterized by a unique combination of low overall hydrophobicity and large net charge [22]. Deposition of aggregated forms of α-synuclein in neuronal or glial cytoplasm is a pathological hallmark of several neurodegenerative diseases, including Parkinson's disease, dementia with Lewy bodies, Lewy body variant of Alzheimer's disease, and multiple system atrophy [23,24]. In vitro, the protein forms fibrils with morphologies and staining characteristics similar to those extracted from disease-affected brain [25–30]. The kinetics of fibrillation are consistent with a nucleation-dependent mechanism [26,31], in which the critical early stage of the structural transformation involves a partially folded intermediate [20]. All these experiments have been performed under relatively idealized conditions, low protein and moderate salt concentrations. However, as mentioned, the inside of the cell is a very crowded environment, and is poorly modeled by dilute solutions. In the present study we analyzed the potential role of molecular crowding in the fibrillation of human α-synuclein.

2. Materials and methods

2.1. Expression and purification of recombinant α-synuclein

The expression (in *Escherichia coli*) and purification of α-synuclein were as described previously [20].

2.2. Materials

Thioflavin T (TFT), dextran (molecular mass of 138 000 Da), polyethylene glycols (with molecular masses of 200, 400, 600 and 3350 Da), Ficolls (70 000 and 400 000) were obtained from Sigma, St. Louis, MO, USA. All other chemicals were of analytical grade from Fisher Chemicals.

2.3. Fibril formation assay

Assay solutions contained 15 μM TFT and α-synuclein at a concentration of 70 μM (1.0 mg/ml) in 20 mM Tris–HCl, 0.1 M NaCl, pH 7.5, and the desired concentrations of the crowding agent. A volume of 100 μl of the mixture was pipetted into a well of a 96-well plate (white plastic, clear bottom) and a 1/8″ diameter Teflon sphere (McMaster-Carr, Los Angeles, CA, USA) was added. Each sample was run in triplicate or quadruplicate. The plate was loaded into a fluorescence plate reader (Fluoroskan Ascent) and incubated at 37°C with shaking at 300–600 rpm with a shaking diameter of 1 mm.

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The fluorescence was measured at 30-min intervals with excitation at 450 nm and emission at 485 nm, and curve-fit as described in [32].

3. Results

3.1. High concentrations of crowding proteins affect α-synuclein fibrillation

Molecular crowding effects may be examined experimentally in vitro by using concentrated solutions of a model 'crowding agent' such as polyethylene glycol, dextran, Ficoll and proteins [7,15]. We have studied fibrillation of human recombinant α-synuclein in model crowded environments using changes in TFT fluorescence. TFT is a fluorescent dye that interacts with amyloid fibrils leading to an increase in fluorescence intensity in the vicinity of 480 nm [33,34]. Fig. 1 shows that high concentrations of inert proteins such as lysozyme (50 mg/ml or 3.5 mM) and bovine serum albumin (BSA; 60 mg/ml or 0.9 mM) dramatically accelerated the process of α-synuclein fibrillation. Incubation of lysozyme or serum albumin alone at high concentrations did not lead to any increase in TFT signal (data not shown).

Interestingly, under the experimental conditions used, BSA showed a larger acceleration effect than lysozyme. We considered the differences in the biophysical characteristics of these proteins as a possible explanation. Lysozyme is a small globular protein with molecular mass 14.3 kDa and p*I* 9.32, whereas BSA has molecular mass of 66.4 kDa and p*I* 5.6. Since both these proteins, with quite different net charges (+8 and −17 for lysozyme and BSA, respectively), both accelerated α-synuclein fibrillation, the effect of specific electrostatic interactions between α-synuclein and the crowding proteins can be eliminated. The stronger effect of BSA may be

