The intracellular deposition of fibrillar aggregates of α-synuclein is a characteristic feature of Parkinson disease. Alternatively, as a result of its unusual conformational plasticity, α-synuclein may fold into an amphipathic helix upon contact with a lipid-water interface. Using spin label ESR and fluorescence spectroscopy, we show here that α-synuclein affects the lipid packing in small unilamellar vesicles. The ESR hyperfine splittings of spin-labeled phospholipid probes revealed that α-synuclein induces chain ordering at carbon 14 of the acyl chains below the chain melting phase transition temperature but not in the liquid crystalline state of electroneutral vesicle membranes. Binding of α-synuclein leads to an increase in the temperature and cooperativity of the phase transition according to the fluorescence anisotropy of the hydrophobic polyene 1,6-diphenylhexatriene and of the fluorescence emission maxima of the amphiphilic probe 6-dodecanoyl-2-dimethylaminophthalene. Binding parameters were obtained from the fluorescence anisotropy measurements in combination with our previous determinations by titration calorimetry (Nuscher, B., Kamp, F., Mehnert, T., Odoy, S., Haass, C., Kahle, P. J., and Beyer, K. (2004) J. Biol. Chem. 279, 21966–21975). We also show that α-synuclein interacts with vesicle membranes containing sphingomyelin and cholesterol. We propose that the protein is capable of annealing defects in curved vesicle membranes, which may prevent synaptic vesicles from premature fusion.

α-Synuclein (S) is a small cytosolic protein with unknown function that is abundant in nerve terminals of the dopaminergic system. This protein is associated with Parkinson disease and other neurodegenerative disorders (1, 2). The hallmark feature in these conditions is the deposition of αS in insoluble intracellular aggregates (Lewy bodies) (3). Three single site αS mutations are known to be associated with rare, early onset variants of the disease (4–6), whereas the overwhelming number of Parkinson cases are of the sporadic type. Recombinant αS remains monomeric and almost entirely unfolded unless its aqueous solution reaches a concentration threshold at which it may form different aggregates such as spherical or doughnut-shaped oligomers and amyloid-like fibrils (7, 8). The formation of large αS fibrils may be preceded by assembly into protofibrillar aggregation intermediates as shown by chromatography and by an investigation of the aggregation kinetics (9).

The interaction of αS with small unilamellar vesicles (SUV) obtained by sonication of a phospholipid suspension reveals particularly intriguing features. Upon binding to the lipid-water interface, the “natively unfolded” protein assumes a conformation characterized by two helical domains interrupted by a short non-helical region (10–12). The vesicles are neither disrupted nor permeabilized by the monomeric protein, whereas influx of metal ions or efflux of dopamine has been observed upon addition of αS protofibrils (13). Protofibrillar intermediates have been therefore frequently considered as the toxic species in the aggregation pathway (14).

The binding of αS to the lipid-water interface of fluid state bilayers requires positive (convex) membrane curvature, as provided by SUV, in addition to the presence of negative interfacial charges. We have shown recently, using isothermal titration calorimetry and CD spectroscopy, that the protein also interacts strongly with SUV composed of neutral (zwitterionic) phospholipids such as 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) below the respective chain melting temperatures (Tm) (15). Titration of the vesicles at T < Tm into an αS solution leads to a considerable heat release that is accompanied by a coil-helix transition as shown by CD spectroscopy. The large exothermic heat can only partially be attributed to the protein folding. Rather, it was assumed that membrane annealing contributes most of the enthalpy when αS binds to defects in the highly curved vesicle membrane.

In this study, we employed ESR and fluorescence spectroscopy to investigate lipid chain dynamics and chain ordering as a result of interfacial αS binding. Protein binding led to increased chain melting temperatures and to enhanced cooperativity of the phase transitions, which was attributed to defect healing in the curved vesicle membranes. Binding constants and binding free energies calculated from fluorescence-derived isotherms were in agreement with earlier results obtained by calorimetry (15).

αS binding accompanied by a random coil-helix transition was observed not only in the presence of synthetic phospholipids with disaturated acyl chains, but also when the vesicles were composed of phospholipids that are typical constituents of synaptic vesicle membranes such as sphingomyelin and cholesterol (16–20). This led us to speculate that stabilization of the synaptic vesicle reserve pool may be related to the intracellular function of αS (15).

