



Interaction of α -synuclein with vesicles that mimic mitochondrial membranes

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ABSTRACT

α -Synuclein, an intrinsically-disordered protein associated with Parkinson's disease, interacts with mitochondria, but the details of this interaction are unknown. We probed the interaction of α -synuclein and its A30P variant with lipid vesicles by using fluorescence anisotropy and ¹⁹F nuclear magnetic resonance. Both proteins interact strongly with large unilamellar vesicles whose composition is similar to that of the inner mitochondrial membrane, which contains cardiolipin. However, the proteins have no affinity for vesicles mimicking the outer mitochondrial membrane, which lacks cardiolipin. The ¹⁹F data show that the interaction involves α -synuclein's N-terminal region. These data indicate that the middle of the N-terminal region, which contains the KAKEGVVAAAEE repeats, is involved in binding, probably via electrostatic interactions between the lysines and cardiolipin. We also found that the strength of α -synuclein binding depends on the nature of the cardiolipin acyl side chains. Eliminating one double bond increases affinity, while complete saturation dramatically decreases affinity. Increasing the temperature increases the binding of wild-type, but not the A30P variant. The data are interpreted in terms of the properties of the protein, cardiolipin demixing within the vesicles upon binding of α -synuclein, and packing density. The results advance our understanding of α -synuclein's interaction with mitochondrial membranes.

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1. Introduction

Parkinson's disease and other neurodegenerative disorders are characterized by cytoplasmic neuronal inclusions known as Lewy bodies [1–4]. α -Synuclein, a 140-amino acid intrinsically disordered protein, is the main component of Lewy bodies [5–8]. The protein (Fig. 1A) comprises a positively-charged N-terminal region (residues 1–60) with an imperfect consensus repeat KTKEGV, a hydrophobic middle segment known as the NAC region (Non Amyloid Component, residues 61–95), and a negatively-charged C-terminal region (residues 96–140) [9,10].

α -Synuclein's exact functions remain obscure, but recent work highlights its roles in neurotransmitter release, mitochondrial dysfunction, and aging. One function involves SNARE-complex assembly at presynaptic vesicles via its C-terminal region while its N-terminal region is anchored to the vesicle [11]. The protein is important for synaptic activity when the nerve terminals repeatedly release neurotransmitters, which requires the assembly and disassembly of the complex.

α -Synuclein localizes not only to the presynaptic neural terminals but also to the mitochondria, whose membranes are unique in eukaryotes because they contain cardiolipin (CL), an anionic phospholipid

with two phosphatidyl glycerol moieties (Fig. 1B). This localization is associated with mitochondria dysfunction [12–14] including impairment of complex I [15,16], oxidative stress [17], mitochondrial lipid abnormalities [16], and mitochondrial fission [18]. The protein is also involved in aging, but the reasons for its age-related loss of function are unknown [11]. Here, we focus on interactions with the mitochondrial membrane.

The interaction of α -synuclein with vesicles of different lipid composition, phospholipid headgroups, sizes, and surfaces has been studied [19–30]. The protein has a higher affinity for small unilamellar vesicles than for large unilamellar vesicles (LUVs), and binds more strongly to vesicles containing anionic phospholipids [10,19,20,31]. However, little attention has been focused on quantitative assessment of interactions with bilayers that mimic the lipid compositions of the mitochondrial inner and outer membranes.

We used fluorescence anisotropy and high resolution nuclear magnetic resonance spectroscopy (NMR) to investigate the interaction of wild-type α -synuclein and one of its familial Parkinson's disease variants [32], A30P, with LUVs having lipid compositions similar to the inner and outer mitochondrial membranes. We evaluated the importance of CL as well as the effect of saturated and unsaturated CL acyl side chains. We also investigated the effect of temperature, and using ¹⁹F-labeled α -synuclein, we determined which region of the protein interacts with LUVs containing CL. The results advance our understanding of α -synuclein's interaction with mitochondrial membranes.

