Stabilization of α-Synuclein Secondary Structure upon Binding to Synthetic Membranes*

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W. Sean Davidson†, Ana Jonas‡, David F. Clayton§, and Julia M. George¶

From the †Department of Biochemistry, University of Illinois, Urbana, Illinois 61801 and the §Department of Cell and Structural Biology, University of Illinois, Urbana, Illinois 61801

α-Synuclein is a highly conserved presynaptic protein of unknown function. A mutation in the protein has been causally linked to Parkinson’s disease in humans, and the normal protein is an abundant component of the intraneuronal inclusions (Lewy bodies) characteristic of the disease. α-Synuclein is also the precursor to an intrinsic component of extracellular plaques in Alzheimer’s disease. The α-synuclein sequence is largely composed of degenerate 11-residue repeats reminiscent of the amphipathic α-helical domains of the exchangeable apolipoproteins. We hypothesized that α-synuclein should associate with phospholipid bilayers and that this lipid association should stabilize an α-helical secondary structure in the protein. We report that α-synuclein binds to small unilamellar phospholipid vesicles containing acidic phospholipids, but not to vesicles with a net neutral charge. We further show that the protein associates preferentially with vesicles of smaller diameter (20–25 nm) as opposed to larger (125 nm) vesicles. Lipid binding is accompanied by an increase in α-helicity from 3% to approximately 80%. These observations are consistent with a role in vesicle function at the presynaptic terminal.

Our studies in songbird brain resulted in the identification of a presynaptic protein, which we called synelfin, whose expression is regulated in the avian song control circuit during the critical period for song learning (1). We hypothesized that this protein might play an important role in the modulation of synaptic plasticity, and have focused on determining its normal cellular and biochemical function.

Independent evidence has emerged that implicates this protein in human neurodegenerative disease. Ueda et al. (2) reported that a fragment of the homologous human protein is an intrinsic component of the amyloid plaques which deposit extracellularly in the brains of individuals with Alzheimer’s disease; they called the protein NACP, for non-amyloid compo-

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† To whom correspondence should be addressed: Dept. of Cell and Structural Biology, B107 Chemical and Life Science Laboratory, 601 S. Goodwin, Urbana, IL 61801. Tel.: 217-244-4525; Fax: 217-244-1649; E-mail: j-george@uiuc.edu.

‡ The abbreviations used are: NACP, non-amyloid component precursor; POPC, 1-palmitoyl 2-oleoyl phosphatidylcholine; POPA, 1-palmi-
toyl 2-oleoyl phosphatidic acid; POPS, 1-palmitoyl 2-oleoyl phosphatidy-
slerine; PS, phosphatidylycerine; PE, phosphatidylethanolamine; SUV, small unilamellar vesicle; LUV, large unilamellar vesicle; MLV, multilamellar vesicle.

EXPERIMENTAL PROCEDURES

Materials

Bovine brain phospholipid extract (Folch extract) and egg ovalbumin were purchased from Sigma (+99% grade). 1-Palmityl 2-oleoyl phosphatidylcholine (POPC), 1-palmityl 2-oleoyl phosphatic acid (POPA), 1-palmityl 2-oleoyl phosphatidylycerine (POPS), 1-palmityl 2-oleoyl phosphatidylethanolamine (POPE), and liver phosphatidylinositol (PI) were obtained from Avanti Polar Lipids (Birmingham, AL). All other reagents were analytical grade.

Methods

Purification of Recombinant α-Synuclein—The canary α-synuclein cDNA was subcloned into the plasmid pET28a (Novagen), which directs
expression in Escherichia coli via an isopropyl-1-thio-β-D-galactopyrano- side-inducible T7 promoter. Purification of α-synuclein was based on a modification of Weinreb et al. (10), who documented the heat stability of the protein; briefly, cell lysates were boiled, and the soluble fraction is precipitated with 60% ammonium sulfate. The pellet is resuspended in 25 mM Tris, pH 9.0, loaded onto a Poros HQ column (Perseptive Biosystems), and eluted with 0–1 M NaCl. α-Synuclein containing fractions are pooled and exchanged into sodium acetate buffer, pH 4.0, loaded onto a Poros HS column (Perseptive Biosystems), and eluted with 0–1 M NaCl. α-Synuclein fractions are finally loaded onto a gel exclusion column (Superose 6, Pharmacia) to confirm purity and achieve a final buffer exchange.