**EXPERIMENTAL PROCEDURES**

**Chemicals—**DMPC, DPPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), egg sphingomyelin, phosphatidylcholine spin labels 1-palmitoyl-2-stearoyl-(5-doxyl)-sn-glycero-3-phosphocholine (5-PCSL), and 1-palmitoyl-2-stearoyl-(14-doxyl)-sn-glycero-3-phosphocholine (14-PCSL) were purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol, bovine brain sphingomyelin (BBSM), egg...
sphingomyelin, lysozyme, and cytochrome c were purchased from Sigma. 6-Dodecanoyl-2-dimethylaminonaphtalene (laurdan) and 1,6-diphenylhexatriene (DPH) were obtained from Molecular Probes (Eugene, OR).

**Vesicle Preparation**—Phospholipids (20 mg) were hydrated in 1 ml of buffer containing 20 mM sodium phosphate, 100 mM KCl, and 0.2 g/liter NaN₃ (pH 7.2) for 15 min at 30 °C. SUV were prepared by sonication of the phospholipid suspensions for 20 min under argon at temperatures −5 °C above the respective transition temperature (Tₘ) of the lipids. A Branson sonifier equipped with a microtip was employed, using a duty cycle of 30%. Lipids and the desired fluorescent compounds or the spin-labeled phospholipids (1%, w/w) were dissolved in 2 ml of chloroform. The solvent was evaporated by a stream of nitrogen, and the lipid film was rehydrated in 1 ml of buffer and hydrated for 1 h. Large unilamellar vesicles (LUV) of DMPC were obtained after freeze/thawing (five times) by extrusion of the phospholipid suspension 20 times at 35 °C through a polycarbonate filter (100-nm pore size) using a manually operated extrusion apparatus (LipoFast™, Armacis GmbH, Weinsheim, Germany). The vesicles were stored well above the Tₘ to avoid fusion.

**Protein Preparation**—Recombinant αS was prepared, purified, and desalted as described (21). Aliquots containing 91 µg of αS from the desalted protein solution (~0.2 mg/ml protein in 1 mM sodium phosphate (pH 7.2)) were lyophilized and stored at −20 °C.

**ESR Spectroscopy**—ESR spectra were recorded on a Bruker ER-200 D-SRC spectrometer equipped with a variable temperature unit. Samples were loaded into glass capillaries (internal diameter of 0.9 mm) with typical sample volumes of 40–50 µl. Spectra were obtained using a TE102 cavity with a modulation frequency of 100 kHz, a modulation amplitude of 0.4 G, a sweep width of 100 G, a center field at 3600 G, and microwave power of 5 milliwatts. The signal-to-noise ratio was improved by accumulation of 4–20 spectra. Samples containing αS were prepared by mixing 50 µl of the vesicle suspension (25 mM phospholipid) with 91 µg of the lyophilized protein to obtain a lipid/protein molar ratio of 200:1. The samples were vortexed gently at room temperature prior to loading into the glass capillaries. Order parameters were determined from the spectra with corrections for the polarity of the environment (22, 23) using hyperfine tensor element values determined for a nitroxyl fatty acid (24).

**Fluorescence Spectroscopy**—Fluorescence experiments were carried out with a Jasco FP-6300 spectrophotometer. Excitation and emission wavelengths of 360 and 430 nm, respectively, were employed for measurements of the fluorescence anisotropy of DPH. The steady-state fluorescence anisotropy is defined as \( r = (I_{||} - I_{\perp})/(I_{||} + 2I_{\perp}) \), where the symbols || and \( \perp \) refer to light intensity obtained with parallel and perpendicular polarizers, respectively. To avoid photobleaching, the excitation shutter was closed after each measurement. The spectrum of laurdan was excited at 360 nm, and the emission was measured from 390 to 550 nm (0.5-nm increments) using a bandwidth of 2.5 nm (both excitation and emission). Spectra were averaged over 5–10 accumulations, and the emission maximum was evaluated using software provided by Jasco.

**CD Spectroscopy**—Spectra were recorded with a Jasco 810 spectropolarimeter. A cuvette with a 0.2-cm path length was filled with 500 µl of the protein solution and titrated with 2 µl of the vesicle suspension. The temperature was controlled with an accuracy of ±0.5 °C.

**RESULTS**

**Spin Label ESR Spectroscopy**—The consequence of αS binding for the membranes of SUV was studied by spin label ESR spectroscopy. Phosphatidylcholines with the doxyl moiety attached to C-5 (5-PCSL) and C-14 (14-PCSL) were chosen to probe the membrane at different depths from the hydrocarbon-water interface. The former label reflects the motional state of the phospholipid acyl chains close to the hydrocarbon-water interface, whereas the latter probe is particularly suited for the investigation of the lipid boundary of transmembrane proteins (25). The time window of the conventional spin label technique is comparable with the rate of phospholipid chain isomerizations, whereas it is rather insensitive for the tumbling motion of the entire vesicle. Qualitative information on the phospholipid chain dynamics was obtained from the ESR line shapes after addition of 1 mol % doxyl-labeled phospholipids.

