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Besides Fibrillization: Putative Role of the Peptide Fragment 71−82 on the Structural and Assembly Behavior of α-Synuclein

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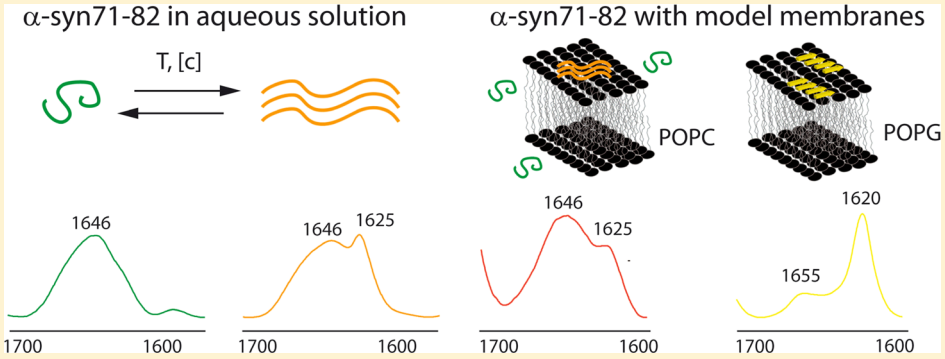
Supporting Information



ABSTRACT: The fibrillization of α-synuclein (α-syn) is involved in Parkinson's disease, a neurodegenerative disorder that affects four million people in the world. The amino acid sequence 71−82 of this protein (VTGVTAVAQKTV) has appeared to be essential for fibril formation. In the present study, we have investigated the secondary structure and thermal stability of the peptide fragment 71−82, α-syn71−82, as a function of concentration and temperature, as well as its interactions with phospholipid model membranes using various spectroscopic techniques. The data show that α-syn71−82 is mainly disordered in solution with the presence of a few β-sheet structure elements. The peptide reversibly forms intermolecular β-sheets with increasing concentration and decreasing temperature, suggesting that it is subjected to a thermodynamic equilibrium between a monomeric and an oligomeric form. This equilibrium seems to be affected by the presence of zwitterionic membranes. Conversely, the influence of the peptide on zwitterionic lipid bilayers is small and concentration-dependent. By contrast, α-syn71−82 is strongly affected by anionic vesicles. The peptide indeed exhibits a dramatic conformational change, reflecting an extensive and irreversible self-aggregation, the majority of the amino acids being involved in a parallel β-sheet conformation. The aggregates appear to be located near the membrane surface but do not perturb significantly the membrane order. Comparing these results with the literature, it appears that α-syn71−82 shares several general properties and structural similarities with its parent protein. These common points suggest that the sequence 71−82 may overall contribute to the behavior and properties of α-syn.

Parkinson's disease is a neurodegenerative disorder that affects four million people in the world, making it the second most common neurodegenerative disease. It is characterized by symptoms such as tremor, stiffness, balance problems, akinesia, and bradykinesia that originate from the drastic reduction of dopaminergic neurons in the substantia nigra of the midbrain.[1,2] To date, no treatment or drug exists to slow or cure this disease.[1]

The specific cause of illness is unknown, but the misfolding and aggregation of α-synuclein (α-syn) are involved.[3,4] α-Syn is a presynaptic protein of 140 amino acids with a molecular weight of 14.5 kDa. Its specific biological function is unknown. It has three major sequence regions: an N-terminal region (residues 1−61) that is amphipathic and α-helical, a hydrophobic middle region (residues 61−95) called the non-β-amyloid component (NAC), and an acidic C-terminal domain (residues 95−140). In solution, α-syn has been proposed to adopt a disordered structure.[5] Fifteen percent of cytosolic α-syn is associated with membranes and adopts an α-helical structure.[6−9] In pathological situations, α-syn forms amyloid-like fibrils, which constitute the major component of Lewy bodies.[7,10] Lewy bodies are pathological intraneuronal protein deposits rich in β-sheet fibrils involved in Parkinson's disease.[3,11,12] These antiparallel β-sheets result from protein aggregation and have been revealed by infrared (IR) and circular dichroism (CD) spectroscopy.[10,13] Parallel β-sheet structures were also reported by nuclear magnetic resonance (NMR)[14] and IR spectroscopy.[15] The environmental con-

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ditions, such as pH, temperature, and presence of water or organic solvents, affect protein aggregation.[16,17]

In vitro, α-syn forms typical cross-β amyloid fibrils[18,19] with characteristics similar to those extracted from the brain.[20] Protofibrils, that is, prefibrillar forms of mature fibrils, and oligomeric species have also been proposed to be formed during the time course of β-aggregation.[19,21−23] It is however still debated whether these particles are on- or off-pathway and whether they actually constitute the neurotoxic species in Parkinson's disease. α-Syn toxicity has been attributed to the interactions between oligomeric forms of the proteins and membranes.[24−29] *In situ*, membranes can also promote fibril formation.[30] α-Syn interacts strongly with anionic phospholipids,[31] as shown by NMR,[7,32] transmission electron microscopy (TEM),[30] SDS-PAGE,[9] fluorescence spectroscopy, and CD.[8] Electrostatic forces thus play a major role in this interaction. Moreover, the protein binds with high affinity when it is embedded in liquid-disordered phases of lipid bilayers, suggesting that not only electrostatic forces are involved but also lipid packing and hydrophobic interactions.[31] The membrane curvature has also an effect on the affinity of α-syn for lipids.[3]

