α-Synuclein Has a High Affinity for Packing Defects in a Bilayer Membrane

A THERMODYNAMICS STUDY*

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A number of neurodegenerative disorders, including Parkinson’s disease, dementia with Lewy bodies, and multiple system atrophy, are characterized by the intracellular deposition of fibrillar aggregates that contain a high proportion of α-synuclein (αS). The interaction with the membrane-water interface strongly modulates folding and aggregation of the protein. The present study investigates the lipid binding and the coil-helix transition of αS, using titration calorimetry, differential scanning calorimetry, and circular dichroism spectroscopy. Titration of the protein with small unilamellar vesicles composed of zwitterionic phospholipids below the chain melting temperature of the lipids yielded exceptionally large exothermic heat values. The sigmoidal titration curves were evaluated in terms of a simple model that assumes saturable binding sites at the vesicle surface. The cumulative heat release and the ellipticity were linearly correlated as a result of simultaneous binding and helix folding. There was no heat release and folding of αS in the presence of large unilamellar vesicles, indicating that a small radius of curvature is necessary for the αS-membrane interaction. The heat release and the negative heat capacity of the protein-vesicle interaction could not be attributed to the coil-helix transition of the protein alone. We speculate that binding and helix folding of αS depends on the presence of defect structures in the membrane-water interface, which in turn results in lipid ordering in the highly curved vesicular membranes. This will be discussed with regard to a possible role of the protein for the stabilization of synaptic vesicle membranes.

α-Synuclein (αS)† is a protein of 140 amino acids that has been identified as a major component of the intracytoplasmic fibrillar deposits (Lewy bodies) associated with idiopathic and inherited forms of Parkinson’s disease (1). The majority of cases are idiopathic, whereas mutations in the αS gene are known to be responsible for rare inherited, early onset variants of Parkinson’s disease (2–4). Although the molecular mode of action of αS and of its homologs is as yet unknown, it was assumed that the protein modulates the dopamine neurotransmission by regulating synaptic vesicle (SV) mobilization from the presynaptic reserve pool (5, 6) or directly regulating the dopamine metabolism (7–9). However, attempts to identify a specific SV-binding protein, e.g. by protein cross-linking, have been unsuccessful so far.

At a certain threshold concentration, αS tends to aggregate into amyloid fibrils (10), whereas the homolog βS, which lacks a stretch of amino acids within the central portion of αS, has a much lower fibrillation propensity (11, 12) and may even inhibit αS fibrillation (13–15), indicating that the hydrophobic central part of αS is essential for its fibrillar aggregation (16). A recent study on the structure of mature fibrillar aggregates, using site-directed spin labeling, indicates that the N terminus of αS is less ordered than the central portion and that the C terminus is completely unfolded in the fibrillar aggregates (17).

According to circular dichroism (CD) and nuclear magnetic resonance spectroscopy, the monomeric αS protein is unfolded in buffer solution (18, 19). In the presence of negatively charged phospholipid vesicles, however, the protein undergoes a transition into a partially α-helical state (20). This has been attributed to the presence of positive amino acid charges in six imperfectly conserved repeats in the N-terminal region of the protein, which may account for the formation of a sided α-helix upon interaction with negatively charged membrane interfaces (19–23). In agreement with this notion, lipid binding and helix formation were barely detectable when αS was added to noncharged vesicles consisting of zwitterionic phospholipids in the liquid crystalline state (20). It was also shown that the surface curvature of the vesicles is important for αS binding, i.e. there was little binding and helix folding in the presence of large unilamellar vesicles containing phosphatidylglycerol, in contrast to small unilamellar vesicles of the same composition (20). This result may be not generally applicable, however, as αS binding to large unilamellar vesicles and even to multilamellar membranes containing negatively charged lipids other than phosphatidylglycerol was shown in later studies (24, 25).

Investigation of the aggregation kinetics revealed the formation of oligomeric intermediates of αS fibrillization (26). Such intermediates (“protofibrils”) potentially incorporate into unilamellar vesicle membranes, resulting in vesicle permeabilization and metal ion influx (27) or release of encapsulated dopamine (28). Of particular interest is the observation that small amounts of an αS-dopamine adduct that forms under
oxidizing conditions stabilize the protofibrillar intermediates (29). Thus, retardation of the protofibril– fibril transition may promote membrane permeation and release of cytotoxic amounts of dopamine from SV. Binding of αS to phospholipid membranes inhibited fibril formation (24, 30), whereas fatty acid binding enhanced the formation of soluble αS oligomers (31–34). Therefore, it will be important to pursue the investigation of the αS–lipid interaction with emphasis on the competition between helix folding and protofibrillar aggregation.

