Remodeling of Lipid Vesicles into Cylindrical Micelles by α-Synuclein in an Extended α-Helical Conformation

Received for publication, March 27, 2012, and in revised form, June 22, 2012. Published, JBC Papers in Press, July 5, 2012, DOI 10.1074/jbc.M112.365817

Naoko Mizuno1,2, Jobin Varkey3, Natalie C. Kegulian4, Balachandra G. Hegde4, Naiqian Cheng4, Ralf Langen5,6, and Alasdair C. Steven4

From the 1Laboratory of Structural Biology, NIAMS, National Institutes of Health, Bethesda, Maryland 20892 and the 5Zilkha Neurogenetic Institute, University of Southern California, Los Angeles, California 90033

Background: Membrane fusion and fission events are effected by remodeling proteins.

Results: Using cryoelectron microscopy, we observed the conversion of large spherical lipid vesicles into narrow protein-coated tubes.

Conclusion: Tubulation is accompanied by α-synuclein switching into an extended α-helical conformation.

Significance: The cylindrical micelles produced resemble a hemi-fission/fusion state of the membrane.

α-Synuclein (αS) is a protein with multiple conformations and interactions. Natively unfolded in solution, αS accumulates as amyloid in neurological tissue in Parkinson disease and interacts with membranes under both physiological and pathological conditions. Here, we used cryoelectron microscopy in conjunction with electron paramagnetic resonance (EPR) and other techniques to characterize the ability of αS to remodel vesicles. At molar ratios of 1:5 to 1:40 for protein/lipid (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol), large spherical vesicles are converted into cylindrical micelles ~50 Å in diameter. Other lipids of the same charge (negative) exhibit generally similar behavior, although bilayer tubes of 150–500 Å in width are also produced, depending on the lipid acyl chains. At higher protein/lipid ratios, discoid particles, 70–100 Å across, are formed. EPR data show that, on cylindrical micelles, αS adopts an extended amphipathic α-helical conformation, with its long axis aligned with the tube axis. The observed geometrical relationship between αS and the micelle suggests that the wedging of its long α-helix into the outer leaflet of a membrane may cause curvature and an anisotropic partitioning of lipids, leading to tube formation.

α-Synuclein (αS)6 is a protein that localizes at neural terminals (1–3). αS is abundant in neural cells, accounting for up to 1% of total protein with estimated concentrations in the hundreds of micromolar range (4–7). Its function(s) has not been precisely defined, but mutations in αS and duplication/triplication of the αS gene have been linked to familial forms of Parkinson disease (8–11). Also, elevated levels of αS cause neurodegeneration in animal models (12–15), reproducing the cytotoxic effects of αS that lead to Parkinson disease in humans. In the brains of Parkinson disease patients, amyloid deposits of αS accumulate in Lewy bodies (16, 17), giving a hallmark of this pathology.

A relationship of αS to neural membrane trafficking has been indicated (18). The majority of αS is found at synaptic terminals (4, 5), where it is seemingly involved in the control of synaptic vesicle formation and maintenance (5, 19). An RNAi screen in Caenorhabditis elegans found 10 genes modifying αS neurotoxicity, four of which turned out to be related to the synaptic endocytosis pathway (20). Synapses of mice knocked out for αS showed diminished levels of synaptic vesicles in the reserve pool (2), whereas overexpression of αS is reported to inhibit re-clustering of synaptic vesicles, thereby reducing neurotransmitter release (15). These and other reports (21–23) suggest that αS interacts directly with membranes to control cycles of synaptic vesicle release.

αS is also involved in defects of other membrane trafficking pathways, with cytotoxic consequences. Overexpression of αS causes disruption of endoplasmic reticulum-Golgi trafficking (14), fragmentation of the Golgi apparatus (24, 25), and distortion of mitochondrial membranes (26), causing them to fission (27). Taken together, these reports suggest that αS interacts widely with multiple organelles, but in all cases, these interactions involve membranes. Thus, it is of fundamental importance to understand the mechanism(s) of αS-membrane interactions.

A number of in vitro studies have found the formation of αS to be highly adaptable. It has long been known that αS is an intrinsically disordered protein in solution (28). This view has recently been challenged by suggesting that the protein exists as...
a natively folded tetramer (30, 31), but further experimentation, including in-cell NMR (29, 72), indicate that αS is an unfolded monomer in vivo. Upon long incubation, it passes through toxic oligomeric states to reach an amyloid conformation (32), rich in parallel in-register cross-β structure (33, 34), which is considered to be its state in Lewy body accumulations. However, in the presence of negatively charged membranes (35), αS adopts an α-helical conformation (36). Its amino acid sequence contains seven 11-residue repeats that are predicted to form amphipathic α-helices that mediate its interaction with membranes; in this respect, it is reminiscent of apolipoprotein (37). Further study of the latter structures detected two forms, depending on the experimental conditions and lipids used, viz. 1) an extended helical form (38–40) like a curved rod; and 2) a “horseshoe”-like form with broken helices that close to hairpins on small vesicles (39–45) and SDS micelles (41, 46, 47). Moreover, these two conformations are reported to co-exist (48, 49) in the same sample preparation, and under some circumstances, more than half of the membrane-interacting region of αS can remain unfolded (50).

Overall, these data suggest that the different membrane-bound forms of αS are energetically similar. Nevertheless, the state of the membrane has been found to correlate with particular structures. EPR spectroscopy revealed that the extended conformation predominates when αS binds to small unilamellar vesicles (SUVs). In this extended conformation, the molecule is further twisted into a right-handed super-coil akin to a coiled-coil structure (39), and the interacted vesicle remain intact. In contrast, horseshoe-like structures are formed when αS binds to vesicles as they are converted into smaller nonvesicular structures (39).