The NAC region is particularly amyloidogenic and easily forms fibrils.[33,34] In the presence of lipid membranes, the NAC region is fully bound to the bilayer surface when the lipid-to-protein molar ratio is high.[35] The sequence motif comprising the residues 71−82 of the NAC region (VTGVTAVAQKTV), denoted α-syn71−82, is particularly sensitive to fibrillization. Deletion of this motif completely inhibits the formation of fibrils, protein aggregation, and its neurotoxicity.[36] If only one amino acid of this motif is substituted, the polymerization rate decreases.[37] Its crucial role is supported by the fact that the NAC region of α-syn differs from β-synuclein only by this 12 amino acid segment. β-Syn, which does not possess this segment, is not present in Lewy bodies and consequently does not aggregate.[38] Previous reports demonstrated the capacity of α-syn71−82 to bind model membranes. Madine et al. have observed the interaction between this peptide and DMPC vesicles using membrane-binding assay and ^{2}H NMR spectroscopy experiments.[39] Moreover, it has been shown that the aggregation rate and fibril morphology are sensitive to pH and temperature.[40] Studies concluded that a six-week incubation at pH 7 and 37 °C is the best way to obtain fibrils and a high rate of fibrillization.[16] Replica exchange molecular dynamics simulations have shown that the peptide can aggregate to form trimers and tetramers with an antiparallel β-sheet conformation.[41]

Despite these studies, the secondary structure, thermal stability, and propensity to aggregate of α-syn71−82 remain to be fully elucidated, especially as a function of concentration. Moreover, it is necessary to characterize the peptide interactions with model membranes in order to better understand the behavior and properties of this peptide and its parent protein. The aim of this work is to study the structure, assembly properties, and interactions of α-syn71−82 with model membranes using complementary spectroscopic techniques. Therefore, we have investigated the conformation of pure α-syn71−82 in solution as well as the effects of membranes on the peptide with circular dichroism and infrared spectroscopy. Conversely, the effect of the peptide on the polar headgroups and acyl chains of phospholipid membranes was evaluated by IR and ^{31}P NMR spectroscopy. Vesicles were made of palmitoyloleoylphosphatidylcholine (POPC) and palmitoyloleoylphosphatidylglycerol (POPG) because these lipids are representative of the lipids contained in the normal human brain.[42,43]

MATERIALS AND METHODS

Materials. POPC and POPG were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and used without purification. Na_2HPO_4 was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA), NaH_2PO_4 was obtained from Fisher Scientific Company (Bridgewater, NJ, USA), and Tris-HCl was acquired from Sigma (St-Louis, MI, USA). Deuterium oxide (D_2O) was purchased from CDN isotopes (Pointe-Claire, QC, Canada). Buffers were prepared with distilled and deionized water provided by a Barnstead NANOpurII system with four purification columns (resistivity of 18.2 MΩ/cm; Boston, MA, USA). All solvents were of reagent or HPLC grade, and used without any further purification. Fmoc-protected amino acids were purchased from Matrix Innovation (Québec, QC, Canada).

Peptide Synthesis. α-Syn71−82 (VTGVTAVAQKTV) was synthesized using a conventional solid-phase synthesis approach on a Wang resin by adding *N*-Fmoc-L-amino acids without acetylation.[44] The Fmoc group was deprotected with 20% piperidine−dimethylformamide (DMF) by a 15 min treatment. Amino acids were activated with *N*,*N*′-diisopropylcarbodiimide (DIC)/hydroxybenzotriazole (HOBt) and *N*,*N*-diisopropylethylamine (DIEA). The products were mechanically shaken for 1 h at room temperature. The resin was filtered and washed three times with DMF, MeOH, DMF, and MeOH. The resin was then dried *in vacuo*. A ninhydrin test was performed to monitor the completion of the coupling reaction. For the peptide cleavage, a 95% trifluoroacetic acid (TFA) solution was added for 2 h. The solution was filtered and allowed to dry by evaporation. The peptide was then solubilized in 10 mM HCl and submitted to lyophilization several times in order to remove all traces of TFA. The product identification and purity were confirmed by LC-MS using an Agilent 6210 time-of-flight mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) with electrospray ionization.

Sample Preparation. *Pure α-Syn71−82 in Solution.* For CD experiments, pure α-syn71−82 was dissolved at a concentration of 1.3 mM in phosphate buffer, pH 7.0, made with H_2O. For IR measurements, the peptide was dissolved at concentrations of 7, 9, 20, and 40 mM in phosphate buffer, pH 7.0, made with D_2O.

Multilamellar Vesicles. Multilamellar vesicle (MLV) samples were prepared by mechanical agitation of a varied amount of phospholipids (POPC or POPG) hydrated with phosphate buffer, pH 7.0, made with H_2O (CD) or D_2O (IR) or with Tris buffer, pH 7.4, made with H_2O (NMR). Preparation of multilamellar vesicles was ensured by repeating five cycles of mechanical agitation and freeze−thaw (liquid nitrogen temperature/37 °C) cycles. The last cycle ended by thawing at ambient temperature. For α-syn71−82-containing samples, the vesicles were prepared by the same procedure except that the lipids were mixed with the peptide dissolved in the appropriate buffer to obtain lipid/peptide molar ratios of 15:1, 30:1, or 50:1.

