The Effect of H50Q Mutation on the Interaction between α-Synuclein and Lipid

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Abstract. The abnormal aggregation of α-synuclein (αSyn) is considered as connection with the development of synucleinopathies such as Parkinson's disease, and lipid could be one of crucial inducers. Familial Parkinson's disease (FPD) mutation of αSyn can change its properties and may impact its interactions with lipid. In this study, using multiple fluorescence techniques, we found that H50Q mutation can increase αSyn binding to synaptic-like vesicle, which could be helpful to uncover the H50Q mutation-related pathology of αSyn.

Keywords: α -synuclein; Lipid; Parkinson's Disease; FPD Mutation; Fluorescence.

1. Introduction

α-Synuclein (αSyn) is an inherently disordered protein with a mass of 14.46 kDa that is prevalent in presynaptic terminals and is encoded by the SNCA gene. αSyn does not have a well-defined 3D structure [1]. Incorrectly folded αSyn will aggregate and form Lewy bodies, the neuropathological hallmark of synucleinopathies such as Parkinson's disease (PD) and Lewy body dementia [2,3]. The physiological function of αSyn is still unknown, but the high proportion of αSyn found in the central nervous system, particularly in presynaptic terminals, suggests that αSyn may play a role in synaptic vesicle trafficking [4,5]. Also, αSyn promotes the formation of presynaptic soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes by directly interacting with the N-terminus of synaptobrevin-2/VAMP protein [6].

αSyn usually binds with lipid membrane to carry out not only in normal function but also in the pathology of PD [6-8]. The seven partially conserved 11 amino acid repeat sequences on N-terminal region enable αSyn to bind with lipid membrane. αSyn prefers to bind with negatively charged lipid such as PS (phosphatidylserine) and PG (phosphatidylglycerol), while is less likely to bind with neutral lipid such as PC (phosphatidylcholine) [1-2], due to electrostatic interaction with specific phospholipid head group. When the N-terminal region initiates the binding to membrane, αSyn will adopts itself to a partial α-helix structure [1, 3]. The hydrophobic residues are present inside the membrane, residues with negative charge exposed to the solvent while positively charge resides bind with hydrophilic head of phospholipid membrane by electrostatic interaction [9-11]. The arrangement of the formed α-helix structure is relevant to membrane curvature. For example, the continuous α-helix that binds with 100nm lipid vesicle can be split into two individual helices and form an extended helix [1,12,13]. Whereas when binds with small detergent micelles or higher binding affinity lipid vesicles, the α-helix will present in a bent shape [1]. In general, the N-terminus of αSyn interacts with membrane instead of C-terminus, which maintains the high flexibility of αSyn [12].

So far, studies show that most of the PD cases are sporadic while only 10% are familial disease. Six mutations including A30P, E46K, A53T, A53E, H50Q and G51D are identified on SNCA gene in familial PD [1,4-9,12]. Interestingly, all of these mutations are found in the N-terminus of αSyn, suggesting that they have an effect on lipid binding. It has been shown that mutations such as E46K and A53T can improve αSyn's lipid binding affinity and promote fibril formation, whereas A30P, G51D, and A53E decrease lipid binding affinity and fibrilisation rate. The varied properties of the protein are caused by different mutation points.

Fluorescence is commonly employed in biophysical research. It offers exact quantitative analysis because to its great precision and sensitivity, providing insight into biophysical interaction mechanisms. Various fluorescent methods have been applied into αSyn studies. For example, a study using fluorescence correlation spectroscopy (FCS) performed by Elizabeth Rhoades et al [18] showed

that the affinity of αSyn with higher proportion of negatively charged lipids is stronger than that with lower proportion. In the work of Adam J. Trelex et al [19], single-molecule FRET was employed to analyze the binding affinity toward micelle and large unilamellar vesicle (LUV) and the structure of protein chain after binding. Fluorescence anisotropy is also used in their study to analyze the binding affinity of αSyn and lipid membrane indicated by the free rotation of fluorophores [19, 20].

αSyn is believed to play a crucial role in PD and Lewy body dementia. Whereas the structure arrangement of αSyn in biomolecular interactions as well as the role in those diseases are still unclear. This project will focus on analyzing H50Q mutation impact on the binding interactions between αSyn and lipid membrane using fluorescence methods , to have a deeper understanding of the structure and pathological features of αSyn.