In the presence of large negatively charged vesicles, αS has been observed to remodel vesicles into tubes (51, 52). Circular dichroism (CD) data have shown that this remodeling event is accompanied by a conformational change in αS from random coil to α-helix (51). However, the underlying structural change of αS and the tubulation mechanism has not been established. In this study, we used cryo-EM, CD, and EPR spectroscopy in combination with more closely investigated αS-induced tubulation of vesicles.

**EXPERIMENTAL PROCEDURES**

**Purification of Wild-type and Mutant α-Synuclein—**αS was prepared as described (51). In brief, wild-type and mutant human αS were expressed in *Escherichia coli* BL21 (DE3) pLysS cells, which were lysed by boiling, followed by acid precipitation. The supernatant was passed through anion exchange columns and eluted with a 0–1.0 M NaCl gradient.

Lipids purchased from Avanti Polar Lipids Inc. (Alabaster, AL) as a solution in chloroform were transferred to a glass tube, and the chloroform was removed by blowing N₂ gas. After the lipids were further dried under vacuum in a desiccator for at least 6 h, buffer (20 mM Hepes, pH 7.4, 100 mM NaCl) was added, and the mixture was immediately used in experiments. Preparation and incubation of lipids with αS were performed above 32 °C to avoid any effects from phase transitions. Nevertheless, consistent results were obtained in all cases with experiments performed at room temperature.

**Phospholipid Vesicle Clearance Assay—**Light scattering was measured as a function of time using a Jasco V-550 UV-visible spectrophotometer to monitor the interaction of αS with large lipid vesicles. A monitoring wavelength of 500 nm was used with a slit width of 2 nm and medium response time. Briefly, lipid vesicles were suspended in 20 mM Hepes (pH 7.4) with 100 mM NaCl at a final volume of 500 µl in a quartz cuvette. Control vesicles (no protein added) did not show any change in light scattering.

**Circular Dichroism (CD)—**All spectra were recorded using a Jasco J-810 spectropolarimeter with a 1-mm quartz cell. A scan rate of 50 nm/min, bandwidth of 1 nm, 0.1 nm time response, and step resolution of 0.5 nm were set for all experiments. For time course experiments, the parameters were set at a 1-s data pitch, 4-s response time, and 1 nm bandwidth. Protein concentration was determined using the extinction coefficient at 280 nm based on the number of Trp and Tyr residues in the protein. Appropriate blanks were collected under similar conditions and subtracted to obtain the final spectra. 10 mM sodium phosphate buffer (pH 7.4) was used in all CD experiments.

**Cryoelectron Microscopy—**5-µl drops were applied to holey carbon grids (Quantifoil) and vitrified in a Vitrobot cryo-station (FEI). The humidity was carefully controlled to avoid drying-related deformation of membranes, and the temperature was set to be above the phase transition temperature (Tₘ) of individual lipids. Specimens were observed with a CM200-FEG electron microscope at nominal magnifications of 38,000 and 66,000, with defocus settings in the range of −1 to −4 µm. Film (SO163, Eastman Kodak Co.) was used for recording images, and digitization was done with a SCAI scanner (Carl Zeiss) at rates of 1.84 or 0.966 Å/pixel.

**Image Analysis—**The contrast transfer function was partially corrected by phase-flipping. For averaging experiments, relatively straight tubes were selected, computationally straightened, and cut into segments using a box size of 270 Å. Image segments were binned 2-fold to increase the speed of the computation. For the analysis of αS-POPG at different protein-to-lipid ratios, stacks of 1787 (1:40 data), 1290 (1:20 data), and 1216 (1:10 data) segments were compiled for classification and averaging. For the analysis of αS bound to various lipids, stacks of 4932 (DMPC), 3325 (POPG), 530 (DOPG), 944 (DLPG), and 111 (DAPG) segments were compiled for analysis of cylindrical micelles and 932 (DOPG), 552 (DLPG), and 359 (DAPG) segments for bilayer tubes.

Reference-free classification (k means) and averaging were performed using SPIDER (53) and EMAN (54). Principal component analysis was also applied for further classification of the aligned images. For the cylindrical micelles, we did not see evident morphological distinctions among averaged images, post-classification, and therefore we chose to display the average of the majority class from each experimental condition.

**Fluorescence Measurements—**Desiccated N,N’-dimethyl-N-(iodoacetyl)-N’-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylendiamine (IANBD) was obtained in amide form from Molecular Probes (Eugene, OR), kept at −20 °C in the dark, and dissolved in DMSO prior to use. αS cysteine mutants were stored in 5 mM dithiothreitol (DTT). Immediately before labeling, each mutant was desalted in a PD-10 column (GE Healthcare) into 20 mM
Remodeling of Lipid Vesicles into Cylindrical Micelles by αS

αS Converts Vesicles into Cylindrical Micelles— αS was added to preformed large spherical POPG vesicles at various molar ratios of protein/lipid in the range of 1:40 to 1:10, and the optical absorbance was monitored at 500 nm. An immediate fall-off was observed in each case (supplemental Fig. S1), indicating that the vesicles were breaking down into smaller entities. CD measurements performed in parallel showed that the conformation of αS was switching from random coil to α-helix (Fig. 3B).

To visualize the accompanying structural changes, samples were vitrified and observed by cryo-EM (Fig. 1). At the lowest αS/lipid ratio (1:40), we found relatively short segments of narrow tubes, opening out into irregular forms at their ends (Fig. 1A, red asterisks). The diameter of the narrow segments (~50 Å) shows them to be cylindrical micelles (see below). Complexes of this kind, often tangled together, covered most fields (supplemental Fig. S2). On increasing the protein content, we observed longer, continuously thin, cylindrical micelles (Fig. 1, B and C). On further increasing the αS/lipid ratio to 1:5, tubes became rarer and were replaced by small discoid particles 70–100 Å across (Fig. 1D).

