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Alpha-synuclein and familial variants affect the chain order and the thermotropic phase behavior of anionic lipid vesicles

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ABSTRACT

Alpha-synuclein (aSN) is a presynaptic protein with a pathological role in Parkinson's disease (PD). The mutants A30P, E46K and A53T are involved in PD early-onset forms. aSN is natively unfolded but can self-assemble to oligomers and fibrils and binds anionic membranes in a helical conformation. We study the influence of wild-type (wt) aSN and familial variants on the chain order and thermotropic phase behavior of anionic dimyristoylphosphatidylglycerol (DMPG) bilayers by using electron spin resonance and calorimetry, respectively. The alpha-helical conformation of the proteins in the membrane-bound state is assessed by circular dichroism thermal scans. wt and mutated aSN upon binding to fluid DMPG vesicles progressively increase chain order. Lipid:protein molar binding stoichiometries correspond to 50 for A30P, 35–36 for aSN and A53T, 30 for E46K. The temperature range over which the variants assume the α-helical fold correlates directly with the density of proteins on vesicle surfaces. All variants preserve the characteristic chain flexibility gradient and impart motional restriction in the lipid chain. This is evident at the first CH_2 segments and is markedly reduced at the chain termini, disappearing completely for A30P. The proteins slightly reduce DMPG main transition temperature, revealing preferential affinity for the fluid phase, and broaden the transition, promoting gel-fluid phase coexistence. The overall results are consistent with protein surface association in which the degree of binding correlates with the degree of folding and perturbation of the membrane bilayer. However, the degree of binding of monomer to membrane does not correlate directly with aSN toxicity *in vivo*.

1. Introduction

Alpha-synuclein (aSN) is a water soluble presynaptic protein (140 residues, 14.4 kDa) belonging to the class of intrinsically disordered proteins, *i.e.*, proteins which are mostly unfolded in their native state and in the absence of binding partners [1]. Yet aSN can acquire secondary structure upon interacting with membranes both *in vivo* [2] and *in vitro* [3–7]. Further, it can self-assemble, both *in vivo* and *in vitro*, into a variety of aggregates including partially or highly ordered oligomers and amyloid fibrils [8–15].

The exact function of aSN within the cells is debated, but certainly aSN aggregation is involved in the development and pathogenesis of neurodegenerative disorders, mainly Parkinson's disease (PD) [16]. In addition to aSN gene duplication [17] and triplication [18], which have been linked to late-stage PD and diffuse Lewy body dementia, three point mutations of aSN, namely A30P, E46K and A53T, causing rare early-onset forms of PD, have been identified [19–21]. More recently, new familial mutations in aSN's coding sequence have been discovered which are linked to neuropathological features of PD and other synucleinopathies. These include H50Q [22,23], G51D [24,25] and A53E [26].

Throughout the aSN primary sequence, three distinct important regions can be identified: 1) the *N*-terminal region (NT, residues 1–102), which bears a net positive charge at neutral pH; 2) the non-amyloid beta component region (NAC, residues 60–95) which forms the core of amyloid fibrils; and 3) the C-terminus (CT, residues 98–140), which is highly negatively charged at neutral pH. The NT domain contains seven 11-residue repeats of the consensus sequence *p*KTKEGV*axa*A, where *p*, *a*, and *x* are a polar, an apolar and any residue, respectively [5]. This motif characterizes class A_2 amphipathic helices of the type involved in the lipid binding domains of serum apolipoproteins [27]. It has been shown that the NT domain binds to membrane-mimetic systems and folds in an α-helical configuration, while the remaining CT residues are unfolded [3,4,6,7,28–33]. Binding is initiated by residues 3–25 that anchor aSN to the membrane and afterwards residues 25–100 undergo the coil → helix transition [34].

