A Study of the Regional Effects of α-Synuclein on the Organization and Stability of Phospholipid Bilayers[†]

Jillian Madine, Andrew J. Doig, and David A. Middleton*

Faculty of Life Sciences, University of Manchester, P.O. Box 88, Manchester M60 1QD, United Kingdom

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ABSTRACT: Associations between the protein α-synuclein (α-syn) and presynaptic vesicles have been implicated in synaptic plasticity and neurotransmitter release and may also affect how the protein aggregates into fibrils found in Lewy bodies, the cellular inclusions associated with neurodegenerative diseases. This work investigated how α-syn interacts with model phospholipid membranes and examined what effect protein binding has upon the physical properties of lipid bilayers. Wide line ^{2}H and ^{31}P NMR spectra of phospholipid vesicles revealed that α-syn associates with membranes containing lipids with anionic headgroups and can disrupt the integrity of the lipid bilayer, but the protein has little effect on membranes of zwitterionic phosphatidylcholine. A peptide, α-syn(10−48), which corresponds to the lysine-rich N-terminal region of α-syn, was found to associate with lipid headgroups with a preference for a negative membrane surface charge. Another peptide, α-syn(120−140), which corresponds to the glutamate-rich C-terminal region, also associates weakly with lipid headgroups but with a slightly higher affinity for membranes with no net surface charge than for negatively charged membrane surfaces. Binding of α-syn(10−48) and α-syn(120−140) to the lipid vesicles did not disrupt the lamellar structure of the membranes, but both peptides appeared to induce the lateral segregation of the lipids into clusters of acidic lipid-enriched and acidic lipid-deficient domains. From these findings, it is speculated that the N-terminal and C-terminal domains of full-length α-syn might act in concert to organize the membrane components during normal protein function and perhaps play a role in presynaptic vesicle synthesis, maintenance, and fusion.

α-Synuclein (α-syn)[1] is a 140 amino acid protein that is concentrated at the presynaptic termini of the central nervous system (*1*). The function of α-syn is unknown, but the upregulation of α-syn expression around human nerve cell bodies during early life (*2*) and the upregulation of the homologous protein synelfin in zebra finch brains during song learning (*3*) suggest that it has a role in learning processes. It has also been suggested that α-syn regulates synaptic vesicle formation (*4*) and vesicle pool size (*5*) by inhibiting phospholipase D_2 and may also control dopamine transport (*6*). In its normal form, α-syn is predominantly unfolded, but the protein can misfold and aggregate into *β*-sheet-rich amyloid fibrils found in cellular inclusions called Lewy bodies that are associated with many neurodegenerative diseases (*7*, *8*).

In presynaptic termini, monomeric α-syn exists in equilibrium between free and plasma membrane- or vesicle-bound states (*9*). The equilibrium is tightly regulated, and from densitometric analysis of rat brain homogenates it has been estimated that approximately 15% of α-syn is membrane-bound within the synaptic termini (*10*). It has also recently been shown that α-syn may interact with membrane microdomains known as lipid rafts and that these interactions are necessary for localizing the protein to synaptic terminals (*11*). The affinity of α-syn for the specific lipid components of neuronal vesicular or raft membranes could be relevant to the native function of the protein and may also contribute to the onset of disease if the equilibrium binding conditions are disturbed (*12*). Neuronal vesicles contain high levels of cholesterol, sphingomyelin, and phospholipids with zwitterionic choline and ethanolamine headgroups and acidic serine headgroups. Studies of α-syn binding to phospholipid vesicles suggested that the protein has a higher affinity for small vesicles than for vesicles of larger diameter, possibly because of the higher surface-to-volume ratio of SUVs (*13*, *14*). The protein binds to vesicles containing a mixture of phosphatidylcholine (PC) and acidic phospholipids such as phosphatidylserine (PS), phosphatidic acid (PA), and phosphatidylglycerol (PG) (*15*), increasing the helical content to around 80% and inhibiting fibril formation (*13*). Examination of binding to brain lipids using thin-layer chromatography showed that α-syn also binds to negatively charged phosphatidylinositol (PI) (*16*). This latter study also showed that binding to vesicles of negatively charged phospholipids was

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* To whom correspondence should be addressed. Tel: +44 161 3064217. Fax: +44 161 2360409. E-mail: david.a.middleton@manchester.ac.uk.

[1] Abbreviations: α-syn, α-synuclein; α-syn(10−48), a peptide comprising residues 10−48 from α-synuclein; α-syn(120−140), a peptide comprising residues 120−140 from α-synuclein; CD, circular dichroism; TFE, 2,2,2-trifluoroethanol; DMPC-d_4, 1,2-dimyristoyl-*sn*-glycerol-3-phosphocholine; DOPG, L-α-dioleoylphosphatidyl-DL-glycerol; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine; BPI, L-α-phospatidylinositol; PE, phosphatidylethanolamine; MLVs, multilamellar vesicles; SUVs, small unilamellar vesicles; LUVs, large unilamellar vesicles; NMR, nuclear magnetic resonance; CSA, chemical shift anisotropy; MAS, magic-angle spinning.

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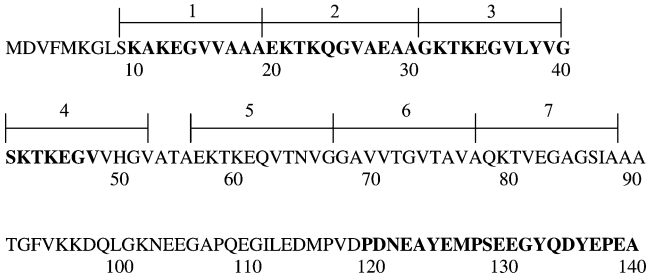

FIGURE 1: The amino acid sequence of human α-synuclein highlighting the 11 amino acid repeat units. The sequences of the peptides studied here, α-syn(10−48), and α-syn(120−140), are shown in bold. The peptide α-syn(10−48) has a net positive charge of 3+ at neutral pH, and α-syn(120−140) has a net negative charge of 8-.

augmented when the vesicles also contained neutral phosphatidylethanolamine (PE). The protein has much lower affinity for vesicles composed only of uncharged lipids, however, and remains unfolded in the presence of zwitterionic phosphatidylcholine membranes (*13*).

The N-terminal residues of α-syn contain seven KT(A)-KE(Q)GV repeat motifs (Figure 1), similar to the amphipathic α-helical domains of apolipoproteins, a family of membrane binding proteins (*17*). Such a similarity suggests that the N-terminal sequence is principally responsible for the ability of α-syn to bind to membranes via electrostatic interactions of the amphipathic helix with the membrane surface (*13*, *18*). The structure of α-syn bound to SDS micelles consists of two long helical stretches spanning V3−V37 and K45−T92, connected by an ordered linker region, and an unstructured C-terminal tail (*18*−*20*). EPR measurements of spin-labeled α-syn bound to phospholipid vesicles indicated that the two N-terminal helices observed in the detergent-bound protein merge into a single unbroken helix when the protein interacts with phospholipids (*21*). The C-terminal domain contains a sequence of acidic amino acids and three tyrosine residues, which are nitrated in Lewy body inclusions (*22*). The C-terminal residues are believed to not participate in membrane binding, presumably being repelled by the negative surface charge of the lipid headgroups. There are no long-range tertiary contacts between the N- and C-terminal regions of α-syn when bound to either detergent micelles or lipid membranes (*21*, *23*).

