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Chaperone-like activity of synucleins

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Abstract Synucleins are a family of small proteins that are predominantly expressed in neurons. The functions of the synucleins are not entirely understood, but they have been implicated in the pathogenesis of several neurodegenerative diseases. Our data show that α-, β- or γ-synuclein suppresses the aggregation of thermally denatured alcohol dehydrogenase and chemically denatured insulin. The A53T but not the A30P mutant α-synuclein was able to inhibit the aggregation of insulin and the chaperone-like activity of α-synuclein was lost upon removal of its C-terminal residues 98–140. These results demonstrate that synucleins with the exception of the A30P mutant possess chaperone-like activity.

Key words: Parkinson's disease; Lewy body; Neurodegenerative disorder

1. Introduction

The human synuclein family includes four homologous proteins, α-, β- and γ-synuclein and synoretin [1–5]. Synucleins are highly soluble proteins with unknown function and different expression profiles. The α- and β-synucleins are predominantly pre-synaptic proteins expressed in neurons of the central nervous system [1–5]. γ-Synuclein is diffusely distributed in the cytoplasm of peripheral neurons, although it is also expressed in the brain [4], while synoretin, which is also found in the brain, is expressed at high levels in the retina [5]. Mutations in the α-synuclein gene (A53T and A30P) have been linked to familial Parkinson's disease (PD)[6–8]. More importantly, wild type α-synuclein is the major component of Lewy bodies (LBs) in sporadic PD, dementia with LBs (DLB) and in a subtype of Alzheimer's disease known as the LB variant of Alzheimer's disease [9–11]. Wild type α-synuclein is also a major component of neuronal and glial cytoplasmic inclusions in multiple system atrophy [9–11] and the α-synuclein peptide (residues 61–95) known as the non-amyloid component of plaques has been implicated in amyloidogenesis in Alzheimer's disease [12,13]. Moreover, antibodies against β-synuclein revealed novel staining of degenerating mossy fiber terminals in the dentate gyrus, the hilar and CA2/3 regions of the hippocampus while antibodies against γ-synuclein stain axonal spheroid-like lesions in the hippocampal region in PD and DLB patients [14]. These new findings suggest that not only α-synuclein but also β- and γ-synuclein may be involved in the pathogenic mechanism responsible for the onset and progression of PD and DLB.

α-Synuclein, the most extensively studied synuclein, is a small acidic protein consisting of 140 amino acids [1]. The circular dichroism spectra of wild type α-synuclein is a typical spectrum of type U (main feature a negative band at 199 nm), which in water is characteristic of an unordered structure in equilibrium with reverse turns [15–17]. α-Synuclein protein contains as little as 3% α-helices and 23% β-sheets and polymerizes to fibrils in vitro [15,16,18]. The formation of polymers is accompanied by an increase in anti-parallel β-sheet conformation and is accelerated by the two known mutations [15,16,18]. Wild type α-synuclein binds to small (hydrodynamic diameter of 20–30 nm) unilamellar vesicles in vitro [17]. The binding of α-synuclein to vesicles is associated with a significant increase in α-helical conformation [17]. The A30P mutant has reduced affinity for vesicles [19]. In addition α-synuclein has been shown to associate with a number of proteins. The association with phospholipase D2 inhibits phospholipase D2 function [20] whereas the association of the α-synuclein with synphilin-1 promotes the formation of cytoplasmic inclusions [21]. The N-terminal portion of α-synuclein (residue 1–61) shares 40% amino acid homology with the family of 14-3-3 proteins [22]. 14-3-3 proteins, like synucleins, are ubiquitously expressed in the brain and have been shown to associate in a chaperone-like manner with PKC, BAD, ERK and Raf-1 [22,23]. α-Synuclein binds to 14-3-3 as well as to PKC, BAD, ERK and the microtubule associated protein tau, but not Raf-1 [22,24]. The interaction of α-synuclein with proteins has led us to hypothesize that synucleins may be functioning as molecular chaperones. This possibility was evaluated in two well-established assays, the thermal-induced aggregation of alcohol dehydrogenase (ADH) and the chemical-induced aggregation of insulin.

2. Materials and methods

2.1. Purification of synuclein proteins

Human α-, β- and γ-synuclein and the A30P and A53T α-synuclein mutant were subcloned into the bacterial expression vector pRK172 and were expressed in *Escherichia coli* BL21 and purified as described previously [18]. The protein concentration was determined using the bicinchoninic acid protein assay (Pierce) using bovine serum albumin as a standard.