The Binding of α-Synuclein to Small (SUV) and Large (LUV) Unilamellar Vesicles—SUV of various lipid compositions were prepared by the sonication technique of Barenholz et al. (14). The SUV were reisolated by ultracentrifugation at 55,000 rpm in a TLA 100.3 rotor for 2 h at 25 °C and exhibited hydrodynamic diameters of 25 ± 5 nm as estimated by gel filtration chromatography (15). LUV were prepared as described by Hope et al. (16). Briefly, phospholipid multilamellar liposomes in buffer were placed into an extrusion device (Lipex Biomedbranes Inc., Vancouver, British Columbia, Canada) which passed the mixture through polycarbonate filters of 100-nm pore size under pressures of 100–500 p.s.i. This procedure routinely produced unilamellar vesicles with hydrodynamic diameters of 125 ± 30 nm. SUV or LUV preparations in Tris buffer were mixed with α-synuclein to determine the affinity of α-synuclein for lipid surfaces of varying curvature. The vesicles and α-synuclein at a 20:1 mass ratio of phospholipid to α-synuclein were incubated in Tris buffer for 2–24 h at 25 °C. The resulting complexes were separated from unreacted lipid and protein on a calibrated Superose 6 (10 mm × 30 cm) gel filtration column (Pharmacia) eluted at 0.5 ml/min with Tris buffer. The protein content of the fractions was determined using the Markwell modification of the Lowry protein assay (17) while phospholipids were determined as inorganic phosphorus by the method of Sokoloff and Rothblat (18). In some experiments, the fractions corresponding to lipid-free protein, SUV and any multilamellar vesicles were pooled and analyzed by immunoblotting with α-synuclein monoclonal H3C, as described in Ref. 1.

Structure Analysis—The structural predictions based on the primary sequence of canary α-synuclein (1) were determined using programs ANTREPROT (19) and the Wisconsin Sequence Analysis program (Genetics Computer Group) running on an IBM PC. Three algorithms, the Levin homologue method (20), the GOR 1 prediction method (21), and the Chou-Fasman prediction method (22) were used for the sequence analysis because of their distinct prediction strategies. Consensus predictions were obtained from the results of all three methods by manual comparison of the predictions for each amino acid in the sequence (see the legend to Fig. 5 for more detail). For more information on the application of these established prediction methods to lipid-binding proteins, see Ref. 23.

Circular Dichroism Measurements and Analysis—The average secondary structure contents of lipid-free and lipid-bound α-synuclein were determined by circular dichroism (CD) spectroscopy using a Jasco J-720 spectropolarimeter. Spectra were taken at 25 °C in a 0.1-cm path length quartz cuvette containing the sample at concentrations of 0.1–0.2 mg/ml of protein in 20 mM phosphate buffer, pH 8.0, without NaCl. The spectra were corrected for buffer and vesicle contributions as appropriate. The percent α-helix was determined from the molar ellipticities at 222 nm (42) as well as by computer fitting to a library of CD spectra from proteins of known structure using the learning neural network program K2D which is based on the algorithm published by Andrade et al. (24).