There is evidence that fibrillization of α-syn is promoted in the presence of lipid membranes (*10*, *24*), which suggests that the association of α-syn with bilayers may present the ideal conditions to seed fibril formation (*12*). Studies by Narayanan and Scarlata (*25*) and Zhu and Fink (*14*) contradict these findings, however, and suggest that membrane binding of α-syn impedes the ability of the protein to self-associate by stabilizing it in a helical conformation. The pathogenic form of α-syn may be soluble, prefibrillar oligomers that, like other amyloid proteins, form channels in cell membranes and cause increased permeability leading to cell death (*26*−*28*). However, atomic force microscopy (AFM) of planar lipid bilayers composed of PS and PC revealed that binding of monomeric α-syn disrupts the lipid bilayer and forms holes at specific defect sites (*16*).

Studies of α-syn−membrane interactions are helping to explain how the protein functions within synaptic termini and are providing insight into how the protein aggregates into toxic species. In the work presented here, ^{2}H and ^{31}P NMR spectroscopy has been used to examine how α-syn interacts with membrane vesicles of different composition and to determine what effect protein binding has upon the order and stability of lipid bilayers. A specific aim of the work was to assess the affinities of the N- and C-terminal regions of α-syn for membranes of different composition and to examine the effects of these regions on the structure and organization of lipid bilayers. This objective was addressed by preparing the peptides α-syn(10−48), which corresponds to residues in the lysine-rich N-terminal region, and α-syn(120−140), which represents the glutamic acid-rich C-terminal region (Figure 1). Experiments were carried out to examine how the full-length protein and the peptide fragments interacted with multilamellar vesicles (MLVs) containing dimyristoylphosphatidylcholine (DMPC), which possesses a zwitterionic polar headgroup, and dioleoylphosphatidylserine (DOPS), bovine phosphatidylinostol (BPI), or dioleoylphosphatidylglycerol (DOPG), which carry anionic polar headgroups. It is shown that both the N- and C-terminal regions of α-syn may contribute to the affinity and selectivity with which the protein interacts with lipid membranes and may also play a role in reorganizing the lipids within the membrane.

MATERIALS AND METHODS

Materials. [1,1,2,2-$^{2}H_4$] L-α-dimyristoylphosphatidylcholine (DMPC-d_4) and [1,1,2,2-$^{2}H_4$-*N*,*N*,*N*-trimethyl-$^{2}H_9$] L-α-dimyristoylphosphatidylcholine (DMPC-d_{13}) were purchased from Avanti Polar Lipids. Unlabeled L-α-dimyristoylphosphatidylcholine (DMPC), L-α-dioleoylphosphatidyl-rac-glycerol (DOPG), L-α-dioleoylphosphatidyl-choline, and bovine L-α-phospatidylinositol (BPI) in chloroform solution, 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (DOPS), and 2,2,2-trifluoroethanol were all purchased from Sigma Chemicals Limited.

Full-length α-syn was purchased from rpeptide (USA). The peptide α-syn(10−48) was synthesized in pure form by Peptide Protein Research Ltd. (U.K.). This specific peptide sequence was selected because it contains four of the KXKXGV repeat units, extending from the first occurrence of this motif in the full-length sequence. The peptide α-syn(120−140) was synthesized in-house using solid-phase fmoc (*N*-(9-fluorenyl)methoxycarbonyl) L-amino acid chemistry, on an Applied Biosystems 433A peptide synthesizer. Cleavage from the resin was achieved using 95% trifluoroacetic acid/2.5% water/2.5% triisopropylsilane and the peptide precipitated and cleaned by ether washing. This peptide was short enough to be amenable to synthesis in high yields, while possessing the salient characteristics of the C-terminal region of α-syn, having a high proportion of acidic residues. Both peptides were prepared without terminal acetylation or amidation. Purification was achieved using high-pressure liquid chromatography (HPLC) with a C18 column, and the product identity was confirmed by MALDI mass spectrometry. Both peptides were over 95% pure, as confirmed by HPLC.

Circular Dichroism. Far-UV circular dichroism (CD) spectra were recorded on a Jasco J-810 spectropolarimeter at 25 °C. Spectra were recorded over the 250−190 nm range at a scan rate of 10 nm/min with step size of 0.1 nm. Spectra were recorded as the average of four scans. For measure-

ments of peptides in trifluoroethanol (TFE) solutions, the peptides were at 40 μM concentration in 10 mM phosphate, pH 7.4 in a 0.1-cm cuvette. TFE was then added directly to the peptide solution in the cuvette to up to 40% by total volume. The final peptide concentration varied from 40 to 24 μM. For measurements of peptides in SUVs of DOPG and DMPC, the peptide was added at 100 μM concentration in sample buffer (1 mM EDTA, 10 mM phosphate, pH 7.4) into a 0.02-cm cuvette either alone or following mixing with the required SUV concentration to achieve a lipid to peptide molar ratio of 50:1 or 20:1.

After recording the spectra, spectral amplitudes were converted to molar elipticity values (θ) after correcting for changes in the initial peptide concentration after adding trifluoroethanol (TFE) and subtracting control spectra containing buffer and any corresponding SUV concentration using the equation:

$$\theta = \frac{\mathrm{CD}}{c \times 10 \times l \times n} \quad (1)$$

where CD is spectral amplitude (mdeg), c is peptide concentration (M), l is path length (cm), and n is number of amino acids. The percentage helix of the peptide was estimated using the equation:

$$\%\ \mathrm{helix} = \frac{\theta_{222}(\mathrm{obs})}{\theta_{222}(\mathrm{max})} \quad (2)$$

where

$$\theta_{222}(\mathrm{max}) = -40000\left(1 - \frac{2.5}{n}\right) \quad (3)$$

This equation can be used when there is a two-state transition between random coil and helix, assumed when there is a visible isodichroic point in the CD spectra (*29*).

Membrane Binding Assay. A preformed multilamellar vesicle (MLV) suspension of DMPC/DOPG (in a 2:1 molar ratio) or DMPC was added to a 180 μL buffer solution (1 mM EDTA, 10 mM phosphate, pH 7.4) containing 0.2 mM of α-syn, α-syn(10−48), or α-syn(120−140), to attain a final lipid/peptide molar ratio of 50:1 or 20:1. Solutions were incubated for 10 min and vortexed and then centrifuged at 80 000 rpm in a Beckman 100 Ti rotor for 60 min to sediment the lipids and any associated peptide. The supernatant was removed and analyzed for remaining peptide concentration from the UV absorbance at 280 nm (A_{280}). Small absorbance values from MLVs added to the buffer solutions in the absence of peptide and centrifuged were subtracted from the absorbances of the peptide solution supernatants. The fraction of bound peptide was calculated from the ratio of the A_{280} values for the initial lipid-free peptide solutions and the corrected A_{280} values for the supernatants. Experiments were carried out in triplicate.