Spectroscopic Techniques. *Circular Dichroism.* Circular dichroism spectra were obtained with a JASCO J-815 spectropolarimeter (Jasco, Easton, MD, USA) at 37 °C. The protein and lipid concentrations were 1.3 and 20 mM, respectively. Spectra were recorded between 190 and 250 nm

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at a scan rate of 100 nm/min, a bandwidth of 1 nm, and a step size of 0.2 nm. Twenty scans were acquired for all samples with a 0.1 mm path length quartz cell. All spectra were smoothed and corrected by subtracting the spectrum of the phosphate buffer or the phosphate buffer with lipids.

Infrared Spectroscopy. IR spectra were recorded with a Nicolet Magna 760 spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) equipped with a nitrogen cooled MCT (mercury−cadmium−telluride) detector. Samples were deposited between CaF_2 windows (BioTools, Wauconda, IL, USA) with a path length of 58 μM. The temperature was thermoelectrically regulated (15−70 °C) using a homemade apparatus, and at each temperature, 128 scans were recorded with a resolution of 2 cm^{-1} using a Happ−Genzel apodization. Spectra manipulations were performed with the Grams/AI 8.0 software (Galactic Industries, Salem, MA, USA). A reference spectrum was subtracted to correct water vapor contributions when necessary. A baseline was subtracted in the 1700−1580 cm^{-1} (amide I′) region using a cubic function.

^{31}P NMR experiments. Proton-decoupled ^{31}P solid-state NMR spectra were obtained with a Bruker Avance 400 MHz spectrometer (Bruker Biospin, Milton, ON, Canada). The spectra were acquired by placing the samples into a 4 mm NMR tube at 15 °C using a frequency of 161.9 MHz with a Hahn echo sequence[45] and TPPM proton decoupling.[46] Phosphoric acid was used as an external reference at 0 ppm. A total of 2000 scans were acquired for each spectrum using 4096 data points. The spectra were acquired with a 90° pulse length of 4.5 μs and a recycle delay of 4 s. The spectral width was 50 kHz, and a line broadening of 50 Hz was applied to all spectra.

RESULTS

Pure α-Syn71−82 in Solution. The conformation adopted by the peptide in solution has been investigated by CD because of its sensitivity to polypeptide secondary structures. Figure 1 shows the CD spectrum of α-syn71−82 in solution at a concentration of 1.3 mM. The spectrum exhibits a strong minimum at 197 nm and weak shoulder at 218 nm, in agreement with previous reports.[16,37,40] The minimum at 197 nm is typical of random coil structures, indicating that the peptide is mainly disordered. The weak minimum at 218 nm suggests the presence of a few β-sheet structures. This spectrum is similar to that of the parent protein α-syn.[5,20,47−49]

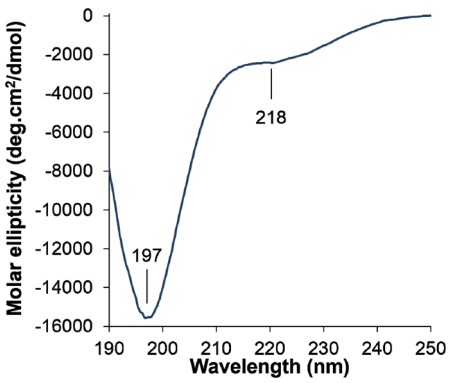

Figure 1. CD spectra of α-syn71−82 at a concentration of 1.3 mM in phosphate buffer pH 7.0 (H_2O) at 37 °C.

IR spectroscopy is also sensitive to protein and peptide secondary structure. Figure 2a shows the IR spectrum of α-syn71−82 in D_2O buffer at pH 7.0 in the amide I′ region as a function of peptide concentration. At the lowest concentration (7 mM), the amide I′ band is broad and characterized by a maximum at 1646 cm^{-1}, indicating that α-syn71−82 is mainly disordered,[50,51] in agreement with the above CD data. A very similar IR spectrum has also been obtained for α-syn in D_2O and has been related to a disordered structure.[10] The amide I′ band of α-syn71−82 is asymmetric, suggesting spectral contributions due to other secondary structure elements. Second derivative calculations (Figure S1, Supporting Information) indeed reveal the presence of three components in the amide I′ region: a main one at 1645 cm^{-1} due to random coils, a weak shoulder near 1668 cm^{-1} assigned to turns, and a small component near 1625 cm^{-1} due to β-sheets.[52] The latter contribution is consistent with CD results. Because CD and IR spectroscopies probe peptide conformation in different ranges of concentrations, the combination of these two techniques suggests that the peptide conformation is basically disordered with a small proportion of β-sheets between 1.3 and 7.0 mM. It is well recognized that α-syn is also mainly disordered in solution.[5] This conclusion has been supported by IR spectroscopy,[20,48,49] the spectrum in solution being very similar to the present spectrum of α-syn71−82 at 7.0 mM.

As the peptide concentration increases, an amide I′ component appears on the raw spectra at 1625 cm^{-1}, while the central band at 1642 cm^{-1} shifts to 1649 cm^{-1} (Figure 2a