RESULTS

Characterization of α-Synuclein with Respect to Apolipoproteins—The identification of several predicted class A amphipathic α-helices in the α-synuclein sequence suggested that α-synuclein might exhibit traits that are characteristic of the plasma apolipoproteins. Experiments showed that recombinant α-synuclein could not clearly interact with dimyristoylphosphatidylcholine liposomes, whereas apoA-I readily cleared them into small disc-shaped particles (data not shown). Furthermore, α-synuclein did not yield protein-lipid complexes using the sodium cholate method of reconstituted high density lipoprotein preparation commonly used with apoA-I (data not shown). Finally, studies using the chemical cross-linking agent BS3 failed to demonstrate self-association of α-synuclein when free in solution (not shown), a common observation with apolipoproteins. Taken together, these results indicate that despite similarities in predicted structure, α-synuclein does not possess the critical properties of apolipoproteins. However, numerous data document a tendency of α-synuclein to colocalize with synaptic vesicles in vivo (6, 25, 26), and the presence of predicted lipid-binding helices suggests a lipid association. We decided to try SUV, which are relatively homogeneous, can be analyzed by gel exclusion chromatography, have sizes in the range of synaptic vesicles, and are compatible with circular dichroism as a means of estimating protein secondary structure. SUV were prepared from a commercially available brain phospholipid extract (Folch fraction I, Sigma) or synthetic POPC, and incubated with recombinant α-synuclein for 2–24 h at room temperature. The incubation mixture was then separated into various size components by gel filtration chromatography (Fig. 1). The resulting fractions were analyzed for protein and phospholipid content. The phospholipid profile from the column runs routinely showed two size populations. The earlier peak (fractions 17–19) was due to large multilamellar vesicles (MLV), whereas the later peak (centered around fraction 25) was due to SUV that were about 20–25 nm in diameter (15). 70% of the α-synuclein that was incubated with brain extract vesicles remained associated with the SUV while the remainder eluted at a volume that was characteristic of the free protein. No α-synuclein was found in the MLV fractions (Fig. 1A). In contrast, α-synuclein did not associate with the synthetic POPC SUV; 100% of the protein eluted in the lipid-free form. Control incubations were performed using egg ovalbumin which has no known lipid surface activity (Fig. 1, B and D). Ovalbumin did not associate with either type of vesicle, indicating that the association of α-synuclein with the brain extract vesicles shown in Fig. 1A was due to non-specific association or trapping of the protein molecules within the vesicles.

Log analysis indicated that Sigma brain extract I contained 50–55% phosphatidylserine (PS), 10–12% PI, and 10% phosphatidyethanolamine (PE), with 23–30% unknown phospholipids. Both PS and PI have a net negative charge compared...
with PE and PC, which have no net charge. To determine if the presence of the acidic lipids was responsible for the preferential binding of α-synuclein to BE1 vesicles versus POPC vesicles, we performed a series of incubations using vesicles composed of 1:1 (w/w) mixtures of purified phospholipids. The incubations were performed exactly as those in Fig. 1. After separation on the column, the fractions corresponding to MLV, SUV, and lipid-free protein peaks were pooled. Equal fractions of each of the pooled peaks were run on an SDS gel and then immunoblotted with the H3C monoclonal antibody to the C terminus of α-synuclein (1) (Fig. 2). In the absence of phospholipid, all of the α-synuclein appeared in the lipid-free fraction as expected. α-Synuclein did not bind to SUV or MLV composed of PC only, nor did it bind to a 1:1 mixture of PC and PE. However, when acidic lipids such as PS, phosphatidic acid (PA), and PI were mixed with either PC or PE, significant amounts of α-synuclein eluted in the SUV fractions but not in the MLV fractions. In the cases of the PA and PI containing vesicles, close to 100% of the α-synuclein was found in the SUV fractions whereas in the case of the PS containing vesicles 10–15% of the α-synuclein remained in the lipid-free fractions. The α-synuclein-vesicle complexes were found to be stable for at least 2 weeks at room temperature. Separate experiments were performed to determine the relative amount of acidic lipid to PC required for efficient α-synuclein binding to SUV. Acidic lipid concentrations of 30–50% of the vesicle weight were required to approach 100% binding under these conditions (data not shown). As the acidic lipid content was reduced to 20% or less, increasing amounts of α-synuclein appeared in the lipid-free form. The incubation with the PI-containing vesicles appeared to produce covalently linked oligomers of α-synuclein when analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 2). The cause of this is not clear, although it is possible that products of lipid peroxidation may be responsible for protein cross-linking. The PI used in these experiments was purified from bovine liver whereas the other lipids were synthetic, monounsaturated lipids that are resistant to lipid oxidation.

