α-Synuclein Association with Phosphatidylglycerol Probed by Lipid Spin Labels[†]

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ABSTRACT: α-Synuclein is a small presynaptic protein, which is linked to the development of Parkinson's disease. α-Synuclein partitions between cytosolic and vesicle-bound states, where membrane binding is accompanied by the formation of an amphipathic helix in the N-terminal section of the otherwise unstructured protein. The impact on α-synuclein of binding to vesicle-like liposomes has been studied extensively, but far less is known about the impact of α-synuclein on the membrane. The interactions of α-synuclein with phosphatidylglycerol membranes are studied here by using spin-labeled lipid species and electron spin resonance (ESR) spectroscopy to allow a detailed analysis of the effect on the membrane lipids. Membrane association of α-synuclein perturbs the ESR spectra of spin-labeled lipids in bilayers of phosphatidylglycerol but not of phosphatidylcholine. The interaction is inhibited at high ionic strength. The segmental motion is hindered at all positions of spin labeling in the phosphatidylglycerol *sn*-2 chain, while still preserving the chain flexibility gradient characteristic of fluid phospholipid membranes. Direct motional restriction of the lipid chains, resulting from penetration of the protein into the hydrophobic interior of the membrane, is not observed. Saturation occurs at a protein/lipid ratio corresponding to ∼36 lipids/protein added. α-Synuclein exhibits a selectivity of interaction with different phospholipid spin labels when bound to phosphatidylglycerol membranes in the following order: stearic acid > cardiolipin > phosphatidylcholine > phosphatidylglycerol ≈ phosphatidylethanolamine > phosphatidic acid ≈ phosphatidylserine > *N*-acyl phosphatidylethanolamine > diglyceride. Accordingly, membrane-bound α-synuclein associates at the interfacial region of the bilayer where it may favor a local concentration of certain phospholipids.

α-Synuclein is a small presynaptic protein that is intimately involved in several forms of neurodegenerative disease (e.g., Parkinson's disease), characterized by the development of pathological cytoplasmic inclusions (Lewy bodies), wherein misfolded aggregated α-synuclein is a prime component (for a review, see ref *1*). The direct pathogenic role of α-synuclein is demonstrated by the existence of missense mutations leading to autosomal dominant early-onset Parkinson's disease (*2*, *3*).

The normal function of α-synuclein is not known with certainty, but it has been implicated in dopamine metabolism and synaptic vesicle homeostasis (*4*). The relation of α-synuclein to the vesicular membrane is not clear in quantitative, qualitative, and dynamic terms because it exists both as a cytosolic protein and as associated with brain vesicles (*5*, *6*). The most detailed information has been obtained from investigations using synthetic membranes. These have shown a requirement for negatively charged phospholipids and demonstrated that the interaction leads to an increase in the α-helical content of α-synuclein (*7*−*10*). Such a structural transition from an unfolded soluble protein to an α-helical membrane-bound species is engendered by the N-terminal domain (residues 1−102) of the protein, which contains seven 11-residue repeats of the consensus sequence pKTKEGVaxaA, where p is a polar residue, a is an apolar residue, and x is any residue (*5*, *8*). This motif characterizes class A_2 amphipathic helices of the type involved in the lipid-binding domains of serum apolipoproteins (*11*). Recent NMR studies have confirmed and extended this prediction by demonstrating that the N-terminal 103 amino acids form a broken amphipathic α-helix with the remaining C-terminal 37 residues being unfolded (*12*). The binding of α-synuclein to vesicles is disturbed by the pathogenic missense A30P mutation (*6*), and prefibrillar α-synuclein oligomers have been found capable of permeabilizing vesicles composed of negatively charged phospholipids (*13*, *14*). It is therefore likely that membrane association may be involved in both the normal and the pathogenic function of α-synuclein.

Electron spin resonance (ESR)[1] spectroscopy of spin-labeled lipids has proved to be a most useful means of studying protein−lipid interactions with peripheral as well as integral proteins in membranes (for a review, see ref *15*). Both the stoichiometry and the selectivity of lipid−protein interactions may be determined by spin-label ESR methods. Surface association can be distinguished from membrane penetration and transmembrane insertion by the characteristic effects on the lipid chain mobility that are registered in the spin-label spectrum. The read-out from the method is related to the labeled phospholipids and reflects the impact of the protein on the lipid component of the membrane. Accordingly, such methodology applied to α-synuclein will comple-

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ment the aforementioned studies that focused on the peptide rather than on the membrane. In the present work, we characterize the interactions of α-synuclein with lipid bilayer membranes by using phospholipid ESR probes that are labeled systematically throughout the length of the *sn*-2 acyl chain. At maximum binding to dimyristoyl phosphatidylglycerol membranes, the N-terminal lipid-associating domains of the protein are less than close-packed. The binding reaction is dominated by surface electrostatics, augmented by the amphiphilicity of the binding domains. Surface association of α-synuclein perturbs the mobility in the upper parts of the lipid chains, with no evidence for penetration of the membrane or transmembrane insertion of the protein. The membrane-bound protein displays selectivity for different species of the spin-labeled phospholipid probes, which suggests that α-synuclein can modify the micromilieu within the outer leaflet of the membrane.