First, SUV of 14-PCSL-labeled DPPC were studied at three different temperatures with and without αS (total lipid/protein molar ratio of 200:1). At 40 °C, αS addition had hardly any effect on the spectrum, i.e. above the phase transition temperature (Tₘ) of the host lipid, both spectra reflect the rapid axial motion of the phospholipid chains in the liquid crystalline state of the membrane (Fig. 1A). According to CD spectroscopy, there was no coil-helix transition of the protein above 40 °C, which confirms the absence of αS-vesicle interaction when \( T > T_m \) (15). At 23 °C, however, the spectrum broadened in the presence of αS, which is most effective for the \( m_z = -1 \) and \( m_z = +1 \) transitions (Fig. 1B). This line shape indicates that the rate of chain reorientation is intermediate with respect to the time scale of ordinary ESR spectroscopy (rotational correlation times between \( 10^{-6} \) and \( 10^{-9} \) s). At 13 °C, the rigidifying effect of αS on the lipid bilayer is shown even more stringently by an increase in the hyperfine splitting and by a concomitant line narrowing of the \( m_z = \pm 1 \) transitions. An order parameter of \( S \approx 0.7 \) can be derived from this spectrum according to a simple model that considers restricted random walk of the long molecular axis within a cone (22). The spectrum recorded without αS displays rather broad lines for the low- and high-field transitions, precluding an analysis in terms of a single order parameter.

**FIGURE 1. Temperature dependence of αS-vesicle interaction as revealed by spin label ESR.** SUV of DPPC with 1 mol % 14-PCSL (lipid concentration, 25 µM) were incubated at 40 °C (A) 23 °C (B), and 13 °C (C) with and without αS (lipid/protein molar ratio 200:1). The spectra were normalized by double integration.

---

**α-Synuclein and Vesicle Bilayers**
with negative net charges in the membrane interface, has little effect on the chain ordering as detected by the nitroxide spin labels. Rather, our observations are in line with the assumption of defect healing in small vesicles that are characterized by the simultaneous presence of ordered and disordered lipid domains and domain boundaries (15). We also note that addition of cytochrome c or lysozyme had no effect on the ESR line shape, indicating that the observed changes were not due to an unspecified lipid–protein interaction.

Protein Binding and Lipid Phase Transition Detected by DPH Fluorescence Anisotropy—DPH was incorporated into the SUV bilayer as a fluorescent probe for the motional anisotropy of the surrounding lipid chains (27). The fluorescence anisotropy (r) reflects the order-disorder transition in the membrane as shown in Fig. 3. Rather large anisotropy values were found in the gel state of DPPC vesicles (r > 0.25), whereas r was <0.04 in the liquid crystalline state. It may be noted that r = 0.4 is the maximum value that will be attained when the DPH long axis is perfectly aligned with the lipid acyl chains in an ordered bilayer (28).

The data in Fig. 3 were fitted using the sigmoidal model function (Equation 1),

\[ r = \frac{a - b}{1 + \exp((T - T_m)/\delta t)} + b \]  

(Eq 1)

where the temperature at the deflection point of the anisotropy (r) is denoted by \( T_m \) (in °C). From this, Equation 2 follows,

\[ \frac{d\Theta}{dT} |_{T_m} = \frac{1}{b - a} \frac{d\Theta}{dr} |_{T_m} = \frac{1}{4}\delta t \]  

(Eq 2)

where \( \Theta = (r - r_s)/(r_f - r_s) \) is the degree of the transition and \( r_f \) and \( r_s \) denote the anisotropy values in the fluid and ordered states of the membrane, respectively. It follows that \( \delta t \) reflects the steepness or, equivalently, the cooperativity of the transition (see “Discussion” and Table 2).

A \( T_m \) of ~38.3 °C was obtained for SUV alone, taking \( T_m \) as the midpoint of the transition. Addition of αS at up to a final phospholipid/protein molar ratio of 200:1 shifted the \( T_m \) to ~39.6 °C, and the slope at \( T_m \) becomes steeper as shown in Fig. 3 (insets). The transition temperatures obtained from DPH fluorescence anisotropy are in good agreement with earlier determinations using differential scanning calorimetry for SUV alone (15, 29) or for SUV in the presence of αS (15, 29).