Association with membranes is believed to be important for aSN function [35–37], toxicity [38,39] and fibrillation [40]. Within this context, growing attention is paid to the study of aSN interactions with synthetic membranes by considering aSN in different aggregation states (monomer, oligomer, fibril) and membranes of different nature and

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composition [3–7,28–34,41–55]. Lipid binding and helix formation occur for monomeric aSN in the presence of lipid vesicles with a net negative surface charge in the fluid state [3–7,29–31]. The protein structure and stability depend on many factors such as protein concentration, protein/lipid molar ratio, pH and ionic strength of the dispersion medium, size, lamellarity and lipid composition of the vesicles. As an example, for 0.2 mg/mL aSN in 0.2 mg/mL of the anionic lipid dimyristoylphophatidylglycerol (DMPG), the coil → helix transition is only observed for vesicles with diameter; >100 nm [31]. aSN also associates to small unilamellar vesicles (SUV) but not to large unilamellar vesicles (LUV) of uncharged lipids in the gel state [7,29] or multilamellar vesicles (MLV) of zwitterionic lipids [41]. In most cases, emphasis is given to the protein rather than to the lipid component of the complexes. However, studies on the influence of aSN species on the molecular properties of lipid membranes can be relevant for understanding the functional and pathological role of the protein.

In this work, we combine different biophysical techniques to investigate the effects of wt aSN and the three familial variants A30P, E45K and A53T in the monomeric state on a suitable membrane model systems consisting of DMPG extruded LUV ($\phi = 200$ nm) dispersed in phosphate buffer solution (PBS) at physiological conditions (20 mM phosphate, 150 mM NaCl, pH 7.4). The study is done with electron spin resonance (ESR) spectroscopy of phosphatidylcholine lipids bearing the nitroxide reporter group at selected carbon atom position, C-*n*, along the s*n*-2 chain, (*n*-PCSL, $n = 5$, 7, 10, 12, and 14), This technique is particularly appropriate for studying lipid-protein interaction with particular respect to lipid dynamics [56,57] and *n*-PCSL have proved to have affinity for PG membranes [41]. Previously, it has been used to characterize the association of wild-type aSN [41] and A30P, A53T, aSN(1–95) and fibrillary forms [45] with MLV of neutral and negatively charged lipids (*i.e.*, DMPC and DMPG). ESR of 5- and 14-PCSL has also been employed to investigate the interaction of aSN with SUV and LUV of zwitterionic DMPC and DPPC lipids [29]. In our work, we expand on previous investigations with a study on the effect of temperature on the binding to anionic lipids of wt-aSN and three PD-associated mutations (A30P, E46K and A53T). These mutations are known to affect aSN aggregation behavior in different ways and are expected to affect the binding to anionic lipid bilayers. In contrast to previous studies, our spin-label ESR measurements are carried out over a wide temperature range that encompasses both the gel-to-fluid phase transition of the lipid vesicles and the coil → helix transition of the membrane-bound proteins. To this end, high-sensitivity differential scanning calorimetry (DSC) at low scan rate is used to monitor the effects of the proteins on the thermotropic phase behavior of DMPG bilayers. In parallel, far-UV circular dichroism (CD) thermal scans are acquired to determine the conformational structure adopted by the proteins upon interacting with DMPG vesicles. How saturating amounts of aSN and mutants affect the rotational mobility, the segmental lipid chain order and the endothermic DMPG phase transitions is presented. In addition, the correlation between the protein-lipid stoichiometry (from ESR) and the extent of protein folding (from CD) on DMPG bilayers is assessed. Possible biological implications of the protein interaction with membranes are discussed.

2. Materials and methods

2.1. Materials

Wild-type aSN and the mutants A30P, E46K, and A53T were expressed and purified as described [12]. The synthetic lipid 1.2-dimyristoyl-*sn*-glycero-3-[phospho-*rac*-(1glycerol)] (DMPG) and the spin-labelled lipids *n*-PCSL, 1-palmitoyl-2-(*n*-doxylstearoyl)-*sn*-glycero-3-phosphocholine ($n = 5$, 7, 10, 12 and 14) were from Avanti Polar Lipids (Birmingham, AL). Reagent grade salts for PBS (20 mM phosphate, 150 mM NaCl, pH 7.4) were from Merck (Darmstadt, Germany). All materials were used as purchased and MilliQ water was used throughout.