2. Methods

2.1 Lipid Vesicle Preparation

Lipid Coagulation Reagent I (DOPE:DOPS:DOPC (5:3:2 w/w)) was provided by Avanti Polar Lipids as lyophilized powders. In preparation, it was dissolved in chloroform, and dried under nitrogen to form lipid films. The lipid films were kept at -20 °C after being exposed to nitrogen flow overnight to completely eliminate any remaining solvent. To produce small unilamellar vesicles (SUVs), the lipid films were hydrated in required buffer with shaking for an hour. The lipid slurry was then extruded 15 times over a polycarbonate membrane with 50 nm holes (Nuclepore, Whatman) using an extruder (Mini-Extruder, Avanti Polar Lipids). The produced SUV suspension may be kept at 4 degrees Celsius for at least two days.

2.2 αSyn Purification and Labelling

αSyn was first expressed though BL21(DE3) E. coli grown in LB, which contain ampicillin (100 μg/ml) at 37 ℃. The expression was induced with the addition of 1 mM IPTG for 3 h at 37 ℃ when the absorbance at 600nm (OD_{600}) reached 0.5. The cells were subsequently collected by centrifugation at 8000 g for 30 minutes in a Beckman Avanti J-26 XP centrifuge with a JLA-10.500 rotor (Beckman Coulter). The cell pellet was resuspended in 20 mM Tris-HCl (pH 7.4) buffer containing 5 mM EDTA and 1 mM protease inhibitor (cOmplete, Roche), and lysed for two minutes using a sonicator. The cell suspension was then boiled at 90 °C for 20 minutes before being centrifuged at 16000 g for 20 minutes with a VWR Micro Star 17 centrifuge (VWR International). The supernatant was then collected and filtered to eliminate any cell debris using an Acrodisc 0.2 m 25mm syringe filter (Pall Corporation). The supernatant was then treated with streptomycin sulfate at a final concentration of 10 mg/ml, and the mixture was agitated for 15 minutes at 4 °C. The supernatant was collected after centrifugation at 16000 g for 20 minutes, and ammonium sulfate was added to 50% saturation. After stirring for 30 minutes at 4 °C, the mixture was centrifuged again at 16000 g. The pellet was recovered and resuspended in 20 mM Tris-HCl with 50 mM NaCl (pH 7.4) before being dialyzed overnight against 2 L of the same buffer. Protein concentration was measured using a PerkinElmer Lambda 25 UV/Vis spectrometer with an absorbance at 280 nm and an extinction value of 5960 M^{-1} cm^{-1} (PerkinElmer).

Dye-labelling of αSyn was performed using a selective thiol-maleimide reaction. The first step was to generate a cysteine mutation at position 7 using a site-directed mutagenesis kit (Phusion, Thermo Fisher Scientific). The dye will attach preferentially at position 7 since the altered residue 7 is the lone cysteine residue in αSyn mutant. After that, the buffer (20 mM Tris-HCl with 50 mM NaCl, pH 7.4) was deoxygenated for 1 hour by bubbling with nitrogen. To decrease disulfide linkages, a 10-fold molar excess of TCEP was added in protein solution. Next, to remove TCEP, the protein solution was eluted with the bubbled buffer through a PD-10 desalting column containing Sephadex G-25 resin (GE Healthcare Life Sciences). The concentration of eluted protein was calculated using an extinction value of 5960 M^{-1} cm^{-1} and the absorbance at 280 nm. Following that, a 3: 1 molar ratio (dye: protein) of Alexa Fluor 488 C5 maleimide (Thermo Fisher Scientific) solution in DMSO was

added to the eluted protein solution . The solution was stirred in the dark for 3 hours. The tagged protein solution was then desalted and concentrated for 5 minutes at 15000 g using a 10K MWCO piercing protein concentrator (Thermo Fisher Scientific). The sample was kept at -80 °C. The absorbance at 495 nm with an extinction value of 72000 M^{-1} cm^{-1} was used to calculate the final concentration of labelled αSyn. The total concentration of protein was determined using the following equation (1) (0.11 is the correction factor for fluorophore's contribution to A_{280})

$$\text{Protein concentration (M)} = \frac{A_{280} - 0.11(A_{495})}{\text{molar } \varepsilon \text{ of protein at 280 nm}} \quad (1)$$

The labelling efficiency was calculated to be 95% based on the ratio of tagged to total protein content.