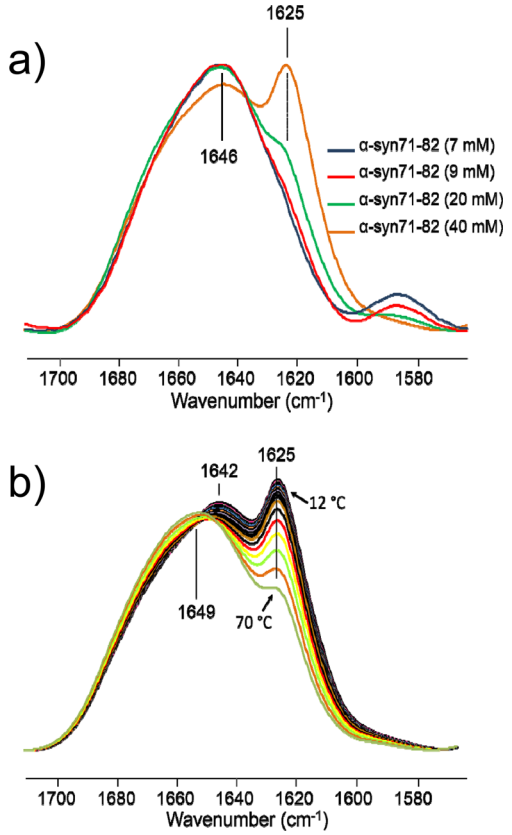

Figure 2. Infrared spectra of α-syn71−82 in phosphate buffer, pH 7.0 (D_2O), (a) as a function of concentration at 37 °C and (b) as a function of temperature for a concentration of 40 mM. Spectra are normalized with respect to the peak height maximum.

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and Figure S1, Supporting Information). These spectral modifications reveal a concentration-dependent conformational change, the development of the 1625 cm^{-1} band being assigned to the formation of intermolecular β-sheets. However, this band is unusual because it is not accompanied by the high-wavenumber component (near 1685 cm^{-1}) that is widely encountered with proteins that undergo self-aggregation upon heating,[53] aging,[54,55] or pH change[56] or in other denaturing conditions.[57−60] It is rather reminiscent of a band that is sometimes observed for native proteins such as β-lactoglobulin,[56] concavalin A,[61] or ribonuclease.[62] Such a component has been related to "exposed" or "strongly bonded" β-sheets[61] or to the β-sheet interface involved in the formation of β-lactoglobulin dimers.[63−65] Thus, it may be hypothesized that the secondary and the quaternary structures of α-syn71−82 in solution are affected by the peptide concentration. This result is reminiscent of the effect of concentration on the extent of α-syn fibrillization upon incubation.[47]

Figure 2b shows the spectrum of α-syn71−82 40 mM in D_2O buffer, pH 7.0, in the amide I′ region as a function of temperature. From 12 to 70 °C, the 1625 cm^{-1} feature decreases in intensity to the benefit of a broad band at 1646 cm^{-1}. It is interesting to note that the opposite trend is generally observed for heat-denatured proteins[53,56,63] for which the formation of β-sheets due to aggregation increases with heating and appears irreversible. In contrast, the present formation of β-sheets by α-syn71−82 is thermally (and partially) reversible. The fact that this peptide forms intermolecular β-sheets with increasing concentrations and decreasing temperature suggests that it behaves like a surfactant molecule. More specifically, it can self-assemble depending on temperature and aqueous conditions, and it seems to undergo a thermodynamic equilibrium between a monomeric and an oligomeric state. The association is characterized by a conformational switch from a disordered to a β-sheet conformation. To avoid any confusion with *β-aggregation*, that is, an irreversible kinetic assembly process that involves a conformational conversion of proteins toward β-sheets, the present phenomenon should be more appropriately designated as *oligomerization* (or *association*), that is, a reversible, thermodynamically driven assembly accompanied by a peptide conformational rearrangement, notably β-sheet formation.

Similar spectra exhibiting a shoulder near 1625 cm^{-1} have also been observed with α-syn, in particular at acidic pH[20,66] or with α-syn mutants.[48,66] This type of spectrum has generally been attributed to a partially folded intermediate, in particular to a species that would be in equilibrium with the natively unfolded conformation.[20] The IR spectra of such β-sheet oligomers formed in the presence of the flavonoid baicalein have been recorded and share strong similarities with the present ones.[67] This suggests that the present α-syn71−82 oligomers might represent the same type of particles. Imaging and SAXS analysis of the samples reveal particles of a size of about 6−10 nm.[19,67] Generally speaking, although α-syn has been found to exist predominantly as a monomeric species,[68] several studies suggest that α-syn can be found in a nonfibrillar oligomeric state[19] in which the secondary structure is rich in β-sheets[21,66] or, putatively, in α-helices.[69,70]

The present results are consistent with molecular dynamics simulations showing that α-syn71−82 can form oligomeric species (dimers, trimers, and tetramers) and adopt an antiparallel conformation,[41,71] although these studies were intended to examine the aggregation process of α-syn71−82. Overall, from the present results, the propensity of α-syn71−82 to oligomerize may have some impact on the behavior of the full-length protein. It seems premature at this stage to draw any definitive conclusion, but it may be put forward that the 71−82 sequence may play a role in the β-sheet oligomerization propensity of α-syn.

In contrast to many proteins and peptides, α-syn71−82 does not undergo irreversible heat-induced β-aggregation (at least up to 70 °C) even at relatively high concentration (40 mM). Thus, although this conclusion may seem paradoxical since α-syn71−82 is known to be highly amyloidogenic, it should also be considered that this monomer adopts a disordered conformation in solution. In other words and using an analogy with polymer physics, water represents a "good solvent". Thus, although many globular proteins unfold and self-aggregate upon heating due to the breaking of intramolecular bonds that stabilize the native structure, α-syn71−82 monomer does not follow this behavior because its preferential conformation is already unfolded. It is important to note however that this conclusion does not exclude the fact that α-syn71−82 can fibrillize with aging as reported previously.[16]

Effect of Membranes on α-Syn71−82. The interactions between α-syn71−82 and phospholipid membranes have been investigated from two points of view. We first present the effect of the presence of membranes on α-syn71−82 secondary structure as deduced from CD and IR measurements and then the impact of α-syn71−82 on phospholipid structure and conformational order as observed by IR and NMR spectroscopy.

