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Effect of Pseudorepeat Rearrangement upon α -Synuclein Misfolding, Vesicle Binding and Micelle Binding

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Abstract

The pathological and physiological hallmarks of the protein α -synuclein are its misfolding into cytotoxic aggregates and its binding to synaptic vesicles, respectively. Both events are mediated by seven 11-residue amphiphilic pseudorepeats and, most generally, involve a transition from intrinsically unstructured to structured conformations. Based upon α -synuclein interactions with aggregation-inhibiting small molecules, a α -synuclein variant termed SaS, wherein the first six pseudorepeats had been rearranged, was introduced. Here, the effects of this rearrangement upon misfolding, vesicle binding and micelle binding are examined in reference to α - and β -synuclein to study the sequence characteristics underlying these processes. Fibrillization correlates with the distinct clustering of residues with high β -sheet propensities, while vesicle affinities depend on the mode of pseudorepeat interchange and loss. In the presence of micelles, the pseudorepeat region of SaS adopts an essentially continuous helix, whereas α - and β -synuclein encounter a distinct helix break, indicating that a more homogeneous distribution of surfactant affinities in SaS prevented the formation of a helix break in the micelle-bound state. By demonstrating the importance of the distribution of β -sheet propensities and by revealing inhomogeneous aS surfactant affinities, the present study provides novel insight into two central themes of synuclein biology.

Keywords

α -synuclein; membrane proteins; NMR spectroscopy; protein misfolding; sodium lauroyl sarcosinate

Introduction

Cytotoxic aggregates arising from the misfolding of the 140-residue intrinsically unstructured protein α -synuclein (aS) have been implicated in the pathogenesis of Parkinson's disease, the second most common neurodegenerative disorder in humans.¹⁻⁵ In a process common to many neurodegenerative diseases, misfolding commences with the formation of small soluble oligomers (dimers, trimers, etc.), which proceed to yield larger protofibrils and eventually form insoluble cross- β sheet fibrils.^{2,3} Misfolding is enhanced by certain posttranslational modifications, oxidative damage, point mutations and certain lipid environments, but, most generally, commences from the free, intrinsically unstructured aS in aqueous solution.¹⁻⁶ An

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unstructured to structured transition also underlies the most tangible physiological function of aS; namely, its ability to bind synaptic vesicles in α -helical conformation.^{7,8} The sequence responsible for both misfolding and vesicle-binding is comprised of seven 11-residue degenerate repeats, which, together with an N-terminal 8-residue flanking sequence and four residues inserted between repeats IV and V, constitute an amphiphilic region (Fig. 1a).^{7,9-12} The remaining highly acidic C-terminal tail slows misfolding and does not associate with vesicles.^{11,13,14}

Several avenues have been pursued to elucidate the molecular basis of aS misfolding. For example, the structural ensemble of unfolded aS,¹⁵⁻¹⁸ the effects of aS mutations, extensions or truncations^{13,19-23}, and its interaction with aggregation-inhibiting small molecules^{24,25} have been characterized. Long-range interactions within the relatively compact structural ensemble of intrinsically unfolded aS¹⁷ have been implicated in the modulation of misfolding.^{14-16,23} Fibrillization kinetics of aS variants can often be predicted based on protein mean β -strand propensity and the content of hydrophobic and charged residues.^{19,20,22} While the general importance of the distribution of β -sheet propensities and sequence positions in protein aggregation has been recognized,^{20,26-28} to our knowledge, no experimental study of the effect of varying the β -sheet distribution on fibrillization is available. In prions, random sequence rearrangement studies revealed that prion fibril formation is dependent on amino acid content rather than sequence.^{29,30} Interestingly, several aggregation-inhibiting small molecules exhibit a remarkable consensus in their interaction with aS, centering on residues 3-18 and 38-51.²⁵ We hypothesized that, if these residues form key interaction sites in the aggregation pathway, a change in the order of pseudorepeats which provides a more dispersed distribution of interactions sites may reduce noxious interactions and misfolding. This led to the design of a shuffled aS variant, termed SaS (Fig. 1b),²⁵ which exhibits protein physico-chemical mean properties identical to those of aS. Using SaS, we show here that the rearrangement of aS pseudorepeats abrogates fibrillization in correlation to the fragmentation of β -sheet propensities, which is accompanied by changes in the structural ensemble of SaS compared to aS.

In a physiological context, the amphiphilic region of aS transitions from unfolded to α -helical conformation upon binding to synaptic vesicles,^{7,8} and any ensuing vesicle stabilization³¹ may relate to the ability of aS to inhibit dopamine neurotransmission.³² Additional functions that implicate aS in membrane-associated processes in the presynaptic terminal are documented⁶ and aS can substitute for the cysteine-string protein- α only in the vesicle-bound state.³³ Remarkably, aS prefers to associate with anionic vesicles of smaller (20-25 nm) rather than larger diameters ($\sim$ 125 nm),^{7,31} placing it in a group of proteins that can sense membrane curvature. By virtue of exhibiting 11-residue pseudorepeats, synucleins are also related to apolipoproteins, which engage lipids by curved, belt-like helices for transport in the bloodstream.³⁴ On the surface of anionic small unilamellar vesicles (SUV), the aS amphiphilic region is characterized by an uninterrupted α -helix.³⁵⁻³⁷ On smaller diameter anionic micelles, aS partitions into two anti-parallel helices,^{18,38-41} which, as judged by their curvature and mutagenesis studies, considerably influence the micelle shape.^{39,42} It is unclear whether the helix break encountered on micelles is simply the effect of a micelle's minimal size, or whether the break reflects a meaningful functional aS property, such as a predetermined helix breaking point. Here, we show that in contrast to aS and β -synuclein (bS), SaS is able to maintain an essentially uninterrupted helix in the presence of a novel micelle system for synucleins (sodium lauroyl sarcosinate; SLAS) in correlation to an apparently more homogeneous distribution of surfactant affinities in SaS.