Sample Preparation for NMR. MLVs containing a mixture of deuterated DMPC and nondeuterated DOPG, DOPS, or BPI in a 2:1 molar ratio were prepared by co-dissolving a total of 1 mg of lipids for full-length α-syn studies and 10 mg of lipids for peptide studies in chloroform/methanol. The solvent was removed under a stream of argon and dried under high vacuum overnight. The lipid film was resuspended in 50 μL of buffer (10 mM phosphate, 1 mM EDTA, in ^{2}H depleted water, pH 7.4), vortexed, and subjected to five freeze−thaw cycles. The samples were transferred to a 4-mm-diameter zirconium magic-angle sample spinner. For neutral MLVs 1 mg or 10 mg of DMPC was suspended in 50 μL buffer as above. The peptide was added in 5 μL of the same buffer following acquisition of control spectra, and the vortex, freeze−thaw process repeated. A heterogeneous mixture of DMPC and DOPG for ^{31}P control experiments was prepared by freeze−thawing individual lipids to form vesicles prior to adding together.

NMR Experiments. Spectra were recorded on a Bruker Avance 400 MHz spectrometer operating at frequencies of 400.13 MHz for ^{1}H, 162.12 MHz for ^{31}P, and 61 MHz for ^{2}H. Wide line ^{2}H NMR spectra of membranes containing DMPC-d_4 were obtained using a double-tuned magic-angle spinning probe without sample spinning. Spectra were recorded as a result of accumulating 10 000−20 000 transients with a 1-s recycle delay. The quadrupole echo sequence ($90_x - \tau - 90_y - \tau -$ acquisition) sequence (*30*) was used with a 90° pulse length of 4 μs and delay τ of 22 μs. In some experiments DMPC-d_{13} was used instead of DMPC-d_4 for reasons of availability. In these experiments, the signals from nine equivalent choline γ deuterons were suppressed by inserting an inversion recovery sequence before the quadrupole echo ($180_x - \tau_1 - 90_x - \tau_2 - 90_y - \tau_2 -$ acquisition*)*. A τ_1 delay of about 32 ms was used to select signals from α and β deuterons only. Wide line, proton decoupled ^{31}P NMR spectra were also recorded using a double tuned magic-angle spinning probe without sample spinning. The samples were contained within a 4-mm zirconia rotor, and spectra were recorded at 30 °C with 50 kHz proton decoupling during the acquisition period. Magic-angle spinning (MAS) ^{31}P NMR spectra were recorded at 4 °C using the same method but with sample rotation at 4 kHz.

RESULTS

Interactions of Full-Length α-Syn with Multilamellar Phospholipid Vesicles. Wide-line ^{2}H NMR was used to screen for interactions between full-length α-syn and membranes composed of phospholipids with zwitterionic and anionic headgroups. The ^{2}H NMR line shape from membranes of headgroup deuterated phospholipids is highly sensitive to changes in the orientation of the sites of deuteration that occur when peptides associate with the membrane surface (*31, 32*). MLVs were used as model membranes in these experiments because the slow tumbling of the vesicles does not average out the quadrupolar anisotropy of the deuterium nuclei and therefore permits site-specific details about the lipid molecules to be observed (*32*).

MLVs were prepared with DMPC containing deuterons at the methylene (α and β) positions (DMPC-d_4), alone or in admixture with the anionic phospholipids DOPG, BPI, or DOPS in a 2:1 molar ratio, with DMPC-d_4 in excess in all samples. Spectra were obtained at 30 °C before and after the addition of α-syn (Figure 2, left). Pake doublets arising from the α and β deuterons of DMPC-d_4 are separated by a quadrupole splitting $\Delta\nu_Q$, which is indicative of the orientation of the choline headgroup. For pure DMPC-d_4 membranes, the quadrupole splittings for the two sets of deuterons are equivalent, but the mixed membranes all give rise to two well-resolved doublets with nondegenerate splitting values (summarized in Table 1).

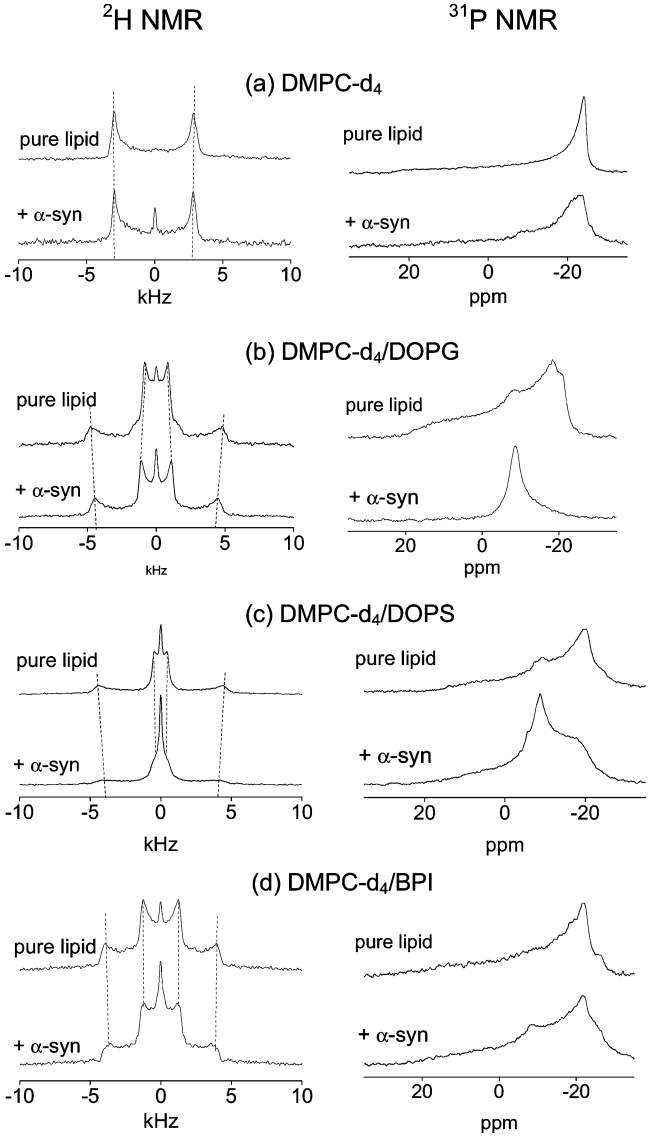

FIGURE 2: Detection of interactions between α-syn and phospholipid MLVs assessed using wide line NMR spectroscopy. Wide-line ^{2}H NMR spectra (left) and ^{31}P NMR spectra (right) were obtained from membrane samples of DMPC-d_4 alone (a) and DMPC-d_4/DOPG (b), DMPC-d_4/DOPS (c), and DMPC-d$_4$/BPI (d) in a molar ratio of 2:1 with DMPC in excess. In each experiment (a−d) spectra were first obtained from the pure lipid samples (top spectra) and again after the addition of α-syn to a 100:1 lipid/peptide molar ratio (bottom spectra). The spectra were recorded at 30 °C. Each sample was prepared from 1 to 2 mg of dry lipid. The quadrupole splittings for deuterons at the α- and β-choline positions of DMPC are denoted by the separation between the dotted lines.