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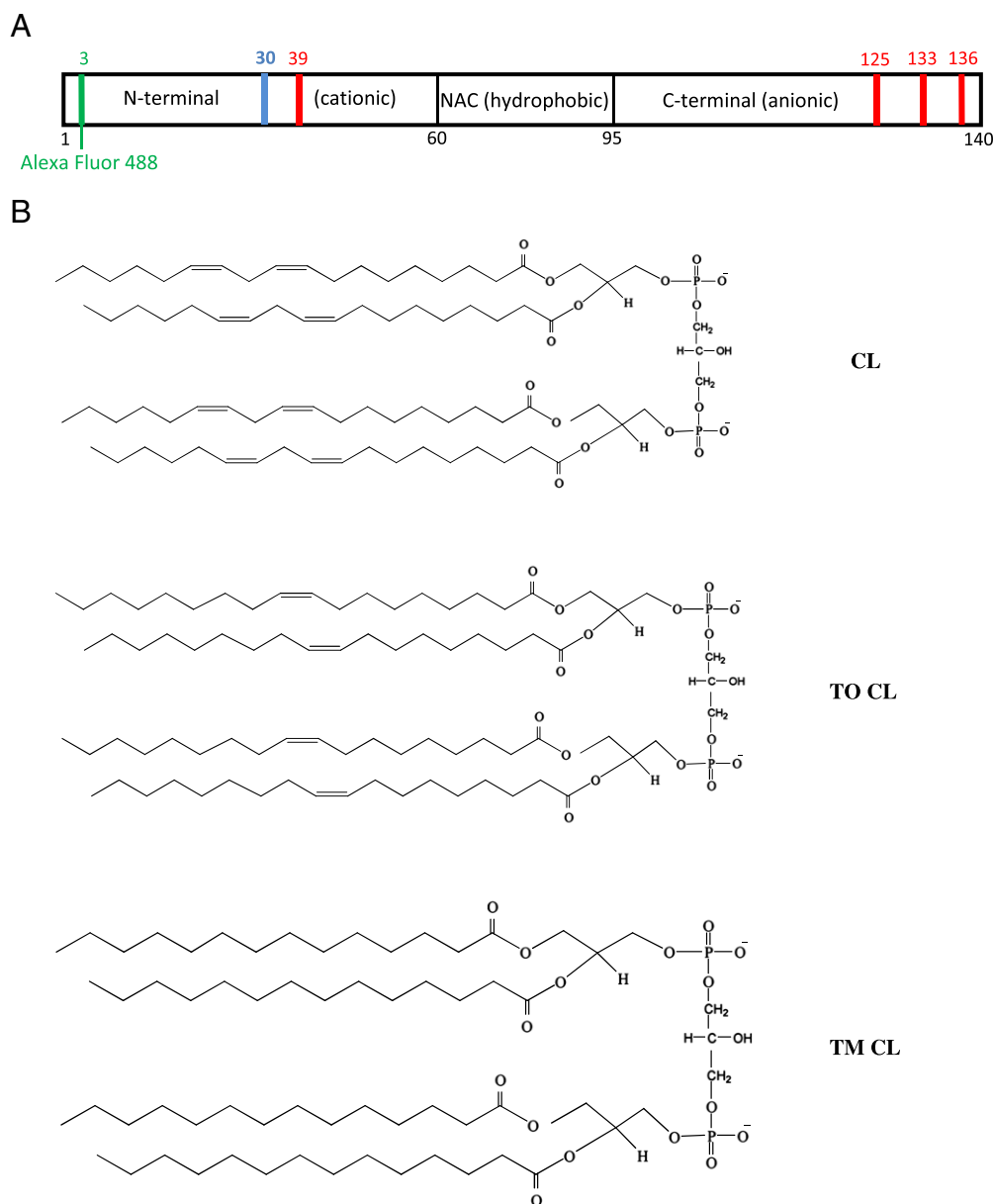


Fig. 1. Schematic representation of α -synuclein (A) showing the four tyrosines (red), the position labeled with dye (green), and the A30P point mutation (blue). (B) Structural formulas of the CLs used to prepare LUVs. NAC refers to the non amyloid component of α -synuclein.

2. Experimental procedures

2.1. Expression, purification, and labeling of human α -synuclein and its A30P variant

Wild-type α -synuclein, A30P α -synuclein, V3C α -synuclein and V3C;A30P α -synuclein were expressed from a T7-7 plasmid in *Escherichia coli* BL21-Gold(DE3) competent cells (Stratagene Cloning Systems, La Jolla, CA). The proteins were purified as described [33,34].

The V3C mutation was made by using a Stratagene site-directed mutagenesis kit. The cysteine was used to attach Alexa Fluor 488 (Invitrogen, Carlsbad, CA). For labeling, 12 mg of V3C α -synuclein were dissolved in sterile, degassed water to a final concentration of 2 mg/mL. Tris(2-carboxyethyl)phosphine and NaHCO_3 were added in a 10-fold molar excess over the protein. The mixture was incubated at room temperature with shaking for 30 min. Next, Alexa Fluor 488 C5-maleimide was added in a 10-fold molar excess over the protein and the mixture incubated at room temperature with shaking for

2 h. The labeled protein was purified with gel filtration chromatography by using a Superdex 75 column eluted with 20% acetonitrile in phosphate buffered saline (NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 1.8 mM). The labeled protein was dialyzed against water.

The extinction coefficient of the dye at 494 nm ($71,000 \text{ M}^{-1} \text{ cm}^{-1}$) was used to quantify the labeled protein. The Lowry method (Pierce, Rockford, IL) was used to quantify the total protein. The degree of labeling was 84% for V3C α -synuclein and 99% for V3C;A30P α -synuclein. Aliquots containing 1 mg of labeled protein were lyophilized and stored at -80°C .

For NMR experiments, wild-type and Y125F α -synuclein labeled with 3-fluoro-L-tyrosine were prepared as described [35].

2.2. Cell culture and mitochondria isolation

Human epithelial carcinoma cells (HeLa) from the ATCC were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented

with 10% fetal bovine serum, penicillin and streptomycin (100 µg/mL). The cultures were incubated at 37 °C in 5% CO₂. Mitochondria were isolated from HeLa cells (2×10^7 cells) by using the Mitochondria Isolation Kit for Cultured Cells from Pierce. Their activity was tested by assessing the cytochrome *c* oxidase activity with the Mitochondria Activity Assay Kit from BioChain Institute, Inc. (Hayward, CA).

2.3. Mitochondrial import of α -synuclein

Freshly isolated mitochondria were incubated with fluorescently-labeled α -synuclein in the presence of energy mixture and transport buffer as described [36]. Trypsin was used to remove α -synuclein attached to the surface of mitochondria. Mito Tracker Red CMXRos (Invitrogen) was used to stain the active mitochondria after protein import and trypsinization. Fused silica and glass microscope slides were prepared as described [37]. Mitochondria containing α -synuclein were allowed to attach to the poly-L-lysine coated slide for 30 min and then rinsed with minimal media. α -Synuclein import was assessed by using confocal microscopy on an inverted laser scanning microscope (Zeiss 510 Meta, Thornwood, NY) equipped with a 63×1.4 NA, Plan-Apochromat, oil immersion objective.

2.4. Vesicle preparation and characterization

Lipids were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL) in chloroform, except cholesterol (CH, from ovine wool), which was dissolved in chloroform to a concentration of 1 mg/mL and stored at -20 °C. The lipids were used without further purification.