MATERIALS AND METHODS

Materials. Recombinant human wild-type α-synuclein was expressed, purified, and characterized as previously described in ref *6*, with the addition of a final reversed-phase high-pressure liquid chromatographic step on a Jupiter C18 (250 × 10 mm) column (Phenomenex) to remove any contaminating polynucleotides. The reversed-phase high-pressure liquid chromatography was carried out using a linear gradient from buffer A to B, the buffers A and B being 0.1% trifluoroacetic acid and 80% acetonitrile in 0.1% trifluoroacetic acid, respectively. The purified proteins were lyophilized using a SpeedVac (Savant). Spin-labeled fatty acids, *n*-SASL, were synthesized as described in ref *16*. Phosphatidylcholines, *n*-PCSL, acylated with *n*-SASL at the *sn*-2 position were prepared as described in ref *17*. Phosphatidylglycerols, *n*-PGSL, and other corresponding spin-labeled phospholipid species were prepared from *n*-PCSL by phospholipase D-catalyzed headgroup exchange as described in the same reference. 5-NAPESL was prepared as described in ref *18* and 5-DGSL as in ref *19*. Spin-labeled cardiolipin, 5-CLSL, was prepared as described in ref *20*, with monolysocardiolipin from Avanti. Dimyristoyl phosphatidylcholine and phosphatidylglycerol (DMPC and DMPG) were obtained from Avanti Polar Lipids (Alabaster, AL).

Sample Preparation. Samples for ESR spectroscopy were prepared as follows. 100 μg of DMPG and 1% (w/w) of the spin label were codissolved in CH_2Cl_2, and a thin film of the lipid was produced by evaporating the solvent with dry nitrogen gas. Final traces of solvent were removed by subjecting the sample to vacuum desiccation for at least 3 h. The sample was then hydrated with 8 μL of 10 mM HEPES, 5 mM EDTA, 150 mM NaCl, pH 7.4 buffer (HBS) and vortexed. The lipid suspension thus obtained was transferred into a 50 μL glass capillary and pelleted in a tabletop centrifuge. Samples containing the protein−lipid complex were prepared in a similar manner, except that the lipid film was hydrated directly with the protein solution in HBS and subjected to at least five freeze−thaw cycles before being transferred to the glass capillaries. With phosphatidylglycerol, it was found that the samples containing protein became optically clear and could no longer be pelleted; therefore, the sample was used as such for ESR studies. In view of this limitation, the volume of the protein solution added was minimized by using a high concentration of protein (20 mg/mL), such that the amount of sample in the ESR cavity was maximized. About 100 μg of lipid and 100 μg of protein were used for the positional dependence and lipid selectivity experiments. Protein concentrations were determined by the method of Lowry et al. (*21*) using bovine serum albumin (1 mg/mL standard from PIERCE) as a standard.

Electron Spin Resonance Spectroscopy. ESR spectra were recorded on a 9 GHz Bruker EMX EPR spectrometer, with a model ER 041 XK-D microwave bridge. Samples were placed in 50 μL glass capillaries and flame sealed. The capillaries were placed in a standard 4 mm quartz sample tube containing light silicone oil for thermal stability. The temperature of the sample was maintained constant by blowing thermostated nitrogen gas through a quartz dewar. Spectra were recorded using the following instrumental settings: sweep width, 120 G; resolution, 1024 points; time constant, 20.48 ms; sweep time, 41.9 s; modulation frequency, 100 kHz; modulation amplitude, 1.0 G; incident power, 5.04 mW. Values of the outer hyperfine splitting, $2A_{max}$, were determined by measuring the difference between the low-field maximum and the high-field minimum.

Hydrophobicity Calculations. The conventional hydrophobic index at residue position *i* is calculated as

$$\Delta G_{tr}(i) = h(i) + h(i \pm 1) + h(i \pm 2) + h(i \pm 3) + h(i \pm 4) \quad (1)$$

where $h(i)$ is the free energy of transfer of residue *i* from water to the membrane polar−apolar interface. Values of $h(i)$ are obtained from the whole-residue interfacial hydrophobicity scale of White and Wimley (*22*). The transfer free energy for one face of an α-helix is calculated according to

$$\Delta G_{tr}^{\alpha}(i) = h(i) + h(i \pm 3) + h(i \pm 4) \quad (2)$$

For an amphipathic α-helix, $\Delta G_{tr}^{\alpha}(i)$ should oscillate between high and low values, with a 3.6-fold periodicity (i.e., between *i* and $i \pm 2$). Both eqs 1 and 2 apply to a nine-residue window (i.e., two turns of an α-helix).