For FRET measurements, a dual labelled S9C-F94C Syn (WT and H50Q mutant) was generated. The selected donor chromophore was Alexa 488 C5 maleimide dye (Thermo Fisher Scientific), while the acceptor was Alexa 594 C5 maleimide dye (Thermo Fisher Scientific). The labeling was done in the same way as single labeling, but with a dye-protein molar ratio of 6: 2: 1 (Alexa 594: Alexa 488: αSyn).

Using a same genetic alteration technique, H50Q mutated αSyn was created. The sequencing result is shown in Fig.1.

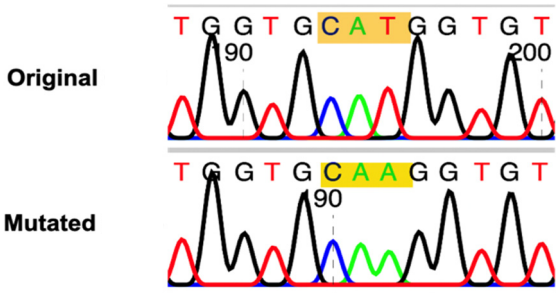


Fig 1. H50Q mutation introduction in vector. Sanger sequencing reveals a histidine 50 (CAT) to glutamine (CAA) codon change

3. Data Analysis

In this project, the equation used to determine binding affinity (K_d) from fluorescence anisotropy is a modified Hill-Langmuir equation:

$$\theta = \frac{A}{1 + K_d/[L]} + C \quad (2)$$

where A is the amplitude of curves, [L] is the ligand concentration and C is the value of the start point.

4. Results

CD measurement was conducted first to study the binding of WT-αSyn and H50Q αSyn with synaptic-like SUV. In this experiment, 15 μM αSyn samples were mixed with 1mM lipid. The result is shown in Fig. 1. Compared to αSyn in the absence of SUV, the spectra of both WT-αSyn and H50Q αSyn in the existence of SUV show a signal decrease at 220 nm, indicating that both WT and H50Q mutated αSyn can bind to SUV and form α-helical structures.

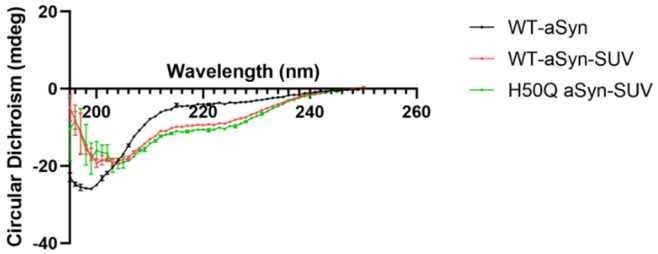


Fig 2. CD spectra of αSyn in the presence and absence of SUV. To measure, 15μM αSyn samples were added to a 1 mm cuvette. This experiment was carried out at 298 K in PBS buffer (pH 7.5). Three repeats were conducted to generate the average of each spectrum.

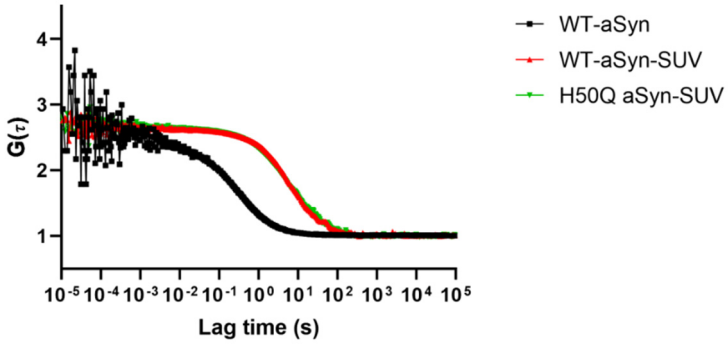


Fig 3. FCS curves of αSyn in the presence and absence of SUV. Both WT and H50Q αSyn can bind to SUV according to the curves. The experiments were carried out at 298K in a buffer solution of 50 mM HEPES and 100 mM NaCl (pH 7.5).

Then, the binding between αSyn and SUV was investigated using FCS technique. 10nM labelled αSyn samples were mixed with 1mM lipid (~40nM SUV). The molecular diffusion time was then detected. The generated result is shown in Fig 2. It can be seen that WT-αSyn and H50Q αSyn in the existence of SUV have longer diffusion times compared to free αSyn, which further validates that both αSyn can bind to SUV.

