^{19}F NMR studies of α-synuclein-membrane interactions

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Abstract: α-Synuclein function is thought to be related to its membrane binding ability. Solution NMR studies have identified several α-synuclein-membrane interaction modes in small unilamellar vesicles (SUVs), but how membrane properties affect binding remains unclear. Here, we use ^{19}F NMR to study α-synuclein-membrane interactions by using 3-fluoro-*L*-tyrosine (3FY) and trifluoromethyl-*L*-phenylalanine (tfmF) labeled proteins. Our results indicate that the affinity is affected by both the head group and the acyl chain of the SUV. Negatively charged head groups have higher affinity, but different head groups with the same charge also affect binding. We show that the saturation of the acyl chain has a dramatic effect on the α-synuclein-membrane interactions by studying lipids with the same head group but different chains. Taken together, the data show that α-synuclein's N-terminal region is the most important determinate of SUV binding, but its C-terminal region also modulates the interactions. Our data support the existence of multiple tight phospholipid-binding modes, a result incompatible with the model that α-synuclein lies solely on the membrane surface.

Keywords: binding; ^{19}F NMR; membranes; α-synuclein

Introduction

α-Synuclein (Fig. 1) is a 140 amino-acid, intrinsically-disordered protein associated with Parkinson's disease and other neurodegenerative disorders[1–8] whose function is hypothesized to involve its interaction with membranes.[8–12] The protein binds lipids and anionic detergents through the seven imperfect, cationic, 11-amino acid repeats located in its N-terminal and hydrophobic regions.[6–8,13–20] Electron paramagnetic resonance (EPR) data on its complex with small unilamellar vesicles (SUVs) suggest that the first ~100 residues of the monomeric protein adopt an α-helical conformation that lies on the membrane surface.[21–25] The last ~40 residues lack defined structure and do not appear to be involved in membrane interactions.[13,21] Recent solution NMR data, however, appear incompatible with this model. More specifically, ^{15}N intensity and relaxation data from titration of SUVs sugget there exists several binding modes in which the first 25 residues adopt a helical state that anchor the interaction with SUVs.[26,27]

A variety of other techniques, including circular dichroism spectropolarimetry,[12,13,19,28] fluorescence spectroscopy,[21,29–31] and EPR[21–25] have been used to study how the phospholipid composition of vesicles

Abbreviations: DMPC, dimyristoyl-phosphatidylcholine; DMPG, dimyristoyl-phosphatidylglycerol; EPR, electron paramagnetic resonance; 3FY, 3-fluoro-*L*-tyrosine; NMR, nuclear magnetic resonance; POPA, palmitoyl-oleoyl-phosphatidic acid; POPC, palmitoyl-oleoyl-phosphatidylcholine; POPE, palmitoyl-oleoyl-phosphatidylethanolamine; POPG, palmitoyl-oleoyl-phosphatidylglycerol; POPS, palmitoyl-oleoyl-phosphatidylserine; SDS, sodium dodecyl sulfate; SUV, small unilamellar vesicle; tfmF, trifluoromethyl-*L*-phenylalanine.

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Figure 1. The amino acid sequence of human α-synuclein. The first 11-amino acid repeat (underlined), the negatively charged amino acids (in blue), the positively charged amino acids (in red), and the four tyrosines (bolded) are indicated. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

affects α-synuclein affinity. Although these studies indicate that the properties of phospholipid membranes (i.e., charge, curvature, size, and the identity of the acyl chains) affect binding, there is no consensus, and some studies are contradictory.[21,22,24–27] We show that ^{19}F NMR is another useful technique for assessing α-synuclein-membrane interactions.

^{19}F is a good reporter of conformational changes due to its sensitivity to the environment and the fact that few natural biological molecules contain fluorine.[32–35] Additionally, adding a few fluorine atoms to a protein has a minimal effect on structure and dynamics.[32,33,35,36] Furthermore, the methods listed above provide only the overall affinity of α-synuclein for membranes. ^{19}F NMR detects α-synuclein-membrane interactions at the level of individual residues.

Here, we use ^{19}F NMR as a probe to monitor systematically the binding of α-synuclein to SUVs with different head groups and acyl chains. There are four tyrosines in α-synuclein (Fig. 1). One is at position 39. The other three are near the C-terminus, at positions 125, 133, and 136. We substituted these tyrosines with either 3-fluoro-*L*-tyrosine (3FY) or trifluoromethyl-*L*-phenylalanine (tfmF). The ^{19}F signal is detectable in the free state. Once the protein binds and exchanges slowly with the free state, the ^{19}F signal is undetectable because the slow tumbling of the large SUV (~20 nm diameter)[37] broadens the resonance into the baseline. The decrease in signal corresponds to binding. We also combine site-directed mutagenesis with ^{19}F NMR to determine which segment of α-synuclein binds to SUVs and to estimate binding affinities.