RESULTS

Interactions with Phosphatidylglycerol and Phosphatidylcholine. The association of α-synuclein with lipid membranes can be detected from the perturbation of the chain mobility of spin-labeled lipids, by using ESR spectroscopy as found for classical water-soluble peripheral membrane proteins (*23*). Figure 1 shows the temperature dependences of the outer hyperfine splittings of spin-labeled phosphatidylglycerol (5-

[1] Abbreviations: DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; *n*-SASL, *n*-(4,4-dimethyloxazolidine-*N*-oxyl)stearic acid; *n*-PGSL, -PCSL, 1-acyl-2-[*n*-(4,4-dimethyloxazolidine-*N*-oxyl)]stearoyl-*sn*-glycero-3-phosphoglycerol, -phosphocholine; 5-PESL, -PASL, -PSSL, 1-acyl-2-[5-(4,4-dimethyloxazolidine-*N*-oxyl)]stearoyl-*sn*-glycero-3-phosphoethanolamine, phosphatidic acid, -phosphoserine; 5-CLSL, 1-(3-*sn*-phosphatidyl)-3-[1-acyl-2-(5-(4,4′-dimethyloxazolidine-*N*-oxyl)stearoyl)-*sn*-glycero(3)phospho]-*sn*-glycerol; 5-DGSL, 1-acyl-2-[5-(4,4′-dimethyloxazolidine-*N*-oxyl)]stearoyl-*sn*-glycerol; 5-NAPESL, 1,2-dipalmitoyl-*sn*-glycero-3-[*N*-5-(4,4-dimethyloxazolidine-*N*′-oxyl)stearoyl]-phosphoethanolamine; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; ESR, electron spin resonance.

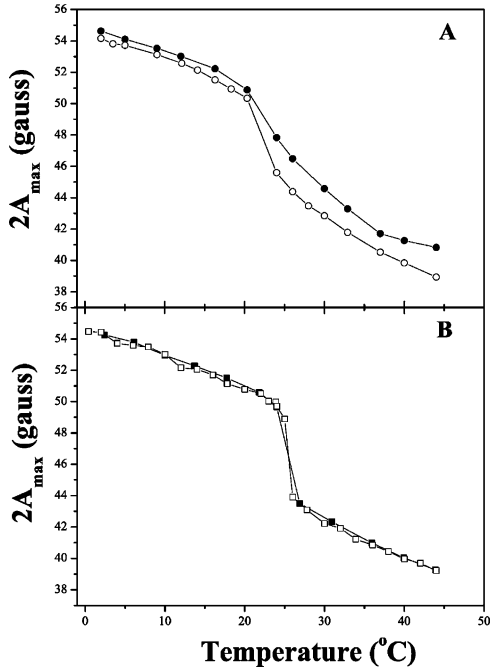

FIGURE 1: Temperature dependence of the outer hyperfine splitting, $2A_{max}$, of 5-position phospholipid spin labels in phospholipid membranes in the presence (solid symbols) and absence (open symbols) of 1:1 wt/wt α-synuclein. (A) 5-PGSL in dimyristoyl phosphatidylglycerol bilayer membranes. (B) 5-PCSL in dimyristoyl phosphatidylcholine bilayer membranes.

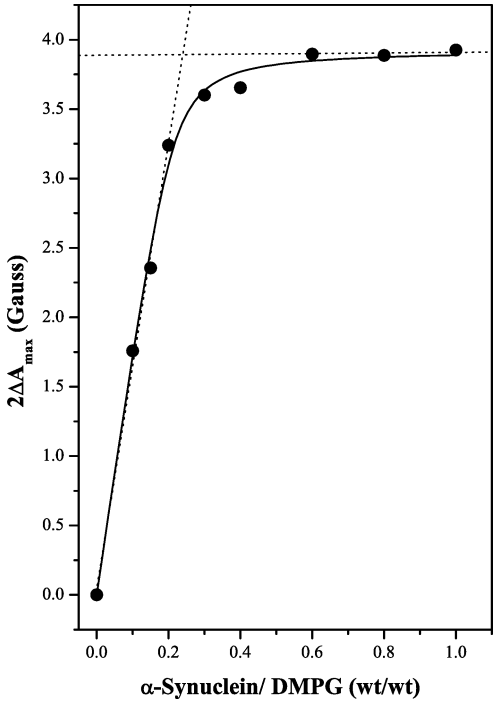

FIGURE 2: Dependence of the increase, $2\Delta A_{max}$, in outer hyperfine splitting of the 5-PGSL phosphatidylglycerol spin label on the α-synuclein/lipid ratio in DMPG membranes at 30 °C. Dashed lines indicate the initial linear slope and constant saturation level.

PGSL) in dimyristoyl phosphatidylglycerol (DMPG) membranes and of spin-labeled phosphatidylcholine (5-PCSL) in phosphatidylcholine (DMPC) membranes. The increase in rotational motion at the chain melting temperature of 23−25 °C is evident for both lipid membranes. Addition of 1:1 wt/wt α-synuclein with respect to lipid decreases the mobility (increases A_{max}) of 5-PGSL in both the gel and the fluid membrane phases of DMPG (see Figure 1A). The larger effect is in the fluid phase. The cooperativity of the DMPG chain melting transition is reduced slightly, but its position remains approximately the same. In contrast, there is no evidence from the spin-label ESR spectra for an interaction with phosphatidylcholine membranes. The spin-label mobility is the same in the presence and absence of α-synuclein for DMPC membranes (Figure 1B). That α-synuclein does not bind to DMPC membranes, under these conditions, was demonstrated by direct protein assays.