Changes in the ^{2}H NMR line shapes were monitored after the addition of α-syn to the membranes at a lipid-to-protein molar ratio of 100:1. In the case of the pure DMPC membranes, no change in the NMR line shape was observed (Figure 2a, left), suggesting that the protein did not perturb the lipid headgroups under the experimental conditions. By contrast, the spectra of the mixed lipid membranes all showed changes in the splitting values after the addition of α-syn, signifying that the protein interacted strongly enough with the membrane surface to affect the orientation of the choline headgroups. Interestingly, the changes in the splittings for the α and β deuterons followed different patterns for the three membrane systems. In all three cases, the outer splitting for the α deuterons decreased, but the inner splitting from the β deuterons increased for the DMPC/DOPG and DMPC/DOPS membranes and decreased for the DMPC/BPI membranes. The origin of these different effects is not clear, but it appears that the change in orientation of the DMPC headgroups occurring after the addition of α-syn is modulated by other lipid species present in the membrane.

Table 1: Summary of ^{2}H Quadrupole Splitting ($\Delta\nu_Q$) Values for the α- and β-Deuterons of DMPC-d_4 Measured from the ^{2}H NMR Spectra of DMPC-d_4/DOPG, DMPC-d_4/DOPS and DMPC-d_4/BPI (in a 2:1 Lipid Molar Ratio) and DMPC-d_4 Alone in Pure Lipid MLVs and after the Addition of α-Syn to Lipid/Peptide Molar Ratio of 100:1

	$\Delta\nu_Q$ α (kHz)		$\Delta\nu_Q$ β (kHz)	
sample	pure lipid	100:1	pure lipid	100:1
DMPC/DOPG	9.8	9.0	1.1	2.2
DMPC/DOPS	8.8	8.3	0.9	1.1
DMPC/BPI	7.9	7.4	2.6	2.3
DMPC[a]	5.7	5.7	5.7	5.7

[a] The splitting values cannot be attributed to α or β deuterons unambiguously.

Wide line ^{31}P NMR was used to determine whether interactions of α-syn with the zwitterionic and anionic membrane surfaces destabilized the lamellar structure of the membranes. The line shape of ^{31}P spectra from lipid assemblies is sensitive to transitions from lamellar phases to inverted hexagonal phases (*33*), or small diameter (< 500 nm) vesicles and inverted micellar structures (*34*). All four membrane samples before the addition of α-syn gave rise to ^{31}P NMR line shapes that were characteristic of bilayer structures at 30 °C (Figure 2, right). After the addition of α-syn to the pure DMPC-d_4 membranes the spectrum remained diagnostic of a lamellar assembly (Figure 2a, right). The spectrum of the DMPC/DOPG membranes underwent a considerable narrowing after the addition of the protein, exhibiting a line shape suggesting that the lamellar membrane had become disrupted, perhaps forming hexagonal phase or to smaller vesicular structures (Figure 2b, right). Electron micrographs of the DMPC/DOPG membranes (not shown) were consistent with the NMR experiments and showed that α-syn fractured the vesicles into smaller structures. A similar narrow component appeared in the NMR spectrum of DMPC/DOPS membranes after the addition of α-syn, although a broad, bilayer-diagnostic component was also present, which indicated that these membranes were not disrupted to the same extent as the DMPC/DOPG membranes (Figure 2c, right). The spectrum of the DMPC/BPI membranes indicated that the lamellar structure was not perturbed by α-syn (Figure 2d, right), even though the ^{2}H NMR spectra indicated that the lipid headgroups were sensitive to the protein.

In summary, these experiments have shown that α-syn interacts with membranes with a negative surface charge and, when phosphatidylglycerol or phosphatidylserine headgroups are present, disrupts the lipid vesicular structure.

Studies of the Association of α*-Syn Peptides with Membrane Surfaces.* The studies on full-length α-syn above support previous conclusions that the protein interacts preferentially with negatively charged membrane surfaces and also revealed that the protein gives rise to considerable reorganization of the lipid molecules within MLVs. Further studies were carried out to examine whether the profound

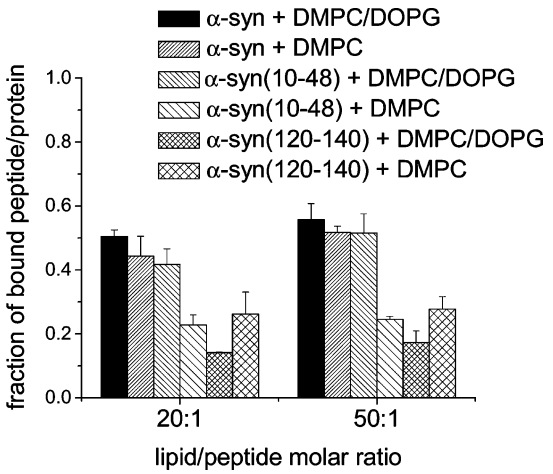

FIGURE 3: Measurement of the fraction of α-syn peptides bound to MLVs of DMPC/DOPG (2:1 lipid molar ratio) or DMPC. The fraction of α-syn(10−48) and α-syn(120−140) bound to MLVs at 30 °C was deduced from measurements of the residual concentration of peptide in the supernatant of the lipid suspensions, as described in Methods and Materials.

effect that α-syn has upon the integrity of lipid bilayers can be attributed to the lysine-rich N-terminal region of the protein. For this purpose, the membrane binding affinity of the peptide α-syn(10−48), corresponding to the region of the full-length protein extending from the first of the KXKXG repeats, was examined. For comparison, studies were also conducted on a second peptide, α-syn(120−140), representing the C-terminal domain of α-syn that is believed not to participate in membrane binding. Vesicles of DMPC/DOPG were chosen as model membranes with a negative surface charge because the NMR experiments shown in Figure 2 indicated that full-length α-syn has a greater effect on the organization of these membranes than on the other membrane systems examined.

The relative affinities of α-syn, α-syn(10−48), and α-syn(120−140) for DMPC/DOPG and DMPC membranes were assessed by determining the fraction of peptide or protein that co-sedimented with lipid MLVs, at initial lipid/peptide (protein) molar ratios of 50:1 and 20:1 (Figure 3). In the case of α-syn, approximately 50% of the protein co-sedimented with DMPC/DOPG membranes at a lipid-to-protein ratio of 50:1. Interestingly, a similar fraction of α-syn co-sedimented with DMPC MLVs, indicating that the protein had little or no specificity for the surface charge of the membranes. Such a lack of specificity has previously been reported in studies of α-syn binding to large unilamellar vesicles of neutral and negatively charged lipids (*25*), but α-syn appears to have a significantly higher affinity for small unilamellar vesicles containing negatively charged lipids (*13*). A similar fraction (∼50%) of the peptide α-syn(10−48) associated with the DMPC/DOPG membranes at the highest lipid/peptide molar ratio but rather less co-sedimented with DMPC, with less than 20% of the peptide bound. Conversely, α-syn(120−140) had a higher affinity for DMPC membranes than for DMPC/DOPG membranes, with ∼25% of the peptide bound to the DMPC membranes at a lipid/peptide molar ratio of 20:1.