The binding of αS to vesicular lipid membranes has been demonstrated by different techniques, including size exclusion chromatography, CD spectroscopy, and atomic force microscopy (20–22, 28). In the present study calorimetric techniques, i.e. isothermal titration calorimetry (ITC) and differential scanning calorimetry (DSC), provide another, more direct proof of protein–lipid interaction, suggesting that not only negatively charged lipids but also defect structures in the membrane water interface are capable of inducing interfacial binding and helix folding of αS. These results will be discussed regarding the putative stabilization of intracellular, e.g. SV membranes by αS.

EXPERIMENTAL PROCEDURES

Materials—Phospholipids, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1-palmitoyl-2-oleyl-sn-glycero-3-phosphocholine (POPC), and 1-palmitoyl-2-oleyl-sn-glycero-3-glycerol (POPG) were purchased from Avanti Polar Lipids (Alabaster, AL). Bovine brain sphingomyelin (BBSM) as 1-palmitoyl-2-oleyl-sn-glycero-3-phosphoethanolamine (LPC) and a LiposoFast™ extruder device (Armatis GmbH, Weinheim, Germany).

ITC—A VP-ITC instrument equipped with a motor driven syringe (MicroCal, Amherst, MA) was employed for titration calorimetry. The volume of the calorimeter cell was 1.41 ml. Small volumes of the vesicle suspensions were injected with the computer-controlled syringe into the protein solution in the calorimeter cell. Typically, 40 injections of 7 μl each were executed and the integration of the calorimeter signals, base-line corrections, and normalization with respect to protein and lipid concentrations were done using MicroCal ORIGIN software. Only five to seven injections were necessary for the determination of the reaction heat by injection of protein into a suspension of lipid vesicles. Control titrations were performed, i.e. vesicles into buffer alone or protein into buffer alone, and the results were subtracted from the experimental data.

The sigmoidal titration curves obtained with gel state vesicles were evaluated assuming independent saturable protein binding sites in the outer vesicle interface. First, the ligand concentration was equated with the total lipid concentration in the syringe, neglecting the aggregational state of the lipids. Second, N independent lipid binding sites on the protein were assumed. The fractional occupancy of lipid binding sites on the protein, Φ, and the free lipid and total protein concentrations [L] and [P], respectively, then yield the microscopic binding constant $K^*$ = $\Phi(1 - \Phi)[L]$ and the total lipid concentration [L] = [L] + [N][P]. The fractional saturation of the protein is related to the total heat Q being released by $Q = N K^* [P] [\Delta H^*_V]$, where $\Delta H^*_V$ and $V_c$ denote the molar enthalpy of ligand binding and the total volume of the cell. After elimination of [L] and Φ, the heat content of the cell can be expressed as a function of the total lipid concentration [L]t, where $a = [L]_t/[N][P]$, $Q = 1/2[\Delta H^*_V] [1 + a(1 + K^*)^{-1}] - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*)^{-1}\right)^{1/2} - \left(1 + a(1 + K^*}
The negative heat flow obtained after each vesicle injection (in μcal/s) indicates that the protein-vesicle interaction is accompanied by an exothermic enthalpy. Integration of the individual calorimeter signals yielded the heat release per titration step (in μcal). In a parallel experiment, vesicles were injected into the buffer solution alone, which accounts for the effect of vesicle dilution. Enthalpy values in terms of kcal/mol of injected lipid were then obtained by dividing the integrated heat values by the respective molar amounts of lipid injected. As shown in Fig. 1B, the magnitude of the exothermic heat decreases monotonically with increasing phospholipid concentration in the calorimeter cell and vanishes when the total molar ratio exceeds 300 mol of phospholipid/mol of protein.

The titration curve in Fig. 1 can be ascribed to a partition equilibrium, i.e. the amount of protein available for vesicle binding decreases with increasing vesicle concentration in the solution. The enthalpy for complete binding, ΔH^p, was measured by titration of a dilute protein solution into a vesicle suspension in the calorimeter cell. Five injections were sufficient for an accurate determination. The data were further analyzed as described in detail by Seelig and colleagues (39, 40). Briefly, the bound fractions, \( X_b \), of the protein were calculated using the cumulative heat values, as shown in Equation 4, where \( \delta h_i \) is the \( i \)th integrated calorimeter peak.

\[
X_b = \sum_{i=1}^{n} \delta h_i / \Delta H^p \tag{Eq. 4}
\]