The effect of increasing ionic strength on the binding to DMPG membranes was also investigated (spectra not shown). In the absence of salt, the increase in outer hyperfine splitting of the 5-PGSL spin label on adding 1:1 wt/wt α-synuclein to DMPG membranes was $2\Delta A_{max} = 4.2$ G, at 38 °C. With 150 mM NaCl, this was reduced to $2\Delta A_{max} = 3.1$ G and with 500 mM NaCl to $2\Delta A_{max} = 0.7$ G. Shielding of electrostatic interactions therefore reduces the binding of α-synuclein by ∼85%. This argues against a significant hydrophobic contribution and underscores the role of ionic interactions.

Protein−Lipid Titration. Figure 2 gives the increase in outer hyperfine splitting, $2\Delta A_{max}$, of the 5-PGSL spin-labeled phosphatidylglycerol in DMPG membranes with increasing α-synuclein concentration. The decrease in lipid chain mobility saturates with a value of $2\Delta A_{max} \approx 3.9$ G at a weight ratio of added α-synuclein to DMPG of approximately 0.6, which corresponds to approximately 36 lipids per protein. The stoichiometry of the interaction can be estimated alternatively by extrapolation of the increase in $2\Delta A_{max}$, on initial tight binding, to the saturation value of $2\Delta A_{max}$. This gives a value of one protein bound per 100 lipids. The latter is likely to be an upper estimate because of a possible nonlinear dependence of $2\Delta A_{max}$ on protein binding. Addition of α-synuclein to DMPG lipid dispersions solubilizes the lipid, which precludes determination of the binding stoichiometry by a conventional centrifugation assay (see Materials and Methods). All subsequent experiments were performed at a 1:1 weight ratio of added protein to lipid, to ensure saturation binding of protein to the lipid.

Chain Flexibility Profiles. Perturbation by α-synuclein binding of the ESR spectra from spin labels at different positions, *n*, in the *sn*-2 chain of the lipid was also investigated. Figure 3 gives the ESR spectra of the *n*-PGSL phosphatidylglycerol spin-label positional isomers in fluid DMPG bilayer membranes, in the presence and absence of a saturating quantity of α-synuclein. In the absence of protein, the outer hyperfine splitting, or the spectral anisotropy (which is given by the difference between the outer and the inner hyperfine splittings), decreases progressively with increasing *n*, as the spin-label position is stepped down the chain toward the center of the membrane. Close to the terminal methyl region of the chain, a quasi-isotropic, sharp, three-line spectrum is obtained for 14-PGSL and 16-PGSL. This flexibility gradient in segmental chain mobility is a characteristic hallmark of the liquid−crystalline state of fluid phospholipid bilayers (see, e.g., ref *24*).

In the presence of bound α-synuclein, the outer hyperfine splitting and spectral anisotropy is increased at all spin-label positions, with the possible exception of 16-PGSL (see Figure 3). Nevertheless, the characteristic flexibility gradient with

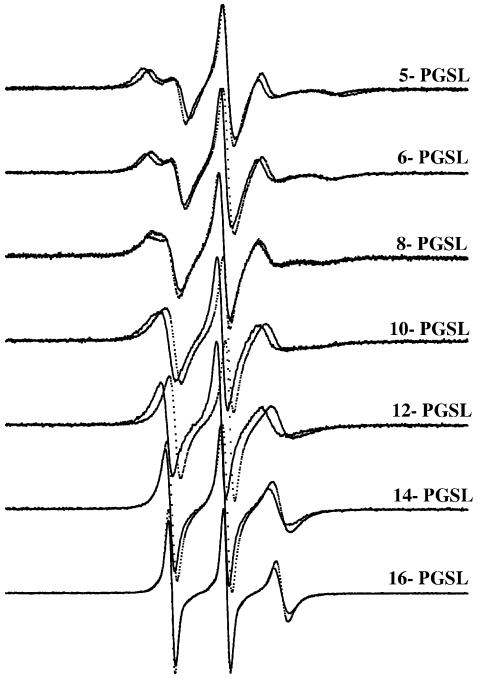

FIGURE 3: ESR spectra of *n*-PGSL positional isomers of spin-labeled phosphatidylglycerol in fluid-phase DMPG bilayer membranes in the presence (solid lines) and absence (dotted lines) of 1:1 wt/wt α-synuclein (1:22 mol/mol). Spin-label position, *n*, in the *sn*-2 chain is indicated on the figure. $T = 38$°C; scan width = 120 G.