2.2. Chaperone-like activity assays

Recombinant human heat shock protein (HSP) 27 was from Stressgen (Victoria, B.C., Canada). Bovine insulin, horse liver ADH and

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bovine pancreatic ribonuclease A (RNAse A) were from Sigma, St. Louis, MO, USA. The following molecular masses for insulin, ADH, α-synuclein, β-synuclein and γ-synuclein, HSP27 and bovine RNAse A were utilized to determine the molar concentration of proteins: 5.7, 80, 14.4, 14.3, 13.3, 22.3 and 13.7 kDa, respectively. The thermal-induced aggregation of horse liver ADH was performed as described previously [25]. ADH, 0.5 mg/ml (6.2 μM), was dissolved in 50 mM phosphate buffer, pH 7.0, and the protein was heated at 45°C. The thermally denatured ADH in the absence of a chaperone aggregates over time. The aggregation of ADH is monitored by the increase in light scattering at 360 nm in a microplate spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA, USA). The effect of synucleins was determined by the addition of different amounts of synucleins.

Bovine insulin dissolved in 20 mM NaOH was diluted in 100 mM phosphate buffer, pH 7.0, to a final concentration of 0.15 mg/ml (26 μM). Insulin was reduced as described previously [26] by the addition of 20 mM DTT. The extent of aggregation of the insulin B chain after reduction was determined as a function of time at 25°C by monitoring the scattering at 360 nm. Each concentration of synucleins was repeated in triplicate for both chaperone activity assays.

2.3. Preparation of truncated α-synuclein

α-Synuclein (5 mg/ml) was digested with 5 μg/ml endoproteinase Asp-N from *Pseudomonas fragi* (Calbiochem, CA, USA) in 50 mM potassium phosphate buffer, pH 7.0, overnight at 37°C. The reaction was stopped by the addition of 0.1 mg/ml of leupeptin. The protein was then separated on a 16.5% sodium dodecyl sulfate (SDS)–polyacrylamide gel and visualized by Coomassie blue R-250.

3. Results and discussion

The chaperone-like activity of synucleins was determined by their ability to prevent the thermally-induced aggregation of ADH at 45°C and the chemically-induced aggregation of insulin at 25°C. These two assays have been extensively utilized to determine the chaperone-like activities of proteins [25–29]. Heating of ADH at 45°C denatures the protein and induces aggregation, which is followed over time at 360 nm (Fig. 1). The addition of purified human α-synuclein to the denatured protein solution reduced the aggregation of ADH (Fig. 1A). A three-fold molar excess of α-synuclein was required for a 50% reduction in the aggregation of ADH whereas a six-fold molar excess of α-synuclein resulted in more than 70% reduction in aggregation (Fig. 1A). Similar to α-synuclein, β- and γ-synucleins are equally effective in abrogating this aggregation (not shown). Under the same conditions, human heat shock protein HSP27 with a known chaperone-like activity was capable of reducing the aggregation of ADH (Fig. 1B). In contrast bovine RNAse A, which is similar in size to the synucleins and does not possess chaperone-like activity, was not able to reduce the aggregation of ADH (Fig. 1C).

Reduction of the disulfide bonds between the A and B chains of insulin with DTT leads to the aggregation of B chains. In the absence of a chaperone, the aggregation of the denatured B chains is monitored over time by the increase in light scattering at 360 nm (Fig. 2). The addition of purified human α-synuclein resulted in a reduction of insulin aggregation (Fig. 2A). A two-fold molar excess of synucleins was required to suppress the aggregation of insulin by more than 50% (Fig. 2A). Both β- and γ-synucleins were capable of preventing the aggregation of insulin (not shown). Two point mutations of α-synuclein linked to familial PD ($Ala^{30} \rightarrow Pro$ and $Ala^{53} \rightarrow Thr$) were also assayed for their ability to suppress the DTT-induced aggregation of insulin. The A53T mutant α-synuclein was capable of preventing the aggregation of insulin similar to the wild type α-synuclein