The results shown in Fig. 2 demonstrate that α-synuclein can bind to SUV which contain negatively charged phospholipids. These data suggested a greater affinity for the SUV versus the MLV, but since the larger vesicles are multilamellar (with a smaller proportion of lipid accessible for surface binding) this observation was inconclusive. To determine whether α-synuclein has a similar affinity for more planar, less curved lipid surfaces, the binding of α-synuclein to LUV was compared with that of the SUV. We chose lipid compositions of either PC/PA (1:1) or PC alone, since these gave the most and least α-synuclein binding, respectively, in the SUV experiments. The LUV had hydrodynamic diameters of 125 ± 30 nm, whereas the SUV measured 20–25 nm. The results are shown in Table I. α-Synuclein did not bind to vesicles that contained only PC, regardless of size. In the case of the PC/PA vesicles, binding to

| Vesicle composition | Binding to SUV (~25 nm diameter) | Binding to LUV (~125 nm diameter) |
|---------------------|----------------------------------|----------------------------------|
| PC/PA 1:1 (w/w)     | 120 ± 10                         | 40 ± 20                          |
| PC only             | 2 ± 2                            | >1                               |

*Values in this table are derived from two independent experiments done in triplicate (*n* = 6).
additional forces must also help stabilize the protein-lipid interaction.

Structural Analysis of α-Synuclein in the Lipid-free and Lipid-bound States—Circular dichroism spectroscopy was used to determine the effects of lipid binding on the secondary structure of α-synuclein. The spectrum of lipid-free α-synuclein is shown in Fig. 4A. The prominent minimum ellipticity at 200 nm was characteristic of a high percentage of random coil. Table II lists the predicted secondary structure compositions based on the CD spectrum from 200 to 241 nm. The data indicate that lipid-free α-synuclein is highly unstructured in solution. This is in strong agreement with the results of Weinreb et al. (10) for human α-synuclein. Upon lipid binding, α-synuclein undergoes a striking change in conformation (Fig. 4A, Table II), shifting to a highly α-helical conformation as evidenced by the characteristic minima at 208 and 222 nm. The helical character of the protein increased from less than 3% to greater than 71% α-helix when bound to lipid. A similar change in conformation was observed when α-synuclein bound to PC/PS vesicles (Fig. 4B), although the helical content was not as high as in the case of the PC/PS vesicles. This difference is due to the spectral contribution of lipid-free α-synuclein, which was present only in the PC/PS incubations (see Fig. 2). The incubation of α-synuclein with PC-only vesicles resulted in a CD spectrum that resembled lipid-free α-synuclein, confirming that α-synuclein did not bind to these vesicles. The secondary structure content of α-synuclein bound to PC/PS vesicles was also measured under conditions of increasing ionic strength. Table III shows that as the NaCl concentration was increased, the degree of α-helix content decreased with a corresponding increase in the estimated amount of random coil content. These changes in structure generally correlated with the decreases in α-synuclein binding shown in Fig. 3. In a single experiment with a small amount of recombinant human NCAP-α-synuclein (a kind gift of Peter Lansbury), we observed a qualitatively similar increase in α-helical content when the protein associated with PC/PS vesicles (data not shown).

To determine the regions of α-synuclein that undergo lipid-induced structural changes, we first performed computer-generated secondary structure predictions using the primary sequence of the protein. Three structural prediction algorithms (see “Methods”) varied in their overall secondary structure predictions (not shown), with consensus values of 56% α-helix, 10% β-sheet, 0% turn, 27% random coil, and 7% undetermined. These values differ significantly from estimates based on circular dichroism measurements in either the presence or absence of vesicles (Table II), probably because the algorithms do not adequately model the stabilization of secondary structure by associated lipids, which appears to be critical in the case of α-synuclein. For this reason, a modeling strategy was applied which was originally developed for analysis of the exchangeable apolipoproteins (13). Like the apolipoproteins, much of the α-synuclein sequence is composed of repetitions of a degenerate 11-mer sequence which can form an amphipathic α-helix with a conserved arrangement of acidic, basic, and hydrophobic residues (1). We subjected the entire α-synuclein sequence to helical wheel analysis, and identified 5 potential amphipathic