The DPH fluorescence was further employed for the determination of the binding constant and binding stoichiometry of αS. A suspension of DPPC vesicles containing 1 mol % DPH was titrated at 37 °C with αS (Fig. 4A). This temperature is just below the phase transition temperature of the vesicles according to Fig. 3. The vesicle surface became saturated with protein when the total lipid/protein molar ratio was ~100:1. Vesicles composed of an equimolar mixture of POPC and POPG were titrated analogously at room temperature, i.e. in the fluid state of the vesicle membranes. The DPH fluorescence anisotropy is two to three times and the αS-induced increase in fluorescence anisotropy is about seven times smaller here than the values obtained for gel state DPPC vesicles (Fig. 4B).

Binding isotherms, i.e. \( \Phi = (r - r_o)/(r_f - r_o) \), were obtained from these data, where \( \Phi \) denotes the fractional occupancy of virtual binding sites and \( r_o \) and \( r_f \) are the fluorescence anisotropies without αS and in the presence of a saturating αS concentration, respectively. A simple equilibrium model that assumes independent binding sites ([L]_i) for the protein on the vesicle surface was employed for the calculation of dissociation constants and the number of lipids associated with one protein molecule. The number of associated lipid molecules (irrespective of

**FIGURE 2. Binding of αS immobilizes lipid molecules in SUV but not LUV.** A, ESR spectra of DMPC-containing SUV (lipid concentration of 25 mg/ml) containing 1 mol % 14-PCSL with and without αS (lipid/protein molar ratio of 200:1; temperature of 10 °C, B, the same conditions as described for A, but with SUV instead of LUV. C, ESR spectra of SUV containing 1 mol % 5-PCSL with and without αS, A and B, are denoted by \( \alpha A \) and \( \alpha B \).}

**α-Synuclein and Vesicle Bilayers**

**FIGURE 3.** Lipid ordering and helix folding of αS. A suspension of αS (15 mg/ml) was titrated into LUV (Fig. 2A) and SUV (Fig. 3A), in agreement with the notion that lipid interfacial order is higher in SUV than in LUV. The line shape effect of αS strongly affected the ESR spectra of SUV with and without αS (Fig. 2B), whereas the ESR spectra of SUV (lipid concentration of 25 mg/ml) containing 1 mol % 5-PCSL with and without αS (Fig. 2C) were not affected by αS addition, as expected.

Analogous results were obtained when the interaction of αS with 14-PCSL-labeled DMPC vesicles was studied (Fig. 2). At 10 °C (i.e. 13 °C below the \( T_m \) (23 °C) of multilamellar DMPC), αS strongly affected the ESR line shape (Fig. 2A). At the same temperature, there was no effect on the ESR line shape when the protein was added to LUV obtained by lipid extrusion (Fig. 2B), in agreement with the notion that lipid interfacial order is higher in SUV than in LUV. The line shape effect of αS was small when 5-PCSL rather than 14-PCSL was introduced as the reporter molecule due to the much broader ESR spectra obtained when the nitroxyl label was close to the lipid–water interface (10, 12, 15). The line shape effect of αS was small when 5-PCSL rather than 14-PCSL was introduced as the reporter molecule due to the much broader ESR spectrum obtained when the nitroxyl label was close to the lipid–water interface (Fig. 2C). At 21 °C, the order parameter as defined above increased slightly from \( S \approx 0.67 \) to 0.71 upon protein addition. Note that this experiment was performed at 21 °C rather than 10 °C, as the effect of αS addition was negligible at the lower temperature.

Lipid ordering was also observed at 21 °C upon addition of the protein to vesicles consisting of BBSM in the presence of 14-PCSL as a significant broadening of the \( m_z = +1 \) transition (data not shown). This lipid has a broad thermal transition centered at 37 °C (26). We were unable to isolate pure spectral components by intersubtraction of the spectra obtained with and without αS, which is most probably due to the heterogeneity of BBSM.