Figure 3 shows the CD spectra of α-syn71−82 in the presence of POPC and POPG membranes. In the presence of

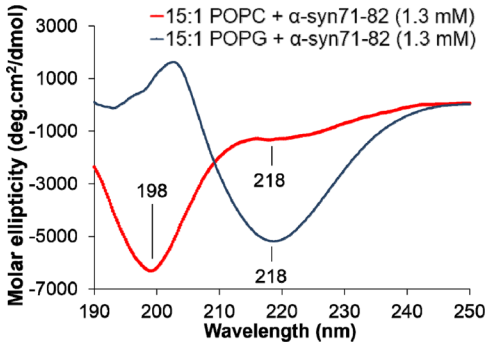

Figure 3. CD spectra of α-syn71−82 at a concentration of 1.3 mM in phosphate buffer, pH 7.0 (H_2O), in the presence of POPC or POPG vesicles at a lipid/α-syn71−82 molar ratio of 15:1 and at 37 °C.

POPC, the spectrum of α-syn71−82 is very close to the spectrum of the pure peptide with a strong minimum at 198 nm and a small one at 218 nm, indicating as above the predominance of disordered structures with some β-sheets. Therefore, zwitterionic membranes have no effect on α-syn71−82. For POPG multilamellar vesicles, the spectrum changes dramatically, with a strong minimum at 218 nm and a maximum near 205 nm, which is characteristic of β-sheet structures. At this stage, it is difficult to determine what types of β-sheets (antiparallel, intermolecular, or reversible) are formed in these conditions, but negatively charged membranes obviously have an important impact on the structure of α-syn71−82. This observation should have been anticipated since the peptide is positively charged (+1) and the membrane

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negatively charged (−1), a situation where electrostatic attraction is promoted.

Figure 4 shows the IR spectra of α-syn71−82 in the amide I′ region in the absence and presence of POPC and POPG

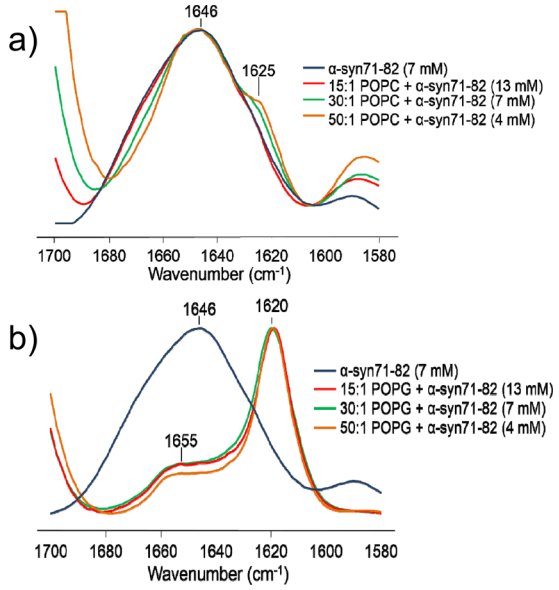

Figure 4. Infrared spectra of α-syn71−82 in phosphate buffer, pH 7.0 (D_2O), in the absence and presence of (a) DOPC and (b) DOPG vesicles at lipid/α-syn71−82 molar ratios of 15:1, 30:1, and 50:1 and at 37 °C. Spectra are normalized with respect to the peak height maximum.

vesicles. In the case of POPC (Figure 4a), the spectrum obtained at a lipid/peptide molar ratio of 15:1 is identical to that of the pure peptide. This result is in agreement with the CD spectrum showing that the peptide is not affected by zwitterionic membranes at the lowest lipid/peptide molar ratio investigated. At higher lipid/peptide molar ratios, the spectrum undergoes no major change except for the development of a shoulder at 1625 cm^{-1}. Thus, the peptide still remains basically disordered although some intermolecular β-sheets appear with increasing the lipid/peptide molar ratio. These results suggest that POPC membranes slightly perturb the peptide and only affect the association equilibrium in a lipid/peptide ratio-dependent manner. In particular, it may seem paradoxical that oligomeric β-sheets are formed as the lipid/peptide molar ratio increases, that is, as the peptide concentration (with respect to water) decreases. This apparently contradictory result can be rationalized considering that the peptide partitions between a membrane-bound and a free state. The amide I′ band probably reflects the contributions of both forms. At low lipid/peptide molar ratio (high peptide content), the peptide is mainly free and in a monomeric form. At high lipid/peptide molar ratio (low peptide content), the majority of the peptides are bound to the membranes, leading to a locally elevated peptide concentration and thus to a partial oligomerization as shown by the appearance of the component at 1625 cm^{-1}. Interestingly, the intensity of the 1625 cm^{-1} band relative to the 1645 cm^{-1} one is not affected by increasing temperature (Figure S2a, Supporting Information), suggesting that the oligomeric form of the peptide is stabilized in the presence of membranes.