![Circular dichroism spectra of recombinant α-synuclein in the lipid-free form and after incubation with various SUV.](image)

**Fig. 4.** Circular dichroism spectra of recombinant α-synuclein in the lipid-free form and after incubation with various SUV. Samples were prepared in 50 mM phosphate buffer, pH 7.6, at 100 μg/ml. The molar ellipticity was measured at 25 °C in a 0.1-cm path length quartz cell. Panel A shows α-synuclein either alone (filled squares) or after incubation with 1:1 POPC/POPA (w/w) small unilamellar vesicles for 2–24 h at 25 °C at a 1:20 weight ratio of α-synuclein to phospholipid (filled circles). Panel B shows α-synuclein incubated with POPC-only SUV (open squares) and with 1:1 POPC/POPS vesicles (open circles). The spectra shown are averaged from two separate experiments each done in triplicate (n = 6).

**Table II**

| Sample | α-Helix content from ellipticity at 222 nm | Secondary structure predictions by K2D |
|--------|------------------------------------------|---------------------------------------|
|        | %                                      | α-Helix | β-Sheet | Random |
| α-Synuclein only | >1 | 3 ± 1 | 23 ± 8 | 74 ± 10 |
| α-Synuclein + PC/PA vesicles | 71 | 82 ± 1 | 0 ± 0 | 18 ± 2 |
| α-Synuclein + PC/PS vesicles | 63 | 77 ± 3 | 1 ± 1 | 22 ± 3 |
| α-Synuclein + PC vesicles | 2 | 5 ± 2 | 35 ± 13 | 53 ± 15 |

*Values in this table are derived from three independent experiments each done in triplicate (n = 9). * Determined from molar ellipticities at 222 nm by the method of Chen et al. (38) ±5% (1 S.D.). * Determined using the K2D learning neural network program (http://www.embl-heidelberg.de/~andrade/K2D.html) using the algorithm published by Andrade et al. (24) (± 1 S.D.).

**Table III**

| Sample | α-Helix content from ellipticity at 222 nm | Secondary structure predictions by K2D |
|--------|------------------------------------------|---------------------------------------|
|        | %                                      | α-Helix | β-Sheet | Random |
| 0 mM NaCl | 71 | 82 | 0 | 18 |
| 100 mM NaCl | 65 | 80 | 1 | 19 |
| 250 mM NaCl | 67 | 79 | 1 | 20 |
| 500 mM NaCl | 50 | 57 | 9 | 34 |

*Values in this table are derived from two experiments done in triplicate (n = 6). * Determined from molar ellipticities at 222 nm by the method of Chen et al. (38) ±5% (1 S.D.). * Determined using the K2D program as in Table II ±5% (1 average S.D.).
α-helices which encompass all of the 11-mer repeats and some adjacent amino acid residues; these are illustrated in Fig. 5.