Results

Pseudorepeat shuffled aS does not fibrillize in correlation to rearranged β -sheet propensities

Because SaS and aS have identical mean physico-chemical properties, differences in their misfolding would have to arise from differences in their amino acid sequence distribution. The initial oligomerization of soluble aS proceeds through β -sheet-rich conformations^{17,43} and the core region of ultimately deposited aS fibrils, consisting of residues 36-98, adopts cross- β sheet architecture.^{9,10} The amino acid sequence of the aS fibril core consists of residues which generally have high β -sheet propensities in contrast to the remainder of the protein (Fig. 2a). Moreover, general protein aggregation prediction algorithms^{26,28} identify aS regions of high β -sheet propensities as aggregation-prone (data not shown). The pseudorepeat rearrangement of SaS, which results in a more homogeneous interaction with aggregation-inhibiting small molecules,²⁵ renders this region of high β -sheet propensities discontinuous (Fig. 2a) and therefore may alter protein misfolding.

Pursuant to our design goal, SaS does not fibrillize *in vitro* (Fig. 3a) and, in this respect, resembles bS, which behaves innocuously *in vitro* and *in vivo*.⁴⁴⁻⁴⁶ Interestingly, bS also exhibits greatly diminished β -sheet propensities compared to aS as a result of several amino acid substitutions and the loss of 11 residues (Figs. 1c and 2b). Electron micrographs confirmed the fibrillar nature of aS aggregates, whereas, for SaS and bS, amorphous precipitates were obtained (Fig. 3b). Soluble oligomers, particularly dimers, were detected for aS in contrast to SaS and bS by Western blots (Fig. 3c). However, cross-reactivity to the antibody A11 was obtained for both SaS and aS, but not for bS, in dot blots (Fig. 3d). A11 binds a unique conformation-dependent structure that is common to many soluble oligomers,⁴⁷ indicating that SaS may still possess the capacity of forming oligomers. In conclusion, aS pseudorepeat shuffling abolished misfolding into insoluble fibrils, but may have left some aggregation-prone aS epitopes intact. The sequence distribution of regions of high β -sheet propensity correlate with the fibrillization propensities of SaS and aS, whereas bS exhibits greatly diminished β -sheet propensities.

SaS forms a more compact structural ensemble than aS with somewhat altered average ensemble conformational propensities

The rearranged amino acid sequence and secondary structure propensities of SaS compared to aS (Figs. 1a-b and 2a) may lead to differences in the properties of the structural ensembles of these intrinsically unfolded proteins. To compare some pertinent ensemble parameter between SaS and aS, NMR spectroscopy was employed. First, the backbone H-N signal broadening of each residue arising from a paramagnetic spin label covalently attached to residue 87, which lies in the unchanged seventh repeat (Fig. 1a-b), was evaluated for SaS and aS. Within the reach of the spin label, broadening patterns differed significantly (Fig. 4a). SaS predominantly experienced stronger broadening than aS, which was indicative of a more compact structural ensemble than that obtained for aS. Interestingly, this applied not only to the amphiphilic region, but to the tail segment as well. Next, the secondary structure propensities of SaS and aS were compared in the form of secondary $^{13}\text{C}^\alpha$ shifts. It is well established that (non-deuterated) aS exhibits positive $^{13}\text{C}^\alpha$ secondary shifts,¹⁸ which implies a preference to sample helical over extended conformations.^{48,49} While this preference was maintained in SaS (after accounting for deuterium isotope shifts), some regions exhibited distinct $^{13}\text{C}^\alpha$ secondary shift changes (Fig. 4b) indicative of altered structural propensities. The 4-residue segment, Ala53-Ala56, interspersed between repeats IV and V, was the most conspicuous. The shuffling of (neighboring) repeats renders it more helical in character, whereas other affected regions, which mostly localize at pseudorepeat borders, showed either decreased or increased $^{13}\text{C}^\alpha$ shifts. The two repeat interchanges that introduce or remove a charge at a repeat-repeat border (I $\leftrightarrow$ V and II $\leftrightarrow$ IV) generally showed larger shift changes than the “neutral” repeat III $\leftrightarrow$ VI

interchange (Fig. 4b). In conclusion, a more compact structural ensemble for SaS relative to aS was detected wherein new neighbor effects, particularly those involving charged residues, most clearly altered average ensemble conformational propensities.

Small unilamellar vesicle binding depends on the order and number of synuclein repeats

Using SaS, the effect of pseudorepeat rearrangement on synuclein vesicle binding was studied. In SaS, the interchange of repeats that occur before the 4-residue insert (Ala53-Ala56) with repeats positioned afterward results in residues that are not positioned at their structurally equivalent aS helix orientations relative to the membrane surface (Fig. 1a-b). For the aS amphiphilic helix, with an average of 3.67 residues per turn,^{35,50} the 4-residue insert shifts the helix register of repeats V-VII relative to repeats I-IV by 0.33 residues or 32° and, strictly speaking, pseudorepeats I-IV and V-VII represent two different repeat systems. For example, some well-conserved pseudorepeat residues, such as threonine at repeat position 3 and lysine at positions 2 and 4, change their orientation relative to the helix face between the two repeat systems (Fig. 1a-b). This also suggests that the occurrence of the 4-residue insert followed the 7-repeat assembly in synuclein evolution. For SaS, this indicated that numerous amino acids changed their orientation orthogonal to the aS helix axis by $\pm 32^\circ$, in addition to a translational shift parallel to the helix axis. Specifically, out of 31 sequence position changes along the hydrophobic helix face, a new residue type was present at 15 positions (Fig. 1b) and the interchange of repeats III and VI also changed the helix orientation of two “snorkeling” lysine residues.^{39,50} A decrease in SaS vesicle binding compared to aS may therefore ensue as a result of less optimal packing interactions of rotated valine, threonine, glutamine, alanine, and glycine residues with the lipid hydrocarbon chains, but also from less favorable lysine-lipid headgroup electrostatic interactions and altered lysine snorkeling depths. The lack of one net pseudorepeat for bS compared to aS, along with the mode of aS/bS amino acid substitutions (Fig. 1c), are expected to result in a lowered vesicle association as well.