Wide line ^{2}H NMR was used to examine how the peptides affect the lipid headgroups of uncharged and negatively charged membrane surfaces. In Figure 4 (left) is shown a

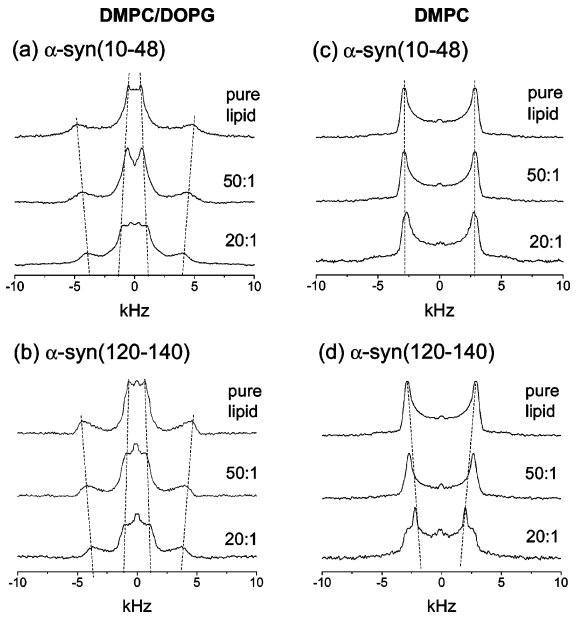

FIGURE 4: Detection of phospholipid headgroup perturbation by α-syn peptides from ^{2}H NMR spectra of MLVs containing deuterated DMPC. Spectra of vesicles containing headgroup deuterated DMPC (DMPC-d_4 or DMPC-d_{13}) and DOPG in a 2:1 molar ratio were obtained before and after the addition of α-syn(10−48) (a) or α-syn(120−140) (b) to a 50:1 and 20:1 lipid/peptide molar ratio. Spectra of vesicles containing deuterated DMPC alone were also obtained before and after the addition of α-syn(10−48) (c) or α-syn(120−140) (d) to a 50:1 and 20:1 lipid/peptide molar ratio. The spectra were recorded at 30 °C. The quadrupole splittings for deuterons at the α- and β-choline positions of DMPC are denoted by the separation between the dotted lines.

Table 2: Summary of Quadrupole Splitting ($\Delta\nu_Q$) Values for the α and β Deuterons of DMPC-d_4 (or DMPC-d_{13}) Measured from the ^{2}H NMR Spectra of DMPC-d_4 (-d_{13})/DOPG (in a 2:1 Lipid Molar Ratio) and DMPC-d_4 (-d_{13}) Alone in Pure Lipid MLVs and after the Addition of α-Syn(10−48) or α-Syn(120−140) to Lipid/Peptide Molar Ratios of 50:1 and 20:1

	$\Delta\nu_Q$ α (kHz)				$\Delta\nu_Q$ β (kHz)			
sample	pure lipid	100:1	50:1	20:1	pure lipid	100:1	50:1	20:1
DMPC/DOPG								
+ α-syn(10−48)	9.8	9.8	9.0	8.0	1.1	1.1	1.5	2.2
+ α-syn(120−140)	9.8	9.8	9.2	7.7	1.1	1.1	1.8	2.3
DMPC[a]								
+ α-syn(10−48)	5.7	5.7	5.7	5.7	5.7	5.7	5.7	5.7
+ α-syn(120−140)	5.7	5.7	5.7	5.7	5.7	5.7	5.5	4.3

[a] The splitting values cannot be attributed to α or β deuterons unambiguously.

series of ^{2}H NMR spectra from MLVs of DMPC-d_4/ DOPG (in a 2:1 molar ratio) obtained at 30 °C in the absence and in the presence of the two α-syn peptides. The quadrupole splittings measured from all the spectra in the presence of the two peptides are summarized in Table 2. Addition of α-syn(10−48) or α-syn(120−140) to the lipid/peptide molar ratio (100:1) used in the experiments on full-length α-syn had no effect upon the splittings (spectra not shown), suggesting that the shorter peptides perturb the lipid headgroups to a lesser extent than does the full-length protein. Upon addition of α-syn(10−48) to the MLVs, first to a lipid/peptide molar ratio of 50:1 and then to 20:1, the splitting for the α deuterons decreased and the β deuteron splitting increased in a peptide concentration-dependent manner

(Figure 4a). The changes in the spectrum indicate that peptide binding to the membrane surface alters the orientation of the choline headgroups. A similar trend was observed when α-syn(120−140) was added to DMPC-d_4/DOPG membranes at the same lipid peptide molar ratios (Figure 4b). These observations suggest that the presence of the C-terminal region of α-syn at negatively charged membrane surfaces perturbs the lipid headgroup orientations.

Further ^{2}H NMR experiments were carried out to observe the effects of the peptides upon membranes containing only DMPC-d_4 and having no net surface charge (Figure 4, right). The quadrupole splittings for the deuterons did not change significantly when syn(10−48) was added to the membranes even at a lipid/peptide molar ratio of 20:1, indicating that the choline headgroup orientation was not perturbed by the peptide (Figure 4c). Similarly, the spectrum of the DMPC-d_4 membranes did not change when α-syn(120−140) was added to a lipid-to-peptide molar ratio of 50:1, but at a ratio of 20:1 a decrease in the splitting for one set of deuterons of DMPC-d_4 could be observed (Figure 4d). These changes suggest that α-syn(120−140) does perturb the neutral surface of the DMPC vesicles, but at much higher lipid-to-protein ratios than were necessary to observe similar effects of full-length α-syn (Figure 2).

In summary, the peptides representing the N- and C-terminal regions of α-syn perturb the structure and orientation of lipid headgroups, but effects were observed at higher concentrations than were required for the full-length protein to give rise to similar effects. Hence, in the full-length protein, the N- and C-terminal regions and other parts of the protein probably have a combined effect on the lipid headgroups.

Effects of Peptide Binding upon Lipid Organization. The changes in the ^{2}H NMR spectra of phospholipid MLVs following the addition of α-syn peptides are reminiscent of the effects caused by peptides that induce the lateral segregation of the membrane into clusters or domains of neutral and negatively charged lipids (*32*, *35*, *36*). Here, the possibility that α-syn(10−48) and α-syn(120−140), and by inference the terminal domains of the parent protein, might also induce lipid clustering in mixed DMPC/DOPG vesicles was investigated using ^{31}P magic angle spinning (MAS) NMR. With this technique, it is possible to observe the effects of peptides on the headgroups of individual lipid species from changes in peak widths and chemical shifts (*37*, *38*).