Helices 1–4 (residues 1–60) conform to the class A2 consensus (see “Discussion”), distinguished by clustered basic residues at the polar/nonpolar interface and positioned ± 100° from the center of the nonpolar face; a preponderance of lysine relative to arginine among these basic residues; and several glutamate residues localized to the polar face. Segrest et al. (13) defines several amphipathic α-helix-terminating amino acids: 1) Pro at any position; 2) Phe, Met, Ile, Leu, Val, Trp, or Cys within 40° of the center of the polar face; 3) Lys, Arg, Glu, or Asp within 40° of the center of the nonpolar face; 4) Gln or Asn within 40° of the center of the nonpolar face; 5) Pro within 40° of the center of the nonpolar face; 6) Gln or Asn within 40° of the center of the polar face; 3) Lys, Arg, Glu, or Asp within 40° of the center of the polar face; 4) Gln or Asn within 20° of the center of the nonpolar face, or a cassette of four or more of the neutral or polar residues Thr, Gly, Ser, His, Gln, and Asn. Helices 1–3 are terminated by residues meeting criterion 2 (Val-16, Leu-38, and Val-49). Although no helix-breaking residues occur between residues 50 and 94 according to the rules of Segrest et al. (13), helix 4 (residues 50–60) and helix 5 (residues 61–94) are plotted separately because the distinctive charge distribution of a class A2 helix is not maintained beyond residue 60. Instead, helix 5 displays a random radial distribution of charged residues to the polar face, which is similar to the class G* helix of Segrest et al. (13). Helix 5 is terminated by Leu-94, according to criterion 2. The remainder of the α-synuclein sequence is non-amphipathic, but the structural predictions algorithms suggest that residues 95–111 and 133–143 also have the propensity to form α-helices that might be indirectly stabilized by lipid binding. This analysis predicts an overall α-helix content of 83%, 63% contributed by the five amphipathic helices, and an additional 20% from the C terminus. This correlates well with the % α-helix estimated by CD for lipid-bound α-synuclein. Our structural predictions are summarized in Fig. 6, along with the primary sequences of canary and human α-synuclein. Note that differences between the two sequences conserve the 5 helices, the putative helix-breaking residues, and the acidic C terminus.

Additional computer analysis using the algorithm of Geurjon and Deleage (19) found that the potential helical segments within α-synuclein were not predicted to be transmembrane helices (not shown). This suggests that α-synuclein interacts with lipid surfaces by virtue of the hydrophobic faces of amphipathic helices and not by threading helical segments through the bilayer.

DISCUSSION

α-Synuclein binds best to vesicles containing at least 30% acidic phospholipid. Because the requirement for acidic phospholipid was determined at a lipid/protein mass ratio of 20:1 (molar ratio ~400:1), there must be an excess of acidic sites available even at 20% acidic phospholipid, suggesting that binding requires multiple interactions.

A preference for acidic phospholipids could be a mechanism for targeting the protein to a particular membrane or vesicle population. Synuclein was first isolated from the electric fish Torpedo because of its association with the abundant cholinergic vesicles of that animal’s electric organ, essentially a mass synaptic vesicle (25), and EM immunohistochemistry suggests that the protein is concentrated in the cytoplasmic matrix surrounding synaptic vesicles in the presynaptic terminal (25, 27). Our studies of primary hippocampal neurons show a tight colocalization of α-synuclein with the synaptic vesicle protein synapsin by immunofluorescence, further indication that the protein associates with this vesicle population (6). However, in subcellular fractionation experiments, α-synuclein does not tightly copurify with synaptic vesicles, and the majority of α-synuclein in cytosolic and synaptosomal subfractions is freely soluble (1, 8, 26). This paradoxical feature of α-synuclein could result from rapid exchange of the protein between membrane

Fig. 5. Helical wheel analysis of α-synuclein structure. The α-synuclein sequence was projected radially at 100° intervals (11) to determine the distribution of charged residues along an idealized α-helix. Nonpolar residues are shaded black, polar residues are gray, and charged residues are unshaded. Residues predicted to be helix breaking are boxed and indicated by an asterisk (*); helices are assumed to terminate on the prior residue. The angle subtended by the nonpolar face is indicated by a dashed line, and the center of the nonpolar face by an arrow. A, helix 1, residues 1–15 plus terminating residue Val-16; B, helix 2, residues 17–37 plus terminating residue Leu-38; C, helix 3, residues 39–48 plus terminating residue Val-49; D, helix 4, residues 50–60; E, helix 5, residues 61–93, plus terminating residue Leu-94. Note that for helix 5, the polar/nonpolar interface is not demarcated by basic residues as in helices 1–4.

Fig. 6. Diagrammatic summary of α-synuclein structure. The primary sequence of canary α-synuclein is shown; differences in the human sequence are indicated by either the substituted residue or — to represent a deletion. The seven 11-residue repeats are indicated above the primary sequence. The five candidate helices derived in Fig. 5 are represented as boxes; (*) represents non-amphipathic residues either punctuating the helices or in the C terminus.
surface and cytosol, modulated by factors that have yet to be identified.