To assess the extent of small unilamellar vesicle (SUV) binding by the synuclein variants, the fraction of protein that can be separated from SUV-containing solutions by gel filtration was quantified.⁷ The separated protein amounts varied significantly (Table 1); almost twice as much protein was separated for SaS in comparison to aS, and, for bS, the majority of protein was obtained as free protein. Synuclein vesicle binding therefore depends on the order and number of pseudorepeats. Assuming similar free energies for the unfolded SaS and aS states, the rearrangement of pseudorepeats in SaS lowered the affinity for SUV. This shows that, compared to aS, the same lipid-protein interactions cannot be entertained. However, SaS is still curvature sensitive and binds large unilamellar vesicles (LUV) almost to the same extent as does aS (Table 1).

An inhomogeneous distribution of surfactant affinities governs α -synuclein-micelle interactions

Micelles composed of sodium dodecyl sulfate (SDS) or lyso-1-palmitoylphosphatidylglycerol (LPPG) act as folding scaffolds for aS, resulting in the formation of two anti-parallel helices.^{18,38-41} While SDS and LPPG micelles exhibit smaller radii than SUV, it is unclear whether the micelle-induced helix break is merely the result of differences in scaffold curvatures, or arises from a localized weakness in surfactant interactions that the micelle can exploit to minimize deformations of its intrinsic micelle shape upon engaging synuclein proteins. The rearranged repeats in SaS provided a unique opportunity to study differences in the synuclein-micelle interplay and, hence, provide information on the distribution of surfactant affinities along the synuclein sequence. At the same time, a new micelle system for reconstituting synucleins, sodium lauroyl sarcosinate (SLAS), was introduced. SLAS combines a C₁ carbonyl functionality and a carboxylate group that confers its negative charge in a relatively small molecule (Fig. 5a). As such, it serves as an alternative to the relatively strong detergent SDS.

To better understand SLAS-synuclein interactions, the micelle properties of free SLAS were characterized first. Translational diffusion measurements of SLAS micelles by pulsed field gradient NMR spectroscopy revealed an unhydrated micelle diameter of 44.4 Å, corresponding to a micelle mass of 30.6 kDa or an aggregation number of ~104 (Fig. 5a). This is somewhat larger than SDS micelle dimensions⁵¹ and 7-9 times smaller than SUV. Titrations of aS, SaS and bS with SLAS reached saturation at a molar ratio of 1:100 (Fig. 5b and data not shown), i.e., close to the SLAS aggregation number, yielding high-quality NMR spectra (Fig. 5c). Thus, SLAS micelles bound synucleins quantitatively; without large rearrangements, however, a spherical SLAS micelle would be too small to bind an uninterrupted synuclein helix in analogy to SDS and LPPG micelles.

To assess differences in the backbone structure and dynamics of the synuclein variants in the presence of SLAS micelles, several NMR parameters were compared. First, a quality factor (q-factor), which scales between 0 (highest quality) and 1 (lowest quality),⁵² calculated from residual dipolar couplings and secondary structure coordinates obtained from molecular fragment replacement⁵⁵ was used to assess the ability to describe a segment of backbone structure using a single conformation, i.e., to measure structural homogeneity. Subsequently, the {¹H}-¹⁵N NOE value and ¹³C^α secondary shift of a residue were evaluated to gauge the degree of local backbone order (pico- to nanosecond dynamics)⁵³ and secondary structure formation,^{48,49} respectively. Sequential H^N-H^N NOE intensities were also evaluated and were found to provide complementary information (data not shown). For aS and bS, these parameters demonstrated structural inhomogeneity, low backbone order and low helical content for parts of repeats II-III (Fig. 6a,d,e), whereas SaS almost uniformly exhibited a homogeneous and well-ordered structure that is generally, but not exclusively, more helical in character (Fig. 6a-c,e-f). Interestingly, for bS, backbone order was even more decreased than for aS despite its 11-residue shorter sequence (Fig. 6d). These data clearly establish the presence of an aS/bS helix break at the border of repeat II and III. This preceded the aS break experienced by SDS-bound aS at Leu38-Thr44³⁹ by a few residues. However, within small changes in the length of the N- and C-terminal helices, the structure of SLAS-bound aS is expected to be similar to the described SDS-bound aS structure.³⁹ Analogously, SLAS-bound bS appeared similar to SDS-bound bS⁵⁴ with again a somewhat earlier helix break in the presence of SLAS. In contrast, SaS maintained low q-factors (<0.3) for a continuous segment of 71 residues. Between rearranged, consecutive SaS repeats V and IV (Fig. 1b), a modest amount of structural inhomogeneity was observed, peaking at q=0.53. At the end of rearranged repeat V, SaS residues 17-19 exhibited relatively low ¹³C^α secondary shifts and residue 20 of the following repeat IV experienced a diminished {¹H}-¹⁵N NOE value (Fig. 6b-c). This indicated that for four residues, that is, one helix turn, helical conformation has frayed and helical content was diminished. However, in contrast to SLAS-bound aS, but also SDS-bound aS,³⁹ molecular fragment replacement⁵⁵ for SaS still retrieved helical conformations throughout the entire amphiphilic region (Fig. 7b), which, together with a maximal q-factor of 0.53, indicated that, on average, helical conformation is still preserved. In conclusion, the synuclein micelle association does not invariably cause a distinct helix break. Rather, the extensive helix break of aS and bS in comparison to SaS must arise from a difference in the protein-micelle interplay as a result of its rearranged sequence. SaS folds more homogeneously, that is, interacts with the micelle surface more homogeneously or, when viewed in terms of free energy changes of discrete sequence segments, exhibits a more homogeneous distribution of surfactant affinities than aS and bS.