The unbound concentration of αS, \( c_p \), and the ratio of bound protein per total lipid in the calorimeter cell, \( X_b = n_b \Delta H_{in} / n_L \), was obtained from \( X_b \) (39), which yielded the binding isotherm (Fig. 1B, inset). A simple partition equilibrium would result in a linear relation, \( X_b = K_p c_p \), from which the binding constant \( K_p \) can be derived. A nonlinear relation was found, however, which must be attributed to electrostatic attraction of the protein by the negative charges at the vesicle surface, resulting in the effective protein concentration being higher close to the membrane-water interface than in bulk solution (Fig. 1, inset). For the binding of small peptides to charged vesicle membranes, the interfacial electrostatics were successfully taken into account using the Gouy-Chapman theory (41). A quantitative analysis along these lines is not feasible for αS, as it requires knowledge of the effective protein charge and of the surface potential of the membrane. Qualitatively, it turns out that the binding ratio \( X_b \) increases in a nonlinear and nonsaturable manner, as can be expected for a partition equilibrium that is modulated by the surface potential of the vesicle membrane.

The conformational rearrangement of αS as a consequence of the protein-vesicle interaction can be conveniently followed by CD spectroscopy (20–22, 30, 42), i.e. a decreasing ellipticity at 222 nm is diagnostic for an increasing contribution of α-helical regions to the overall conformational equilibrium. Fig. 2 shows CD titrations of wt- and A30P-αS with vesicles consisting of binary mixtures of POPC and POPG at molar ratios 1:1 and 2:1, respectively. The CD data are presented in terms of the mean residue ellipticities ([θ]222) to account for slightly different concentrations of the protein solutions in the cuvette. At low vesicle concentrations, the ellipticities decrease almost linearly as a function of the total lipid/protein molar ratio. The ratio of the initial slopes obtained with vesicles composed of POPC and POPG at molar ratios 2/1 and 1/1, respectively, is ~1.4 for both the wt and the mutant protein. This compares favorably with the ratio of the POPG concentrations of the two different vesicle preparations (1.34), indicating that helix formation is a function of the interfacial charge density. More notably, the initial slope is significantly smaller for A30P-αS versus wt-αS for either vesicle composition, suggesting a lower affinity of the mutant protein for the charged membrane interface. In the presence of equimolar POPC/POPG vesicles, the ellipticities approach constant values at total lipid/protein molar ratios >200:1. Lipid/protein ratios resulting in maximum helix folding of the proteins were difficult to attain at the lower POPG content, as scattering from the more concentrated vesicle suspension resulted in unacceptably noisy CD spectra.

Earlier work suggested that a highly curved membrane interface as obtained by sonication of phospholipid dispersions is required for vesicle binding and concomitant α-helix folding of monomeric αS (20). Our calorimetric data are in line with this observation but are at variance with later reports (24, 25). Negligible heat values were obtained upon titration of αS with large unilamellar POPC/POPG vesicles generated by lipid extrusion (molar ratios 1:2 and 1:1). After subtraction of a control (vesicles titrated into buffer alone), the initial heat values were typically less than 0.01 kcal/mol of protein. Titration at temperatures other than 30 °C (18 and 24 °C) yielded the same
Almost linear relations were found between the cumulative heat values (Equation 4) and the mean residue ellipticities measured at 222 nm, \([\theta]_{222}\), for vesicles consisting of DPPC in the gel state and for liquid crystalline POPC/POPG vesicles, indicating that the heat being released is associated with an equivalent increment in protein helicity (Fig. 3C). The slopes of the linear regression lines are 4 times larger for DPPC than for POPC/POPG, whereas the mean residue ellipticity reaches similar values for both vesicle systems, suggesting that the large exothermic heat values observed in the presence of the DPPC vesicles cannot be solely because of the coil-helix transition.

The ITC curves obtained by injection of gel state vesicles into an \(\alpha\)S solution are characteristically different from those observed in the presence of POPC/POPG vesicles (cf. Figs. 1 and 3). Therefore, an evaluation in terms of a partition equilibrium may be not applicable here. In fact, the shape of the titration curves is reminiscent of what one observes upon specific ligand binding by an enzyme or receptor protein (45). Thus, saturable binding sites were assumed to account for the present titration results and the data was evaluated according to Equation 1 (see “Experimental Procedures”). Fig. 4 shows fitting results (solid lines) that were obtained when \(\alpha\)S was titrated with DPPC vesicles at different temperatures in the gel state of the vesicular membranes. Table I summarizes the thermodynamic parameters for \(\alpha\)- and A30P-\(\alpha\S) titrated with different pure lipid vesicles and with vesicles containing cholesterol. The table also includes estimated helicities, i.e. the percentage of the entire protein sequence in an \(\alpha\)-helical state, determined for a total lipid/protein ratio of 450 mol/mol according to Equations 2 and 3 (see “Experimental Procedures”).