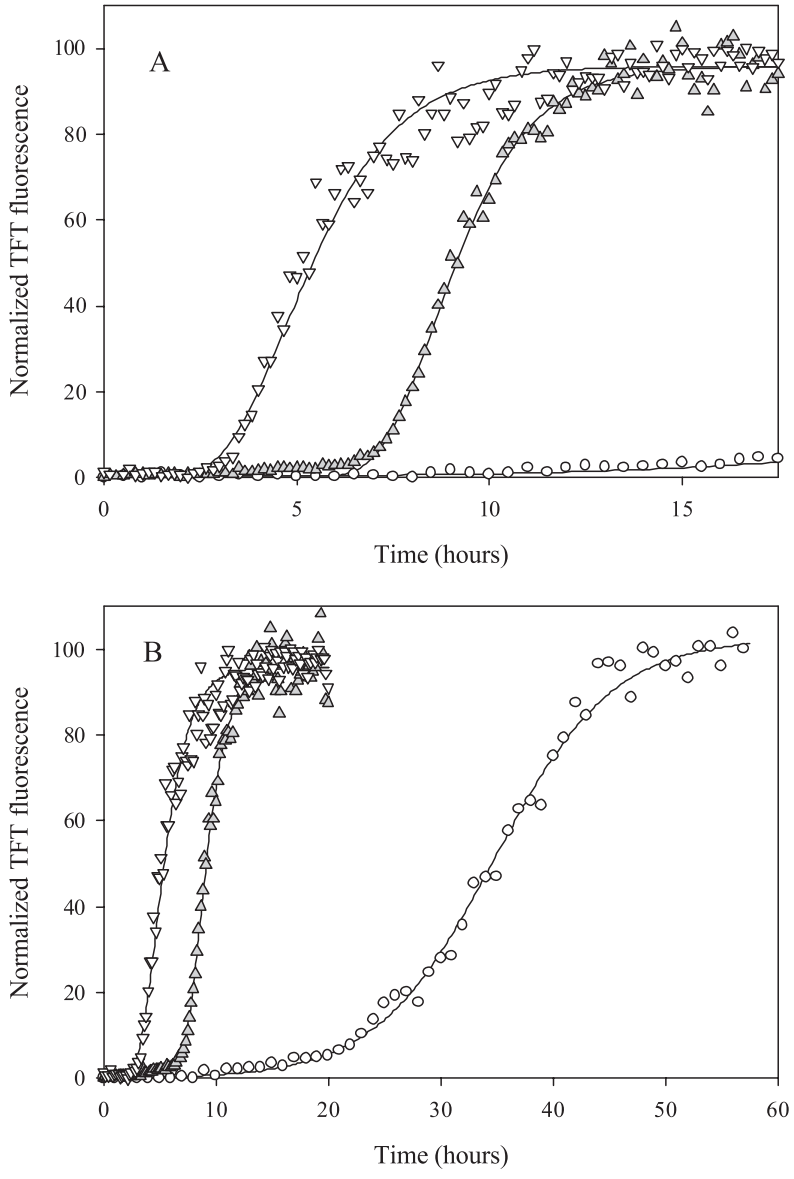

Fig. 1. Effect of high concentrations of lysozyme (50 mg/ml; triangles) and BSA (60 mg/ml; reversed open triangles) on fibrillation of human recombinant α-synuclein. A: Limited time range; B: extended time range. The time course of TFT changes for α-synuclein alone (the control) is shown for comparison (open circles). The symbols represent TFT fluorescence intensities determined experimentally, and the lines are results of data fitting according to [32]. α-Synuclein concentration was 1 mg/ml (70 μM) throughout these experiments. Conditions were 25 mM sodium phosphate buffer, pH 7.5, 100 mM NaCl, 37°C.