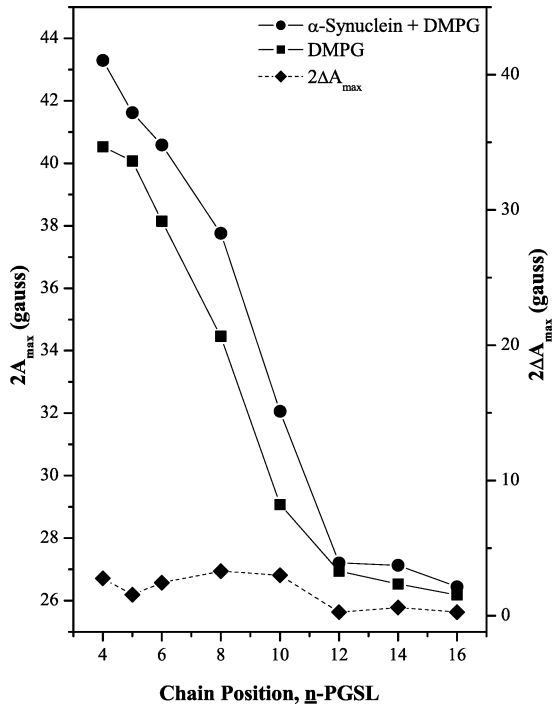

FIGURE 4: Dependence on spin-label position, *n*, of the outer hyperfine splitting, $2A_{max}$, of the *n*-PGSL phosphatidylglycerol spin labels in fluid-phase membranes of DMPG with (circles) and without (squares) 1:1 wt/wt α-synuclein (1:22 mol/mol). $T = 38$ °C. Dashed line (diamonds) and right-hand scale: increase, $2\Delta A_{max}$, in outer hyperfine splitting on adding α-synuclein.

the chain position of the fluid lipid bilayer membranes is preserved. Figure 4 shows the dependence of the outer hyperfine splitting, $2A_{max}$, on chain position, *n*, for the *n*-PGSL spin labels in fluid DMPG membranes, with and without a saturating amount of α-synuclein bound. The outer

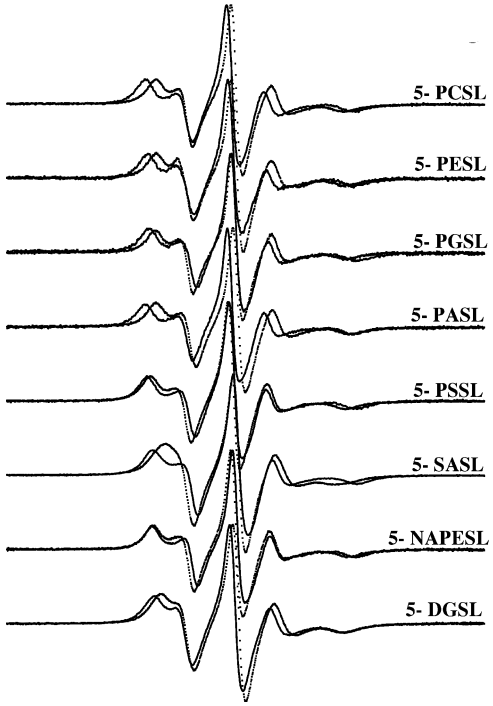

FIGURE 5: ESR spectra of different 5-position spin-labeled lipid species (5-PXSL, 5-SASL, 5-CLSL, 5-NAPESL, and 5-DGSL, as indicated) in fluid-phase DMPG bilayer membranes in the presence (solid lines) and absence (dotted lines) of 1:1 wt/wt α-synuclein (1:22 mol/mol). $T = 38$ °C; scan width = 120 G.

hyperfine splitting is increased to roughly comparable extents at the different chain positions down to C-10. As for the binding of other peripheral proteins, this indicates that surface association of α-synuclein increases the chain packing density in the membrane (see, e.g., ref *25* and references therein). There is no appearance of a second, more motionally restricted component in the spectra of the spin labels positioned toward the terminal methyl ends of the chains. This is evidence that α-synuclein binds solely at the membrane surface and does not penetrate the membrane interior, as does, for instance, the myelin basic protein or apocytochrome *c* (*23*).

Selectivity of Lipid−Protein Interaction. The selectivity of interaction of different lipids with α-synuclein bound to DMPG was determined by using probe amounts of lipids spin labeled on the 5-C atom of the *sn*-2 chain. Figure 5 gives the ESR spectra of the different spin-labeled phospholipids, 5-PXSL, and of spin-labeled stearic acid, 5-SASL, in fluid bilayer membranes of DMPG in the presence and absence of a saturating amount of α-synuclein. Also included in Figure 5 are spectra of spin-labeled *N*-acyl phosphatidylethanolamine, 5-NAPESL, in which the spin label is at the 5-position of the *N*-acyl chain, and of spin-labeled diglyceride, 5-DGSL, which lacks the phospholipid headgroup, but where the spin label is still at the 5-position of the *sn*-2 chain.