Results and Discussion

Binding of 3FY labeled α-synuclein to spherical micelles, rod-like micelles, and SUVs

SDS micelles are generally used as membrane mimics. At low salt concentrations, they form spherical micelles with diameter of ~5 nm with highly curved surfaces.[38] Rod-like micelles form at high salt concentrations.[24,38]

Three ^{19}F peaks are observed from 3FY-labeled α-synuclein in buffer [Fig. 2(A)]. The middle peak is twice as large because the resonance from residues 39 and 125 overlap.[32] In 200 m*M* SDS, all four ^{19}F resonances are observed [Fig. 2(B)]. Upon binding micelles, the resonance from 3FY39 decreases and shifts from −59.9 ppm to −60.2 ppm. As shown in Figure 2(C), increasing the salt concentration to 250 m*M*[24] broadens the position 39 resonance beyond detection, but the resonances from the three C-terminal residues remain unchanged. Increasing the temperature to 50°C, causes the 3FY39 resonance to reappear, although it is broad [Fig. 2(D)]. The resonances from the C-terminal residues shift to lower field due to strong temperature-sensitivity of ^{19}F chemical shifts.

In contrast to SDS micelles, the 3FY39 resonance was barely observed in palmitoyl-oleoyl-phosphatidylcholine (POPC)/palmitoyl-oleoyl-phosphatidylserine (POPS) SUVs, even at 50°C [Fig. 2(E–G)]. The resonances from the C-terminal residues do not change at molar protein/lipid ratios of 1/250 and 1/1000 [Fig. 2(E–G)].

SDS binding induces a conformational change in α-synuclein.[28] Our data [Fig. 2(B)] are consistent both with this conclusion and with conclusions based on ^{15}N NMR data, which show that the N-terminal region of the protein binds SDS while the C-terminal region remains disordered.[13,20,24,28,31,32]

Solution NMR is a powerful tool for accessing processes that occur over a range of timescales.[26] In 250 m*M* NaCl, SDS forms larger rod-like micelles, decreasing the tumbling rate of the α-synuclein-micelle complex. The slow tumbling results in the absence of a detectable resonance for residue 39 [Fig. 2(C)]. Increasing the exchange and tumbling rates by increasing the temperature facilitates detection of the resonance [Fig. 2(D)]. The 3FY39 resonance was not observed even at 50°C in SUVs [Fig. 2(E–G)] because tight binding leads to slower exchange and because the large SUVs tumble more slowly than micelles. The chemical shift of free 3FY (−59.6 ppm) is close to that observed for the C-terminal 3FY resonance region of the protein under all conditions, confirming that C-terminal region of α-synuclein is disordered. The data in Figure 2 show that the 3FY labeled protein provides important qualitative information, but quantification is difficult because of the overlap of the resonance from 3FY39 and 3FY125.

Influence of lipid head groups probed with tfmF labeled α-synuclein

To overcome the incomplete resolution of the 3FY resonances, we used an orthogonal t-RNA synthase system[39] to label the protein specifically with tfmF at position 39 and then analyzed the influence of membrane charge on binding. As described previously,[33] we used mass spectrometry to confirm the identity of the labeled protein. We prepared SUVs containing neutral (PC, PE) or negatively charged (PS, PG, and PA) head groups. Two samples with the same amount of ^{19}F labeled α-synuclein were prepared, one with the desired SUV and one without