Lipid binding and helix folding of αS were originally observed in the presence of SUV composed of neutral and acidic phospholipids in the fluid state (10, 15). Thus, one may expect that the protein affects the chain order in vesicles consisting of an equimolar mixture of POPC and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG) at 30 °C. In the presence of 14-PCSL, however, there was only a marginal increase in the hyperfine splitting (~0.7 G), whereas the ESR line shape barely changed when the membrane was labeled with 5-PCSL (data not shown). Thus, αS binding to an entirely liquid crystalline vesicle, even
their location in the inner or outer leaflet of the vesicle) is \( N = \frac{[L_{un}]}{([L_g] + [L_p])} \), where \([L_g]\) denotes the concentration of unoccupied sites, \([L_p]\) is the concentration of bound protein molecules, and \([L_{tot}]\) is the total lipid concentration. This yields the apparent dissociation constant of the lipid-protein complex, \( K_d = \frac{(1 - \Phi)}{\Phi} \left[ \frac{[P_{tot}]}{(\Phi[L_{tot}]/N)} \right] \), with the total protein concentration ([P_{tot}]) added during the titration. The binding free energy (\( \Delta G \), as obtained from \( K_d \)) and the number
TABLE 1

Thermodynamic parameters of αS binding to SUV as obtained by DPH fluorescence anisotropy (Fig. 4)

| Lipid                  | ΔG      | ΔH      | TΔS     | N  |
|------------------------|---------|---------|---------|----|
|                        | kcal/mol| kcal/mol| kcal/mol|    |
| DPPC                   | −10.7   | −425.1  | −414.4  | 37 |
| POPC/POPG (1:1)        | −10.1   | −73.9   | −63.8   | 208|

*Data from isothermal titration calorimetry (15); TΔS from the Gibbs equation.*

FIGURE 5. Fluorescence emission spectra of laurdan in BBSM vesicles. Normalized fluorescence emission spectra of laurdan (0.5 mol % in the vesicle membrane) were acquired in the fluid and ordered states of BBSM containing SUV (lipid concentration, 250 μM). Dotted lines, without αS at (a) 23 °C and (b) 45 °C; solid lines both at 32.5 °C without (c) and with αS (d). a.u., arbitrary units.

DISCUSSION

Proteins without a defined secondary structure are referred to as natively unfolded (36). The characteristic feature of αS, which is a rather abundant member of this protein family, is the ambivalence of its folding and aggregation propensities. On the one hand, αS tends to aggregate via an unknown number of nucleation intermediates into large fibrillar structures, depending on protein concentration, temperature, and pH (37, 38). Such structures are typically found in the disease-associated Lewy bodies (39). The primary structure of αS reveals six imperfect repeats with a KTKEGV consensus sequence. The amphipathic N-terminal region of the protein is reminiscent of the exchangeable apolipoproteins (10). Earlier observations indeed led to the conclusion that αS binds to negatively charged vesicles in the fluid state (10, 40, 41).

We have shown recently that the protein strongly interacts with SUV
below the chain melting phase transition of the phospholipids (15). The small diameter of SUV (≈30 nm) leads to curvature stress in the bilayer, which results in rather broad phase transitions centered at 4–5 °C below the $T_m$ of the respective unstressed membranes, e.g., DMPC and DPPC vesicles undergo the melting transition at 19 and 36 °C rather than at 23 and 41 °C, respectively (42, 43). Similarly, SUV containing BBSM have a broad transition at 35 °C (cf. Table 2) rather than 41 °C.

Curvature stress is absent when vesicles are obtained by phospholipid extrusion (LUV). The larger average diameter of LUV compared with SUV (≈100 versus 30 nm) allows for stress-free lipid packing, even when the lipid chains assume a partially ordered state below the chain melting temperature (cf. Fig. 2B). It is therefore conceivable that packing defects are the inevitable consequences when ordered domains are present in the highly curved SUV membrane (44). Small gel state domains may coexist with few disordered lipids in the defect zones in vesicles consisting of a single lipid species. Likewise, there may be liquid ordered and disordered domains in the vesicle membrane when the vesicles are made up of a lipid mixture containing cholesterol. The formation of rigid and virtually flat membrane patches in vesicles below the $T_m$ has been demonstrated by cryoelectron microscopy (45). Thus, ordered lipid domains in SUV may be pictured as colliding ice floes surrounded by fluid lipids. Binding of the sided αS helix in the defect zones seems to compensate for curvature stress, which promotes ordering of the perturbed lipids. This interaction results in an exothermic heat as shown previously by titration calorimetry (15).