For all spin-labeled lipids tested, the outer hyperfine splitting, $2A_{max}$, is greater for the protein-bound membranes than for the free lipid membranes. The increase in $2A_{max}$ differs, however, for the different spin-labeled lipids. This reflects a selectivity of interaction of α-synuclein with the different lipid headgroups. Table 1 gives the increase, $2\Delta A_{max}$, in outer hyperfine splitting for the protein-bound membranes for each of the spin-labeled lipids tested. The

Table 1: Outer Hyperfine Splittings ($2A_{max}$) at 38 °C of Phospholipid Spin Labels Labeled at the Fifth Position of the *sn*-2 Chain, Incorporated in DMPG Bilayers and α-Synuclein/DMPG Complexes[a]

	$2A_{max}$ (G)		
spin label	DMPG	DMPG + α-syn	$2\Delta A_{max}$ (G)
5-SASL	35.5	42.8	7.3
5-CLSL	35.5	41.9	6.4
5-PCSL	39.3	43.7	4.4
5-PESL	39.6	43.1	3.6
5-PGSL	40.0	43.5	3.5
5-PASL	40.7	43.7	3.0
5-PSSL	40.9	43.8	2.9
5-NAPESL	41.0	42.8	1.8
5-DGSL	37.9	38.4	0.5

[a] Buffer: 10 mM HEPES, 5 mM EDTA, and 150 mM NaCl, pH 7.4.

largest increase is for the single chain charged lipid, 14-SASL, and for the charged double-headgroup lipid, 14-CLSL. The smallest increase is for the neutral lipid, 5-DGSL.

DISCUSSION

Figure 6 shows the hydropathy profile of α-synuclein calculated using a nine-residue window (i.e., eq 1) with free energies of transfer to the membrane interface from White and Wimley (*22*). This forms a useful basis for discussion of the interactions with lipid membranes. Experiments with deletion and fusion proteins have demonstrated that the lipid binding capacity of α-synuclein is distributed across exons 3, 4, and 5, encoding residues 1−102 (*8*). NMR studies also indicate that only the N-terminal domain (residues 1−102) designated in Figure 6 binds to lipid membranes (*27*). This domain has a net charge of +5 at neutral pH and contains six-residue KTKEGV consensus sequences, which form the core for 11-residue repeats of class A_2 amphipathic helices (*7*). The amphipathic character of the helices in the N-terminal domain is indicated by the four-residue oscillatory periodicity of the dotted line in Figure 6. This is calculated according to eq 2 to give the free energy of transfer for just one face of a nine-residue stretch of α-helix (see also ref *28*). The C-terminal domain (residues 103−140) is highly acidic, with a net charge of −15, and does not bind to membranes (*8*, *27*).

Binding and Chain Mobility. α-Synuclein perturbs the lipid chain motion of membranes composed of the anionic lipid phosphatidylglycerol but not of the zwitterionic lipid phosphatidylcholine. This correlates with previous findings that α-synuclein binds only to negatively charged lipids and not to electroneutral lipids (*7*, *9*, *13*). The net positive charge of the N-terminal lipid-binding domain is responsible for the requirement for negatively charged lipids. Unlike serum apolipoproteins (*29*), or bee venom melittin (*30*), the amphipathic nature of the N-terminal helical domain is insufficient to achieve binding of α-synuclein to phosphatidylcholines. The lack of α-synuclein binding to zwitterionic lipids is in agreement with the predictions of Figure 6. Almost no segments of appreciable length have a favorable free energy of transfer, $\Delta G_{tr}^{\alpha} < 0$, to the membrane interface (dashed line in Figure 6). This is in contrast to the amphiphilic helices in serum apolipoproteins A-I and A-II, for instance, which are predicted to have a favorable free energy of transfer (data not shown).

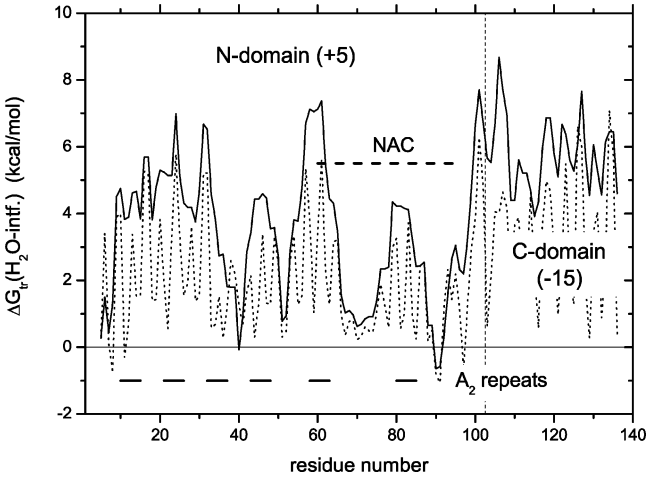

FIGURE 6: Free energy of transfer, ΔG_{tr}, from water to the membrane interface for α-synuclein (*26*), calculated using a nine-residue window (solid line) with the hydrophobicity scale of White and Wimley (*22*). The dotted line is the hydrophobicity calculated for one side of an amphipathic helix, according to eq 2. The vertical dashed line divides the α-helical N-terminal membrane-binding domain from the unstructured acidic C-terminal domain (*27*). Short horizontal bars represent the KTKEGV consensus repeat motif (*5*); the long dashed bar indicates the NAC peptide found in amyloid plaques (*26*).

The interaction of α-synuclein with phosphatidylglycerol membranes is largely abolished at high ionic strength, as registered by the effect on lipid chain mobility. This is a characteristic feature of electrostatic binding that is shared in common with peripheral basic proteins (*31*, *32*). A reduction in binding of α-synuclein to phosphatidic acid-containing membranes (*7*), and to phosphatidylserine-containing membranes (*9*), at high ionic strength has been found previously. However, binding to small sonicated vesicles was only reduced by 30% in the former study, in contrast to the latter study and the present results with lipid dispersions.