To visualize the cylindrical micelles in greater detail, we first cut the images into 270-Å segments (this procedure minimizes the effects of curvature on the ensuing analysis). We then aligned them and classified them computationally to obtain relatively homogeneous sets (Fig. 2). The segments were then averaged in each class to reduce the noise level. Tubes formed at each of three αS/lipid ratios were analyzed separately. In each case, the averaged images show two peak densities, on either side of the tube. Some class averages show slight bends (e.g. Fig. 2, 3rd panel, top row and 4th panel, middle row), however, the main source of variability is in width. The thinnest tubes (48–54 Å) were formed at all three protein/lipid ratios, but there is a greater prevalence of slightly wider tubes at ratios of 1:20 and 1:40 (widths summarized in Fig. 2 legend were mea-
Remodeling of Lipid Vesicles into Cylindrical Micelles by αS

Cryo-EM observations of structures produced upon adding increasing amounts of αS to a fixed amount (400 μM) of POPG membranes, viz. A, 10 μM (1:40); B, 20 μM (1:20); C, 40 μM (1:10); D, 80 μM (1:5). A, flattened membranes are seen (two examples are marked with red asterisks). In that experiment, images were taken with higher defocus to boost contrast. Some small discoid particles are marked in D with red arrowheads. Contaminating ice particles (white arrowheads) are denser and more sharply defined.

![Image 1](https://example.com/image1)

**FIGURE 2.** Selected class average images of the tubes formed at 1:40, 1:20, and 1:10 ratios of protein/lipid (POPG). The widths of the averaged tubes are as follows: 54, 54, 62, 67, and 75 Å (average 62 Å, S.D. 9.1 Å) for 1:40 (protein/lipid); 54, 53, 56, 58, and 58 Å (average 56 Å, S.D. 2.3 Å) for 1:20 (protein/lipid); and 50, 50, 52, 48, and 51 Å (left to right, average 50 Å, S.D. 1.6 Å) for 1:10 (protein/lipid).

The average width of all the remodeled tubes measured in the 1:40 experiment was 62 Å (S.D. 9.1 Å). The corresponding values were 56 ± 2.3 Å for the 1:20 experiment and 50 ± 1.6 Å for the 1:10 experiment. We take the two peaks to mark the positions of the lipid headgroups, in which case the observed dimensions are consistent only with the tubes being micellar (a single lipid leaflet is ~20 Å thick (58)). The density peaks should also reflect, to some extent, protein bound to the outer surface. However, no definite protein-associated pattern is seen, and it appears that signal from the protein is smeared out and affects the observed density profiles primarily by damping the white interference fringes along the sides of the tubes. These fringes result from phase-contrast imaging. Moreover, the inferred α-helices are only about 10 Å thick and are likely to be partially submerged into the lipid layer (see EPR data below and "Discussion").

**FIGURE 3.** Phospholipid vesicle clearance by αS. A, the clearance of vesicles (large nonextruded vesicles) in the presence of increasing amounts of αS was monitored by recording the absorbance at 500 nm. DMPG, DOPG, DLPG, and DAPG vesicles (600 μM) incubated with 1.5, 30, and 60 μM of αS are shown with the blue, red, and green traces, respectively. B, secondary structure of αS changes on interacting with vesicles. Circular dichroism was used to distinguish whether the observed vesicle clearing effect was accompanied by an increase in αS α-helicity. αS (30 μM) was incubated for 5 min with vesicles. A 1:20 protein-to-lipid molar ratio was used in all cases. αS alone is shown in black; αS with DMPG vesicles is shown in red; αS with POPG vesicles is shown in green. The percentage helicity of αS was obtained by fitting the spectra with the program K2D2, yielding numbers in the range of 70–80%. C, mean residue ellipticity at 222 nm is plotted as a function of time. A protein-to-lipid ratio of 1:20 was used. αS with DMPG vesicles are shown in red; POPG vesicles are shown in green; DOPG vesicles are shown in purple; DLPG vesicles are shown in blue; and DAPG vesicles are shown in orange. The spectral change at 222 nm is indicative of α-helix formation within the first 5 min.

αS-induced Membrane Remodeling Occurs with Lipids with Various Acyl Chains—The central core of a cylindrical micelle, seen in the density profiles as the part between the two peaks, is occupied by the acyl chains. Based on simple geometric considerations, short and saturated acyl chains should be more amenable to micellar tube formation, although longer and bulkier acyl chains might favor the formation of bilayer tubes. To test this hypothesis, we investigated the interaction of phosphoglycerol lipids with various acyl chains, ranging from the short chain 1,2-dimyrstoyl-sn-glycero-3-phospho-(1'-rac-glycerol) to (DMPG) to the long chain and polyunsaturated 1,2-diaraclidonoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (DAPG) (supplemental Fig. S3).

As with POPG vesicles, αS reduced the light scattering from vesicles of various other phosphoglycerol lipids within minutes of addition and did so in a concentration-dependent manner (Fig. 3), again reporting the conversion of large vesicles into smaller entities. The most rapid remodeling was observed for DMPG vesicles, where the light scattering fell precipitously within a few seconds (Fig. 3A). These remodeling events were...
accompanied by αS switching to an α-helical conformation (Fig. 3, B and C).