By contrast, the amide I′ band is dramatically affected by the presence of POPG bilayers (Figure 4b) and is independent from the POPG/peptide molar ratio. The spectra are characterized by a strong and sharp component at 1620 cm^{-1} (width of 15−16 cm^{-1}) with a weaker shoulder near 1655 cm^{-1}, suggesting a very organized structure. Second derivative calculations (Figure S3, Supporting Information) show well-defined components at 1620 and 1660 cm^{-1} and reveal the putative presence of smaller components near 1646 and 1636 cm^{-1}, although they are too ill-defined to be able to ascertain their presence without uncertainty. They however suggest the presence of slightly contributing secondary structures, probably disordered structural elements. The doublet at 1620 and 1660 cm^{-1} is characteristic of intermolecular β-sheets, which is consistent with the CD data. The amide I′ band is unaffected by heating or cooling (Figure S2b, Supporting Information), showing that the peptide conformation is irreversibly formed and results from a typical β-aggregation process. This type of aggregates fundamentally differs from the oligomers found in solution or in the presence of PC, either at the structural level or based on the formation process mechanism. It thus appears that the type of particles (aggregates vs oligomers) formed by the peptide varies depending on the type of membranes (i.e., charged vs neutral).

From these data only, it cannot be determined whether fibrils or aggregates with other microstructures are formed. However, a fibrillar organization is very likely since similar IR spectra of α-syn have been associated with amyloid fibrils.[11,18,20,49] These spectra have been attributed to β-sheets[20,49] or antiparallel β-sheets.[11,18] However, as recalled above, the amide I′ band corresponding to β-sheets resulting from protein aggregation is usually dominated by a strong component near 1620 cm^{-1} with a smaller one near 1685 cm^{-1} (in D_2O).[53,56,72] Both modes result from a vibrational splitting (of about 60−65 cm^{-1}) due to dipolar coupling between amide groups and are attributed to antiparallel β-sheets.[73] In the case of parallel β-sheets, the splitting is reduced (~35−40 cm^{-1}),[73−75] which precisely corresponds to what is observed here for α-syn71−82 as well as for α-syn fibrils.[20,49] Interestingly, a similar spectrum has been observed with another short peptide related to neurodegenerative diseases, the β-amyloid fragment peptide 25−35.[76] More generally, the formation of (in-register) parallel β-sheets by amyloid peptides and proteins is well-known[14,15,77,78] and has been suggested to represent a general structural feature of amyloid fibrils.[79] The formation of parallel β-sheets by α-syn71−82 is particularly consistent with the fact that the core of the parent protein α-syn forms amyloid fibrils with parallel β-sheets.[78−81] A qualitatively similar appearance of intermolecular β-sheets has also been observed by IR spectroscopy for α-syn in the presence of dimyristoylphosphoglycerol (DMPG), although the amount was lower as judged from their amide I band.[10] In the present case, apart from the two components at 1620 and 1660 cm^{-1} mentioned above, only very small contributions arise in the amide I′ region of α-syn71−82, suggesting that the majority of the amino acids are involved in β-sheets.

Thus, it seems that α-syn71−82 has an affinity for POPG membranes and that the resulting spatial confinement of the peptide near the lipid surface promotes its self-aggregation and the formation of parallel β-sheets.

Effect of α-syn71−82 on Phospholipid Membranes. To investigate the effects of α-syn71−82 on phospholipid membranes, the temperature-dependence of the position of the CH_2 symmetric stretching ($\nu_s(CH_2)$) and C═O stretching (ν(C═O)) bands of POPC and POPG has been measured in the absence and presence of α-syn71−82. The $\nu_s(CH_2)$ vibrational mode is sensitive to the lipid chain order and shift

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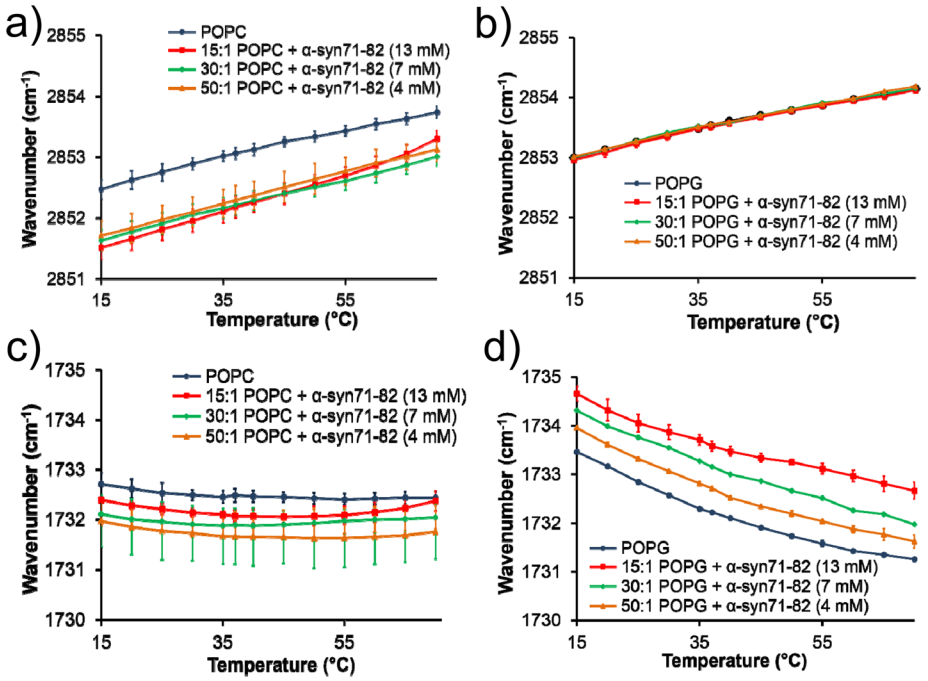