The strategy for detecting lipid domain formation exploits the NMR properties of DOPG and DMPC arising from their different hydrocarbon chain compositions. The effects of these NMR properties are illustrated in Figure 5 by spectra of two DMPC/DOPG membrane samples obtained in the absence of α-syn peptides at 4 °C. A homogeneously mixed DMPC/DOPG MLV sample gives rise to a ^{31}P MAS NMR spectrum exhibiting peaks at −0.9 ppm from DMPC and at 0.4 ppm from DOPG (Figure 5a). Both peaks are rather narrow (width at half-height $\Delta\nu_{1/2} \sim 0.3$ ppm) because the unsaturated chains of DOPG maintain an overall high bilayer fluidity at 4 °C. The spectrum obtained from the same lipids prepared in an identical molar ratio, but as a mixture of preformed DMPC MLVs and preformed DOPG vesicles (Figure 5b), exhibits a similarly narrow peak for the fluid DOPG, but the peak for DMPC is significantly broader ($\Delta\nu_{1/2}$

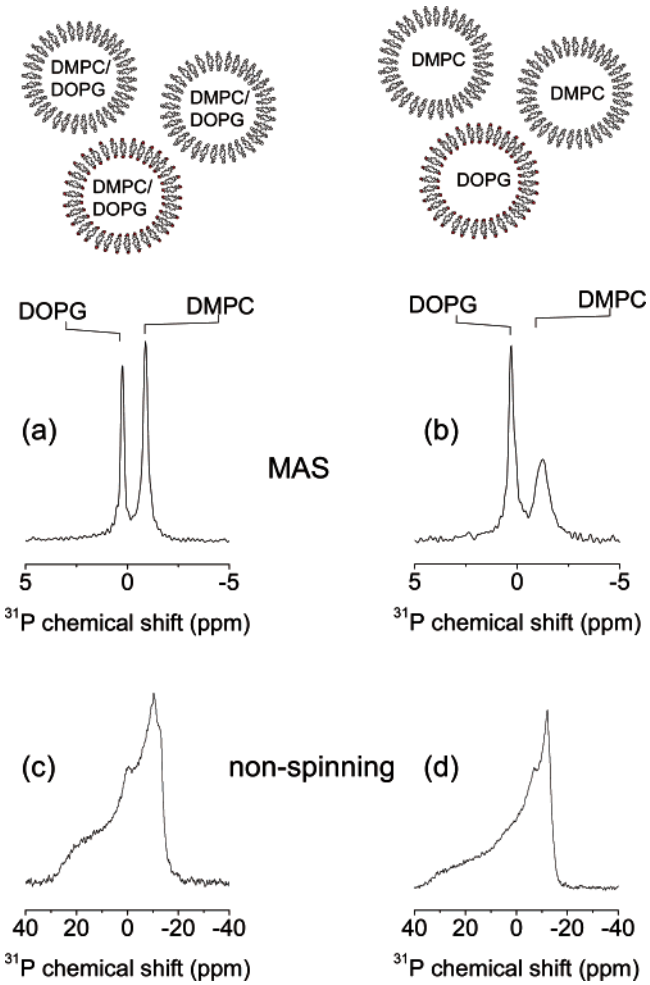

FIGURE 5: An illustration of the effect of lipid phase separation on the ^{31}P MAS NMR spectra of DMPC/DOPG membranes. The diagram at the top illustrates the composition of the homogeneously mixed vesicles prepared from DMPC/DOPG in a 2:1 molar ratio (sample I, left) and from a mixture of preformed DMPC and preformed DOPG vesicles in the same ratio (sample II, right). Spectra were obtained at 4 °C with sample spinning at 4 kHz from sample I (a) and sample II (b) after the accumulation of 512 transients. The assignments of the two peaks to DOPG and DMPC are as stated. Spectra were also obtained at 30 °C without sample spinning from sample I (c) and sample II (d) after the accumulation of 20 000 transients. The recycle delay was 1 s.

$\sim$ 0.77 ppm). The difference in the line widths occurs because the saturated chains of the DMPC molecules in the preformed vesicles are below the melting temperature ($\sim$23 °C) and, in the absence of unsaturated DOPG, are able to pack together into a gel phase. These lipids undergo slow or intermediate rates of rotational and lateral diffusion, which consequently broadens the ^{31}P NMR peak. Hence, this extreme example of lipid segregation leads to the formation of different lipid phases with distinct NMR properties. Similar effects may be observed if the α-syn peptides bind to the homogeneous DMPC/DOPG membranes and induce lipid clustering. Nonspinning ^{31}P NMR spectra of the same membrane samples at 30 °C (Figure 5c,d) confirm that both sets of membranes have a lamellar structure.

High resolution (MAS) and wide line ^{31}P NMR spectra of homogeneously mixed DMPC/DOPG membranes were obtained before and after the addition of α-syn(10−48) and α-syn(120−140) to a lipid-to-peptide molar ratio of 20:1 (Figure 6). The wide line spectra (Figure 6a−c) indicated

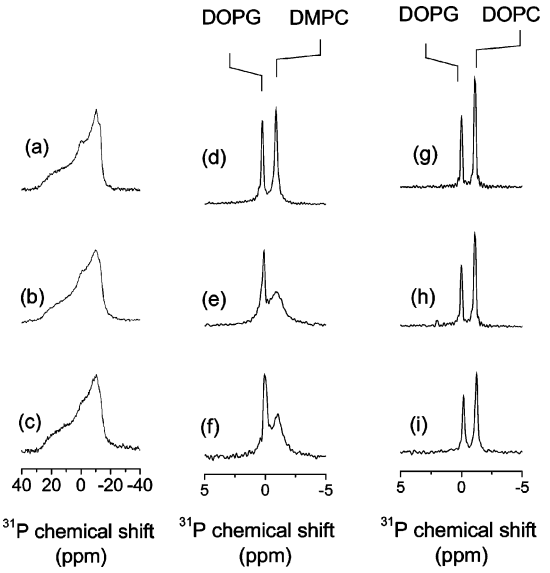

FIGURE 6: An experiment to detect lipid phase separation as a result of α-syn(10−48) and α-syn(120−140) binding to MLVs of DMPC/DOPG. Wide line ^{31}P NMR spectra at 30 °C (a−c) and MAS ^{31}P NMR spectra at 4 °C (d−f) were obtained from homogeneous vesicles (defined as sample I in Figure 5) of DMPC/DOPG in a 2:1 molar ratio before (a,d) and after the addition of α-syn(10−48) (b,e) or α-syn(120−140) (c,f) to a lipid/peptide molar ratio of 20:1. Also shown are the MAS spectra from homogeneous vesicles containing dioleoylphosphatidylcholine (DOPC) and DOPG in a 2:1 molar ratio before (g) and after the addition of α-syn(10−48) (h) or α-syn(120−140) (i). The peak assignments are stated above the MAS spectra. The sample spinning rate was 4 kHz, and a 1-s recycle delay was used.

Table 3: Summary of ^{31}P NMR Isotropic Chemical Shifts (σ_i) and Line Widths ($\Delta\nu_{1/2}$) at 4 °C and CSA Values at 30 °C for Homogeneous MLVs of DMPC/DOPG (in a 2:1 Molar Ratio) and Pure DMPC before and after the Addition of α-Syn(10−48) or α-Syn(120−140) to a Lipid to Peptide Molar Ratio of 20:1

	$\sigma_{i\,(ppm)}$		$\Delta\nu_{1/2}$ (ppm)		
sample	DMPC	DOPG	DMPC	DOPG	CSA (ppm)
DMPC/DOPG alone	−0.9	0.3	0.3	0.2	34[a]
+ α-syn(10−48)	−0.9	0.1	1.4	0.2	34[a]
+ α-syn(120−140)	−1.1	0.0	1.0	0.3	34[a]
DMPC alone	−0.5	N/A	0.8	N/A	43
+ α-syn(120−140)[a]	−0.6	N/A	0.9	N/A	43

[a] CSA values were measured from the outer edges of the spectra and cannot be attributed to a specific lipid component.

that all the membrane samples fully retained their bilayer composition after the addition of the peptides, in contrast to the pronounced phase changes that occurred after the addition of full-length α-syn (Figure 2). After the addition of the peptides, the peak from DOPG remained relatively narrow, but in all cases the peak from DMPC broadened considerably. Upon addition of α-syn(10−48), the peak width for DMPC broadened from ∼0.3 to ∼1.4 ppm (Figure 6e), and the chemical shift for DOPG moved upfield slightly (Table 3). Similarly, upon addition of α-syn(120−140) the DMPC peak width broadened, although slightly less from 0.3 to 1.0 ppm (Figure 6f). In this case, the chemical shifts for both lipid components were affected by peptide binding. The spectrum of pure DMPC (not presented) did not show any changes in line width or chemical shift after the addition of α-syn(10−48) or α-syn(120−140).