**Acyl Chain Bulkiness Affects Tube Morphology**—To compare the morphological changes in membranes with different acyl chains, we observed tubes formed at a fixed protein/lipid ratio (1:20) by cryo-EM (Fig. 4, B–F). Two types of tubes were observed as follows: cylindrical micelles and wider tubes, with varying diameters. It is apparent, both from the images directly and from averaged density profiles, that the wider tubes are tubular bilayer membranes (Fig. 5, bottom row). The ratio of micellar versus bilayer tubes correlated with acyl chain size. In the case of DMPG, which contains the shortest acyl chains (14 carbons), we observed exclusively cylindrical micelles. As the bulkiness of the acyl chains (i.e. chain length and degree of unsaturation) increased, more bilayer tubes were observed (72% with DAPG). The averaged images for the major classes of cylindrical micelles obtained with the respective lipids yielded widths of 60 ± 1.3 Å for DMPG, 56 ± 2.3 Å for POPG, 44 ± 1.9 Å for DLPG, 52 ± 2.9 Å for DOPG, and 40 ± 0.8 Å for DAPG (Fig. 5). The thicknesses of the membranes of the bilayer tubes, measured in similar fashion, were 48 ± 2.0 Å for DLPG; 49 ± 1.0 Å for DOPG; and 46 ± 1.6 Å for DAPG. With DMPG and POPG, no bilayer tubes were observed.

It is not clear why there are differences in tube widths for different kinds of lipids; nevertheless, in general, the width of bilayer tubes increased for lipids containing more unsaturated acyl chains. This trend led even to ameba-like shapes on adding DAPG (Fig. 4F), which has eight double bonds in
its acyl chains (supplemental Fig. S3). We also performed some preliminary experiments with other anionic lipid compositions and observed similar effects (supplemental Fig. S4).

αS Switches to an α-Helical Conformation on the Membrane Surface—Next, we addressed the conformational change in αS that accompanies vesicle tubulation. The CD spectra indicate that αS increases in α-helicity (Fig. 3, B and C). To further characterize this transition, we performed fluorescence spectroscopy. αS was labeled with the polarity-sensitive IANBD at various positions along its amino acid sequence. In all cases, the fluorescence was generally low in solution (for example, residue 31, Fig. 6A, black dotted line). Upon exposure to membranes, the fluorescence of all N-terminal sites (residues 31, 48, 52, 70, and 76) increased markedly (Fig. 6A), suggesting that this region interacts directly with the membrane. In contrast, two C-terminally labeled derivatives (at positions 124 and 136) did not show any detectable change in fluorescence, implying that the C-terminal region does not interact with the membrane. A minor change was detected when residue 100 was labeled, suggesting that the end of the membrane-interacting region is close to this position. These data are consistent with the structure of αS in its vesicle-bound form, where the N-terminal but not the C-terminal regions interact with the membrane (39).

αS Adopts an Extended α-Helical Conformation—The N-terminal region of αS harbors seven 11-residue repeats and previous studies of vesicle-bound αS demonstrated that each repeat forms three α-helical turns (39). Within a given repeat, positions I, V, and IX were found to face away from the membrane surface, whereas positions III, VII and XI face into its interior (Fig. 6B). To further investigate the conformational change of αS and possible similarities between tubule-bound and vesicle-bound αS, we employed site-directed spin labeling together with continuous wave-EPR spectroscopy and R1-spin labeling of the residues shown in Fig. 6B. The respective X-band EPR spectra indicate a transition from a highly dynamic structure in solution to an ordered structure on the tubulated membrane, as with vesicle-bound αS (supplemental Fig. S5). The spectra are missing strongly immobilized components, indicating the lack of tertiary or quaternary contacts. These spectra are completely different from those produced by the amyloid fibrillar form of αS in which a cross-β conformation is adopted (32, 34).

Next, we performed accessibility measurements using the paramagnetic colliders NiEDDA ([NiEDDA]) and O2 ([O2]). Accessibility to the more hydrophobic O2 molecule is strongly enhanced in lipid phases, although NiEDDA preferentially partitions into the aqueous phase. Using these reagents, we calculated the depth parameter ϕ (ln([O2]/[NiEDDA])), an established measure of membrane immersion depth (59). ϕ-values were plotted for each residue in both tube-bound and vesicle-bound situations (Fig. 6C). First, we observed a clear positive correlation in ϕ-values between tube-bound and vesicle-bound
Remodeling of Lipid Vesicles into Cylindrical Micelles by αS

αS. This means that the two conformations are nearly identical. Second, the $\phi$-values can be grouped into two sets (green and red circles in Fig. 6C). Residues 31 and 76 in the green circles in Fig. 6C have reduced $\phi$-values, located in positions IX, V, and I on the helical wheel (Fig. 6B, colored in green) on the solvent-exposed surface. Residues 37, 41, 44, 48, 52, and 70 have larger $\phi$-values (Fig. 6C, red circle) and are membrane-inserted. Of particular note are residues 41 and 44 that are $\alpha$-helical in the extended conformation but are part of an inter-helical loop in the broken conformation (47). The former conformation is assumed when αS binds to vesicles and the helix breaks into a horseshoe shape upon exposure to small amounts of SDS. These results indicate that on tubular membranes αS assumes an extended, amphipathic, and $\alpha$-helical conformation, as in its vesicle-bound mode.

To further test the way in which αS takes up an extended structure on tubes, we measured the distance between two spin-labeled sites by 4-pulse DEER experiments. Three key distances were measured between residues 11R1/26R1, 22R1/52R1, and 63R1/81R1 (Fig. 7A). Fits of the time evolution data (Fig. 7B), performed using Tikhonov regularization (56, 57), yielded distance distributions consistent with those expected for an extended helical structure. To confirm the robustness of these distance distributions, we also used Gaussian fits. The peaks moved only by small amounts (Fig. 7, legend), attesting to their robustness. Of particular note, the 22R1–52R1 spacing

![Image](https://via.placeholder.com/150)