Aliquots of lipids in chloroform, were mixed in glass vials and the solvent was removed overnight in a vacuum concentrator. The dried mixtures were suspended in 1 mL of 50 mM sodium phosphate buffer, pH 7.4, to a final lipid concentration of 2 mM. The following lipids were used for preparing LUVs: CH (cholesterol), CL (bovine heart), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1',3'-bis[1,2-dimyristoyl-*sn*-glycero-3-phospho]-*sn*-glycerol (TM CL), and 1',3'-bis[1,2-dioleoyl-*sn*-glycero-3-phospho]-*sn*-glycerol (TO CL). Fig. 1B shows the structures of CLs.

Vesicles of composition DOPC:DOPE:CL:CH 2.0:1.3:1.0:0.6 molar ratio and DOPC:DOPE:CH 4.0:2.0:0.9, corresponding to inner and outer membrane of mitochondria [38], respectively, were prepared. LUVs with different ratios of CL (1.0, 0.8, 0.5, and 0) or different CL acid side chains (TM and TO) were also prepared.

LUVs were prepared by multiple extrusion [20,31,39] through a 0.1 µm polycarbonate membrane (Whatman Inc., Sanford, ME).

For NMR experiments, the vesicles were prepared with the same protocol, but at a concentration of 4 mM. The dried mixture corresponding to the inner mitochondrial membrane was resuspended in 50 mM sodium phosphate buffer, pH 7.4, containing 10% D₂O. The final concentration of ¹⁹F labeled protein in the NMR tube was 100 µM and the protein:lipid molar ratio was ~1:100. Protein in buffer alone was used as a reference.

Vesicles size and its distribution were characterized with dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments, Southborough, MA). Measurements were performed on freshly prepared LUVs and LUVs stored for 72 h at 4 °C. The LUV concentration was 2 mM in 50 mM sodium phosphate buffer, pH 7.4. The measurements were performed at 25 °C with the viscosity and refraction index set to those for water.

2.5. NMR

¹⁹F spectra were acquired at 25 °C on a Varian Inova 600 MHz spectrometer equipped with a 5 mm triple resonance probe. The spectra comprised 2048 transients with a 30 kHz sweep width. The chemical shift was referenced to trifluoroethanol at 0 ppm. The experiments were conducted in triplicate.

2.6. Fluorescence anisotropy of labeled proteins

The experiments were conducted on a FluoroLog®-322 spectrofluorometer (HORIBA Jobin Yvon Inc., Edison, NJ) with an excitation wavelength of 495 nm and an emission wavelength of 519 nm. Four hundred µL of 100 nM protein solution in 50 mM sodium phosphate buffer, pH 7.4, were titrated with LUVs at 25 or 37 °C.

Each point is the average of five measurements, and each condition was tested in triplicate. Control measurements using only LUVs or unlabeled wild-type α -synuclein in the same buffer were performed to assess background fluorescence, which was negligible.

The anisotropy was calculated as described [40]. The dissociation constants (K_d) were calculated by fitting the data to a one site model (SigmaPlot 11.0).

2.7. DPH fluorescence anisotropy

1,6-Diphenyl-1,3,5-hexatriene (DPH) was incubated with LUVs for 1 h at room temperature either in the presence or absence of protein. Samples comprised 100 nM protein and 300 µM LUVs in 50 mM phosphate buffer, pH 7.4. The final molar lipid to DPH ratio was 300:1. Fluorescence anisotropy was recorded at 25 °C by using an excitation wavelength of 360 nm and an emission wavelength of 440 nm. Fluorescence anisotropy was also measured at 37 °C for CL inner membrane LUVs in the presence or absence of wild-type or A30P α -synuclein. The result for each condition is the average of five measurements.

3. Results

3.1. In vitro mitochondrial import of α -synuclein

Fluorescently-labeled α -synuclein was localized in isolated mitochondria (Fig. 2A). Mito Tracker Red CMXRos is an indicator of active mitochondria because its accumulation is related to membrane potential. The red fluorescence shows that mitochondria are active after protein import and trypsinization (Fig. 2B). Co-localization of fluorescently-labeled protein and Mito Tracker Red indicates that

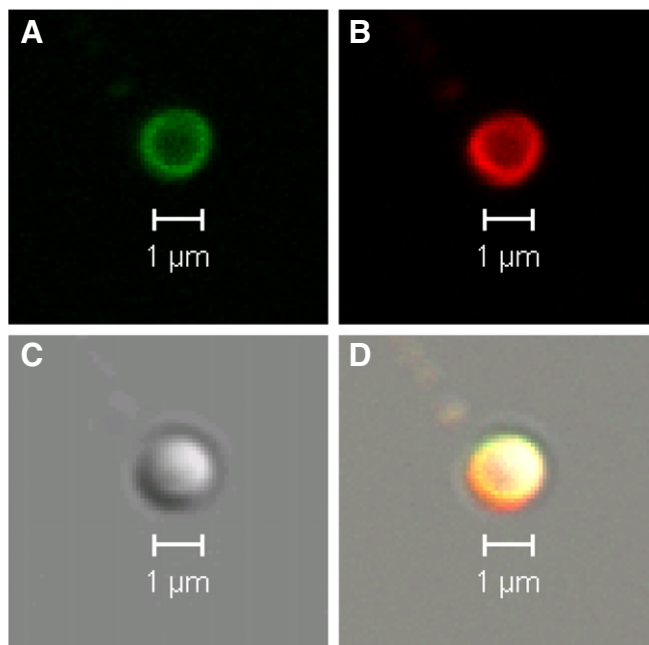


Fig. 2. Isolated mitochondrion from HeLa cells incubated with fluorescently-labeled α -synuclein (A), and Mito Tracker Red (B), differential interference contrast (C), and the merged images (D).

much of the protein is located outside the matrix (Fig. 2D). These findings are consistent with those of Devi et al. [13]. To understand the distribution of α -synuclein in the inner and outer membrane, we used lipid vesicles with a composition similar to these membranes.