The average composition of synaptic vesicles is estimated to be 0–2% PA, 40–48% PC, 24–36% PE, 4–12% sphingomyelin, 7–12% PS, 3–4% PI, with a cholesterol:phospholipid ratio of ~0.5–0.6 (28–30); the proportion of acidic phospholipids (10–18%) is somewhat less than the 30% acidic phospholipid required for maximal binding of α-synuclein to our synthetic membranes. However, these estimates of composition do not take into account the asymmetric distribution of phospholipid species between the two leaflets of the membrane, a critical property of many biological membranes, including synaptic vesicles. Determination of phospholipid asymmetry in synaptic vesicles has been somewhat confounded by methodological concerns, but it is estimated that 100% of the PI in synaptic vesicles is localized to the cytoplasmic face (30). Also, it is increasingly clear that phospholipids can become locally concentrated within a single membrane leaflet, with important consequences for protein localization. For example, microdomains of PS form in response to increased [Ca(2+)], encouraging translocation and activation of protein kinase C (31). Bilayer asymmetry and/or phospholipid microdomains might be critical to α-synuclein localization in vivo, yet difficult to reproduce in vitro.

The observation that α-synuclein binds small (20–25 nm) in preference to large (~125 nm) unilamellar vesicles of the same composition most likely results from differences in phospholipid packing on the vesicle surface. The phospholipid bilayer of an LUV is an essentially planar surface in which the fatty acyl side chains are motionally restricted (32), compared with the curved surface of an SUV (33). It has been demonstrated that the initial insertion of the amphipathic α-helical regions of plasma lipoproteins into a phospholipid bilayer is facilitated by the existence of lipid packing defects caused by extreme membrane curvature or by the differential packing properties of different phospholipids within the membrane (34). It is likely that these factors also affect the partitioning of α-synuclein between the lipid-bound and lipid-free states. This observation may be important for in vivo targeting of α-synuclein to vesicles or membranes with irregular or curved surfaces.

The binding of α-synuclein to acidic phospholipid vesicles (PC/PA or PC/PS) is accompanied by a large increase in its α-helical content; no such increase is observed in the presence of PC vesicles, to which α-synuclein does not bind (Table II). α-Helicity declines in parallel with lipid binding as salt concentration is increased (Table III). These observations suggest that lipid binding is mediated by α-helical structures which are themselves stable only in the presence of lipid, and are consistent with our initial identification of consensus domains for lipid-binding amphipathic α-helices in the α-synuclein sequence (1). We have now extended our analysis of the α-synuclein sequence, and have identified 5 potential amphipathic α-helical domains representing 2 different classes, in accordance with the criteria established by Segrest et al. (13) for analysis of the exchangeable apolipoproteins.

A key feature of the α-synuclein peptide sequence is a set of 7 degenerate 11-residue repeats. 11-mer repeats are also a hallmark of the amphipathic α-helices of the exchangeable apolipoproteins, which mediate a variety of lipid and protein interactions. These α-helices are divided into classes depending upon the distribution of residues to the polar and nonpolar faces of the helices (13). Class A helices are typically lipophilic, and are characterized by a clustering of basic residues at the polar/nonpolar interface and acidic residues at the center of the polar face. Class G* helices are implicated in protein interactions; this class is less well defined and is typically characterized by a random radial distribution of charged residues to the polar face of the helix. Class Y helices (which are rare) also mediate lipid interactions (35, 36), and are characterized by 2 clusters of negative residues separating 3 clusters of positive residues on the polar face of the helix.

Class A helices are further subdivided as class A1, A2, or A4. Class A1 is least common and is distinguished by clusters of basic residues ±120° from the center of the nonpolar face. Class A2 helices typically have arginines positioned ±90° from the center of the nonpolar face, with leucines at the center of the nonpolar face. Class A4, the motif which is best defined and correlated with the highest lipid affinity, is characterized by lysines positioned ±100° from the center of the nonpolar face, and glutamates clustered in the center of the polar face. Segrest et al. (12) proposed a model for the interaction of class A helices with phospholipid membranes, wherein positively charged lysine or arginine residues positioned at the polar/nonpolar interface interact with negatively charged phosphate groups in the phospholipid backbone. The amphipathicity of these amino acid residues, particularly lysine with its longer aliphatic side chain, allows the hydrophobic face of the helix to penetrate more deeply into the bilayer interior, stabilizing the lipid-protein interaction (12).