FIGURE 6. Fluorescence spectroscopy and continuous wave-EPR analysis of singly labeled αS derivatives indicate the formation of an ordered and continuous helical structure. A, normalized fluorescence spectra for 15 μM IANBD-labeled αS and 300 μM POPG vesicles. Black dotted line, αS-31C-IANBD fluorescence before addition of lipid. All other lines represent αS-IANBD fluorescence –1 h after addition of POPG: blue broken line, αS-136C-IANBD; red dotted line, αS-124C-IANBD; green dashed line, αS-100C-IANBD; orange solid line, αS-76C-IANBD; blue solid line, αS-70C-IANBD; red solid line, αS-31C-IANBD; green solid line, αS-48C-IANBD. Green circles show the solvent-exposed residues, and red circles show lipid-exposed residues. B, green solid line depicts the positions of the cysteine mutants that were tested for membrane accessibility using continuous wave-EPR. The roman numerals mark the positions of amino acids in the 11-amino acid repeat. Green circles show the solvent-exposed residues, and red circles show lipid-exposed residues. C, $\varphi$ on small unilamellar vesicles (SUVs) plotted against $\psi$ on tubes. The ratios of the accessibilities to O2 and NiEDDA for residues are expressed by the depth parameter $\varphi = \ln(I_{O2}/I_{NiEDDA})$, with increasing values indicating deeper membrane immersion.

![Image](https://via.placeholder.com/150)

FIGURE 7. Spin-label intramolecular distances from 4-pulse DEER experiments. A, positions of double mutants in a bent helix (SDS-bound structure of αS) versus extended helix (SUV-bound structure of αS). B, 1st column depicts the dipolar evolution for each of the indicated spin label pairs. 1st column, black traces denote background-corrected experimental data and the red curves depict fits made using Tikhonov regularizations. 2nd column shows the resulting inter-molecular distance distributions. To optimize signal-to-noise ratios, short acquisition times were used for longer distances, and long acquisition times were used for shorter distances for which dipolar evolutions are of lower frequency. For shorter distance scans, the base-line correction was verified by scans of longer time base. Because of significantly better signal-to-noise ratios, however, shorter scans were used to carry out Tikhonov regularization. The increased uncertainty arising from shortened time scans was compensated by enhanced signal-to-noise ratios. Because the spectra were somewhat noisy, we also calculated the distance distributions by Gaussian regularization (data not shown) and found that the peaks moved only by small amounts, from 26 to 27 Å, 50 to 45 Å, and 28 to 27 Å, respectively. C, comparison of experimental spin label inter-electron distances obtained for αS bound to tubes with an ideal helix, αS bound to an SUV, SDS micelle, or SLAS micelle.

AUGUST 24, 2012•VOLUME 287•NUMBER 35 JOURNAL OF BIOLOGICAL CHEMISTRY 29307
was determined to be 45–50 Å. The corresponding distance was measured to be 23 Å for the broken helix (Fig. 7C, SDS/SLAS) and 49 Å for the extended helix (Fig. 7C, ideal helix). These distances also showed consistency between the tube-bound αS and the vesicle-bound αS (Fig. 7C) (39). In sum, these data strongly indicate that αS forms an extended helical structure on tubes, like that of vesicle-bound αS. Similar results were obtained for all phosphoglycerol tubes that we tested (other data not shown).

**DISCUSSION**

Previously, we showed that when large POPG vesicles are exposed to αS, the protein adopts an α-helical formation as it remodels the vesicles into tubes (51). This study characterizes the tubulation reaction further. We have found with protein/lipid ratios between 1:10 and 1:40 that most tubes produced are cylindrical micelles, a membrane topology previously described for lipid/detergent mixtures (60–62) and for some lipoprotein tubules induced by the endocytic protein endophilin (63). The cylindrical micelles cannot be mistaken for amyloid αS fibrils because of the following. 1) Cryo-EM shows them to have a low density core, unlike αS amyloid fibrils (see Fig. 1A in Ref. 33). 2) They are observed to diverge continuously into wider tubes (Fig. 4E), again unlike amyloid fibrils. 3) Tubes form within a few seconds of incubation whereas amyloid fibrillation takes many hours. 4) Our CD and EPR experiments reveal the presence of α-helix as opposed to the β-sheets of amyloid, consistent with the previously reported lack of thioflavin T fluorescence (51). 5) EPR shows the αS to be interacting with a membrane, whereas fibrils are made of αS alone.

With some dependence on the protein-to-lipid ratio used and on the acyl chains of the lipids, we found that the majority species formed in each case was cylindrical micelles. In these assemblies, the lipid is organized in what is often referred to as the “hemifusion” or “hemifission” states. These states have been much discussed but seldom observed directly. Our study shows that, under appropriate conditions, they can be an abundant and stable assembly form.

Although the inferred transition from initial to final state was not captured in detail, we observed hints as to how it may proceed. When specimens were vitrified immediately after mixing αS and POPG vesicles, an initial elongation of vesicles seemed to precede tube formation (supplemental Fig. S6). The observation of bilayer tubes at lower protein/lipid ratios suggests that they could be intermediates in a pathway that leads at higher protein concentration to micellar tubes.

**Anisotropic Interaction of αS along the Tube Surface**—The surface of αS-induced tubes appears rather smooth, even though the protein is present on the tube surface, according to gold labeling EM (51) and the present EPR and fluorescence data. The extended amphipathic structure indicated by the latter data accounts for the difficulty to visualize the protein. The assigned α-helix length is ~140 Å. Given that the cylindrical micelles are only about 50 Å in diameter, the helix should run nearly parallel to the tube axis if it is to remain in continuous contact with the membrane (Fig. 8). A single α-helix generates little contrast in cryo-EM and that of αS may be partly submerged in the lipid layer, providing an explanation for why we do not explicitly visualize coating molecules.

When αS attaches to the membrane surface at the lowest αS concentration used (1:40), we observed some slightly wider tubes with less uniform diameters (Fig. 2, 1:40, 2nd panel). This feature could indicate that the more uniform width of the narrower tubes reflects a denser (saturating) packing of αS molecules on the surface of the tube.