The gel state ESR spectra of SUV labeled with 14-PCSL (Fig. 1) suggest that the spin-labeled lipids undergo rather unrestricted motions in

### Table 2

| Lipid       | Probe | $T_m$   | $\beta t$ | $\beta t^{-2}$ |
|-------------|-------|---------|-----------|----------------|
| DPPC        | DPH   | 38.3    | 2.04*     | 0.24           |
| DPPC + αS   | DPH   | 39.6    | 1.87      | 0.29           |
| BBSM        | DPH   | 34.9    | 5.28      | 0.036          |
| BBSM + αS   | DPH   | 36.5    | 4.69      | 0.045          |
| DPPC        | Laurdan | 39.2   | 1.15      | 0.76           |
| DPPC + αS   | Laurdan | 41.8   | 0.78      | 1.64           |
| BBSM        | Laurdan | 30.4   | 2.92      | 0.12           |
| BBSM + αS   | Laurdan | 33.0   | 1.83      | 0.30           |

*See Equations 1 and 2 and “Discussion.”

---

**FIGURE 6.** Binding of αS affects the lipid phase transition of DPPC and BBSM as shown by laurdan fluorescence. Fluorescence emission maxima of laurdan in vesicles bilayers (0.5 mol %) were recorded as a function of temperature without (A) and with (B) αS (lipid concentration of 250 μM; lipid/protein molar ratio of 200:1). Insets, first derivatives of the fitting functions obtained with (solid lines) and without (dashed lines) αS (cf. Fig. 3).
the vesicle bilayer, even at 13 °C (≈−23 °C below the phase transition temperature for DPPC vesicles). Thus, because of its bulky doxyl moiety, the spin-labeled phosphatidylcholine may be excluded from the ordered domains upon freezing of the majority of the host lipids. The ESR line shapes observed after αS addition clearly support the assumption that binding of the protein leads to annealing of bilayer defects in the vesicle membrane when $T < T_m$ (Fig. 1). The spectra obtained at 23 °C in the presence of αS and without the protein at 13 °C display very similar line shapes. These line shapes are most probably due to a broad distribution of motions of spin-labeled molecules within the defect regions. At 13 °C, αS addition eventually results in a spectrum suggesting that the probe now experiences an environment of ordered lipid molecules, i.e. the outer hyperfine splitting of 59 G is typical for 14-PCSL in a gel state bilayer (46).

When DMPC-containing SUV were labeled with 14-PCSL at 10 °C (Fig. 2A), i.e. −10 °C below the $T_m$, the spectrum appears to consist of two superimposed spectral components characterized by smaller and larger hyperfine splittings. The more restricted component increases upon addition of αS. Protein addition had no effect on the ESR line shape when LUV (100 nm) rather than SUV were employed as a control, which confirms the notion that αS binding requires a curved lipid-water interface. Notably, a spectral component with a large hyperfine splitting prevails here (Fig. 2B), indicating that the majority of the lipids had entered an ordered state. Finally, a more uniform line shape was obtained when the doxyl moiety was attached at C-5 of the sn-2 chain (Fig. 2C), i.e. closer to the membrane-water interface. A slight increase in the outer hyperfine splitting upon addition of αS (≈1.4 G at 21 °C) rather than a change in the line shape indicates that different spectral components are not resolved.

It has been demonstrated that the fluorescence anisotropy of the non-polar probe DPH reflects the fluid-gel state phase transition of single component phosphatidylcholine or sphingomyelin multibilayer liposomes (47, 48). It also turned out that the phase transition temperature is only slightly diminished in the presence of DPH (49). The fluorescence of the polar probe laurdan shows a large red shift and a decreasing amplitude when the host membrane undergoes the transition (30). The sensitivity of DPH and laurdan for the bilayer phase transition is based on different physical principles, i.e. the DPH fluorescence anisotropy detects the lipid acyl chain disorder, whereas the laurdan emission is a function of the polarity of the surroundings as predicted by the Lippert equation (28). The shift of the laurdan emission maximum has been attributed to the penetration of water into the bilayer interface in the fluid state of the membrane (31). It may therefore be assumed that these probes provide evidence for the onset and completion of the phase transition at different levels of the bilayer.

We have used the DPH and laurdan fluorescence emissions to map the phase transitions of SUV, assuming that the fluorescence spectra reliably report the molecular changes induced within the vesicle bilayer by αS binding. Addition of the protein raises the transition temperature of the vesicles (cf. Figs. 3, 5, and 6), which is in line with our earlier notion that αS binding results in healing of packing defects in the highly curved vesicle membranes (15). Notably, the laurdan emission shows a drastic blue shift within the phase transition region, e.g. by 22 nm at 40 °C for DPPC and by 15 nm at 32 °C for BBSM, as a result of cooperative chain ordering in these vesicles. We further note that our simple approach of evaluating the laurdan spectra is insensitive to bleaching of the probe, which represents an advantage of using the wavelength variation alone rather than a generalized polarization as proposed by Parasassi et al. (30).