Several features of the results summarized in Table I are noteworthy. The \(\Delta H^\circ\) values are extraordinarily large and negative, which corresponds to an extremely exothermic process. It is not unusual that peptide or protein binding to membranes is accompanied by an exothermic enthalpy, however with a much lower heat release per amino acid. For a number of 23 residue peptides, Seelig and co-workers (46–48) found a total enthalpy per residue of 0.4–0.7 kcal/mol, which may be contrasted with the total heat release per residue of 1.4–1.7 kcal/mol for \(\alpha\)S and A30P-\(\alpha\S (140 residues). Further, a large negative entropy compensates for the exothermic enthalpy, whereas the free energy values are rather similar, irrespective of whether the \(\alpha\) or the mutant protein is being titrated or whether cholesterol is present in the membranes. Finally, the ellipticities obtained under saturating conditions depend only marginally on whether \(\alpha\S interacts with zwiterionic or with charged lipids. It will be shown that these results are not compatible with a model that merely includes contributions from protein folding and interfacial protein binding (see “Discussion”).

Almost no reaction heat was detectable when the \(\alpha\)- or the A30P-\(\alpha\S proteins were titrated above the respective chain melting temperatures of the zwiterionic lipids, i.e. at 42°C for DPPC (see Fig. 4, open triangles) and BBSM and at 25°C for DMPC. Simultaneous CD titrations yielded only negligible changes of the ellipticities, indicating that there was neither vesicle binding nor helix folding. Likewise, there was no calorimetric or spectroscopic response when titrations were performed at these temperatures with large rather than small unilamellar vesicles in the liquid crystalline state of the membrane. The question then arises whether \(\alpha\S interacts with large vesicles in the gel state. Thus vesicles of 100 or 200 nm diameter were prepared by extrusion of DPPC at 45°C, followed by rapid cooling to the desired temperature (30°C), which avoids damage of the polycarbonate membrane of the extruder device (see “Experimental Procedures”). Again, al-

result, indicating that \(\Delta H\) does not accidentally change sign at 30°C. Moreover, the small CD signal at 222 nm hardly changed upon vesicle addition, which further confirms the absence of \(\alpha\S binding and helix formation (data not shown). The same result was obtained when \(\alpha\S was titrated with small or large unilamellar vesicles consisting of POPC alone, i.e. without net negative charges in the membrane interface.

Interaction of \(\alpha\S With Gel State Membranes—\)Phospholipids with unsaturated acyl chains were usually employed in previous studies on \(\alpha\)-lipid interaction, which implies that the vesicle membranes were in the liquid-crystalline state. Using ITC, we obtained an unexpected result when vesicles consisting of phospholipids with saturated chains were titrated into an \(\alpha\S solution below the lipid chain melting temperature \(T_m\). Fig. 3A shows the titration of gel state small unilamellar vesicles prepared from DPPC (45 mM) at 30°C into a solution of \(\alpha\S (9.7 \mu M) in a cuvette with a cell volume of 1.41 ml). The well established chain melting transition for multilamellar dispersions of this phospholipid occurs at 41°C (43), whereas a broad phase transition was observed for sonicated small DPPC vesicles at 38°C (44). The salient feature of the experiment is the sigmoidal shape of the titration curve obtained by integration of the heat flow signals (Fig. 3A, bottom panel). This may be contrasted with the hyperbolic titration curves that were obtained when liquid crystalline vesicles containing lipids with net negative charges were titrated into the protein solution (cf. Fig. 1). Similar sigmoidal curves were found when titrations were performed with lipids other than DPPC below their respective phase transition temperatures, e.g. DMPC (\(T_m = 23°C\) or BBSM (\(T_m \sim 38°C\)). Likewise, the A30P mutant yielded sigmoidal ITC curves when vesicles were added below the chain melting temperature of the phospholipids (see below).

CD spectra were recorded in parallel with the calorimetric titrations using a second aliquot of the same protein solution. A strict analogous titration protocol as in the ITC runs was employed with respect to protein and lipid concentration in the cuvette. CD spectra characterized by distinct ellipticity minima around 210 and 220 nm were obtained after subtraction of the corresponding vesicle spectra in buffer alone, indicating that the protein acquires an \(\alpha\)-helical conformation with increasing lipid/protein ratio (Fig. 3B). At a total lipid/protein molar ratio of 400, the ellipticities reached a lower limit, which corresponds to the deflection point in the ITC curve (Fig. 3A).

Fig. 2. Mean residue ellipticities of \(\alpha\)- and A30P-\(\alpha\S in the presence of small unilamellar POPC/POPG vesicles versus total lipid/protein molar ratio. The proteins were titrated with 5-\(\mu\)l aliquots of the vesicle suspensions (POPC/POPG molar ratio 1/1 and 2/1, respectively; total lipid concentration, 45 mM).
most no reaction could be detected, indicating that strong interaction of monomeric αS with zwitterionic lipids in the bilayer membrane requires a small radius of curvature and lipids in an ordered state.