We can estimate the protein/lipid ratio at saturating binding of αS to tubular micelles with partially embedded 140-Å-long α-helices aligned with the tube axis and separated by single rows of lipid molecules. The observed widths of the cylindrical micelles and that of an α-helix (~10 Å) suggest that there would be a maximum of 8–10 α-helices in cross-section. If we allow ~15 lipids per molecular length of αS, we obtain molar ratios in the range of 1:15. Although this estimate awaits experimental testing, it is consistent with our observation that further increasing the content of the lipophilic protein correlates with switching the system toward a different kind of complex (the discoid particles) that is more protein-rich.

**Mechanism of Curvature Induction**—An extended helical structure running almost parallel to the tube axis would provide a plausible mechanism for the induction of membrane curvature. As indicated in Fig. 8, we expect the lipids to be arranged with their acyl chains facing inward into a somewhat crowded interior, so that increasing curvature would push the head groups apart, circumferentially. By running parallel to the axis of the cylindrical micelle, αS would fill this gap between head-
groups and effectively stabilize the curved lipid arrangements. In fact, the extended helical structure would provide a highly anisotropic curvature constraint that would promote the induction of curvature perpendicular to the tubule axis. It remains to be tested whether the great length of the αS helix contributes to the remarkable longevity of the cylindrical micelles, which are stable for days (data not shown), or whether shorter helices might also be able to cause the formation of stable cylindrical micelles.

A number of membrane curvature-inducing proteins, including BAR domain-containing proteins (64–66), insert amphipathic helices into the membrane. However, they often contain additional scaffolding domains, making it more difficult to assign individual contributions to curvature induction. Inasmuch as αS does not present a pre-existing scaffold but folds up as a consequence of its interaction with the membrane, the present data demonstrate that amphipathic helices can be sufficient to induce stable bilayer tubes as well as cylindrical micelles.

Effect of Lipid Bulkiness on Tube Formation—We observed that the propensity for forming micellar or bilayer tubes is modulated by the length and bulkiness of the lipid acyl chains. Although smaller acyl chains might help to reduce crowding at the center of the cylindrical micelles, long and bulky acyl chains might destabilize the cylindrical micelle in favor of other curved structures. This expectation is largely borne out by the data. The overall trend is that short acyl chains, like those of dimer-istoyl (DM) or palmitoyl oleoyl (PO), favor tubulation into cylindrical micelles whereas bulkier acyl chains result in more bilayer tubes. With diarachidonoyl (DA), a very bulky lipid with eight double bonds, the remodeling products were larger and had a more bulbous morphology.

Implications of αS-coated Cylindrical Micelles and Lipoprotein Particles in a Cellular Context—The membrane remodeling phenomena that we observe in vitro take place at relatively high concentrations of the protein principle (αS). For them to be operative in a cell would require some mechanism to achieve the needed local concentrations. However, as αS is an abundant protein accounting for 1% of total protein mass in neural cells (4, 5), there appear to be realistic prospects that such is the case. Molecular crowding in the cellular milieu (67) could also promote the reaction. However, further in situ data are needed to clarify the status of this hypothesis.

As yet, there has been no demonstration that αS-coated cylindrical micelles are formed in situ, although they form readily and persist in vitro. However, there have been reports of thin filamentous structures in neural tissues of humans and a mouse model that are αS-positive according to immuno-gold EM with anti-αS antibodies (68, 69). The present findings raise the possibility that they may represent cylindrical micelles instead of or as well as amyloid fibrils.

The main focus of this study was to gain structural and mechanistic insight into the ability of αS to induce curvature in tubular membranes. In the process, we also discovered that at higher protein/lipid ratios, αS induces the formation of discoid particles, 70–100 Å across (Fig. 1D). Whether these structures derive from tubular precursors and what structure αS may assume in them remain to be established. If it is important to maximize contact between the αS molecule and the lipids, a bent or segmented helical structure would be preferred to an extended one (Fig. 8). Regardless of the details, the ability to induce discoid membrane particles could be of physiological relevance. It may also be another property that αS shares with apolipoproteins (70, 71), a family of proteins involved in the transport of lipids whose ability to form similarly sized membrane discs is well established.

REFERENCES

1. Abeliovich, A., Schmitz, Y., Fariñas, I., Choi-Lundberg, D., Ho, W. H., Castillo, P. E., Shinsky, N., Verdugo, J. M., Armanini, M., Ryan, A., Hynes, M., Phillips, H., Sulzer, D., and Rosenthal, A. (2000) Mice lacking α-synuclein display functional deficits in the nigrostriatal dopamine system. Neuron 25, 239–252.

2. Cabin, D. E., Shimazu, K., Murphy, D., Cole, N. B., Gotschalch, W., McIlwain, K. L., Orrison, B., Chen, A., Ellis, C. E., Paylor, R., Lu, B., and Nussbaum, R. L. (2002) Synaptic vesicle depletion correlates with attenuated synaptic responses to prolonged repetitive stimulation in mice lacking α-synuclein. J. Neurosci. 22, 8797–8807.

3. Greten-Harrison, B., Polydoro, M., Morimoto-Tomita, M., Diao, L., Williams, A. M., Nie, E. H., Makani, S., Tian, N., Castillo, P. E., Buchman, V. L., and Chandra, S. S. (2010) αβ synuclein triple knockout mice reveal age-dependent neuronal dysfunction. Proc. Natl. Acad. Sci. U.S.A. 107, 19573–19578.

4. Shibayama-Imazu, T., Okahashi, I., Omata, K., Nakajo, S., Ochiai, H., Nakai, Y., Hama, T., Nakamura, Y., and Nakaya, K. (1993) Cell and tissue distribution and developmental change of neuron-specific 14-kDa protein (phosphonpeuroprotein14). Brain Res. 622, 17–25.