Stoichiometry of Lipid−Protein Interaction. High-resolution NMR experiments have shown that only the N-terminal domain, residues 1−102 (see Figure 6), binds to anionic micelles and lipid vesicles (*27*). When bound, the N-terminal domain adopts an α-helical configuration. In this conformation, an amphipathic helix lying on the surface of the membrane would cover approximately 25−30 lipids (of cross-sectional area 0.62 nm^2), depending on the effective diameter of the helix (1.0−1.2 nm). From the results of Figure 2, it was deduced that the saturating stoichiometry for α-synuclein was in excess of 36, and maximally 100, lipids per monomer. It therefore seems likely that the N-terminal amphipathic helices are not closely packed on the membrane surface. This contrasts with the binding of basic peripheral proteins, such as cytochrome *c* (*31*, *33*) and myelin basic protein (*32*), to anionic lipids, which are approximately close-packed at saturation. The unbound, nonstructured C-terminal domains of α-synuclein must inhibit close-packing of the N-terminal domains at the membrane surface. Saturation binding of α-synuclein to 1:1 mixtures of palmitoyl-oleoyl phosphatidylcholine and phosphatidylserine is found at lipid/protein ratios ∼5−10:1 w/w (*9*), which also corresponds to a high stoichiometry of 50−100 negatively charged lipids per protein.

Membrane Binding Site. The pattern of perturbation of the lipid chain motion by α-synuclein allows conclusions to be drawn on the nature of the membrane binding site. Comparison with ESR studies on the binding of other soluble proteins and peptides to charged lipid membranes is helpful in this respect. Only basic peptides and proteins that do not penetrate the membrane, such as pentalysine, polylysine (*34*), and cytochrome *c* (*35*), bind to negatively charged lipids without abolishing the bilayer chain melting phase transition. The effect of α-synuclein on the thermotropic behavior of DMPG bilayers (see Figure 1A) is similar to that for these classical basic peptides and proteins. The lipid chain mobility is reduced to a limited extent, both in the fluid phase and in the gel phase, by binding α-synuclein (cf., ref *34*). This suggests that α-synuclein binds to the membrane surface without penetrating the hydrocarbon chain region. The preservation of the characteristic chain flexibility gradient, and the lack of any second motionally restricted spin-labeled lipid component in the spectra from labeling positions $n = 12-16$ (see Figure 3), also supports this conclusion. For instance, the transmembrane insertion of α-lactalbumin at low pH drastically changes the shape of the chain flexibility profile and results in the appearance of a population of motionally restricted lipids (*36*), quite unlike the situation with α-synuclein. The profile of perturbation by α-synuclein binding that is shown in Figure 4 indicates effects on the lipid mobility only down to chain position $n = 10$. A similar, although less complete, cutoff is also observed in the binding of melittin to phosphatidylglycerol membranes (*30*). For other basic proteins, such as myelin basic protein (*32*), avidin (*25*), and cytochrome *c* (Görrisen and Marsh, unpublished), the perturbation extends deeper, to the $n = 12$ and 14 positions. Possibly, the surface orientation of an amphipathic helix results in suboptimal interaction with the charged lipid headgroups; hence, perturbation of the chain packing density extends less deeply into the membrane.

Lipid Selectivity. The lipid selectivities reported in Table 1 are for the association of spin-labeled lipids at probe amounts with α-synuclein that is prebound to dimyristoyl phosphatidylglycerol membranes. A model has been presented for the selectivity (see Appendix) in which the spin-labeled lipids compete for specific sites, with relative association constants, K_r (*37*). Relative to phosphatidylglycerol (PG), the association constants are then related to the increase, $2\Delta A_{max}$, in outer hyperfine splitting by

$$\frac{K_r}{K_r^{PG}} = \frac{\Delta A_{max}{}^{o}/\Delta A_{max}^{PG} - 1}{\Delta A_{max}{}^{o}/\Delta A_{max} - 1} \qquad (3)$$

It is assumed that there is a rapid exchange of the lipid between free and bound sites and that the intrinsic value of ΔA_{max} for the bound species is $\Delta A_{max}{}^{o}$. The latter is not known, but the value of ΔA_{max} for 5-SASL provides an approximate (lower) estimate. With this assumption, the values of K_r/K_r^{PG} are ∼1.6 for PC, ∼1.1 for PE, ∼0.7 for PA and PS, ∼0.4 for NAPE, and ∼0.1 for DG. The value of ΔA_{max} for 5-CLSL is too close to that for 5-SASL to provide a reliable estimate of K_r/K_r^{PG} for this lipid.