The amplitudes of the titration curves in Fig. 4 reveal the strong temperature dependence of the total enthalpy of the αS-vesicle interaction, whereas the curve profiles indicate that the effective binding constants and the total number of lipids associated with the protein are less sensitive to temperature. It may be assumed that knowledge of the overall heat capacity, ∆C_p of the reaction facilitates an estimate of the relative contributions of protein folding, interfacial protein-lipid interaction, and structural changes in the vesicular membranes. Thus, titrations were performed (in steps of 3 or 2 °C) with DPPC vesicles over a broad temperature range, including the phase transition temperature. According to Fig. 5, ∆H plethora decreases almost linearly between 15 and 30 °C, which amounts to ∆C_p = −7.3 kcal/mol K in this temperature range. The entropy values, ∆S, decrease so that T ∆S largely compensates for the exothermic heats. At the same time there is also a slight decrease in ∆G (Fig. 5, inset), as obtained from the effective binding constant K (Equation 1). A particularly notable feature is the large decrease in enthalpy and entropy at 36 °C, i.e. just below the transition temperature of the small vesicles (−38 °C; Ref. 44), without an abrupt change in ∆G. In the actual temperature range of the phase transition (38–41 °C), the integrated ITC curves assumed a biphase shape, which precluded an evaluation according to Equation 1 (data not shown).

The temperature dependence of the mean residue ellipticities, [θ]_222, obtained with wt-αS in the presence of DPPC vesicles at a total lipid to protein molar ratio of 450 is shown in Fig. 6. The ellipticities barely change throughout the heating scan up to 30 °C and increase moderately between 30 and 37 °C. There is an abrupt increase of [θ]_222 in a narrow temperature range above 37 °C, which obviously corresponds to the phase transition. It is important to note that the sudden drop in ∆H and ∆S (Fig. 5) occurs at 36 °C when the helicity of the protein is still large. A downscan performed immediately after the heating scan showed the refolding of the protein, although the reversibility was not perfect (Fig. 6, open symbols). The CD spectra in the inset to Fig. 6 indicate that there is an isodichroic point at 205 nm, suggesting that the temperature induced unfolding of αS is a two-state process (cf. Fig. 3B). A parallel fluorescence dequenching assay, using vesicles with encapsulated calcein, showed no dye release in the entire temperature range of the experiment (15–42 °C; data not shown), proving the integrity of the vesicles during the measurements.

The total molar enthalpy, ∆H, of the αS-vesicle interaction was also obtained in an alternative manner by injecting the protein into a vesicle suspension, i.e. the syringe now contained the protein solution, whereas the vesicles were placed into the calorimeter cell. Dividing the corrected average heat values (see “Experimental Procedures”) by the moles of protein per injection then gave the molar enthalpies of αS in the gel state (DPPC, BBSM) yields 4–5 times larger enthalpy values per mole of protein than injection into liquid crystalline vesicles (POPC/POPG). This is in excellent agreement with the slopes of the ITC-CD correlation plots shown in Fig. 3C. Further, A30F-αS produces slightly less exothermic enthalpy values than wt-αS upon injection into any of the vesicle suspensions. Finally, the absolute heat values obtained
by this protocol are 25–30% larger than those obtained from the vesicle into protein titrations.

An additional experiment (data not shown) was performed, using βS instead of αS, to exclude the possibility that protein aggregation provides a substantial contribution to the heat release observed by ITC. The total enthalpy obtained upon titration of βS into a DPPC vesicle solution at 30 °C was 262.4 ± 4.8 kcal/mol, which is comparable with what has been observed for αS, whereas the mean residue ellipticity reached a somewhat lower value of −11,963 ± 380° cm² dmol⁻¹ (corresponding to a helicity of 31.6%). The aggregation propensity of βS is much lower than that of αS (12, 13), indicating that the observed enthalpy must be attributed to effects other than protein aggregation.

The enthalpy measured in the ITC experiment must be considered as a composite quantity, including contributions from interfacial binding, helix folding, and changes in lipid packing as a consequence of the strong interfacial interaction. Therefore, we conclude that a sizeable fraction of the exothermic heat is the result of an energetic stabilization of the gel state vesicle membranes. Membrane stabilization was shown directly by DSC (Fig. 8). DPPC vesicles were prepared by sonication at 45 °C, and DSC scans were performed without and with addition of wt-αS. Downscans and upscans were performed with similar results in the temperature range from 29 to 43 °C. The lipid/protein molar ratio (200:1) was chosen so as to warrant saturation of the vesicle interface with protein. There is indeed a drastic narrowing of the transition in the presence of αS, which is in line with the notion that protein binding relieves curvature stress in the vesicular membrane.