3.2. Interaction with LUVs having lipid compositions similar to the inner and outer mitochondrial membrane

Mitochondria contain 25.3% phospholipid by mass [38]. In terms of composition, the mitochondria comprise 40.8% phosphatidyl choline, 37.4% phosphatidyl ethanolamine, 19.1% CL, and ~3% phosphatidyl inositol. The major phospholipids of the outer membrane are phosphatidyl choline (55.2%), and phosphatidyl ethanolamine (25.3%). The major phospholipids of the inner membrane are phosphatidyl choline (44.5%), phosphatidyl ethanolamine (27.7%), and CL (21.5%). Although the amount of CL in the outer membrane is small, the exact value remains controversial [41,42]. Nevertheless, there is at least 40 times more CL in the inner membrane than in the outer membrane [43].

Fluorescence anisotropy was used to quantify the interaction of fluorescently-labeled V3C α -synuclein and V3C:A30P α -synuclein with LUVs. Alexa Fluor 488 dye was chosen because of its high photostability [44]. Labeling at position 3 was selected because this region is highly dynamic, decreasing the possibility that the modification will disrupt the native conformation [45]. The average diameter of the LUVs, as determined by dynamic light scattering, is ~140 nm, consistent with values for vesicles extruded through membranes with 100 nm pores [20,31]. The vesicles were stable for at least three days if kept at 4 °C in 50 mM sodium phosphate buffer, pH 7.4. Nevertheless, the vesicles were used within 48 h. The presence or absence of CH or CL did not affect vesicle size.

First, we studied the interaction of fluorescently-labeled proteins with LUVs having a molar DOPC:DOPE:Cholesterol (CH) ratio of 4.0:2.0:0.9, which corresponds to the composition of the outer membrane. No change in the anisotropy was noted when titrating 100 nM protein with LUVs, suggesting limited affinity of fluorescently labeled V3C or V3C:A30P α -synuclein for this type of vesicle (Fig. 3A and B).

LUVs with a molar ratio corresponding to the inner membrane (DOPC:DOPE:CL:CH, 2.0:1.3:1.0:0.6) gave strikingly different results (Fig. 3); both the wild-type protein and the A30P variant bind with sub mM dissociation constants. As shown in Table 1, V3C α -synuclein binds slightly more strongly (K_d 130 μ M) than the V3C:A30P variant (K_d 210 μ M). Since only the inner membrane contains CL, these observations implicate CL in the binding of α -synuclein and are consistent with studies showing that α -synuclein interacts with vesicles containing anionic phospholipids [10,20,24,46].

3.3. Importance of CL

α -Synuclein is also mostly associated with the inner membrane of mitochondria in brains from patients with Parkinson's disease [13,15]. In terms of phospholipids, brains of mice lacking α -synuclein have reduced levels of only CL and phosphatidylglycerol (a CL precursor) [16]. Since CL is mitochondria-specific, we decided to investigate α -synuclein binding to LUVs containing different amounts of this phospholipid. The data are shown in Fig. 4, and the K_d values are compiled in Table 1.

LUVs containing DOPC, DOPE, and CH in molar ratios corresponding to the inner mitochondrial membrane were prepared with 100%, 80%, 50%, and 0% CL, where 100% represents the normal amount in the inner membrane. For V3C α -synuclein, decreasing the CL by 20% causes less than a two-fold increase in K_d , but halving the CL increases K_d by a factor of six. No binding was noted when CL was absent (Fig. 4A). These findings show that CL is essential for the interaction

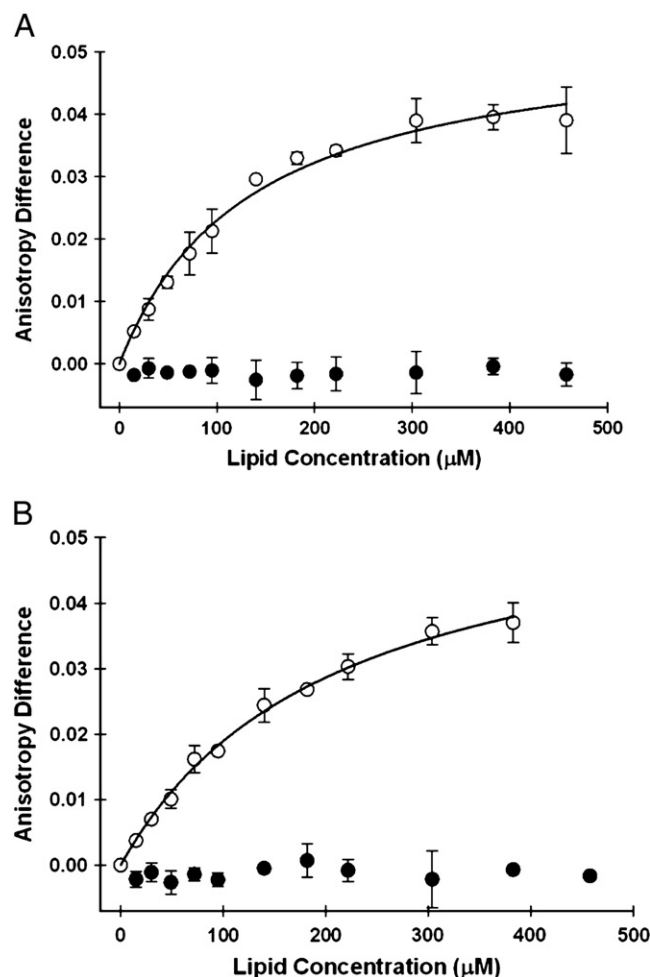


Fig. 3. Interaction of fluorescently-labeled V3C (A) and V3C:A30P (B) α -synucleins with LUVs having a composition similar to that of the inner (○) and outer (●) membranes. Error bars represent the standard deviations from triplicate experiments.

between α -synuclein and the inner mitochondrial membrane and that the affinity increases with CL concentration.