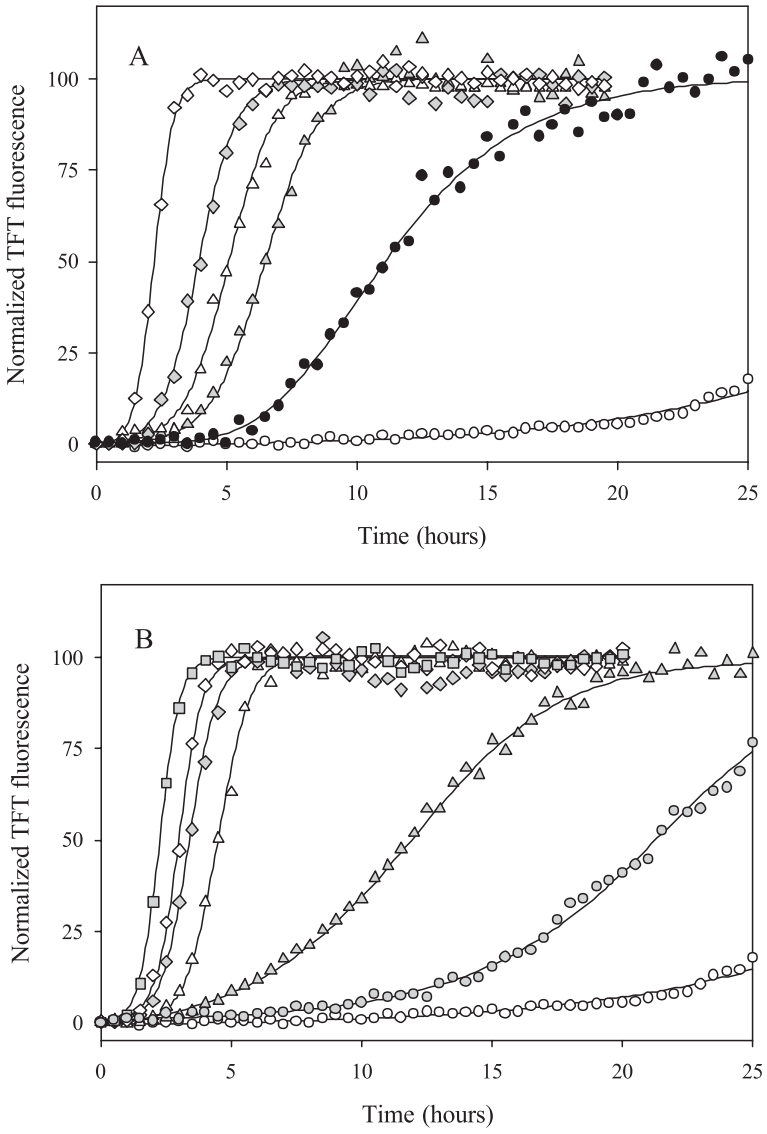

Fig. 2. Effect of polyethylene glycols on fibrillation of human recombinant α-synuclein. A: Fibrillation of α-synuclein in the presence of 150 mg/ml of PEG-200 (gray triangles), PEG-400 (open triangles), PEG-600 (gray diamonds) and PEG-3350 (open diamonds). Data for 150 mg/ml ethylene glycol (molecular mass of 62.1 Da) are shown for comparison (black circles). The control, α-synuclein alone, is shown by the open circles. B: Fibrillation of α-synuclein in the presence of different concentrations of PEG 3350: 25 mg/ml (gray circles), 50 mg/ml (gray triangles), 75 mg/ml (open triangles), 100 mg/ml (gray diamonds), 125 mg/ml (open diamonds) and 150 mg/ml (gray squares). Other conditions were the same as in Fig. 1.

due to the difference in their excluded volumes; BSA and lysozyme have Stokes radii, R_S, of 33.9 and 19.0 Å, respectively [35], which corresponds to a 5.7-fold difference in their molecular hydrodynamic volumes. Thus, the total excluded volume of BSA in our experiments is ~1.5-fold larger than that of lysozyme, as the molar concentration of latter is 3.9-times higher than that of former. Thus, the effective concentration of α-synuclein was ~1.5-times higher in the solution of BSA than in the presence of lysozyme. We attribute the increased rate of fibrillation with BSA due to this excluded volume effect, since we have shown that the rate of fibrillation is concentration dependent [36].

3.2. *Polyethylene glycols accelerate α-synuclein fibrillation in a concentration- and chain length manner*

Fig. 2A shows that α-synuclein fibrillation was dramatically accelerated in the presence of high concentrations of polyethylene glycols (150 mg/ml). The data indicate that the polyethylene glycol length plays a crucial role in the acceleration of fibril formation by α-synuclein, with longer polymers inducing faster α-synuclein fibrillation. We assume that this is due to the fact that longer polymers will have larger excluded volumes, giving rise to higher values for the effective α-synuclein concentration in solution. In these experiments the viscosity increased linearly with the increase in PEG length from 1.536 ± 0.004 to 4.659 ± 0.008 cP for 150 mg/ml solutions of ethylene glycol and PEG-3350, respectively. Such an increase, although relatively small, may affect the rates of macromolecular diffusion and consequently affect the rates of fibril formation. Thus, the increased viscosity in the presence of polyethylene glycols with longer chain length could slow down the α-synuclein fibrillation. However, we have established that

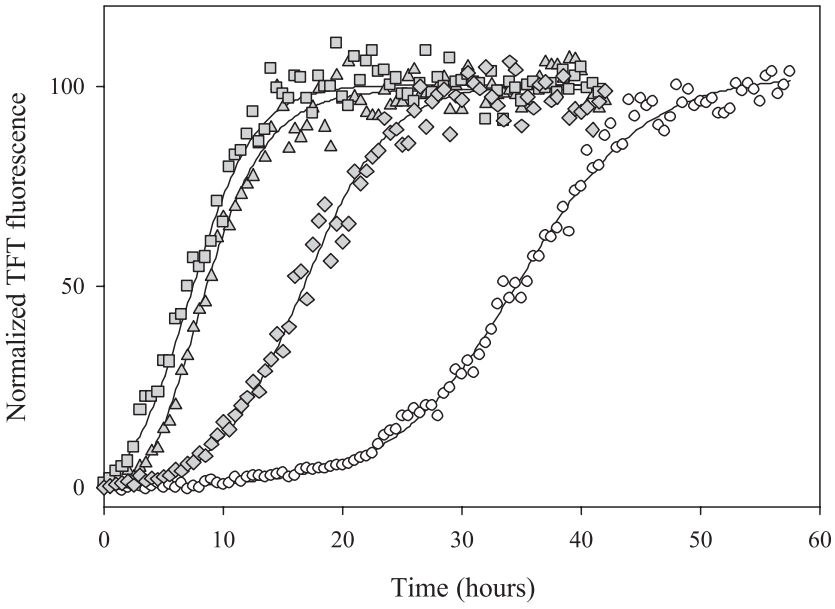

Fig. 3. Effect of high concentrations of polysaccharides on fibrillation of human recombinant α-synuclein. Symbols correspond to fibrillation of α-synuclein alone (open circles), or in the presence of 150 mg/ml of dextran-138000 (gray diamonds), Ficoll-70000 (gray triangles) or Ficoll-400000 (gray squares). The symbols represent TFT fluorescence intensities determined experimentally, and the lines are results of data fitting according to [32]. Other conditions were the same as in Fig. 1.