SaS exhibits homogenous, but high helix dynamics in contrast to aS and bS

Up to now, the micelle-induced folding of synuclein secondary structure was examined. Backbone dynamics of folded synuclein segments, which takes place on the nano- to millisecond timescale, may provide further insight into synuclein-micelle interactions and was

examined next. Dynamics on this intermediate timescale modulate the magnitude of another NMR parameter, the alignment tensor magnitude, D_a .⁵⁶ A backbone segment that exhibits a lower D_a value than a reference segment experiences enhanced fluctuations of its backbone orientation relative to the reference segment. Moreover, segments that encompass disordered secondary structure, such as part of repeats II-III in aS and bS (Fig. 6a,d), will invariably experience low D_a values. The distributions of D_a values along the synuclein sequences revealed that SaS exhibits fundamentally different intermediate timescale dynamics than aS and bS (Fig. 7a). Dynamics were relatively uniform for SaS, whereas aS and bS exhibited highly non-uniform behaviors in correlation to the above outlined patterns of surfactant affinity distributions. Local sequence variations in the ratio of association and dissociation rates may directly contribute to these observed dynamics, but significant contributions from differential helix dynamics of micelle-associated backbone segments are also expected. For example, the bS sequence encompassing repeats IV-V exhibited less intermediate timescale dynamics than the corresponding aS region (Fig. 7a) despite a virtually identical degree of backbone order (Fig. 6d). If, based on comparable backbone orders, comparable micelle associations are assumed, it follows that, on the micelle surface, the helix orientation of repeats IV-V fluctuates less for bS than for aS. For SaS, it can be attempted to illustrate the fluctuations of helix orientations (helix dynamics) based on the backbone fragments retrieved by molecular fragment replacement.⁵⁵ SaS structural states ranging from highly curved, almost anti-parallel to elongated, linear structures with accompanying SLAS micelle shapes can thus be envisioned (Fig. 7b). The range of sampled SaS helix orientations is probably higher than for aS/bS since SaS consistently exhibits lower D_a values for well-folded regions under identical alignment conditions (Fig. 7a). It is also noted that gel filtration profiles of aS and SaS are indistinguishable in the presence of SLAS (data not shown), indicating that, like aS, one SaS molecule associates predominantly with only one micelle. In conclusion, intermediate timescale dynamics confirmed a homogeneous appearance of the SaS amphiphilic region in contrast to the pronounced heterogeneity encountered for aS and bS, but, at the same time, suggest that SaS samples a wider range of helix orientations than aS and bS.

Discussion

The present study sought to address two main questions. First, does the fragmentation of documented aS binding sites of several aggregation-inhibiting small molecules²⁵ abrogate aS misfolding and, if so, what is the underlying reason? Second, are synuclein-micelle interactions dominated by amino acid sequence properties, or rather, by a limited size and plasticity of the micelle? If dominated by amino acid sequence properties, what can be learned about the underlying properties of the well conserved synuclein amino acid sequence?⁵⁷

The pseudorepeat rearranged SaS synuclein variant, which exhibits a more homogeneous interaction with aggregation-inhibiting small molecules than aS,²⁵ does not readily fibrillize as revealed by thioflavin T fluorescence measurements and electron microscopy but rather forms amorphous aggregates over time as does innocuous bS.⁴⁴⁻⁴⁶ On the level of aS amino acid sequence, a fragmentation of the extensive cluster of residues of high β -sheet propensities congruent with the aS fibril core (residues 36-98)^{9,10,58} is noted for SaS. This includes the fragmentation of the Glu61-Val95 sequence, known as NAC (non-A β -amyloid component) region,⁵⁹ which encompasses the region of highest consecutive β -sheet propensity in aS (Fig. 2a). It appears that the probability of forming a fibrillization-competent, β -sheet rich nucleation species is sensitive to the distinct clustering of regions of high β -sheet propensities in synucleins. This interpretation agrees with the absence of bS misfolding, which exhibits strongly reduced β -sheet propensities compared to aS as a result of the loss of a 11-residue stretch of high β -sheet propensity and the net substitution of five β -branched residues for non- β -branched residues (Figs. 1c and 2b). Between SaS and aS, steric and geometric factors, such as altered side chain packing possibilities, may also reduce the success of forming β -sheet-rich

SaS oligomers. However, in the presence of certain divalent metal ions, even bS will fibrillize,⁶⁰ suggesting that steric factors alone would not be sufficient to prevent SaS fibrillization. The sensitivity of fibrillization to synuclein sequence differs from the results obtained for prions where, upon random sequence rearrangements, amino acid content rather than sequence governed fibrillization propensities.^{29,30} However, the prion domain responsible for misfolding is highly enriched in Gln and Aln residues,^{29,30} which may explain its relative insensitivity to sequence.

Fibrillization of aS can also be suppressed without changing the canonical aS pseudorepeat sequence; for example, by N-terminal sequence extension²¹ or by point mutations in the C-terminal tail.²³ While these alterations invariably change the amino acid content and, thus, protein physico-chemical properties, they suggest that the properties of the structural ensemble of intrinsically unfolded aS may play a role in modulating the kinetics of aS fibrillization. Further support for this assessment is provided by studies of the structural ensemble of the wild-type protein^{15,16} and the dependence of fibrillization kinetics on solution conditions.^{17, 43} For SaS, NMR spectroscopy showed that, compared to aS, its structural ensemble is more compact and exhibits altered average ensemble conformational propensities particularly at repeat-repeat borders. Although these changes are expected to result primarily from the extensive fragmentation of β -sheet propensities in SaS, reduced SaS misfolding may also benefit from changes in ensemble properties such as altered contacts with the acidic C-terminus (Fig. 4a).^{13-16,23}

In vivo, synucleins exist in two co-existing populations: a cytosolic reservoir pool and a vesicle-bound functional pool.^{7,8} Although lipid environments can promote aS misfolding,⁶¹ physiological synaptic vesicle binding and concomitant synuclein folding is likely to impede misfolding since the concentration of unfolded, cytosolic aS decreases. The distinct pattern of amino acid substitutions and losses that lead to the reduced β -sheet propensities of bS in comparison to aS suggests that bS has experienced stronger evolutionary pressure to reduce its misfolding potential than aS which, in our view, would imply a higher cytosolic protein fraction than present for aS. We have therefore compared the amount of synuclein that is separable from SUV by gel filtration. For bS the majority of protein was separable from SUV, confirming a high fraction of cytosolic, free protein⁶² in contrast to aS which was found to be predominantly associated with SUV.⁷ In this sense, aS is viewed as being more optimized for vesicle binding than bS, but less optimized to behave innocuously in the cytosol. Moreover, since bS binds vesicles to a lesser degree than aS, it is also expected to serve as a weaker physiological inhibitor of vesicle fusion than aS. The characteristic pattern of amino acid substitutions and losses between bS and aS (Fig. 1c) also suggests that bS is evolutionarily derived from an aS precursor. For SaS, binding to SUV was significantly reduced compared to aS, highlighting an interesting aspect of the synuclein sequence. Namely, the 32° shift in helix register between pseudorepeats I-IV and V-VII by the intervening 4-residue insert. As a result, on the hydrophobic face of SaS, 15 residue types have altered their orientation relative to the aS helix face (Fig. 1b) and two “snorkeling” lysines^{39,50} have shifted their helix orientation and immersion depth, together contributing to the reduced vesicle association detected.