The selectivity pattern for the lipid spin labels that is shown in Table 1 is in the following order: SA > CL > PC > PE ≈ PG > PA ≈ PS > NAPE > DG. This characteristic pattern differs considerably from those obtained in comparable experiments with peripheral basic proteins such as cytochrome *c* and myelin basic protein (*37*) or with basic peptides such as pentalysine and polylysine (*34*). For these classical basic proteins and peptides, the interaction with phosphatidylserine and phosphatidylglycerol is greater than that with phosphatidylcholine or phosphatidylethanolamine, in the characteristic order PS ≥ PG > PC ≥ PE. This is the reverse situation to that for α-synuclein. The inverted order, in which the zwitterionic lipids exhibit a stronger perturbation than do the anionic lipids, is found, however, with melittin (*30*) and to a lesser extent with lysozyme (*37*). Both of these latter proteins have helices with amphipathic character.

The values of K_r reflect in-plane molecular selectivities of the prebound protein and therefore are not related directly to the ability of membranes of a particular lipid to bind the protein (see ref *23*). Phosphatidylcholine is a case in point: this lipid is able to associate with surface-bound α-synuclein, although the protein does not bind to membranes of DMPC alone. This molecular interaction with 5-PCSL may, however, be related to the ability of α-synuclein to bind molecules of phosphatidylcholine when the protein is in large excess (*10*). A possible origin for the selectivity of this zwitterionic lipid may lie in the presence of negatively, as well as positively, charged residues in the N-terminal lipid-binding domain of α-synuclein. The preferential interaction with zwitterionic phosphatidylethanolamine is not as strong as that with phosphatidylcholine, although phosphatidylethanolamine in admixture with negatively charged lipids is found to support interaction in a way that phosphatidylcholine does not (*9*). This preference was interpreted not as a lipid selectivity but rather in terms of the tendency of phosphatidylethanolamine to induce spontaneous membrane curvature.

The apparently strong interaction with stearic acid is because binding to α-synuclein involves an upward vertical translation of this single chain lipid such that it is located closer to the polar−apolar interface (see ref *38*). Other selectivities do correlate to some extent with binding capacity. Dioleoyl phosphatidylserine vesicles are not permeabilized by α-synuclein, whereas vesicles of dioleoyl phosphatidylglycerol are permeabilized (*14*). In 1:1 mixtures with palmitoyl-oleoyl phosphatidylcholine, vesicles containing palmitoyl-oleoyl phosphatidylserine bind α-synuclein to a slightly lesser extent than do those containing palmitoyl-oleoyl phosphatidic acid (*7*, *8*). These latter results reflect the trends seen in Table 1.

The spin-label data support a biphasic model for α-synuclein's interaction with, and action on, the membrane. First, for the interaction to take place specifically requires negatively charged phospholipids, as previously described in detail (*7*−*9*). Second, bound α-synuclein only penetrates the outer bilayer leaflet very superficially but is here capable of recruiting lipid species that not are able to support the initial interaction per se. Such a modification of microenvironments on the membrane may have functional significance for the role of α-synuclein in maintaining the vesicular pools at nerve terminals.

APPENDIX

In-Plane Lipid Associations with Membrane-Bound Protein. It is assumed that the bound protein has n_p lipid sites

at its membrane-facing surface. Different lipid species can have differing selectivities or occupancies at these sites, relative to the host lipid. The local interaction of the lipids at any one of these sites is not necessarily responsible for the strong binding of the protein as whole to the membrane. The latter may be governed primarily by longer-range electrostatic interactions. The exchange association of a spin-labeled lipid L* can be depicted by the following equilibrium:

$$L^* + LP \leftrightarrow L^*P + L \qquad (A.1)$$

where L is the host lipid, and P represents an association site at the protein. The association constant, K_r, of the spin-labeled lipid relative to the background lipid, is then given by

$$K_r = \frac{[L^*P][L]_f}{[LP][L^*]_f} \qquad (A.2)$$

where subscript f indicates the free lipid. For probe amounts of the spin-labeled lipid, the fraction *f* that is associated at one of the protein sites is given by (*39*)

$$f = \frac{K_r}{n_t/n_p - 1 + K_r} \qquad (A.3)$$

where n_t is total lipid/protein ratio of the host (or background) lipid.

Assuming fast exchange between free and protein-associated sites, the averaged hyperfine splitting, A_{max}, of the spin-labeled lipid is given by

$$A_{max} = fA_{max}^{o} + (1 - f)A_{max}^{f} \qquad (A.4)$$

where A_{max}^{o} and A_{max}^{f} are the values for the bound and free lipid, respectively. The increase in hyperfine splitting, $\Delta A_{max} = A_{max} - A_{max}^{f}$, on lipid−protein interaction is therefore given by

$$\Delta A_{max} = f\Delta A_{max}^{o} \qquad (A.5)$$

where $\Delta A_{max}^{o} = A_{max}^{o} - A_{max}^{f}$ is the intrinsic difference in hyperfine splitting between free and protein-associated sites. The condition for fast exchange is that the rate of exchange is greater than ΔA_{max} (in frequency units). Combining eqs A.3 and A.5 gives the following expression for the relative association constant of the spin-labeled lipid:

$$K_r = \left(\frac{n_t}{n_p} - 1\right)\frac{1}{\Delta A_{max}^{o}/\Delta A_{max} - 1} \qquad (A.6)$$

The latter equation leads immediately to eq 3 that is used in the text.

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