We also studied the interaction of the A30P variant with these LUVs. The decrease in affinity is even stronger for the variant than it is for the wild-type protein (Fig. 4B, Table 1).

3.4. Effect of acyl group

For the studies described above we used bovine heart CL, whose side chains contain two double bonds, one between C_6 and C_7 , the other between C_9 and C_{10} (Fig. 1B). In brain CL, however, oleic acid, which has only the former double bond, is the most abundant fatty acid [47]. To examine the effect of side chain saturation on α -synuclein binding, LUVs with a molar ratio corresponding to the lipid composition of the inner membrane were prepared by using TOCL with its four oleic acid chains (Fig. 1B).

The results are shown in Fig. 5, and the K_d values are compiled in Table 1. The V3C variant binds LUVs with the more abundant side chain more strongly (K_d 67 μ M) than LUVs containing two double bonds (K_d 130 μ M).

The affinity of A30P α -synuclein for LUVs containing one double bond per side chain (K_d 153 μ M) is nearly the same as its affinity for LUVs with two (K_d 210 μ M). We conclude that α -synuclein prefers the LUVs with compositions that most closely mimic those from brain mitochondria.

Table 1
Dissociation constants (μM) of fluorescently-labeled α -synucleins.*

	OM	CL 100% (IM)	CL 80% (IM)	CL 50% (IM)	CL 0% (IM)	TO CL (IM)	TM CL (IM)	CL 100% (IM) 37 °C
V3C	-	130 \pm 20	210 \pm 20	850 \pm 120	-	67 \pm 3	3000 \pm 4000	59 \pm 3
V3C;A30P	-	210 \pm 20	680 \pm 150	1200 \pm 700	-	153 \pm 7	-	230 \pm 20

* Data were acquired at 25 °C unless otherwise specified; OM, outer membrane; IM, inner membrane; 100%, 80%, 50%, and 0%, LUVs with IM compositions of 100%, 80%, 50% and 0% CL respectively; TO CL and TM CL, LUVs of IM lipid composition with saturated CL (TM) or CL possessing one double bound (TO); -, Kd could not be estimated.

3.5. Positional information

Since V3C α -synuclein and V3C;A30P α -synuclein are labeled with the dye at a single position (Fig. 1A), the fluorescence anisotropy studies provide information about protein binding, but not positional information.

^{19}F NMR was used to test the effect of chain saturation and to determine if the specific regions of α -synuclein bind differently. ^{19}F is sensitive to its environment, such that small changes in the structure of a labeled region are easily detected.

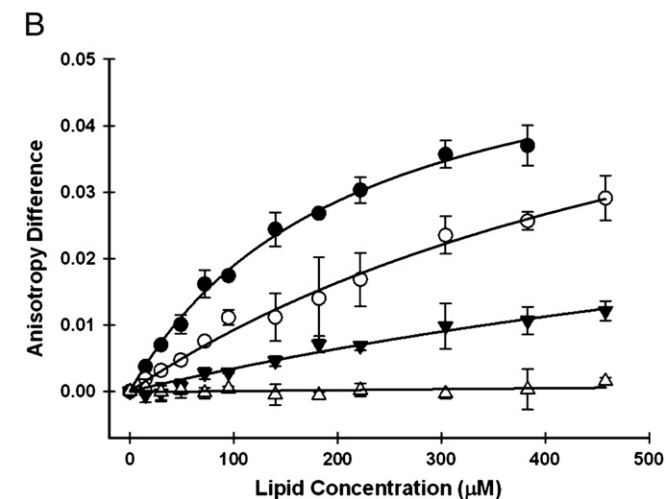
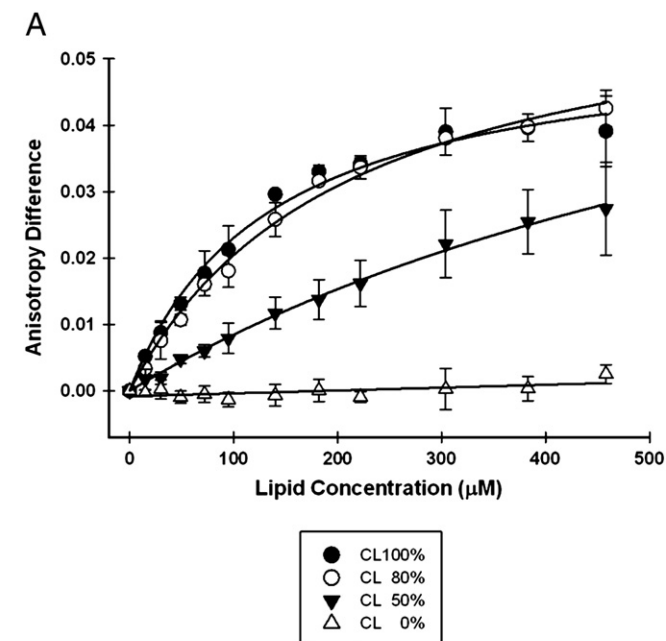


Fig. 4. Differential binding affinity of fluorescently labeled-V3C (A) and -V3C;A30P (B) α -synucleins to LUVs having a composition similar to the inner membrane but varying amounts of CL. Error bars represent the standard deviations from triplicate experiments.