The aS amphiphilic region binds to SUV via an uninterrupted α -helix,³⁵⁻³⁷ whereas SDS and LPPG micelles induce the formation of two separate helices in aS and bS.^{18,38-41,54} Micelles of the detergent tested here, sodium lauroyl sarcosinate (SLAS), for which an aggregation number of 104 was determined by translational diffusion measurements, are no exception to this trend as revealed by the examination of backbone structural and dynamic parameter of aS and bS by NMR spectroscopy. Except for one frayed helix turn, SLAS micelle-bound SaS is able to maintain well-defined α -helical conformation throughout the entire amphiphilic region, demonstrating that the aS and bS helix break is not merely a consequence of the limited size

and plasticity of the micelle. Rather, synuclein-micelle interactions depend on the way synuclein can deform the micelle and on the ability of the micelle to influence the synuclein structure. The micelle can exploit an inhomogeneous distribution of surfactant affinities along the aS and bS amphiphilic sequences to “break” this region into two helices in order to minimize its own distortions. For SaS, a more even distribution of affinities was revealed leading to a more uniform helix strength, which is not easily broken by micelle interactions. These properties are also reflected by the helix dynamics of micelle-bound SaS, aS and bS. Compared to the heterogeneous dynamics of aS and bS, SaS exhibited more homogenous helix dynamics. Interestingly, dynamics on an intermediate timescale (nano- to milliseconds) are generally higher for SaS than for aS and bS, suggesting that SaS is able to sample a wider range of helix orientations than aS and bS. This flexibility may aid micelle binding via an essentially uninterrupted helix. Thus, although aS interactions with spherical micelles result in non-physiological structures, this comparative study of SaS, aS and bS has allowed the identification of an inhomogeneous distribution of sequence surfactant affinities in both aS and bS in addition to providing general insight into protein-micelle interactions.

In terms of aS function, an inhomogeneous distribution of aS surfactant affinities may provide a predetermined helix break of significance during vesicle fusion or when binding the highly crowded synaptic vesicles.⁶³ Regarding the sequence characteristics that underlie surfactant affinity variations, synucleins exhibit an almost contradicting contrast of amphiphilic helix stabilizing and destabilizing elements. The presence of numerous glycines and β -branched amino acids, in particular valine and threonine on the membrane-facing helix side (Fig. 1a), incur relatively high entropic penalties upon adopting a helical conformation.⁶⁴ In contrast, the numerous lysines and hydrophobic moieties facing the membrane interact favorably with anionic lipid/detergent headgroups and hydrocarbon tails, respectively, leading to successful folding. At present, the meaning of this conspicuous pattern, which directly correlates synuclein folding and misfolding, is difficult to interpret without further experimental data. A recent EPR study reported different membrane affinities between residues 9/18 and 69/90.⁶⁵ However, with careful corrections for unspecific background spin labeling of aS, less conspicuous differences were obtained.^{11,35} In general, the balance of amphiphilic helix stabilizing and destabilizing sequence elements may allow synucleins to dynamically bind synaptic vesicles, which takes place in a cooperative manner,⁶⁶ and facilitate the transition between its cytosolic and vesicle-bound states. In addition, a relation to its curvature-sensing ability is conceivable. For one prominent β -branched residue type, threonine, an interesting property can be noted in this context. Threonine has the unusual property of influencing backbone conformation via its side chain rotameric state. Threonine in $\chi_1 = +60^\circ$ (*gauche*⁻) side chain conformation, but not in the more common $\chi_1 = -60^\circ$ (*gauche*⁺) conformation, induces or stabilizes a helix bending angle of 3–4° relative to alanine.⁶⁷ In SLAS-bound aS, χ_1 torsion angles are, for the most part, averaged (data not shown) and unavailable for vesicle-bound aS. However, upon setting χ_1 to +60° for only five threonines, an aS helix curvature is obtained that closely matches the vesicle-bound state (Fig. 8). The synuclein threonine rotameric states can therefore stabilize the binding of curved synaptic vesicle and *vice versa*, and this may also apply to other threonine- and serine-rich membrane curvature-sensing proteins such as ArfGAP1.⁶⁸

Materials and Methods

Fibrillization kinetics, A11 antibody reactivity, Western blots and electron microscopy

The synuclein variants aS, bS and SaS were prepared as described.²⁵ Protein concentrations were measured by UV spectroscopy ($\epsilon_{\text{aS/SaS}} = 5120 \text{ M}^{-1}\text{cm}^{-1}$, $\epsilon_{\text{bS}} = 5960 \text{ M}^{-1}\text{cm}^{-1}$). Samples were assayed in 25 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, pH 7.4, 0.02% NaN_3 solution and passed through a 100 kDa cut-off filter prior to use. A thioflavin T (Calbiochem, Inc.) stock solution of 100

μM was prepared in MilliQ- H_2O and filtered through a $0.2\ \mu\text{M}$ cellulose acetate filter. Protein samples were incubated at concentrations of $100\ \mu\text{M}$ and $37\ ^\circ\text{C}$ in an orbital shaker (25 mm radius, 150 rpm). Thioflavin T fluorescence was sampled every 4 h by removing a $10\ \mu\text{l}$ volume, diluting it to $950\ \mu\text{l}$ using $50\ \text{mM}$ Glycine- NaOH , pH 8.5, and adding $50\ \mu\text{l}$ of the thioflavin T stock solution. Fluorescence measurements were performed immediately on a Jasco FP-6500 fluorescence spectrophotometer using an excitation wavelength of 446 nm (5-nm slit width). Emission was recorded from 450 to 600 nm (20-nm slit width) and reported at 482 nm. A11 antibody reactivity was tested as described in the literature.⁴⁷ For the detection of soluble oligomers, the synuclein variants were incubated as described above for the thioflavin T measurements. Oligomer formation was assessed by Western blots using the Syn202 antibody (Abcam Inc.) and anti-aS antibody (BD Biosciences Inc.), respectively, following SDS-PAGE using 4-20% gradient gels. For electron microscopy, $10\ \mu\text{l}$ sample aliquots were coated on formvar- and carbon-coated copper specimen grids (Electron Microscopy Sciences, Inc.) for 10 min and subsequently stained with 6% uranyl acetate solution for 10 min. Images were taken on a Jeol JEM 1400 electron microscope operated at 100 kV.