The spectra of the mixed DMPC/DOPG membranes after the addition of the α-syn peptides were reminiscent of the spectrum of mixed preformed vesicles shown in Figure 5b and suggested that the peptides bind to the membrane surface and separate the lipids into DOPG-rich and DMPC-rich domains. An alternative interpretation of the selective broadening is that the peptides associate directly with the choline headgroups and restrain the mobility of DMPC without reorganizing the lipid bilayer. This possibility was ruled out by conducting a control experiment in which spectra of DOPC/DOPG membranes were monitored before and after the addition of the peptides. These membranes present the same surface charge density to the peptides as do the DMPC/DOPG membranes, but all the hydrocarbon chains are unsaturated and the lipids will remain fluid at 4 °C even if they are separated into clusters. Selective line broadening of the peak for DOPC can only occur if the peptides interact directly with the choline headgroups and constrain their mobility. The two peaks in the spectra of the DOPC/DOPG membranes remain equally as narrow before and after the addition of the peptides (Figure 6g−i), confirming that the mobility of the choline headgroups is not restrained directly through interactions with the peptides.

Secondary Structure of α*-Syn Peptides in the Presence of Lipid Vesicles.* CD spectroscopy was used to examine whether the secondary structures of α-syn(10−48) and α-syn(120−140) in the presence of DMPC/DOPG membranes conformed to the structures of the corresponding regions of the parent protein observed when bound to detergent micelles (*19*, *23*). First, a TFE titration experiment was carried out to assess the intrinsic propensity of the peptides to adopt defined secondary structures, (*39*). It is well established that full-length α-syn can adopt a predominantly helical conformation upon association with lipid membranes (*13*). Spectra of the peptides alone (Figure 7a,b; black lines) were consistent with the two peptides being predominantly random coil in aqueous solution. Further spectra of the peptide solutions were then obtained during the titration of TFE into the solutions. The spectra of α-syn(10−48) indicated that the peptide underwent a progressive structural change toward a higher helical content as the volume of TFE was increased to 45% (v/v) (Figure 7a, red line). No further change in the spectrum was observed upon increased TFE addition (data not shown). Analysis of the spectrum at 45% TFE suggested that α-syn(10−48) adopted 10−15% helical structure. There is a clear isodichroic point within the spectra providing strong evidence that this secondary structural change is a two-state transition at the residue level from random coil to helix. The spectra of α-syn(120−140) suggests that it remains predominantly unfolded even at 45% TFE (Figure 7b, red line) and hence has little or no propensity to form an α-helix.

Similar, although less pronounced, structural changes of the peptides were observed when SUVs of DMPC/DOPG were titrated into the peptide solutions (Figure 7c,d, blue lines). A progressive increase in the helical structure of α-syn(10−48) was observed as the lipid-to-peptide ratio was increased, indicating that binding of the peptide to the membranes was accompanied by a marked change in its secondary structure (Figure 7c). By contrast, little or no change in the initial random coil structures of α-syn(120−140) (Figure 7d) was observed, and it is probable that the

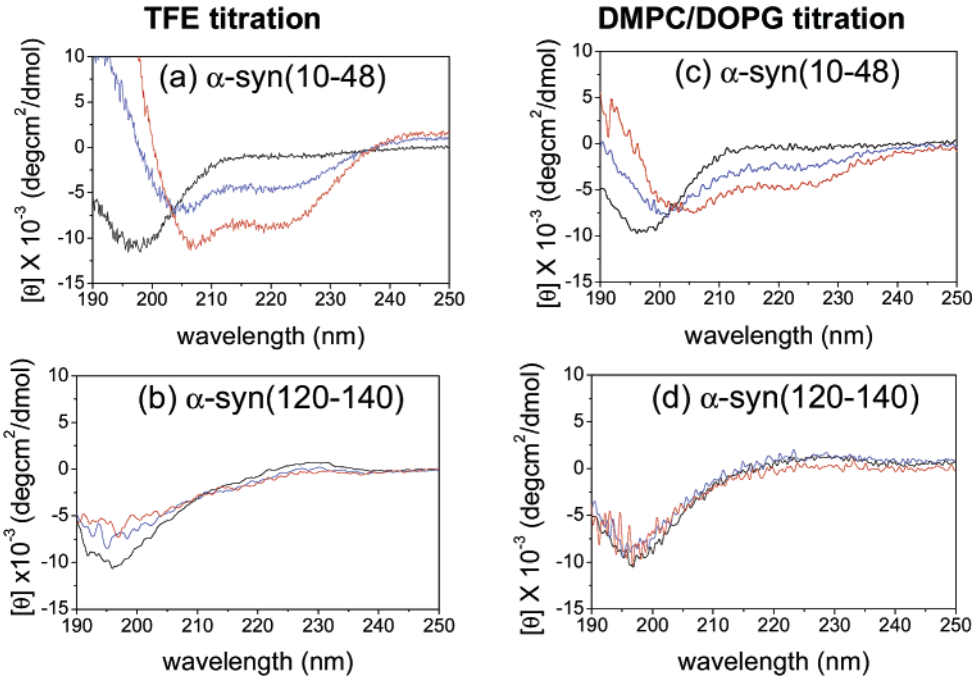

FIGURE 7: Analysis of the secondary structures of α-syn peptides in aqueous/TFE solutions and in the presence of SUVs of DMPC/DOPG using CD spectroscopy. CD spectra were obtained in the absence of lipids from aqueous solutions of α-syn(10−48) (a) and α-syn(120−140) (b) in the absence of TFE (black) and after the addition of TFE to 29 vol % (blue) and 41 vol % (red). The molar elipticities were calculated after correcting for dilution by TFE. Spectra were also obtained in aqueous (TFE-free) solution for α-syn(10−48) (c) and α-syn(120−140) (d) in the presence of SUVs of DMPC/DOPG at lipid/peptide molar ratios of 50:1 (blue) and 20:1 (red) and in the absence of lipid (black). The spectra were corrected for interference from the SUVs by subtracting the spectra of peptide-free lipid suspensions at the appropriate concentrations.

association of this peptide with the vesicles does not induce the formation of defined secondary structure or that the association is too weak to induce a stable structure.