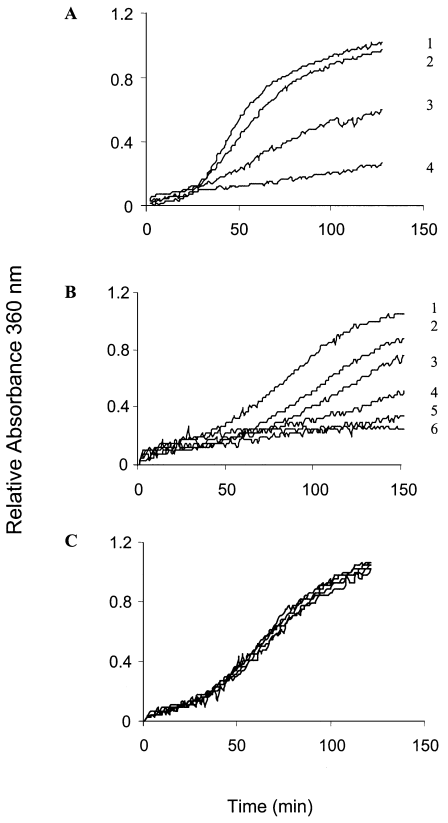

Fig. 1. Chaperone-like activity of α-synuclein assessed by the ADH assay. A: The reduction in the aggregation of ADH (6.2 μM) in 50 mM phosphate buffer, pH 7.0, at 45°C is shown as a function of time in the absence or presence of different concentrations of α-synuclein. Lines 1–4: 0, 3.5, 17.5 and 35 μM α-synuclein, respectively. B: Thermal aggregation of ADH (6.2 μM) in the presence of HSP27. Lines 1–6: 0, 0.024, 0.060, 0.120, 0.24 and 0.72 μM HSP27, respectively. C: Thermal aggregation of ADH (6.2 μM) in the presence of 0, 3.5, 7.0, 27.6 and 55 μM RNAse. Each concentration of protein was repeated in triplicate and a representative tracing is shown.

(Fig. 2B). In contrast, the A30P mutant α-synuclein did not suppress the aggregation of DTT-treated insulin B chains (Fig. 2B). The same results were obtained using the ADH thermal-induced aggregation assay (data not shown). The A30P mutant protein has been found incapable of binding vesicles [19] whereas the A53T mutant protein was found to form α-synuclein polymers faster than wild type and A30P mutant proteins [15,17,18] and showed an increased interaction with synphilin-1 [21]. The data herein indicate that the A30P mutant is also incapable of chaperone-like association with denatured proteins and suggests that the A30P mutant may promote the formation of protein polymers by association with aggregation-prone proteins. HSP27 but not RNAase A were able to reduce the aggregation of insulin (Fig. 2C–D).

A unique feature of chaperone proteins is a flexible hydrophilic tail region in the C-terminal. Nuclear magnetic resonance spectroscopy has revealed that proteins with chaperone-like activities, such as α-crystallin and HSP25, have unstructured flexible solvent-exposed C-terminal extensions [27,28]. The polar and flexible C-terminal tail is thought to promote the interaction of the chaperone with the hydrophobic region in the denatured protein and thus play a critical role in substrate–chaperone association [27,28]. The mutation