A small laurdan blue shift (≈1.5 nm) was also observed when αS was added to vesicles composed of an equimolar mixture of POPC and POPG at 30 °C (data not shown). This suggests that αS induces somewhat tighter lipid packing of the bilayer interface, leading to reduced water penetration, in agreement with Fig. 4B and with the slight increase in the ESR hyperfine splittings mentioned under “Results.”

Ramakrishnan et al. (50) recently demonstrated an increase in the $T_m$ in the presence of αS by spin label ESR spectroscopy in dispersions of dimyristoylphosphatidylglycerol. A direct comparison with our present data is difficult, as these authors used multibilayers composed of a single negatively charged lipid species rather than vesicle/lipid mixtures. It is also interesting to note that evaluation of the hyperfine splittings yielded a lipid/protein stoichiometry of ≈100 mol/mol, which is smaller than the total number of lipids associated with the protein as derived from the present DPH data for disaturated lipids or for the lipid mixture.
containing POPG (cf. Table 1). Using a number of positional isomers of the spin-labeled phosphatidylglycerol, these authors observed a significant increase in hyperfine splitting upon αS addition almost over the entire acyl chain length, which is in broad agreement with the enhanced DPH fluorescence anisotropy observed here upon αS binding to SUV containing charged or partially ordered lipids.

The cooperativity of the phase transition can be qualitatively deduced from the slopes of the experimental data in the transition region or, equivalently, from the parameter $\delta T$ which is related to the width of the transition (Equation 2). Using this criterion, both the static DPH fluorescence anisotropy and the shift of the laurand emission wavelength yielded an increasing cooperativity of the transition in the presence of αS. A more quantitative insight may be gained from a two-dimensional version of the Zimm-Bragg theory of cooperative chain melting (51, 52). This statistical approach considers the free energy of solid, fluid, and interfacial lipid species and provides a parameter (σ) that is inversely related to the cooperativity of the transition. This yields the temperature dependence of the transition degree, $(d\sigma/dT)_{RT_s} = (1/4\sqrt{\sigma})(\Delta H_m/RT_m^2)$, where $\Delta H_m$ is the calorimetric transition enthalpy (51). From Equation 2, it follows that $1/\Delta T = (1/\sqrt{\sigma})(\Delta H_m/RT_m^2)$, i.e., the relation $\delta T \propto 1/\sigma$ may be valid as long as changes in the enthalpy are negligible.

We have shown previously that addition of αS to gel state DPPC-containing SUV results in an exothermic heat that accounts for ~7% of the calorimetric transition enthalpy (15). This is nearly balanced by the increase in the $T_m$ by 1–3 K upon αS addition (Table 1). The parameter $\delta T^2$ was chosen here to characterize the transition cooperativity, as it increases with increasing slope, $(d\sigma/dT)_{RT_s}$, which is intuitively more satisfying than using $\delta T$ directly (Table 2). Thus, $\delta T^2$ may indeed be considered as a reliable indicator of the protein-induced changes in the chain melting cooperativity of SUV.

The data summarized in Table 2 suggest that the variation of the laurand fluorescence maxima reflects the phase transition of the small vesicles more reliably than the DPH fluorescence anisotropy. The rigid rod-like DPH structure probably evokes more stress in the highly curved vesicle membrane compared with the flexible laurand molecule, which results in a slightly lower transition temperature and in diminished transition cooperativity. It follows from the DPH fluorescence anisotropy that $\delta T^2$ increases upon αS addition by 21 and 25% for DPPC and BBSM, respectively, whereas the effect of αS on the laurand fluorescence maximum amounts to 116 and 150%. Because of the different physical principles involved, one cannot expect the same $\delta T$ parameters to be obtained from the DPH and laurand experiments. It is remarkable, however, that both techniques yield almost the same relative increase in the parameter $\delta T^2$ for BBSM versus DPPC (Table 2). Different absolute values for $\delta T^2$ may be also due to a different degree of perturbation in the vesicle membranes by the rigid DPH and by the more flexible laurand molecule. Somewhat broader phase transitions are indeed detected by DPH than by laurand (7.9 and 19.8 °C versus 3.0 and 6.3 °C as obtained from the first derivatives for DPPC and BBSM, respectively). This difference suggests that the deeply embedded rigid DPH perturbs its local environment in the highly curved SUV bilayers. The observation that neither DPH (49) nor laurand (53) strongly affects the phase transition in large or multimembrane vesicles supports this assumption.