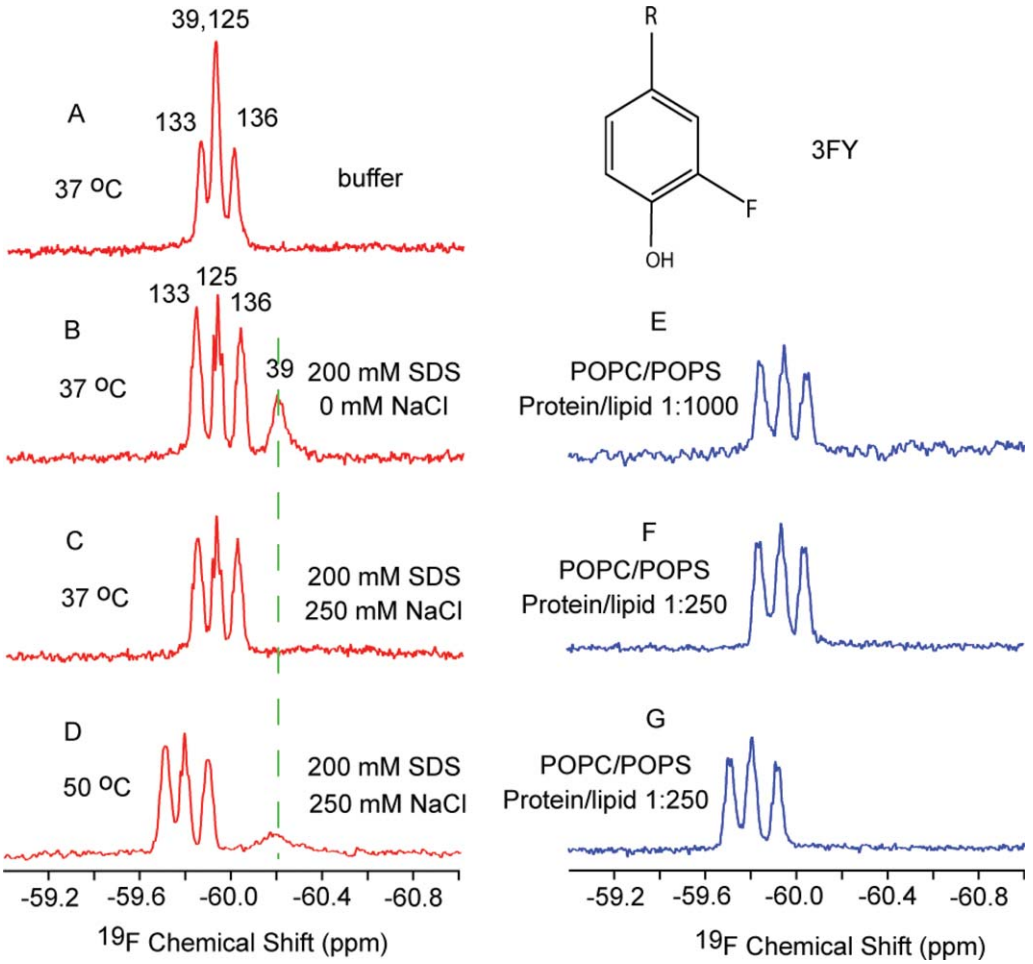

Figure 2. Spectra of 3FY-labeled α-synuclein in buffer (A), in the presence of spherical micelles (B), rod-like micelles (C and D), and small unilamillar vesicles (E–G). The assignments[32] are indicated above the spectra. The protein concentration was 250 μ*M*. The structure of the 3FY side chain is shown above panel E. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

it. After acquiring a ^{19}F spectrum of 150-μ*M* α-synuclein in buffer, the same parameters were used to acquire the spectrum with SUVs at a protein-to-lipid ratio of 1/100. The results are shown in Figure 3.

SUVs attenuate the tfmF resonance, but its chemical shift remains unchanged, indicating slow exchange between the free and bound states. The resonance from the bound state is broadened beyond detection because of the slow tumbling of the SUVs. The decrease in the area under the resonance corresponds to the bound population. Thus, comparing the decreases for different SUVs provides information about the affinity of α-synuclein for the vesicles.

In SUVs made from a 7:3 mixture of the zwitterionic lipids, POPC and palmitoyl-oleoyl-phosphatidylethanolamine (POPE), 62% of the original α-synuclein signal is observed, indicating that 38% is vesicle-associated [Figs. 3(A) and 4]. Changing POPE to a negatively charged lipid, increases the bound fraction to 50%, 70%, 75%, and 100% in 7:3 mixtures of POPC/POPS, POPC/palmitoyl-oleoyl-phosphatidylglycerol (POPG), POPC/palmitoyl-oleoyl-phosphatidic acid (POPA), and POPG alone [Fig. 3(B–E)]. The results are summarized in Figure 4.