α -Synuclein has four tyrosines, one at position 39 in the N-terminal region and the others near the C-terminus at positions 125, 133, and 136 (Fig. 1A). The ^{19}F NMR spectrum of 3-fluoro-tyrosine-labeled wild-type α -synuclein in buffer alone exhibits only three resonances (Fig. 6A). As shown by Li et al., [35], the middle resonance is a composite of resonances from tyrosines 39 and 125. We used the Y125F variant to eliminate the overlap (Fig. 6 B).

Spectra acquired in the presence of vesicles containing CL with two (CL), one (TO), and no (TM) double bonds are shown in Fig. 6 C, D, and E, respectively. The spectra in panels C, D and E are similar despite the differences in affinity (Table 1). This is because the high concentrations required for NMR result in almost complete binding

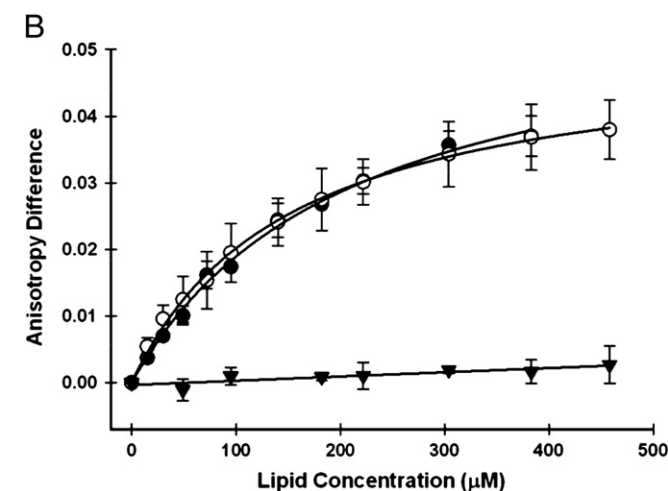
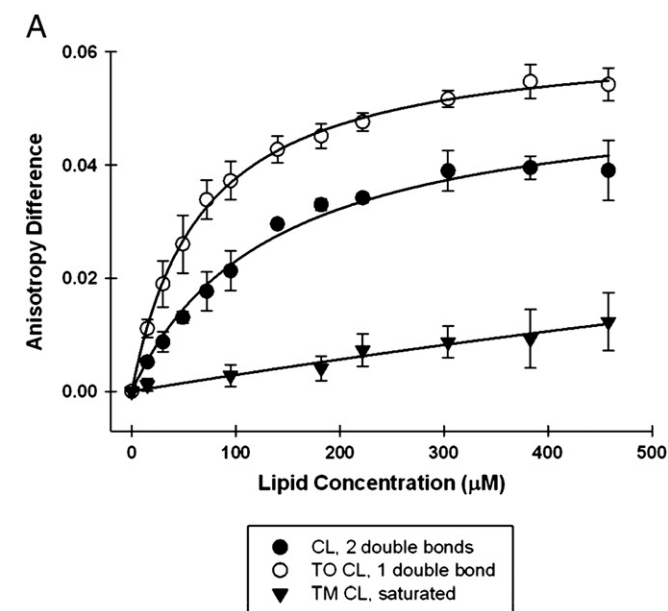


Fig. 5. Binding of fluorescently labeled-V3C (A) and -V3C;A30P (B) α -synucleins to LUVs with a composition similar with inner membrane but different degrees of side chain saturation. Error bars represent the standard deviations from triplicate experiments.

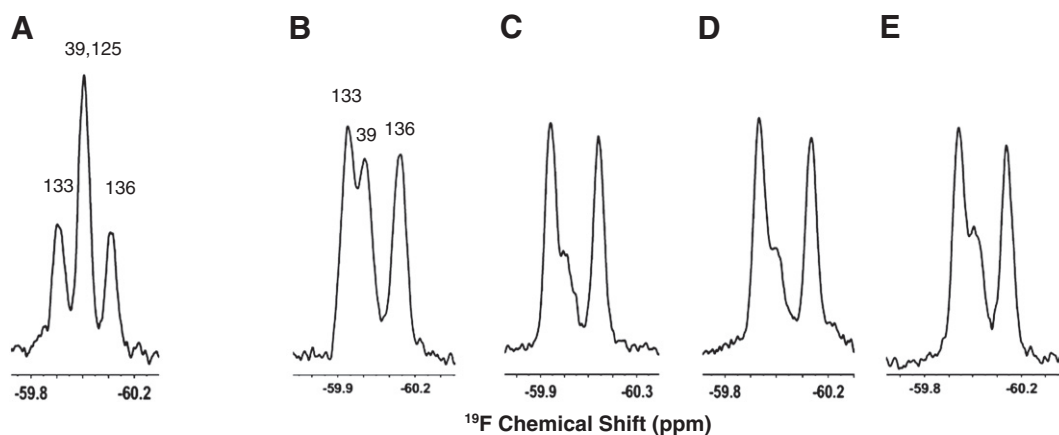


Fig. 6. ¹⁹F NMR spectra of tyrosine labeled wild-type (A) and Y125F (B) α -synuclein in buffer and the labeled Y125F variant in the presence of LUVs containing 100% CL (C), TO CL (D), and TM CL (E). Assignments are shown above the resonances [35]. The molar ratio of protein to lipid is \sim 1:100.

for all samples despite the difference in K_d values. The fact that the resonances from positions 133 and 136 are unchanged by the LUVs, but the resonance from position 39 decreases (Fig. 6C–E), indicates that N-terminal region of the protein interacts with the vesicles.