We suggest that membrane binding of αS, accompanied by a structural transition, stabilizes synaptic vesicles and protects them from premature fusion as a result of curvature stress. This is in line with observations reported for αS knock-out mice indicating that the protein is required for the maintenance of the synaptic vesicle reserve pool (54). Thus, membrane-protein interaction may be considered as the defining property with regard to the physiological function of αS. To prove this assumption, it was necessary to demonstrate binding and helix formation of the protein in the presence of model membranes with a lipid composition that is more closely related to the in vivo situation. The expected structural transformation is shown in Fig. 7 as a function of the spinlabeling and cholesterol concentration in the vesicle bilayer, clearly indicating that an increasing proportion of these lipids promotes helix folding of αS. The lipid-induced structure formation may be a consequence of demixing of the membrane components, resulting in bilayer microdomains and domain boundaries, a situation that may be analogous to the appearance of grain boundaries in SUV below the chain melting phase transition temperature. The concomitant effect of protein binding on the membrane as detected by DPH fluorescence anisotropy is less prominent here and is only noticeable at low temperature for the lipid mixture containing BBSM, which may be due to the heterogeneity of the fatty acid composition and the absence of a well defined thermal transition in these vesicles.

The association of αS with microdomains or lipid rafts was demonstrated recently in a cell culture model (55) and in an in vitro assay (56). Interestingly, the A30P mutation disrupts this interaction, in agreement with our previous observation that αS(A30P) has a lower binding affinity in the presence of SUV compared with the wild-type protein (15). The mutant forms of αS were not studied here, as the emphasis of this work was on the annealing effect of αS. It is interesting to note that membrane microdomains may be involved in other neurodegenerative disorders with amyloid deposition, e.g. in Alzheimer disease, microdomains are suspected to promote the amyloidogenic processing of the amyloid precursor protein (57–59), although the issue is still highly controversial (58). Likewise, targeting of the prion protein to membrane microdomains may have a critical role in the process of infection (60). It has been shown that this raft interaction is independent of the presence of a glycosylphosphatidylinositol anchor, suggesting that the N-terminal ectodomain may bind directly to the membrane interface. These observations, as well as our present results, strongly support the conclusion that lipid metabolism may be an important factor that must not be neglected when striving for a thorough understanding of the molecular basis of neurodegeneration.

Acknowledgments—We are grateful to Drs. Philipp Kahle and Sabine Odoy for providing α-synuclein and to Brigitte Nüscher for technical assistance.

REFERENCES
1. Dev, K. K., Hofele, K., Barbieri, S., Buchman, V. L., and van der Putten, H. (2003) Neuropharmacology 45, 14–44
2. Moore, D. I., West, A. B., Dawson, V. L., and Dawson, T. M. (2005) Annu. Rev. Neurosci. 28, 57–87
3. Spillantini, M. G., Schmidt, M. L., Lee, Y. M.-Y., Trojanowski, J. Q., Jakes, R., and Goedert, M. (1997) Nature 388, 839–840
4. Polymeropoulos, M. H., Lavedan, C., Leroy, E., Ide, S. E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., Steneros, E. S., Chandrasekharappa, S., Athanassadou, A., Papapetropoulos, T., Johnson, W. G., Lazzarini, A. M., Duvoisin, R. C., Di Iorio, G., Golbe, L. I., and Nussbaum, R. L. (1997) Science 276, 2045–2047
5. Kruger, R., Kuhn, W., Muller, T., Wotzalla, D., Graeber, M., Koen, S., Przuntek, H., Eggen, J. T., Schols, L., and Riess, O. (1998) Nat. Genet. 18, 106–108
6. Zarranz, J. I., Alegre, J., Gomez-ESTeban, J. C., Lezcano, E., Ros, R., Ampuero, I., Vidal, L., Hoenicka, J., Rodriguez, O., Atares, B., Llorens, V., Gomez-Tortosa, E., del Ser, T., Munoz, D. G., and de Yebenes, J. G. (2004) Ann. Neurol. 55, 164–173
7. Uversky, V. N. (2003) J. Biomol. Struct. Dyn. 21, 211–234
8. Ding, T. T., Lee, S.-J., Rochet, J.-C., and Lansbury, P. T., Jr. (2002) Biochemistry 41, 10209–10217
9. Conway, K. A., Lee, S.-J., Rochet, J.-C., Ding, T. T., Williamson, R. E., and Lansbury, P. T., Jr. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 571–576
10. Davidson, W. S., Jonas, A., Clayton, D. F., and George, J. M. (1998) J. Biol. Chem. 273, 9443–9449