DISCUSSION

Sonicated Vesicles as in Vitro Model Systems—It is generally accepted that αS undergoes a transition from an unstructured into a partially folded state upon binding to phospholipid membranes. How αS binding affects the membrane has been largely

### Table I

Thermodynamic parameters and maximum helicities for the binding of wt- and A30P-αS to SUV of different lipid composition

| Protein and phospholipids | ΔH° kcal/mol | TΔS° kcal/mol | AG° kcal/mol | Helicitya % |
|--------------------------|-------------|---------------|--------------|------------|
| wt-αS + DPPC            | −216.9 ± 2.0| −206.6 ± 2.3  | −10.2 ± 0.1  | 47.4       |
| wt-αS + BBSM            | −239.1 ± 27.0| −226.4 ± 27.5 | −10.1 ± 0.3  | 43.2       |
| A30P-αS + DPPC          | −219.1 ± 23.6| −208.7 ± 14.0 | −10.2 ± 0.1  | 48.4       |
| A30P-αS + BBSM          | −237.6 ± 4.0 | −228.4 ± 5.0  | −9.1 ± 0.1   | 44.0       |
| wt-αS + DMPC            | −160.7 ± 5.4 | −151.5 ± 5.5  | −9.2 ± 0.2   | 42.9       |
| wt-αS + DPPC + cholesterolb | −215.6 ± 3.0| −205.7 ± 3.0  | −9.8 ± 0.0   | 45.0       |
| wt-αS + BBSM + cholesterolb | −296.9 ± 17.7| −287.0 ± 16.9 | −9.8 ± 0.8   | 40.7       |
| wt-αS + POPC/POPGc      | −73.9 ± 4.3 | ND            | ND           | 43.8       |
| A30P-αS + POPC/POPGc    | −55.3 ± 9.3 | ND            | ND           | ND         |

a Helicities were determined according to Equations 2 and 3 at lipid/protein ratios of 500 mol/mol.
b 20 mol%.
c ΔH° determined by protein into vesicle titration. POPC/POPG, 1 mol/mol.
neglected, however. Motional restriction of the lipid acyl chains upon αS binding has been observed recently, but only for a single lipid/protein ratio (49). The majority of previous binding studies was carried out with phospholipids bearing a negative net charge, and it was argued that electrostatic interaction between positively charged amino acid residues and the acidic lipids targets the protein to the membrane interface (20). Based on the 11-mer repeat sequence of the protein, a helical wheel analysis led to the conclusion that membrane binding results in a helical wheel structure of the protein, a helical wheel representation of the different models of αS-membrane interaction.

Phospholipid vesicles are more suitable model systems for an investigation of αS binding and folding than micelles, considering the small micellar lifetime and the high monomer concentration of the surfactants. Therefore, we used small unilamellar phospholipid vesicles with diameters ≤40 nm as obtained by sonication of a lipid suspension. The average size of the sonicated vesicles falls within the reported size range for vesicles (51). It is believed that αS is involved in the regulation of SV mobilization at nerve terminals (6). The phospholipid composition of SV is characterized by a high proportion of sphingomyelin (12 mol% of the total phospholipid content) and a high cholesterol/phospholipid molar ratio (0.63), suggesting that there may be lateral heterogeneity in the bilayer membrane of SV (52). The high thermotropic phase transition temperature of brain sphingomyelins (53) is of particular interest with regard to the present observations of αS interaction with gel state vesicle membranes. Unilamellar vesicles obtained by sonication may be therefore well suited as in vitro model systems.

Sonicated vesicles are metastable because of their extreme surface curvature. This creates lateral packing stress in the inner and outer leaflets of the bilayer membrane (54), resulting in considerable broadening of the main phase transition and in a shift of the transition temperature, e.g., for DPPC from 41 to 38 °C (44). The imperfection of the stressed vesicle bilayer is also reflected by the reduced transition enthalpy of 5.5 versus 8.1 kcal/mol in multilamellar DPPC liposomes (55). Sonicated vesicles below Tm are prone to fusion, which probably represents another consequence of the curvature stress in these membranes (56). It has been shown that the fusion products are mainly stable unilamellar vesicles of diameter ~70 nm (57) and that the fusion process accelerates with decreasing temperature (58). A half-life time of 45 h was observed for DPPC vesicles at 31 °C (44). Therefore, in the present study the lipids were sonicated and the vesicles stored at temperatures 3–5 °C above Tm of the pure phospholipids and the titrations were performed within 3 h after sonication.