NMR spectroscopy

All NMR experiments were carried out on a TXI cryoprobe-equipped Bruker Avance 700 spectrometer. Free aS and SaS were studied at concentrations of $0.5\ \text{mM}$ in $25\ \text{mM}$ HEPES- NaCl , pH 7.4, $50\ \text{mM}$ NaCl , 0.02% NaN_3 solution at $15\ ^\circ\text{C}$. Proteins were exchanged into the same buffer solution by four ultrafiltration-dilution cycles (1:10 dilution). ^{15}N -labeled S87C mutants of both proteins were (1-Oxyl-2,2,5,5-tetramethyl-3-pyrroline-3-methyl) methanethiosulfonate (MTSL) spin-labeled as described.³⁹ Backbone assignments of the free proteins were described earlier.²⁵ In the presence of SLAS micelles, $^2\text{H}/^{13}\text{C}/^{15}\text{N}$ -labeled aS, SaS and bS were studied at concentrations of $0.75\ \text{mM}$ in $25\ \text{mM}$ $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, pH 7.4, 0.02% NaN_3 solution at $25\ ^\circ\text{C}$ and a molar aS:SLAS ratio of 1:100, which is saturating (Fig. 5b and data not shown). The NMR experiments described in detail for SDS-bound aS³⁹ were performed to achieve backbone assignments and the measurements of the reported structural and dynamic parameters. For the measurement of RDC, the protein-micelle complex was aligned relative to the magnetic field in a stretched, negatively charged polyacrylamide gel⁶⁹ of 5.2% (w/v) with an acrylamide (AA) to bisacrylamide (BIS) ratio of 39:1 (w/w), a molar ratio of AA to 2-acrylamido-2-methyl-1-propanesulfonate (AMPS) of 95:5, and a 6.0/4.2 stretch ratio. With ^2H splittings of 2.7, 3.0 and 3.1 Hz, the alignment strength varied by 13% between the aS, bS and SaS samples, respectively. Chemical shift assignments have been deposited at the Biological Magnetic Resonance Data Bank with accession numbers 16300 (free aS), 16301 (free SaS), 16302 (SLAS-bound aS), 16303 (SLAS-bound SaS), and 16304 (SLAS-bound bS), respectively.

Translation diffusion measurements of SLAS micelles

The translational self-diffusion constant, D_s , of SLAS was measured as a function of weight fraction by BPP-LED experiments⁷⁰ employing a TXI cryoprobe with an experimentally determined⁷⁰ z-gradient strength of $52.1\ \text{G/cm}$. To account for the diffusion contribution from free SLAS molecules, the concentration dependence of D_s was corrected as described in the literature⁷⁰ to yield the micellar self-diffusion constant, D_m . Specifically, linearization of D_s to obtain D_m is optimal, as judged by a maximum in Pearson's correlation coefficient for D_m as a function of weight fraction, for a free SLAS concentration of $5\ \text{mM}$ (Fig. 5a and data not shown). This value is intermediate between the reported critical micelle concentrations of $10.2\ \text{mM}$ and $3.5\ \text{mM}$ between 0 and $100\ \text{mM}$ NaCl , respectively,⁷¹ in good agreement with the conditions of the present solution ($25\ \text{mM}$ $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, pH 7.4). Extrapolation of the concentration dependence of D_m to infinite dilution yields a diffusion constant, D_0 , of $8.15 \times 10^{-11}\ \text{m}^2\text{s}^{-1}$. Relative to lysozyme ($D_0 = 11.3 \times 10^{-11}\ \text{m}^2\text{s}^{-1}$), an unhydrated micelle radius of $22.2\ \text{\AA}$ is thus obtained. Using an SLAS density of $1.11\ \text{g/cm}^3$, calculated from the

experimentally determined partial molar volumes of its sarcosine, carbonyl and hydrocarbon moieties^{72,73} using Traube's additivity rule,⁷³ an average micelle mass of 30.6 kDa and an aggregation number of 104 are obtained, respectively.

Calculation of q-factor and alignment tensor magnitude

To determine the backbone conformation that fits the experimental dipolar couplings best, molecular fragment replacement (MFR)⁵⁵ using N-H, C'-C α , C'-N residual dipolar couplings and N, C α , C β , C' chemical shifts was employed using a fragment length of seven residues. For each fragment, the ten best candidates in the MFR database⁵⁵ were selected. Backbone dihedral angles were then determined by averaging over all selected candidates of all (overlapping) fragments containing any given angle, but excluding fragments where this angle is located in its N- or C-terminal residue. The alignment tensor magnitudes, D_a , calculated for this average structure varies little with fragment lengths ranging from 7-11 residues (data not shown), indicating that backbone dynamics is not significantly impacting the fragment structures over this length range. Singular value decomposition using the DC program of the NMRpipe package⁷⁴ was employed to calculate the five independent alignment tensor parameters using, for example, 21 N-H, C'-C α , C'-N dipolar couplings for a seven residue fragment and the average secondary structure obtained by MFR. Using these tensor parameter and secondary structure, the q-factor of a given 7-residue fragment is then obtained by back-calculating the dipolar couplings and relating them to the experimental ones as described in the literature.⁵² Calculating the alignment tensor parameter and q-factors using ideal helix coordinates (Fig. 1a-b) yields analogous results (data not shown).