Figure 5. Temperature-dependence of the (a, b) $\nu_s(CH_2)$ and (c, d) $\nu(C{=}O)$ wavenumbers of (a, c) POPC and (b, d) POPG vesicles in the absence and presence of α-syn71−82 at lipid/α-syn71−82 molar ratios of 15:1, 30:1, and 50:1 in phosphate buffer, pH 7.0 (D_2O).

to higher wavenumbers as the acyl chains incorporate *gauche* conformers when the temperature increases.[82,83] In the case of POPC and POPG, the gel-to-fluid phase transition cannot be monitored since it is below 0 °C. However, information relative to the lipid chain order in the fluid phase can be obtained. As can be seen in Figure 5a, $\nu_s(CH_2)$ of POPC progressively increases with temperature due to an increase in the chain disorder. In the presence of α-syn71−82, the $\nu_s(CH_2)$ follows the same trends, but the value is lower, which suggests that the lipid chains are slightly more ordered in the fluid phase, thus indicating that peptide molecules alter the bilayers. For POPG vesicles, no effect is observed on the $\nu_s(CH_2)$ band whatever the molar ratio (Figure 5b).

The $\nu(C{=}O)$ mode of phospholipid vesicles is composed of two bands located at 1627 and 1640 cm^{-1} assigned to hydrogen-bonded (hydrated) and free (dehydrated) carbonyl groups, respectively.[84] As a consequence, a shift to lower wavenumbers is interpreted as an increase in the amount of the hydrogen-bonded C=O groups. Figure 5c shows that for pure POPC vesicles, the position of the $\nu(C{=}O)$ band is almost constant and slightly decreases due to a higher hydration of the interfacial region of the bilayer by water molecules upon heating. In the presence of α-syn71−82, the $\nu(C{=}O)$ band is progressively shifted to lower wavenumbers as the lipid/peptide molar ratio decreases. This result suggests that the peptide induces a slightly higher hydration of the carbonyl groups. In the case of POPG vesicles, there is an increase in the wavenumbers in the presence of α-syn71−82, which decreases with the lipid/peptide molar ratio (Figure 5d), indicating that the C=O groups are less hydrated.

Phosphorus-31 is a nucleus of choice to probe the perturbations induced by α-syn71−82 on the polar headgroups of phospholipids because of its 100% natural isotopic abundance and its spin of 1/2.[85−87] The dominant interaction is the chemical shift anisotropy (CSA). The spectral width can be used to determine the lipid order, while the spectral line shape informs on the vesicle shape in the magnetic field. Figure 6 shows the ^{31}P NMR spectra of POPC and POPG vesicles in the absence and presence of α-syn71−82 for different molar ratios. For POPC vesicles, we observe a small increase of the spectral width, associated with increased order of the phospholipid headgroups, and a spectral line shape characteristic of more spherical vesicles. These effects are more

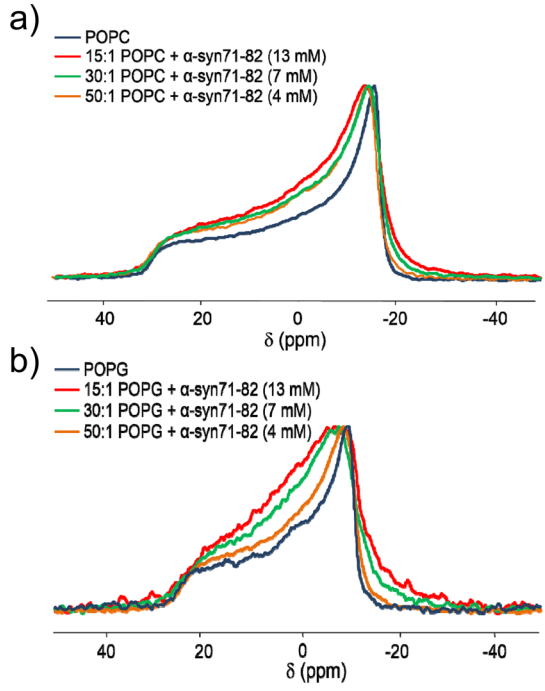

Figure 6. ^{31}P NMR spectra of (a) POPC and (b) POPG vesicles in the absence and presence of α-syn71−82 at lipid/α-syn71−82 molar ratios of 15:1, 30:1, and 50:1 in Tris buffer, pH 7.4, at 15 °C. Spectra are normalized with respect to the peak height maximum.

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pronounced at higher peptide concentration. For POPG vesicles, the increase of spectral width is more pronounced and the spectral line shapes obtained in the presence of a-syn71−82 reflect less mobile phospholipid headgroups. These effects are also more pronounced at higher peptide concentration.

Taken together, the data show that in the presence of α-syn71−82, the POPC chain order and carbonyl and phosphate groups are only slightly affected by the peptide and that the peptide structure is slightly modified by the membranes. These results suggest that some peptide molecules can interact and slightly alter the zwitterionic membranes although a proportion of the peptide molecules seem to remain in solution. Alternately, α-syn71−82 is strongly modified by POPG vesicles and seems to have a strong affinity for the negatively charged interface, but it does not perturb the acyl chain order. It only induces a slightly less hydrated system, probably because the accessibility of water molecules to the membranes interface is reduced due to the presence of the protein aggregates.

DISCUSSION

We have investigated in the present study the structure and membrane interactions of α-syn71−82. The data show that depending on the conditions, this peptide can self-assemble into two types of structures characterized by amide I′ spectral patterns that can be associated with different types of β-sheets. One type of assembly is represented by the reversible formation of oligomers; the second corresponds to the irreversible β-aggregation of the peptide. In the latter case, the structure of the aggregates is unknown, but a fibrillar form is likely.