Figure 7. Molar enthalpies of αS-vesicle interaction as obtained by protein into lipid titration. The concentrations of protein in the syringe and of phospholipid in the calorimeter cell were 13.1 μM and 9.9 mM for DPPC and BBSM and 17.9 μM and 11.3 mM for the mixed vesicles containing equimolar amounts of POPC and POPG, respectively. Fifteen injections of 5 μl each were executed, and the resulting exothermic heat values were averaged.

Figure 8. Effect of αS on the thermal transition of DPPC vesicles. Figure shows DSC without (dashed line) and with wt-αS. Lipid concentration was 2.5 mM. Lipid/protein ratio was 200 μmol/mol. The protein was added at 45 °C immediately after sonication. The scan rate was 15°/h.
values associated with αS binding to noncharged gel state vesicles (Table I and Fig. 7).

The protein into lipid titrations (Fig. 7) yielded molar enthalpies that were larger by −25−30% than those obtained when the lipid vesicles were titrated into the protein solution (cf. Table I). The assumption of independent binding sites for the protein on the vesicle surface may be therefore not strictly valid, i.e. the system behaves nonideally close to saturation of the vesicle interface with protein molecules. Injecting small quantities of the vesicles into an excess of the protein (lipid into protein titration) results in instantaneous saturation of the vesicle interface, whereas the reverse titration (protein into lipid titration) avoids saturation of the vesicle interface with protein molecules. Thus, the data in Table I represent averages over the entire binding process, whereas the enthalpy obtained by protein into lipid injection corresponds to the heat release under nonsaturating conditions. It may be further argued that the nonideal behavior of the system near saturation of the vesicle membranes prevents resolution of small enthalpy differences between wt- and A30P-αS binding, i.e. under the nonsaturating conditions of the protein into lipid titration, there is a small but significant difference in the interaction enthalpies (Fig. 7), whereas the lipid into protein titration yields similar exothermic heat values for wt- and A30P-αS (Table I). The average free energy of binding as shown in Table I is also similar for both αS variants. The binding constants for wt- and A30P-αS seemed indeed very similar in the presence of gel state DPPC vesicles (30 °C) as CD titrations yielded almost superimposable titration curves (data not shown). This may be contrasted with the results obtained for liquid crystalline POPC/POPG vesicles (Fig. 2), where the slopes of the titration curves are indicative of a reduced membrane affinity for the mutant protein. Thus, a reduced membrane affinity for the mutant protein can be expected in a natural membrane where both charged lipids and lipids close to the phase transition contribute to αS binding.

The total entropy change that accompanies the αS-vesicle interaction is essentially negative and therefore opposes the enthalpy of the protein-membrane interaction. Thus, the overall process of protein binding and folding is driven by enthalpy. The decline in entropy is again larger than expected from helix formation alone. An estimate of the entropy change per residue for helix folding in an aqueous environment yielded −4.1 cal/mol K (62). Even lower coil-helix entropy values were found for amphiphilic peptides in a membrane interface, e.g. −1.9 cal/mol K/residue was reported recently for vesicle binding of the 23-residue antibiotic peptide magainin (48). These estimates would account for 39 and 19%, respectively, of the total observed entropy loss of wt-αS binding, considering that the helicity of αS reaches 47% upon titration with DPPC vesicles (Table I).

Membrane Annealing as a Result of αS Binding—The thermotropic transition of small unilamellar vesicles from the liquid crystalline to a partially ordered state inevitably leads to defect structures or grain boundaries as a result of the extreme curvature of the vesicular membranes. It may be argued that αS preferentially binds at such defect zones in the vesicular lipid-water interface, which leads to condensation of the perturbed lipids in the vicinity of the grain boundaries. This binding and “defect healing” will be accompanied by an exothermic heat, equivalent to the freezing enthalpy of the lipids involved. We propose that this effect accounts for the astoundingly large heat being released upon αS binding to vesicles in the gel state, i.e. the $\Delta H^\circ$ and $\Delta S^\circ$ values obtained by calorimetry embody a substantial contribution as a result of lipid ordering in the vesicle membrane.

The assumption of lipid ordering as a consequence of αS binding is strongly supported by DSC, as shown in Fig. 8. The remarkable narrowing of the transition indicates that αS binding leads to an increase of the chain melting cooperativity. This is not a result of fusion or enlargement of the vesicles, as shown independently by dynamic light scattering. Therefore, we conclude that αS binding relieves stress in the highly curved vesicle membrane below $T_m$, which makes substantial contributions to the total changes of the molar enthalpy and entropy. Thus, enthalpy-entropy compensation results in rather invariable binding constants, $K$ (Table I), and overall free energy values, $\Delta G = -RT \ln K$, which has been frequently observed upon membrane binding of small amphipathic peptides (46, 48, 63). It may be noted, however, that the earlier reports did not explicitly consider the effects of peptide binding on lipid packing and chain ordering.