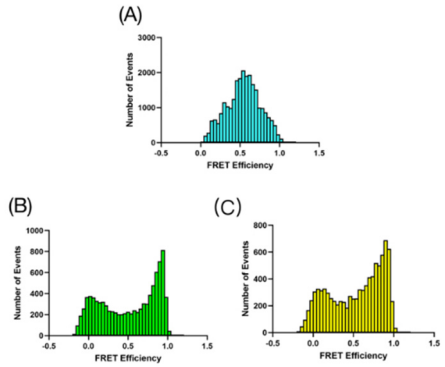


Fig 4. smFRET histograms of αSyn in the presence and absence of SUV. (A) Free αSyn in buffer. (B) H50Q αSyn in the companionship of SUV. (C) WT-αSyn in the presence of SUV. At 298 K, the experiments were carried out in a buffer solution of 50 mM HEPES and 100 mM NaCl (pH 7.5).

smFRET measurement was also performed to further study αSyn-SUV binding interaction. 5 nM dual-labeled WT-αSyn was blended with 1 mM lipid (40 nM SUV) in the experiment. Before measuring, the solution was incubated for 10 minutes and diluted ten times. Figure 3 depicts the FRET efficiency histograms that were produced. Compared to the histogram of free αSyn, the histogram of αSyn monomer bound on SUV shows several peaks at low and high FRET efficiency,

indicating that αSyn monomer are able to bind with SUV via mixed extended or bent helical configurations. In addition, the histogram shape of WT-αSyn bound on SUV is quite similar to that of H50Q αSyn bound on SUV, this consistent with the above results that H50Q mutation does not impact the SUV binding structure of αSyn.

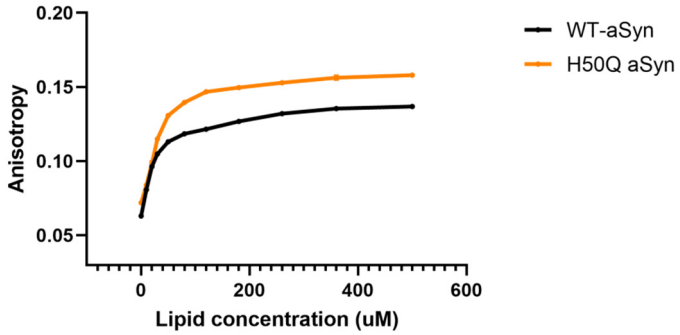


Fig 5. Fluorescence anisotropy titration of αSyn binding to SUV. Ten repetitions were conducted to generate the average of each data point. At 298 K, the experiments were carried out in a buffer solution of 50 mM HEPES and 100 mM NaCl (pH 7.5).

Fluorescence anisotropy measurement was finally carried out to determine the binding affinity between αSyn and SUV. 100nM αSyn samples were mixed with various lipid concentrations. The anisotropy values were recorded by a fluorometer and fitted by Hill-Langmuir equation (Fig. 4). The equilibrium dissociation constant (K_d) value of H50Q αSyn was determined to be 18 ± 1 μM, while WT-αSyn has 24 ± 2 μM. This result suggests that H50Q mutation can actually increase the binding affinity of αSyn with lipid vesicle.

5. Summary

Synuclein misfolding and aberrant aggregation are closely associated to synucleinopathies, including Parkinson's disease. Lipid is thought to be an important inducer of such aggregation. Additionally, it is becoming clear that a growing number of SNCA genetic variants will cause familial Parkinson's disease. in which the H50Q mutation is the latest discovered one. The interactions between H50Q mutated αSyn and lipids so far is not well understood. It is consequently necessary to explore this area.

In this study, using a mimic synaptic vesicle system, we found that H50Q mutated αSyn can also easily bind to synaptic-like SUV based on CD and FCS measurements. Furthermore, thanks to an ultra-sensitive smFRET technique, we found that H50Q mutated αSyn can bind synaptic-like SUV via mixed extended and bent helical structures, which is actually similar to that of WT-αSyn. Finally, we determined that H50Q mutated αSyn (K_d = 18 ± 1 μM) can bind synaptic-like SUV tighter than WT-αSyn (K_d = 24 ± 2 μM).

Overall, it is thought that the pathophysiology of Parkinson disease as well as its function depend critically on the interplay between αSyn and Lipid. Exploring relationships between αSyn and lipid can help to build a deeper understanding of its biomolecular function and designing effective drug against neurodegenerative disorders.

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