Vesicle binding

Unilamellar vesicles were prepared from 1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine (POPC) and 1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-[Phospho-L-Serine] (POPS) (Avanti Polar Lipids, Inc.) at a molar ratio of 7:3. The lipids were dissolved in a 2 ml mixture of chloroform/methanol (19:1 v/v), followed by the evaporation of all solvent under a stream of nitrogen gas and drying of the lipids under a vacuum. The resulting lipid film was hydrated in 25 mM KH₂PO₄/K₂HPO₄, pH 7.4, 100 mM KCl, 0.02% NaN₃, or in 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.02% NaN₃, to obtain a total lipid concentration of 25 mM. For the preparation of SUV, the suspension was bath sonicated at 42 kHz (four times 10 min with 5 min breaks at room temperature) and the remaining larger particles were removed by ultracentrifugation to obtain a clear solution. For the preparation of LUV, the lipid suspension was freeze-thawed several times and extruded through a 0.1 μ m Polycarbonate membrane. Synuclein protein at 75 μ M was incubated with vesicles at a molar protein-to-lipid ratio of 1:250 for 1 h at room temperature, then subjected to gel filtration on a Superose 6 10/300 GL column (GE Healthcare, Inc) at a flow rate of 0.4 ml/min. The peak volume, detected at 280 nm, at the elution position of free synuclein was integrated and related to the amount of protein obtained in the absence of SUV (Table 1).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

aS	α -synuclein
bS	β -synuclein
LPPG	lyso-1-palmitoylphosphatidylglycerol
LUV	large unilamellar vesicle
MTSL	(1-Oxyl-2,2,5,5-tetramethyl-3-pyrroline-3-methyl) methanethiosulfonate
NMR	nuclear magnetic resonance
SaS	shuffled α -synuclein

SDS	sodium dodecyl sulfate
SLAS	sodium lauroyl sarcosinate
SUV	small unilamellar vesicle

Fig. 1.

Amino acid sequence and pseudorepeat arrangement of wild-type α -synuclein (aS), shuffled α -synuclein (SaS), and β -synuclein (bS). (a-b) For aS and SaS, helical models exhibiting 3.67 residues per turn ($\phi = -58.4^\circ$ and $\psi = -48.5^\circ$) illustrate the effects of rearranging the pseudorepeats upon the orientation of C^α - C^β vectors of the colored residues, which line the hydrophobic helix face of wild-type aS. There are 15 positions in SaS that exhibit changed residue types on the hydrophobic face compared to aS, shown boxed in *magenta*, and 16 positions whose swap leaves the residue type unchanged. The interchange of repeats III and VI also changes the orientation of two “snorkeling” lysine residues.^{39,50} A star marks spin-labeled residue 87 in repeat VII. (c) Amino acid differences between aS and bS are highlighted in *magenta*. In addition to the elimination of the 11-residue stretch depicted, the amino acid substitutions result in the loss of six β -branched residues and gain of one such residue compared to aS.

Fig. 2. Comparison of synuclein variant β -sheet propensities. The tabulated β -sheet propensity of each amino acid residue⁷⁵ is averaged over a nine-residue segment and denoted by its center residue. Segments are shifted along the sequence by one-residue increments.

Fig. 3.

Comparison of aS, bS and SaS misfolding. (a) Fibrillization kinetics of α -synuclein (aS), shuffled α -synuclein (SaS) and β -synuclein (bS) detected by thioflavin T fluorescence.⁷⁶ The initial lag in aS fibrillization corresponds to the nucleation phase, and the exponential part to fibril growth. (b) Morphology of the obtained aggregates after 120 h visualized by electron microscopy. (c) Soluble dimeric oligomers are detectable for aS, but not for bS and SaS, by Western blots following SDS-PAGE. The Syn202 antibody (Abcam Inc.) recognizes residues 130-140 in aS, bS and SaS. Rat aS residues 15-123 represent the immunogen for the anti-aS antibody (BD Biosciences Inc.), which recognizes aS and SaS, but not bS. (d) Detection of soluble aggregates using the A11 antibody reactive to a universal conformation-dependent structure of soluble oligomers.⁴⁷ Positive reactivity was observed for aS and SaS, but not for bS, at all times, even at zero hours (100 kDa filtered samples). Shown is a representative dot blot of the samples at 24 h.

Fig. 4.

Comparison of structural ensemble size and secondary structure propensities of unfolded aS and SaS. (a) ^1H - ^{15}N HSQC signal intensity ratios in the presence and absence, I/I_0 , of an MTSL paramagnetic label at residue 87 are compared for aS and SaS, respectively. The largest I/I_0 value in each broadening pattern has been set to 1 and the remaining values have been scaled linearly. (b) Comparison of secondary $^{13}\text{C}\alpha$ chemical shifts between aS and SaS.

Secondary $^{13}\text{C}\alpha$ chemical shifts, defined as the difference between the observed and tabulated random-coil $^{13}\text{C}\alpha$ shift of a residue, correlate well with backbone torsion angles.^{48,49} The tail residue (101-140) chemical shifts are indistinguishable and have been omitted for clarity. The SaS shifts are depicted in the sequence numbering of aS (Fig. 1a) to facilitate a comparison between the two proteins. The repeat numbers for aS and SaS are noted in *black* and *red* Roman numerals, respectively. Depicted secondary $^{13}\text{C}\alpha$ chemical shifts have not been corrected for the $\sim +0.5$ ppm isotope shift that resulted from perdeuteration of the carbon-attached hydrogens.⁷⁷ Taking this offset into account would result in mostly positive secondary $^{13}\text{C}\alpha$ chemical shifts. Shifts were measured by TROSY-HNCA experiments with a maximum evolution time of 27.2 ms employing a constant-time scheme. Measurements were carried out twice for each SaS and aS using independently prepared samples. Representative error bars are shown that depict the maximal shift difference from the mean chemical shift. For both SaS and aS an average error of 3 ppb was obtained.

Fig. 5.