3.6. Temperature and binding

Table 1 shows the affinities at 25 °C (Fig. 3A and B) and 37 °C (Fig. 7) of fluorescently-labeled V3C α -synuclein and V3C;A30P α -synuclein for LUVs with a composition corresponding to the inner membrane. The affinity of V3C α -synuclein is two fold greater (K_d 59 μ M) at 37 °C than it is at 25 °C (K_d 134 μ M), but the affinity of the V3C;A30P protein is unaffected.

3.7. DPH fluorescence anisotropy

The fluorescence anisotropy of DPH was used to examine the packing density of LUVs with different CL acyl side chains. The anisotropy depends on the type of phospholipid and its degree of saturation with higher packing density causing larger anisotropy.

The data (Fig. 8) indicate that in buffer, at 25 °C, LUVs containing CL with one double bond (TO CL) are as well packed as those containing two double bonds (CL), but both are better packed than LUVs with completely saturated cardiolipin (TM CL).

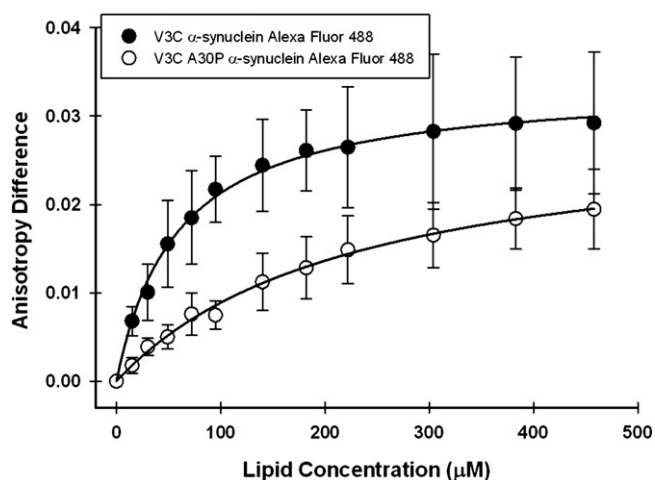


Fig. 7. Interaction of fluorescently-labeled-V3C α -synuclein and -V3C;A30P α -synuclein with LUVs having a composition similar to the inner membrane at 37 °C. Error bars represent the standard deviations from triplicate experiments.

Wild-type and A30P α -synuclein reduce the anisotropy for all three types of LUVs. The decrease is comparable for wild-type and A30P α -synucleins. Studies conducted at 37 °C also show a decrease in anisotropy for CL IM vesicles in the presence of both wild-type and A30P α -synuclein (Fig. 8, inset). These data show that α -synuclein perturbs the packing density.

4. Discussion

α -Synuclein interacts with isolated mitochondria from HeLa cells and is imported in the proximity of the inner membrane (Fig. 2). The protein has no affinity for LUVs with the lipid composition of the outer mitochondrial membrane (Table 1), which lacks CL. This observation is consistent with studies showing that α -synuclein enters mitochondria via the protein import channel [15] rather than through lipid interactions.

A significant affinity was noticed for both the wild-type protein and the A30P variant for LUVs with a composition similar to that of the inner mitochondrial membrane, with the V3C α -synuclein having slightly higher affinity (Fig. 3). Vesicles with the lipid composition of the inner membrane, except that they lack CL, do not bind α -synuclein. Consistent with this conclusion, decreasing the CL content reduces α -synuclein affinity (Fig. 4A and B). These observations show the importance of CL in the inner mitochondrial membrane for α -synuclein localization.

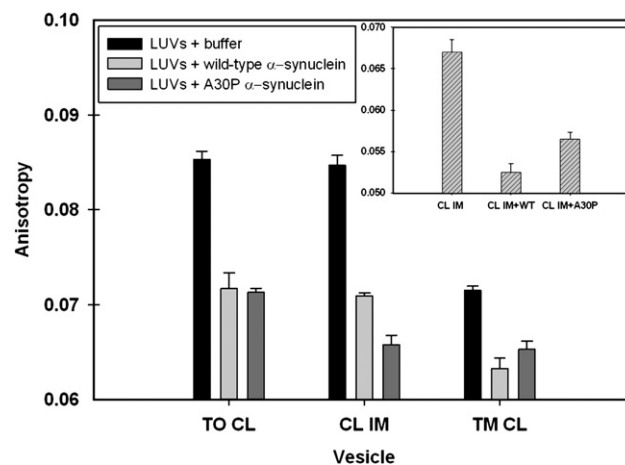


Fig. 8. DPH anisotropy upon the interaction of α -synucleins with LUVs having different CL acyl side chains at 25 °C. The inset shows the DPH anisotropy in CL IM LUVs in the presence and absence of α -synucleins at 37 °C. Error bars represent the standard deviations from 5 experiments.

Our data and those of others show that α -synuclein binds more tightly to vesicles containing anionic phospholipids such as phosphatidylglycerol or phosphatidylserine showing that anionic character is important for protein binding [10,19,20,30,31]. The fact that the protein has a higher affinity for smaller vesicles suggests that membrane curvature [21] or packing also plays a role. Unfortunately, we cannot tease these apart because cardiolipin is not just negatively charged, it also affects membrane curvature [17,48], has limited miscibility in bilayers [49], and can cause protein clustering [50]. Furthermore, charge neutralization upon α -synuclein binding also accentuates curvature and causes demixing [29]. In addition, the protein may be binding membrane defects arising from the poorer packing of vesicles. For these reasons, we cannot distinguish the effects of charge alone from the effects of charge combined with changes in curvature, demixing, and packing.