5. Iwai, A., Masliah, E., Yoshimoto, M., Ge, N., Flanagan, L., de Silva, H. A., Kittel, A., and Saitoh, T. (1995) The precursor protein of non-β component of Alzheimer disease amyloid is a presynaptic protein of the central nervous system. Neurobiol. 14, 467–475.

6. Uversky, V. N., Li, J., and Fink, A. L. (2001) Metal-triggered structural transformations, aggregation, and fibrillation of human α-synuclein. A possible molecular NK between Parkinson disease and heavy metal exposure. J. Biol. Chem. 276, 44284–44296.

7. Uversky, V. N., Li, J., Bower, K., and Fink, A. L. (2002) Synergistic effects of pesticides and metals on the fibrillation of α-synuclein. Implications for Parkinson disease. Neurotoxicology 23, 527–536.

8. Polymeropoulos, M. H., Lavedan, C., Leroy, E., Ide, S. E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., Stenroos, E. S., Chandrasekharappa, S., Athanassiadou, A., Papapetropoulos, T., Johnson, W. G., Lazzarini, A. M., Duvoisin, R. C., Di Iorio, G., Golbe, L. I., and Nussbaum, R. L. (1997) Mutation in the α-synuclein gene identified in families with Parkinson disease. Science 276, 2045–2047.

9. Krüger, R., Kuhn, W., Müller, T., Woltall, D., Graebner, M., Kösel, S., Przuntek, H., Epplen, J. T., Schols, L., and Riess, O. (1998) Ala30Pro mutation in the gene encoding α-synuclein in Parkinson disease. Nat. Genet. 18, 106–108.

10. Singleton, A. B., Farrer, M., Johnson, J., Singleton, A., Hague, S., Kacher, D., Sagara, J., Hulihan, M., Poirier, D., O’Dowd, B. M., Duvoisin, R. C., Duvoisin, R. C., Duvoisin, R. C., and du Voisin, R. C., and du Voisin, R. C. (2000) Overexpression of human α-synuclein causes dopamine neuron death in rat primary culture and immortalized mesencephalon-derived cells. Brain Res. 866, 33–43.
Remodeling of Lipid Vesicles into Cylindrical Micelles by αS

14. Cooper, A. A., Gitler, A. D., Cashikar, A., Haynes, C. M., Hill, K. J., Bhullar, B., Liu, K., Xu, K., Strathearn, K. E., Liu, F., Cao, S., Caldwell, K. A., Caldwell, G. A., Marsischky, G., Kolodner, R. D., Labar, I., Rochet, J. C., Bonini, N. M., and Lindquist, S. (2006) α-Synuclein blocks ER-Golgi traffic and Rab1 rescues neuron loss in Parkinson models. Science 313, 324–328

15. Nemani, V. M., Lu, W., BERGE, V., Nakamura, K., ONO, B., Lee, M. K., CHAUDHRY, A. F., NICOLL, R. A., and Edwards, R. H. (2010) Increased expression of α-synuclein reduces neurotransmitter release by inhibiting synaptic vesicle recuitement after endocytosis. Neuron 65, 66–79

16. LEE, F. H. (1912) in Handbuch der Neurologie (Lewandowski, M., ed) pp. 920–933, Springer-Verlag, Berlin, Germany

17. Spillantini, M. G., Schmidt, M. L., Lee, V. M., Trojanowski, J. Q., Jakes, R., and Goedert, M. (1997) α-Synuclein in Lewy bodies. Nature 388, 839–840

18. Outeiro, T. F., and Lindquist, S. (2003) Yeast cells provide insight into α-synuclein biology and pathobiology. Science 302, 1772–1775

19. Jensen, P. H., Nielsen, M. S., Jakes, R., Dotti, C. G., and Goedert, M. (1998) Binding of α-synuclein to brain vesicles is abolished by familial Parkinson disease mutation. J. Biol. Chem. 273, 26292–26294

20. Kuwahara, T., Koyama, A., Koyama, S., Yoshina, S., Ren, C. H., Kato, T., MITANI, S., and IWATSO, T. (2008) A systematic RNAi screen reveals involvement of endocytic pathway in neuronal dysfunction in α-synuclein transgenic C. elegans. Hum. Mol. Genet. 17, 2997–3009

21. Gaugler, M. N., Genc, O., Bobela, W., Mohanna, S., Ardah, M. T., El-haramamaiah, G. M. (1990) Amphipathic helix motif. Classes and proper-ties. Proteins 8, 103–117

22. Jao, C. C., Der-Sarkissian, A., Chen, J., and Langen, R. (2003) Structural analysis of α-synuclein. Insights from site-directed spin labeling EPR studies of membrane-bound α-synuclein. J. Biol. Chem. 278, 32486–32493

23. Jao, C. C., Der-Sarkissian, A., Chen, J., and Huber, M. (2008) Spin label EPR on α-synuclein reveals differences in the membrane binding affinity of the two antiparallel helix conformations of membrane-bound α-synuclein. Mol. Biol. 106, 4080–4082

24. Jao, C. C., Huber, M. (2008) Antiparallel arrangement of the helices of vesicle-bound α-synuclein. J. Am. Chem. Soc. 130, 7796–7797

25. Drescher, M., Godschalk, F., Veldhuis, G., van Rooijen, B. D., Subrama-niam, V., and Huber, M. (2010) Antiparallel arrangement of the helices of vesicle-bound α-synuclein. J. Am. Chem. Soc. 132, 4080–4082