2.2. Sample preparation

Lyophilized aSN (wt or mutant) was dissolved in PBS, filtered (0.2 μm) and the concentration determined with a Jasco 7850 spectrophotometer by using an extinction coefficient of 0.412 $(mg/ml)^{-1}\ cm^{-1}$ at the wavelength of 280 nm [12,13]. Large unilamellar vesicles (LUV) of DMPG were obtained by extruding a solution of multilamellar vesicles (MLV) prepared as follows. DMPG lipids were dissolved in chloroform-methanol 2:1 v/v. The solvent was evaporated with a nitrogen gas stream and residual traces removed by drying under vacuum overnight. The lipid films were fully hydrated in PBS by heating at 50 °C, which is well above the lipid phase transition temperature (23 °C), and periodically vortexing for *ca.* 30 min. The hydrated lipid dispersions were subjected to ten cycles of freezing in liquid nitrogen ($t = -196$ °C) and thawing at 50 °C. To obtain LUV, the MLV solutions were extruded eleven times through a 200 nm polycarbonate membrane filter by using a LiposoFast-Basic extruder (Avestin, Canada).

2.3. ESR spectroscopy

DMPG LUV for ESR measurements were prepared as described above at a lipid concentration of 20 mM (13.8 mg/mL) except that the lipids were co-dissolved with 1 mol% of the desired spin-labelled lipid *n*-PCSL. Freshly prepared buffered protein solutions were then titrated into DMPG vesicles at different weight ratios in a final volume of 25 μL. Samples were loaded in glass capillaries, flame sealed, inserted in a standard ESR 4-mm quartz tube containing light silicone oil for thermal stability, and centered in a Bruker (Karlsruhe, Germany) TE_{102} ER 4102ST rectangular ESR cavity. The spectra were collected on an ESP 300 Bruker spectrometer operating at 9-GHz with 100-kHz field modulation equipped with a Bruker ER 4111 VT variable temperature control unit. They were analyzed by measuring the outer hyperfine splittings, $2A_{max}$ and $2A_{min}$ (see Fig. 1), using the Bruker data system for spectral acquisition and processing.

2.4. Circular dichroism

Samples of proteins for CD spectra and of protein:lipid complexes for CD thermal scans were prepared daily at 0.4 mg/mL and at 1:2 wt/wt in PBS, respectively ([DMPG] = 1.2 mM, [protein] = 28 μM). Circular dichroism measurements were performed on a Jasco J-810 CD spectrometer (Jasco Spectroscopic Co. Ltd., Hachioji City, Japan) using a 1-mm cuvette. The CD spectra were recorded in the wavelength range of 190–250 nm, using a bandwidth of 2 nm and a time constant of 2 s at a scan speed of 50 nm/min. Temperature scans (5–95 °C) were monitored at 220 nm with a scanning speed of 60 °C/h.

2.5. Differential scanning calorimetry

Samples for calorimetry were either multilamellar vesicles or extruded large unilamellar vesicles of DMPG at a final concentration of 1 mg/mL in the presence or absence of protein (aSN or a mutant) at 0.5 mg/mL ([DMPG] = 1.5 mM, [protein] = 35 μM). DSC experiments were carried out on a VP-DSC MicroCalorimeter (MicroCal, Inc. MA, USA) with a cell volume of 0.52 mL and a temperature resolution of 0.1 °C. The samples were degassed before the scans and the thermograms were recorded while heating at a scan rate of 5 °C/h as described in [58]. The scans are reported in terms of excess heat capacity, C_{pex}, as a function of temperature and the data analyzed by using the MicroCal Origin software package. Phase transition temperature was determined as temperature of the maximum heat capacity. Enthalpy was determined by integration of the area under the transition peak.