Conflicting results have been reported for the effect of head group charge on α-synuclein binding. Negatively charged head groups were reported to have a higher affinity in some studies,[15,19,30,40] but not in others.[41] Others report that α-synuclein binds weakly to phospholipids with neutral head groups, such as PC and PE.[30,40] Our data show that α-synuclein prefers negatively charged phospholipids over neutral phospholipids. Nevertheless, the strength of α-synuclein-membrane interaction varies, even for acid phospholipids with the same charge, indicating that head group charge is not the only factor.[30] We also find that α-synuclein prefers POPA-containing SUVs to POPS-containing SUVs, which agrees with earlier reports.[15,19] This observation reinforces the idea that although electrostatic interaction plays a significant role, other types of interactions are also involved. The preference of α-synuclein for negatively charged lipids can be explained by the fact that N-terminal region

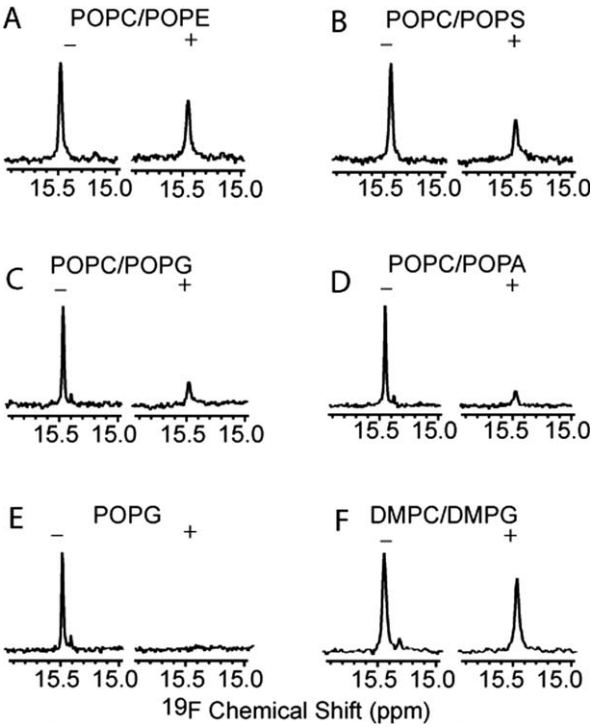

Figure 3. Spectra of α-synuclein with tfmF labeled at position 39 in the absence (–) and in the presence (+) of SUVs. The protein concentration was 150 μ*M*. The molar ratio of protein to lipid was 0.01. The protein concentration is the same in all experiments. Day-to-day differences in shimming account for the small difference in width at half-height for the spectra acquired in the absence of lipids.

of α-synuclein contains many positively charged residues (Fig. 1). The C-terminal region of the protein remains unstructured upon membrane binding because this region contains many negatively charged residues (Fig. 1).

Influence of the acyl chain on binding probed with tfmF labeled protein

We compared ^{19}F data from SUVs made with PC and PG head groups containing unsaturated (POPC/POPG) or saturated dimyristoyl-phosphatidylcholine (DMPC)/dimyristoyl-phosphatidylglycerol (DMPG) acyl chains. The data [Fig. 3(C,F)] show that ~70% of the α-synuclein binds POPC/POPG SUVs, but only ~20% binds DMPC/DMPG SUVs.

The dramatic effect of acyl chain saturation indicates that hydrophobic interactions modulate α-synuclein binding. The experiments were performed at 37°C, where DMPC/DMPG and POPC/POPG SUVs are in the liquid crystalline phase. Neutron diffraction data show that in this phase DMPC/DMPG and POPC/POPG bilayer hydrophobic thicknesses are ~26Å and ~39Å, respectively.[42] The presence of the double band in the POPC/POPG also makes the membrane more dynamic.[43,44] In summary, increasing the hydrophobic thickness and dynamics of the acyl chain lead to higher affinity.

In the α-synuclein-membrane interaction model,[21–25,31] ~100 N-terminal residues lie on the membrane surface as an extended helix, and the remaining residues are disordered. Accordingly, the buried acyl chain should have little effect on α-synuclein interactions. This supposition, however, is inconsistent with our data, which agree with the conclusion of Bodner et al. that the first 25 residues adopt a helical structure state which anchors the interaction to SUVs.[26,27] In that model, α-synuclein prefers SUV defects, which are affected by head group size, charge, and the hydrophobic thickness. The reduced binding of α-synuclein to DMPC/DMPG SUVs is due to the increase in the curvature that arises as a consequence of the acyl chain, which agree with the conclusion of Nuscher et al.[45] As we observe, these properties affect α-synuclein-membrane interactions.