In aqueous solution and at low peptide concentration (7 mM and below), α-syn71−82 is mainly disordered and contains a small proportion of β-sheets. At high peptide concentration (20 mM and above), the peptide reversibly forms intermolecular β-sheets, reflecting the presence of oligomers that can progressively be dissociated upon heating. The monomer−oligomer equilibrium seems to be perturbed in the presence of POPC membranes. By contrast, the peptide readily, largely, and irreversibly self-aggregates in the presence of POPG membranes as revealed by the predominance of intermolecular β-sheets. The majority of the amino acids are involved in β-sheets exhibiting a parallel configuration. Thus, whereas α-syn71−82 has a strong propensity to β-aggregate in the presence of anionic membranes, it is not the case in solution or in the presence of zwitterionic membranes (although aging experiments have not been carried out).

It may be wondered whether the present oligomerization can be seen as a preliminary step leading to aggregation. From the present data, it seems difficult to establish a definitive conclusion regarding this hypothesis. However, two important clues may provide a beginning of an answer. First, oligomers and aggregates have a different structure at a molecular level as suggested by the difference in the shape of the amide I′ band, especially the position of the β-sheet component (1625 cm^{-1} for oligomers, 1620 cm^{-1} for aggregates). Second, the two types of particles result from different formation processes. Oligomers seem to be formed by a reversible thermodynamic association mechanism whereas aggregates are formed by an irreversible kinetic process. Third, these particles are formed in very different conditions, that is, in solution or in the presence of zwitterionic membranes for oligomers and in the presence of anionic membranes for aggregates. Such differences suggest that oligomers do not necessarily represent a preliminary species for fibrillization.

Electrostatic attractions obviously promote α-syn71−82/membrane interactions since the peptide interacts slightly with zwitterionic membranes and strongly with anionic ones. Similar conclusions have been drawn for α-syn.[7,10,88] The present study shows that these interactions result in peptide aggregation near the membrane surface so that the aggregates slightly limit the access of water to the polar headgroups as indicated by the decrease in the hydration of the C═O groups. The presence of aggregates does not seem however to induce strong alteration of the hydrophobic core of the bilayer. Nevertheless, part of α-syn71−82 molecules can also interact with lipid membranes even when electrostatic interactions are not favorable, although the perturbations are small. As a matter of fact, POPC membranes stabilize the monomer−oligomer equilibrium of α-syn71−82 whereas the peptide slightly increases the chain disorder, C═O hydration, and vesicle rigidity. The binding of α-syn71−82 vesicles has also been observed with DMPC vesicles.[39]

The present results regarding α-syn71−82, when compared to those found in the literature regarding the parent protein, show that both polypeptides share several common points: (1) Their conformation is mainly disordered in aqueous solution (for the monomeric species). (2) They can associate in solution and form oligomeric β-sheet-rich structure. (3) They do not have a high affinity for zwitterionic (phosphatidylcholine) membranes and interact strongly with anionic (phosphatidylglycerol) membranes, promoting intermolecular β-aggregation. (4) They form parallel β-sheets.

It is remarkable that this 12 amino acid peptide behaves so similarly compared with the full protein, and it is tempting to assert that several properties of α-syn are (partly) determined by this short sequence region. One has nevertheless to keep in mind that the peptide and its parent protein are structurally different due to the difference in molecular weight, the charges present at the N- and C-termini for the peptide, and the presence of different parts of the protein (especially its basic N-terminal domain). All these parameters may influence the properties and membrane interactions of α-syn. Despite these structural differences, it has already been shown that the 71−82 sequence motif is a key element necessary for the structure and assembly of human α-syn into filaments[37] and that it can form fibrils of similar morphology to α-syn.[16] The fact that both the peptide and the parent protein form parallel β-sheets supports this finding. Besides this result, it may similarly be anticipated that this sequence motif may play a crucial role in the oligomeric state of α-syn and that the quaternary structure of the protein may be modulated by local concentration and lipid membranes, thus influencing fibrillization. Further work is needed to elucidate more precisely the structural and pathological role of the α-syn71−82 peptide in Parkinson's disease.

ASSOCIATED CONTENT

Supporting Information

Second derivative spectra of α-syn71−82 as a function of concentration, infrared spectra of α-syn71−82 in phosphate buffer pH 7.0 (D_2O) in the presence of (a) POPC and (b) POPG vesicles as a function of temperature at a lipid/α-syn71−82 molar ratio of 50:1, and second derivative spectra of α-syn71−82 in the presence of POPG vesicles as a function of

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concentration. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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ABBREVIATIONS

CD, circular dichroism; DIC, *N*,*N*′-diisopropylcarbodiimide; DIEA, *N*,*N*-diisopropylethylamine; DMF, dimethylformamide; DMPC, dimyristoylphosphatidylcholine; DMPG, dimyristoylphosphatidylglycerol; Fmoc, fluorenylmethyloxycarbonyl; HOBt, hydroxybenzotriazole; HPLC, high performance liquid chromatography; IR, infrared; LC-MS, liquid chromatography−mass spectrometry; MCT, mercury−cadmium−telluride; MLV, multilamellar vesicles; NAC, non-β-amyloid component; α-Syn(71−82), peptide fragment 71−82 of α-synuclein; NMR, nuclear magnetic resonance; POPC, palmitoyloleoylphosphatidylcholine; POPG, palmitoyloleoylphosphatidylglycerol; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TEM, transmission electron microscopy; TFA, trifluoroacetic acid; TPPM, two pulse phase modulation

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