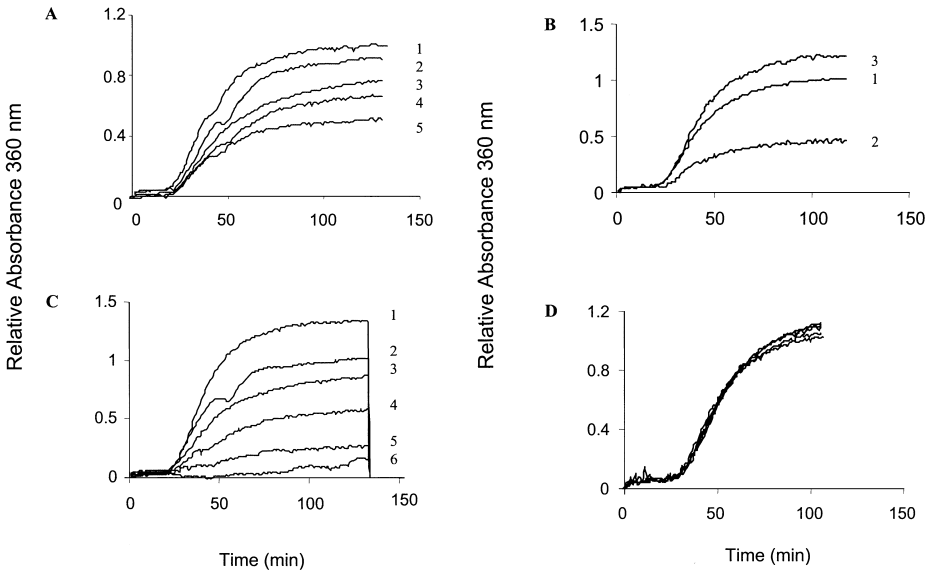

Fig. 2. Chaperone-like activity of synucleins assessed by the insulin assay. A: The reduction in the aggregation of insulin (26 μM) B chains in 0.1 M phosphate buffer, pH 7.0, at 25°C is shown as a function of time in the absence or presence of different concentrations of synucleins. One representative tracing from three independent experiments is depicted. Lines 1–5: 0, 3.5, 8.6, 17.5 and 35 μM α-synuclein, respectively. B: Aggregation of insulin (26 μM) B chains induced by 20 mM DTT was followed in the absence (line 1) or in the presence of 52 μM of A53T α-synuclein (line 2) and 52 μM of A30P α-synuclein (line 3). C: Aggregation of insulin (26 μM) B chains in the presence of HSP27. Lines 1–6: 0, 0.024, 0.060, 0.120, 0.24 and 0.72 μM HSP27, respectively. D: Aggregation of insulin (26 μM) B chains in the presence of 0, 3.5, 7.0, 27.6 and 55 μM RNAse.

or deletion of 17 residues from the C-terminal of α-crystallin caused a marked reduction in the chaperone-like activity of this protein [27]. The C-terminal of the synuclein proteins is rich in aspartic and glutamic acid residues similar to proteins with chaperone-like activity such as tubulin and the mitochondrial chaperonin CNP60 protein. The flexible negatively-charged C-terminal of tubulin was recently found to be critical for the ability of this protein to interact with other proteins in a chaperone-like fashion [29]. To test if the C-terminal of α-synuclein is required for the chaperone-like activity, α-synuclein was digested with endoproteinase Asp-N. Endoproteinase Asp-N has been shown to cleave the aspartic residues in α-synuclein [30] leading to a truncated protein corresponding to residues 2–97, with a predicted molecular mass of 9.5 kDa (Fig. 3A). The data in Fig. 3B indicate that truncated α-synuclein (2–97) does not possess a chaperone-like activity suggesting that the C-terminal of α-synuclein plays a role in conferring this activity. The highly-charged C-terminal of α-synuclein may be important for maintaining its solubility since α-synuclein with a truncated C-terminal (i.e. deletion of residues 110–140 or 120–140) was found to aggregate faster than full-length α-synuclein under the same conditions [31]. Thus, it is possible that the truncated (2–97) α-synuclein interacts with the hydrophobic exposed region of insulin or ADH but the complexes may not be stable or water soluble as the C-terminal of α-synuclein may be required in order to maintain the solubility of the protein and, potentially, the protein complexes.

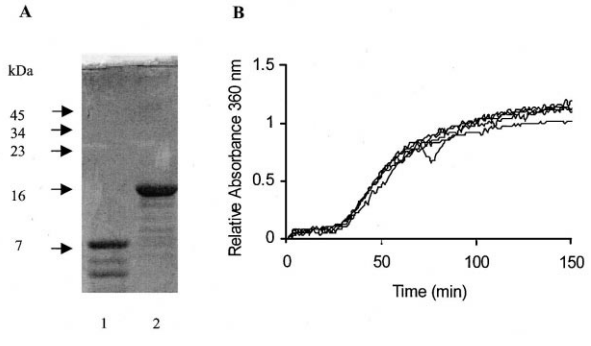

Fig. 3. Deletion of the C-terminal of α-synuclein abolishes its chaperone activity. A: SDS–PAGE stained with Coomassie blue R-250 of human α-synuclein digested with Asp-N (1) and untreated α-synuclein (2). B: The aggregation of insulin (26 μM) was monitored at 360 nm in the absence and presence of different concentrations of Asp-N cleaved α-synuclein 0, 3.5, 17.5, 27.6 and 52 μM.

Both α-synuclein and a central hydrophobic peptide thereof, termed NAC, have been shown to aggregate alone and with other amyloidogenic proteins to form fibrils. The NAC peptide, although natively unfolded, is predicted to form a β-sheet structure that promotes self-aggregation, binding to amyloid and the formation of amyloid fibrils [17,32]. The interaction of the NAC region of α-synuclein with synphilin-1 appears to facilitate the formation of cellular inclusions [21]. Moreover, overexpression of α-synuclein in dividing cells may increase their susceptibility to environmental aversion, which may be due to its interaction with BAD, ERKs or other proteins involved in signal transduction [22]. Therefore the interaction of the NAC peptide or even of full-length α-synuclein with some 'inappropriate' protein partner as a result of overexpression may be detrimental to cell viability. However, protein–protein interaction of α-synuclein requiring the C-terminal may be beneficial since this association produces stable water-soluble complexes with denatured proteins. Although the data herein support a chaperone-like function for the synucleins it remains unknown if the synucleins retain this activity in vivo.

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