26. Bortolus, M., Tombolato, F., Tessari, I., Bisaglia, M., Mammì, S., Babucco, L., Ferrari, A., and Maniero, A. L. (2008) Broken helix in vesicle and micelle-bound α-synuclein. Insights from site-directed spin labeling EPR experiments and MD simulations. J. Am. Chem. Soc. 130, 6690–6691

27. Varkey, J., Issa, J. M., Mizuno, N., Jensen, M. B., Bhatia, V. K., Jao, C. C., Petrlova, J., Voss, J. C., Stanford, C. A., and Langen, R. (2010) Membrane curvature induction and tubulation are common features of synucleins and apolipoproteins. J. Biol. Chem. 285, 32486–32493
52. Pandey, A. P., Haque, F., Rochet, J. C., and Hovis, J. S. (2011) α-Synuclein-induced tubule formation in lipid bilayers. J. Phys. Chem. B 115, 5886–5893
53. Shaikh, T. R., Gao, H., Baxter, W. T., Asturias, F. J., Boisset, N., Leith, A., and Frank, J. (2008) SPIDER image processing for single-particle reconstruction of biological macromolecules from electron micrographs. Nat. Protoc. 3, 1941–1974
54. Ludtke, S. J. (2010) 3-D structures of macromolecules using single-particle analysis in EMAN. Methods Mol. Biol. 673, 157–173
55. Altenbach, C., Greenhalgh, D. A., Khorana, H. G., and Hubbell, W. L. (1994) A collision gradient method to determine the immersion depth of nitroxides in lipid bilayers. Application to spin-labeled mutants of bacteriorhodopsin. Proc. Natl. Acad. Sci. U.S.A. 91, 1667–1671
56. Chiang, Y. W., Borbat, P. P., and Freed, J. H. (2005) The determination of pair distance distributions by pulsed ESR using Tikhonov regularization. J. Magn. Reson. 172, 279–295
57. Jeschke, G., Chechik, V., Ionita, P., Godt, A., Zimmermann, H., Banham, J., Timmel, C. R., Hilger, D., and Jung, H. (2006) DEER Analysis 2006 – A comprehensive software package for analyzing pulsed ELDOR data. Appl. Magn. Res. 30, 472–498
58. Dickey, A., and Faller, R. (2008) Examining the contributions of lipid shape and headgroup charge on bilayer behavior. Biophys. J. 95, 2636–2646
59. Hubbell, W. L., Gross, A., Langen, R., and Lietzow, M. A. (1998) Recent advances in site-directed spin labeling of proteins. Curr. Opin. Struct. Biol. 8, 649–656
60. Vinson, P. K., Talmon, Y., and Walter, A. (1989) Vesicle-micelle transition of phosphatidylcholine and octyl glucoside elucidated by cryo-transmission electron microscopy. Biophys. J. 56, 669–681
61. Walter, A., Vinson, P. K., Kaplan, A., and Talmon, Y. (1991) Intermediate structures in the cholate-phosphatidylcholine vesicle-micelle transition. Biophys. J. 60, 1315–1325
62. Edwards, K., Gustafson, J., Almgren, M., and Karlsson, G. (1993) Solubilization of lecithin vesicles by a cationic surfactant: intermediate structures in the vesicle-micelle transition observed by cryo-transmission electron microscopy. J. Colloid Interface Sci. 161, 299–309
63. Mizuno, N., Joo, C. C., Langen, R., and Steven, A. C. (2010) Multiple modes of endophilin-mediated conversion of lipid vesicles into coated tubes. Implications for synaptic endocytosis. J. Biol. Chem. 285, 23351–23358
64. Takei, K., Slepmen, V. I., Haucke, V., and De Camilli, P. (1999) Functional partnership between amphiphrin and dynamin in clathrin-mediated endocytosis. Nat. Cell Biol. 1, 33–39
65. Farsad, K., Ringstad, N., Takei, K., Floyd, S. R., Rose, K., and De Camilli, P. (2001) Generation of high curvature membranes mediated by direct endophilin bilayer interactions. J. Cell Biol. 155, 193–200
66. Ford, M. G., Mills, I. G., Peter, B. J., Vallis, Y., Praefcke, G. J., Evans, P. R., and McMahon, H. T. (2002) Curvature of clathrin-coated pits driven by epsin. Nature 419, 361–366
67. Ellis, R. J., and Minton, A. P. (2003) Cell biology. Join the crowd. Nature 425, 27–28
68. Kanazawa, T., Adachi, E., Orimo, S., Nakamura, A., Mizusawa, H., and Uchihara, T. (2012) Pale neurites, premature α-synuclein aggregates with centripetal extension from axon collaterals. Brain Pathol. 22, 67–78
69. Rieker, C., Dev, K. K., Lehnhoff, K., Barbieri, S., Ksiazek, I., Kauffmann, S., Danner, S., Schell, H., Boden, C., Ruegg, M. A., Kahle, P. J., van der Putten, H., and Shimshak, D. R. (2011) Neurpathology in mice expressing mouse α-synuclein. PLoS One 6, e24834
70. Silva, R. A., Huang, R., Morris, J., Fang, J., Gracheva, E. O., Ren, G., Kontush, A., Jerome, W. G., Rye, K. A., and Davidson, W. S. (2008) Structure of apolipoprotein A-I in spherical high density lipoproteins of different sizes. Proc. Natl. Acad. Sci. U.S.A. 105, 12176–12181
71. Segrest, J. P. (1977) Amphipathic helices and plasma lipoproteins. Thermodynamic and geometric considerations. Chem. Phys. Lipids 18, 7–22
72. Fauvet, B., Fares, M. B., Samuel, F., Dikiy, I., Tandon, A., Eliezer, D., and Lashuel, H. A. (2012) Characterization of semisynthetic and naturally N-acetylated α-synuclein in vitro and in intact cells: implications for aggregation and cellular properties of α-synuclein. J. Biol. Chem. 287, 28243–28262