Helices 1–4 of α-synuclein are clearly class A, and most resemble the class A4 consensus in the positioning of the interfacial basic residues at ±100° from the center of the nonpolar face, in the preponderance of lysines over arginines, and in the presence of glutamate residues on the polar face. These domains would be predicted to mediate the binding of α-synuclein to phospholipid bilayers. Helix 5 resembles the class G* helix, and thus is a candidate for protein-protein interactions. Interestingly, this putative helix (residues 61–93) corresponds almost exactly to the non-amyloid component peptide (residues 61–95) purified from the Alzheimer’s senile plaque.

A few notable features distinguish the putative amphipathic α-synuclein helices from those in the exchangeable apolipoproteins. The threonine residues at the center of the nonpolar faces of helices 2–4 are unique to α-synuclein and are perfectly conserved among sequences from canary, human, and rat (1), which might represent a site for regulation by phosphorylation. Threonine, although polar, can reside on the nonpolar face of the helix due to its relatively long aliphatic side chain (13). Phosphorylation of these residues would introduce negative charges which would be expected to disrupt lipid binding. Evidence exists that a closely related isoform of this protein, β-synuclein, is phosphorylated in vivo and in vitro (37), although the precise sites remain to be mapped.

Another unique aspect of the α-synuclein sequence is the absence of prolines among the 11-mer repeating domains. The amphipathic α-helical motifs of the exchangeable apolipoproteins are typically divided into anti-parallel 22-residue segments by proline residues, which introduce helix-breaking hairpin turns. The resulting short helical segments function cooperatively, and this cooperativity enhances many of the functions of the apolipoproteins, including their dynamic ability to bind and solubilize lipid particles (for a review, see Ref. 13) In contrast, the α-synuclein helices 1–4 seem to be punctuated at various points by nonpolar residues that are predicted to disrupt the amphipathicity of a helix (Fig. 6). It is not known whether these residues mediate breaks to form distinct helices or merely introduce kinks that do not totally disrupt a single, long helix.

Borhani et al. (38) have recently solved the x-ray crystal structure of a fragment of apoA-I. These authors suggest that the helices in apoA-I are not arranged in an antiparallel fashion when associated with spherical lipid particles, in contrast
with the evidence for an antiparallel orientation in discoidal particles (39). The intrahelical regions in this model form kinks that are significantly less than 180° which allows the protein to wrap around the sphere of lipid in a ring or horseshoe topology. Thus, apoA-I may have the ability to adjust the degree of turn between adjacent helical segments, with turns approximating 180° in discs but of lesser degree in spherical particles.

The absence of proline-induced hairpin turns may explain why α-synuclein can bind spherical and planar surfaces, yet fail to form discoidal particles. It should be noted that among the many protein/lipid incubations performed in the course of the work described here, we never detected structures in the size range of discs (7–12 nm). In addition, α-synuclein failed to clear multilamellar dimyristoylphosphatidylcholine liposomes, which are readily cleared into discoidal particles by apoA-I (data not shown), and did not form discs using the sodium cholate dialysis method, which is well established for the preparation of reconstituted high density lipoprotein with apoA-I (data not shown).

Clearly, identification of the physiological substrates for α-synuclein binding will be critical to discerning its function in vivo. It is interesting to note that the class A helices are typically stabilizing to membranes, inhibiting membrane fusion and lysis by relieving negative monolayer curvature typically stabilizing to membranes, inhibiting membrane fusion and lysis by relieving negative monolayer curvature. It is interesting to note that the class A helices are typically stabilizing to membranes, inhibiting membrane fusion and lysis by relieving negative monolayer curvature.

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