DISCUSSION

Although the physiological role of α-syn is not fully understood, there is much evidence that the function of the protein is coupled to its association with plasma, vesicular, or raft membranes at the synaptic terminal (*5*, *40*). Mice with impaired α-syn expression exhibited reduced synaptic response to stimulation and also had fewer synaptic vesicles than in control animals (*41*). Observations in vivo, combined with evidence gained from studies of liposome binding, have led to the speculation that α-syn regulates, *inter alia*, the plasticity, mobilization, and distribution of synaptic vesicles. Moreover, the increased helical structure of α-syn accompanying its association with membranes appears to affect the propensity of the protein to assemble into the fibrils associated with neurodegeneration (*9*, *14*, *42*). Systematic studies of the effects of α-syn on lipid bilayer order and stability are useful to further define the biological and pathological significance of α-syn-lipid interactions in the synaptic terminus.

Here, NMR studies of the interactions of α-syn with MLVs support atomic force microscopy studies on planar lipid bilayers (*16*) by showing that the association of the protein with negatively charged membranes can have a profound effect upon the integrity of bilayers containing anionic phospholipids. Under the conditions of our experiments, the protein prompted the formation of nonbilayer or small vesicular structures when added to membranes of DMPC mixed with DOPG, DOPS, and BPI, but had little or no effect upon pure DMPC membranes. The extent of disruption of the mixed membranes followed the order DMPC/DOPG > DMPC/DOPS > DMPC/BPI, which suggests that the chemical structure of the anionic lipid headgroup moiety as well as its formal charge may influence the effect of protein binding on membrane order. The disruptive effect on the MLVs at the relatively high protein-to-lipid ratio studied here is likely to be exaggerated compared to how α-syn modulates synaptic membranes. The results strongly suggest, however, that the protein pays a role in modulating the organization of membrane lipid components.

We examined in further detail how α-syn interacts with phospholipid vesicles and affects the structure and organization of the lipid bilayer by observing the membrane interactions of peptides representing the N-terminal and C-terminal domains of the full-length protein. Conclusions about the membrane binding properties of the N- and C-terminal regions of α-syn may be drawn from studies of the two peptides α-syn(10−48) and α-syn(120−140) if is assumed that in the full-length protein these regions are independent of each other and there are no tertiary contacts between these and other regions of the protein that could modulate their lipid-binding properties. This assumption is validated by recent independent structural studies of α-syn in the presence of detergent micelles, all of which suggest that the membrane-bound protein adopts a largely helical conformation in the N-terminal region and an extended C-terminal region, excluding long-range tertiary contacts between sequentially distant sites (*19*, *20*, *23*). Moreover, our CD spectra of α-syn(10−48) and α-syn(120−140) suggest that the structures of the peptides in the presence of SUVs parallel those of the unfolded C-terminal and helical N-terminal regions of micelle

bound α-syn (Figure 7). Taken together, these results provide a strong argument for examining the membrane interactions of the terminal regions independently of each other, as the lipid environment appears to provide stabilizing forces that exceed the forces of attraction between the terminal regions of the protein.

Full-length α-syn and the peptides α-syn(10−48) and α-syn(120−140) exhibited a stable interaction with the surfaces of phospholipid MLVs as determined by a sedimentation experiment (Figure 3). The affinity of α-syn for charged (i.e., DMPC/DOPG) vesicles was similar to its affinity for vesicles with no net surface charge (i.e., DMPC). Previous studies of the membrane binding properties of full-length α-syn have shown that the protein binds strongly to small unilamellar vesicles (SUVs) of acidic phospholipids (*20*), but not to SUVs of phosphatidylcholine (*13*). By contrast, α-syn binds to large unilamellar vesicles (LUVs) of phosphatidylcholine with similar affinity as for LUVs of acidic lipids (*25*). The membrane binding affinity of α-syn is therefore likely to be influenced by differences in the surface area and curvature of SUVs and LUVs (and MLVs), as well as the surface charge. Interestingly, α-syn had a profound destabilizing effect upon DMPC/DOPG bilayers but showed little or no effect on DMPC bilayers (Figure 2). This striking disparity in the effects of α-syn on the two vesicle compositions is clearly not a consequence of differences in the membrane binding affinity of the protein and suggests that α-syn interacts with DMPC/DOPG membranes and DMPC membranes with rather different mechanisms. The peptide α-syn(10−48) binds preferentially to membranes possessing a negative surface charge density and has a much lower affinity for membranes composed only of zwitterionic lipids (Figure 3). Such behavior is predicted from the imperfect repeat units comprising the KXK sequence, which confer amphipathicity on the N-terminal region if it is folded as an α-helix. Interestingly, the peptide α-syn(120−140) interacts preferentially with the surface membranes composed of pure DMPC (Figure 3), suggesting that the glutamic acid-rich C-terminal region of membrane-bound α-syn is able to associate weakly with positively charged choline headgroups. Previous studies of full-length α-syn bound to lipid vesicles or SDS micelles did not detect any interactions of the C-terminal region with the lipids or detergent (*18*−*20*). The previous studies were conducted with vesicles or micelles having a highly negative surface charge, however, which would strongly disfavor interactions with the glutamate-rich C-terminal region. The results here suggest that such interactions may be more favorable with membranes having a low proportion of acidic phospholipids.

Together these observations suggest that both the N- and C-terminal regions of α-syn may both participate in membrane binding and may help to regulate the binding affinity of the protein according to membrane composition. Synaptic membranes are mainly composed of predominantly neutral PC (41%), PE (25%), plasmenylethanolamine (12%), and sphingomyelin (12%), with acidic PS (7%) and PI (4%) distributed throughout (*43*). The distribution of phospholipids within neuronal vesicles is asymmetric, with most of the anionic lipids found in the cytosol-facing leaflet of the membrane bilayer. The results presented here suggest that binding of α-syn to such membranes is driven predominantly by electrostatic interactions between anionic lipids and the basic N-terminal region of the protein. The low negative surface charge density of the vesicles may also permit interactions between the C-terminus and zwitterionic lipids such as PC and PE, however. Such interactions are likely to be weaker when the membrane contains higher proportions of anionic lipids.

^{31}P MAS spectra of DMPC/DOPG membranes in the presence of α-syn(10−48) or α-syn(120−140) suggest that the peptides affect lipid organization within the plane of the bilayer, possibly clustering specific lipid types into peptide-bound and peptide-free domains. Such a phenomenon might explain why full-length α-syn destabilizes the lipid bilayer to form smaller vesicular or nonbilayer structures. The N- and C-terminal regions of the full-length protein may act in concert to laterally separate the anionic and neutral lipids into domains, thereby placing an unfavorable strain on the lipid bilayer leading to breakdown of the vesicles. Lipid domain formation has been observed in a number of membrane binding peptides and proteins, but the biological role is not well understood. For example annexins, calcium binding proteins thought to be involved in cellular trafficking, bind rapidly to membrane surfaces and induces lipid clustering (*44*). Annexins are also able to bind to specific regions of cellular membranes, and it has been suggested that they play a role in caveolae organization (*45*). The ability of α-syn to organize lipids into clusters is therefore consistent with the theory that the protein plays a role in synaptic vesicle formation or in cellular trafficking, perhaps by recruiting lipids to sites of endocytosis. The results presented here provide good justification for conducting further experiments to examine in more detail the effects of α-syn on membrane lipid organization.

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