40% glycerol, which has a similar viscosity to 150 mg/ml PEG-3350, leads to six-fold slower kinetics of α-synuclein fibrillation compared to PEG-3350. Thus, viscosity is not the primary limitation, and we conclude that excluded volume is the critical factor in accelerating α-synuclein fibrillation.

Fig. 2B depicts the effect of increasing the PEG-3350 concentration on the rate of α-synuclein fibrillation. The process of fibril formation was significantly accelerated by the presence of 25 mg/ml of PEG-3350, and subsequent increases in the polyglycol concentration lead to further increases in the rate of α-synuclein fibrillation. Interestingly, concentrations of PEG-3350 above 75 mg/ml showed relatively similar accelerating effects on α-synuclein fibril formation, possibly due to compensating effects of the increased viscosity, which will work in the direction opposite to that of the excluded volume (see above) and decrease the acceleration due to the excluded volume effect. In other words, without the offsetting viscosity effect the rates of fibrillation would continue to increase with increasing concentrations of PEG above 75 mg/ml.

3.3. Polysaccharides facilitate fibril formation of α-synuclein

Fig. 3 illustrates the effect of different polysaccharides, such as dextran-138000 (gray diamonds), Ficoll-70000 (gray triangles) or Ficoll-400000 (gray squares), on the rate of α-synuclein fibrillation in vitro. The rate of fibril formation increases significantly with 150 mg/ml neutral sugar polymers. Several important observations can be made based on these data. First of all, high concentrations of polysaccharides show comparable effects to those of ethylene glycol (the monomer), and smaller effects than PEGs (cf. Figs. 2 and 3). Although the Ficolls are very different in their molecular masses, they show relatively similar accelerating effects, with Ficoll-400000 being a slightly better accelerator than Ficoll-70000. Among the polysaccharides, dextran-138000 had the smallest accelerating effect on α-synuclein fibrillation. These last two observations may be due to the relatively high viscosities of the polysaccharide solutions; the viscosities were 4.471 ± 0.008, 11.171 ± 0.012 and 20.276 ± 0.018 cp for Ficolls 70000, 400000 and dextran, respectively.

4. Discussion

Our results demonstrate that molecular crowding causes substantial acceleration of human α-synuclein fibrillation in vitro. Interestingly, the stimulation was observed in the presence of high concentrations of both charged and neutral polymers, namely proteins, polysaccharides and polyethylene glycols. The magnitude of the accelerating effect depended on the nature of the polymer, its length and concentration. In particular, increasing polymer concentration or polymer size decreased the length of the initial lag in fibrillation, and increased the rate of fibril growth. The kinetics of protein aggregation, involving an initial lag followed by exponential growth of aggregates, are usually ascribed to nucleated polymerization, in which the lag corresponds to the formation of a critical nucleus. It is assumed that formation of the nucleus is a rare event involving the simultaneous assembly of several monomeric intermediate species. Once formed, however, the nucleus rapidly transforms to fibrils which grow exponentially through elongation.

Our data are most consistent with the major effect on the acceleration of fibrillation being due to excluded volume effects, which will favor self-association of α-synuclein due to the effective increased protein concentration. We have previously shown that increasing the concentration of α-synuclein leads to increased rates of fibrillation [20]. The decreased water activity in the polymer solutions is also likely to make a contribution to the acceleration, due to decreasing the solubility of the protein, and thus also favoring self-association. In contrast, these accelerating effects are offset to a limited extent by the increased viscosity of the polymer solutions at high concentration, which will decrease the rate of formation

of the fibril nucleus and the rate of fibril growth due to the decreased diffusion rates. The major effect of the high concentrations of polymers was to decrease the length of the lag, corresponding to formation of the critical nucleus. This effect is consistent with an excluded volume effect and not the increase in viscosity.

As discussed, a high concentration of macromolecules is a characteristic feature of the living cell, and most physiological fluids [1–4]. Our results confirm that volume exclusion effects are general phenomena, and that it is important to take them into account. The modulating role of excluded volume effects in the rate and extent of amyloid formation in vivo has been previously suggested [6,14]. The validity of this hypothesis has been confirmed recently for the in vitro fibrillation of human apolipoprotein C-II [15], and now, in this paper, for fibril formation by human α-synuclein.

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