Identifying the region that binds SUVs by using tfmF as a probe

^{19}F spectra of proteins labeled at positions 39 and 133 are shown in Figure 5. The tfmF 39 signal completely disappears in the presence of SUVs made from POPG, while the signal from tfmF 133 decreases only ~30%. These data show that the N-terminal region interacts strongly with POPG SUVs while the C-terminal region is involved in weaker membrane interactions, a conclusion consistent with other ^{15}N NMR studies.[26,27]

Conclusions

Binding mainly involves the N-terminal region of α-synuclein, and both the head group and the acyl chain of phospholipids are important. Although the protein prefers negatively charged lipids, other properties, including the saturation of the lipid also affect α-synuclein membrane interactions. Hydrophobic thickness and acyl chain saturation dramatically

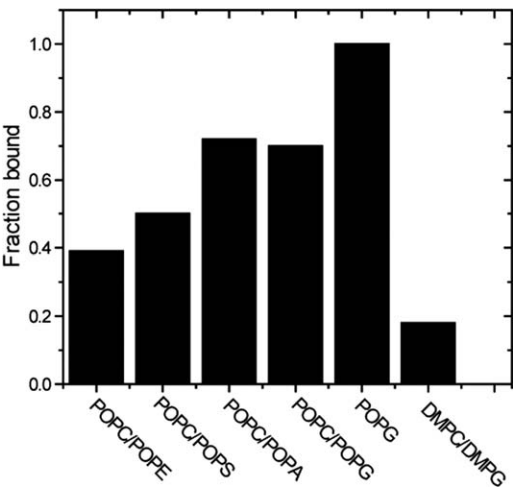

Figure 4. Histogram of the fraction of bound α-synuclein versus lipid composition. The typical uncertainty is ±0.05.

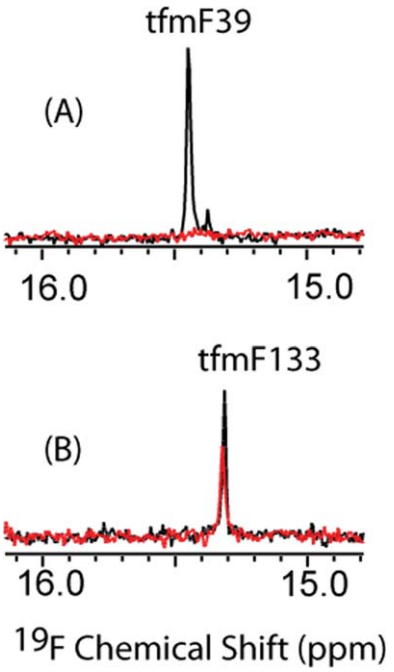

Figure 5. Spectra of labeled α-synuclein in the absence (black) and presence of (red) POPG SUV. The protein concentration was 150 μ*M*. The molar ratio of protein to lipid was 0.01. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

affect protein-membrane interactions. Our data support the idea of multiple tight binding modes.[26,27]

^{19}F labeling is well suited for monitoring protein-membrane interactions, and the method described here should be applicable to other membrane associated proteins. Using this approach, it is easy to quantify how much protein is membrane bound by comparing the signal intensity in the presence or in the absence of SUVs. In addition, site-specific labeling with tfmF provides a simple way to determine which protein segments bind the membrane.

Materials and Methods

3FY and tfmF labeled α-synuclein

The labeled proteins were prepared as described.[32,33]

SDS micelle-bound protein samples. Spherical micelles and rod-like micelles were prepared as described.[24] 3FY α-synuclein was added to solutions of SDS in 10 m*M* Na_2HPO_4 (pH 7.4) with or without 250 m*M* NaCl to a final protein concentration of 250 μ*M*.

SUV-bound protein samples. POPC, POPE, POPS, POPG, POPA, DMPC, and DMPG were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. SUVs were prepared as described.[25] Briefly, the lipids were weighed to produce the desired ratios and dissolved in $CHCl_3$. The solvent was evaporated by using a gentle stream of N_2 (g). The resulting film was dried overnight under vacuum. Dulbecco's phosphate buffered saline (DPBS, 1x free of calcium and magnesium ions 14190-144, GIBCO) was added to the container of lipid film. The sample was vortexed, incubated for 15 min, and then tip sonicated (2W) for 30 min. The sonicated sample was centrifuged for 20 min at 16 000*g* at room temperature. The supernatant was recovered and mixed with labeled α-synuclein. The final protein concentration was 150 μ*M*.

NMR spectroscopy

^{19}F spectra were acquired at 37°C on a Varian Inova 600-MHz spectrometer equipped with a 5 mm ^{19}F *z*-gradient probe. The spectra comprised 512 transients, a 30 kHz sweep width, with a 2 s delay between transients. ^{19}F chemical shifts are referenced to trifluoroethanol at 0 ppm.

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