The ^{19}F NMR data suggest that only the N-terminal region interacts with the vesicles. Others have shown that this region interacts with phospholipid head groups forming an α -helix, while the C-terminus remains unstructured [20,21,24,25]. The A30P variant has a lower affinity for anionic lipid vesicles compared to the wild-type protein (Table 1) as has been reported by others [26,27,30,51]. These observations are consistent with the idea that proline at position 30, interrupts the helix in the vesicle bound protein [46].

The increased affinity at higher temperature observed for the wild-type protein indicates that binding is endothermic, which means the interaction of the protein with membranes is entropically favorable. This observation together with the NMR data lead to the suggestion that the nonpolar strip in the amphipathic N-terminal helix region of the protein [25] adds to the protein's affinity for vesicles via hydrophobic interactions. However, the temperature dependence could also be brought about by demixing (i.e., differential enrichment of lipids).

In summary, α -synuclein and the A30P variant have a significant affinity for lipid vesicles with the composition of the inner mitochondrial membrane, but they do not interact with the vesicles mimicking the outer membrane. The interaction with vesicles mimicking the inner membrane probably involves electrostatic interactions between the cationic lysines in the consensus repeat KTKEGV and the anionic cardiolipin.

The increased affinity of α -synuclein for LUVs made from phospholipids with unsaturated side chains was confirmed [16]. However, in our study α -synuclein binds more strongly to the TO CL, which contains one double bond per acyl side chain compared to the CL, which contains two. This observation is interesting in light of the fact that most of the CL in brain mitochondria possesses one saturated and one unsaturated side chain [47], and is consistent with a role for CL- α -synuclein interactions in Parkinson's disease.

To better understand the affinity of α -synucleins for CL vesicles containing different fatty acids we investigated the vesicle packing density by using DPH fluorescence anisotropy. The fluorescence anisotropy of DPH embedded in a LUV provides information about membrane fluidity, and hence the packing density of its acyl side chains. High packing density lowers fluidity, increasing DPH anisotropy [52].

The DPH anisotropy decreases when wild-type or A30P α -synucleins were added to LUVs containing CL with one, two, or no double bonds, and the percent decrease is similar in both instances (Fig. 8). Specifically, a 16% decrease was noted for TO CL in the presence of wild-type α -synuclein while an 11% decrease was noted for TM CL. Also, given the dissociation constants (Table 1), more protein interacts with TO CL LUVs than with TM CL LUVs. From these observations we suggest that the binding of α -synuclein to TO CL vesicles induces demixing of CL, causing defects at the membrane-water interface (unsaturated acyl chains are more dynamic than saturated chains [53]). We also suggest that the higher affinity of α -synuclein for TO CL arises from the formation of an amphipathic α -helix, with

the nonpolar residues interacting with nonpolar lipids in the defects, as has been observed [28].

The DPH anisotropy decreases when wild-type or A30P α -synucleins were added to LUVs containing CL with one, two, or no double bonds (Fig. 8). The trend in DPH fluorescence anisotropies follows the trend in binding constants (Table 1) indicating that stronger binding leads to decreased DPH anisotropy. We suggest that this trend follows from the geometry of CL, which has two small head groups and four acyl chains. The combination of the small head groups and the large volume of the chains forces the molecule into a cone-shaped structure [54]. This arrangement enhances structural rigidity [53], allowing other molecules in the membranes to penetrate the bilayer. For TM CL, we conjecture that its saturated chains interact with the acyl side chains from the other lipids and the cholesterol to maintain the membrane in a more solvent exposed structure, while for TO CL and IM CL, the unsaturated acyl chains are more dynamic and able to adjust to a more compact, better packed structure upon mixing with other components.

Increasing the temperature increases the fluidity of the vesicles, reducing their packing density. Binding of wild-type protein is stronger at 37 °C than it is at 25 °C. The stronger binding at higher temperature is consistent with our conclusion about membrane destabilization by α -synuclein binding. However, as discussed above, the temperature dependence is also consistent with the idea that hydrophobic interactions play an important role in binding.

To our knowledge, this is the first study to investigate the affinity of α -synuclein for lipid vesicles with a composition similar to mitochondrial membranes. The data explain why α -synuclein has a high affinity for the inner membrane (it contains CL), show that the interaction involves the N-terminal region of the protein, and suggest that the protein destabilizes the inner membrane. An interesting and potentially biologically important caveat to more detailed interpretation of our data arises from results showing the limited miscibility of CL in bilayers [49]. Specifically, we cannot know whether the interactions involve the entire vesicle surface or distinct CL-rich domains — i.e., so-called lipid rafts [55].

The combination of techniques used here, can also be applied to monitor the binding of α -synuclein to isolated mitochondria and mitoplasts. Such investigations should help elucidate the role of α -synuclein in the mitochondria dysfunction associated with neurodegenerative diseases.

Abbreviations

CH	cholesterol
CL	cardiolipin
DOPC	1,2-dioleoyl- <i>sn</i> -glycero-3-phosphocholine
DOPE	1,2-dioleoyl- <i>sn</i> -glycero-3-phosphoethanolamine
DPH	1,6-diphenyl-1,3,5-hexatriene
K_d	dissociation constant
LUV	large unilamellar vesicle
NMR	nuclear magnetic resonance
TM CL	1',3'-Bis[1,2-dimyristoyl- <i>sn</i> -glycero-3-phospho]- <i>sn</i> -glycerol
TO CL	1',3'-bis[1,2-dioleoyl- <i>sn</i> -glycero-3-phospho]- <i>sn</i> -glycerol.

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