Micelle properties of sodium lauroyl sarcosinate (SLAS). (a) Translational self-diffusion constant, D_s , of SLAS as a function of weight fraction (*open circles*) obtained from BPP-LED experiments.⁷⁰ Linearization of D_s to yield the self-diffusion coefficients of SLAS micelles alone, D_m ,⁷⁰ (*solid circles*) is optimal for a free SLAS concentration of 5 mM (c.f. Materials and Methods). Extrapolation of the concentration dependence of D_m to infinite dilution yields a micellar diffusion constant, D_0 , of $8.15 \times 10^{-11} \text{ m}^2 \text{ s}^{-1}$. Relative to lysozyme ($D_0 = 11.3 \times 10^{-11} \text{ m}^2 \text{ s}^{-1}$), an unhydrated micelle radius of 22.2 Å is thus obtained, which corresponds to an average micelle mass of 30.6 kDa and an aggregation number of 104. (b) aS-SLAS binding stoichiometry. ^1H chemical shift changes of Lys10 and Gly41 as a function of molar aS:SLAS ratio. A starting aS concentration of 0.1 mM was employed. (c) TROSY-HSQC spectrum of 0.75 mM $^2\text{H}/^{13}\text{C}/^{15}\text{N}$ -labeled aS at an aS:SLAS molar ratio of 1:100 in 25 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, pH 7.4, 0.02% NaN_3 solution at 25 °C, and a ^1H frequency of 700 MHz. Well-resolved resonances are labeled. SaS and bS spectra are of comparable quality.

Fig. 6.

Backbone structural and dynamic parameters of aS, bS and SaS. (a) Agreement between residual dipolar couplings (N-H, C'-C $^{\alpha}$, C'-N) and the effective average backbone conformation obtained by molecular fragment replacement⁵⁵ expressed by the q-factor. The q-factor was computed for backbone segments of seven residues shifted along the synuclein repeat region by one-residue increments. Segment numbers denote the central residue number of each segment. The q-factor scales between 0 and 1, where a value of 0 indicates perfect agreement between observed and calculated dipolar couplings, and a completely random structure yields a value of 1.⁵² Average backbone conformations were obtained by molecular fragment replacement⁵⁵ using N-H, C'-C $^{\alpha}$, C'-N residual dipolar couplings and N, C $^{\alpha}$, C $^{\beta}$, C'

chemical shifts, which renders the q-factor a measure of the ability to fit the residual dipolar coupling to a single conformation (structural homogeneity). A q-factor calculation using ideal helix coordinates (Fig. 1a-b) yields analogous results (data not shown). For bS, q-values >1 are obtained for segments 30-35 arising from dipolar couplings being close to zero (Fig. 7a) and close to the measurement error. (b,d,e) The $\{^1\text{H}\}$ - ^{15}N NOE value of each residue, which is sensitive to H-N bond vector fluctuations on the pico- to nanosecond timescale, is depicted to compare backbone order⁵³ for SaS, aS and bS. For comparison, SaS data is shown in consecutive residue numbering (Fig. 1b) as well as in aS numbering (Fig. 1a). (c,f) Comparison of secondary $^{13}\text{C}^\alpha$ chemical shifts between SLAS-bound aS and SaS. Secondary $^{13}\text{C}^\alpha$ chemical shifts, defined as the difference between the observed and tabulated random-coil $^{13}\text{C}^\alpha$ shift of a residue, correlate well with backbone torsion angles.^{48,49} The tail residue (106-140) chemical shifts are indistinguishable and have been omitted for clarity. Depicted secondary $^{13}\text{C}^\alpha$ chemical shifts have not been corrected for the $\sim +0.5$ ppm isotope shift that resulted from perdeuteration of the carbon-attached hydrogens.⁷⁷ For comparison, SaS data is shown in consecutive residue numbering (Fig. 1b) as well as in aS numbering (Fig. 1a).

Fig. 7.

Intermediate timescale aS, bS and SaS backbone dynamics and micelle-bound SaS structural model. (a) Alignment tensor magnitudes, D_a , for backbone segments of seven residues shifted along the synuclein repeat region by one-residue increments. Segment numbers denote the central residue number of each segment. D_a values were computed from N-H, C'-C $^\alpha$, C'-N residual dipolar couplings and the effective average backbone conformation obtained from molecular fragment replacement.⁵⁵ Within a 13% variation in ^2H quadrupolar splittings, the same alignment conditions were achieved for aS, SaS and bS, allowing a direct comparison of D_a values between the synuclein variants. (b) Illustration of SaS backbone plasticity. Backbone torsion angles compatible with the range of backbone fragments obtained by molecular fragment replacement⁵⁵ allow a wide range of helix curvatures. Shown are five structures with residues 2-90 in helical conformations, which were aligned on residues 74-81. Although the viewing angle emphasizes spatial fluctuations of backbone segments along the helix axis, fluctuations perpendicular to the helix axis, i.e., on the micelle surface, are equally populated. The size of an unhydrated SLAS micelle is shown for comparison in spherical and spheroid shapes. The spheroids exhibit axes ratios of 1:2 and 1:3, respectively, with volumes identical to a sphere of 44.4 Å diameter, corresponding to a free SLAS micelle (Fig. 5a).

Fig. 8.

Effect of threonine side chain rotameric state on α S backbone conformation. The curvature of the EPR-derived vesicle-bound α S structure³⁵ (*red*) is compared to a helical model of the α S amphiphilic region (*blue*), where ϕ and ψ torsion angles are set to -58.4° and -48.5° , respectively, for all residues except five threonines. The ϕ and ψ angles for threonines at positions 22, 33, 44, 59 and 81, which line the hydrophobic membrane face (Fig. 1a), were adjusted by $\Delta\phi = -6.5^\circ$ and $\Delta\psi = +6.2^\circ$.⁶⁷ This results in similar radii of helix curvatures of 177 and 167 Å for the vesicle-bound and modeled α S structures, respectively. The helix faces do not fully align along the elongated helix, but this could easily be corrected by making minor adjustments to the helix twist of a few residues of the modeled helix. The average numbers of residues per turn are 3.67 ± 0.12 and 3.67 ± 0.03 for the vesicle-bound and modeled α S structures, respectively.

Table 1

Vesicle separable fractions of examined synuclein variants

	SUV separable fraction [%] ^a buffer 1 ^b	SUV separable fraction [%] ^a buffer 2 ^c	LUV separable fraction [%] ^a buffer 1	LUV separable fraction [%] ^a buffer 2
aS	19.2	28.4	90.4	100
SaS	42.8	60.5	93.7	100
bS	60.1	73.7	100	100

^a Fraction of total protein detected as eluate in the absence of SUV/LUV.^b Buffer 1 was 25 mM KH₂PO₄/K₂HPO₄, pH 7.4, 100 mM KCl.^c Buffer 2 was 50 mM Tris-HCl, pH 7.4, 100 mM NaCl.