The temperature dependence of αS binding indicates that the reaction occurs with a remarkably negative heat capacity of −7.3 kcal/mol K in the temperature range from 15 to 27 °C. At the same time there is a strong decrease of $\Delta S$, suggesting that the overall process involves an ordering of the phospholipid acyl chains, in agreement with the assumption of membrane annealing upon αS binding. This may be contrasted with a recent report where a positive heat capacity was found for the interaction of magainin and magainin analog peptides with electroneutral POPC membranes (47). Positive $\Delta C_p$ values between 0.12 and 0.15 kcal/mol K at 25 °C for these peptides and $\Delta S$ increased from −43 to −26 cal/mol K between 8 and 45 °C. The sudden drop in $\Delta H^\circ$ at 36 °C may be a consequence of critical area fluctuations in the vesicle membrane close to the phase transition temperature (64), i.e. the caloric effect of membrane annealing by αS binding will be particularly large in this temperature region.

It may be argued that nonpolar amino acid side chains partially penetrate into the bilayer, thereby adding a hydrophobic effect to the favorable free energy of the αS-vesicle interaction. However, this would be accompanied by positive rather than negative enthalpy and entropy values, as a result of disruption and disordering of the gel state bilayer. An estimate of the enthalpy associated with membrane annealing, in terms of the heat released per mole of lipid, can be obtained if one assumes that the nonhelix contribution is entirely the result of the reorganization of the vesicular membrane. Taking account of the average number of phospholipid molecules associated with one αS molecule (357 ± 10 at 30 °C as obtained from Equation 1), this yields $\Delta H_{lip} = 0.45$ kcal/mol of lipid, which accounts approximately for 7% of the chain melting enthalpy determined previously for the phase transition of small unilamellar DPPC vesicles (44).

There are distinct structural similarities between αS and the exchangeable apolipoproteins (apoA I, apoA II, and apoC I–III) with respect to the 11-mer sequence repeats that define the amphiphilicity of these proteins (20, 50). Lipid binding has been studied extensively for apoA I (63, 65–67). Strong interaction of this apoprotein with zwitterionic phospholipid membranes, accompanied by increasing helicity, occurs in the vicinity of the phase transition temperature, whereas binding to acidic phospholipids is stable over a broad temperature range (66). It has been also shown that small vesicles are much more reactive than large vesicles or multilamellar bilayers (65). Although this behavior bears resemblance to the αS-membrane interaction, there are notable differences, i.e. apoA-I binding is strongly diminished below $T_m$ and the protein solubilizes the bilayer membrane, which results in the formation of discoidal micelles (66, 68). Moreover, titration calorimetry showed an endothermic process upon binding of the apoprotein to large
unilamellar acidic phospholipid vesicles, indicating that the process is driven by entropy (67). In contrast, binding of amphiphilic apoA-I model peptides yielded exothermic interaction enthalpies and revealed a preference for binding to small vesicles (63).

Other proteins have been shown to bind preferentially to gel state membranes, e.g., the sperm adhesive protein bindin (69, 70) or an apolipoprotein from pulmonary surfactant (71). Relatively large enthalpy and entropy values (~58 kcal/mol and ~254 cal/mol K, respectively) were measured for the binding of the latter protein to DPPC vesicles slightly above T_m, which was attributed to an immobilization of the bilayer upon protein binding. Binding of amphiphilic protein structures at packing defects in the membrane that exist close to or below the chain melting temperature may be considered as a common denominator of these earlier results and of the present observations.

Conclusions—Binding and helix folding of αS at highly curved, electroneutral membrane interfaces in a partially disordered gel state has not been described so far. The present results offer a new explanation for the physiological role of the protein regarding the interaction with SV membranes. Curvature stress in the SV membrane may be considered as an essential condition for the rapid, protein-mediated fusion with the presynaptic membrane. At the same time, there is a high proportion of up to 10% of sphingomyelin in SV membranes (72–74). Broad phase transitions in the physiological temperature range have been found for brain sphingomyelins because of their high content of long chain fatty acids (reviewed in Ref. 75). Therefore, it can be expected that nonideal mixing of sphingomyelin or sphingomyelin–cholesterol complexes with the major glycerophospholipid components results in the formation of microdomains and packing defects in the SV membrane, which also may assist effective SV fusion. Thus, stabilization of the SV membrane by αS may be required for the proper tuning of the fusion propensity of the SV reserve pool.

The sphingomyelin content in various tissues has been shown to increase substantially with age (76). It will be also important to investigate the αS interaction with lipid mixtures more closely related to the SV membrane or with SV preparations, although the caloric effects that can be expected may be more subtle than those obtained in the present study with single component vesicle systems.

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