The Dual Dependency of Varying Liposomes and Protein on Available αS Conformers

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Abstract:

α-Synuclein (αS) is a protein commonly found in protein aggregates associated with Parkinson's disease (PD). This intrinsically disordered protein is known to regulate synaptic vesicle (SV) trafficking in the pre-synaptic clefts of most neurons. Improper trafficking of SVs results in miscommunication between neurons, which could lead to symptoms of PD such as muscular tremors. Despite the prevalence of PD, much is still left unknown about the mechanism that causes protein aggregation due to αS binding to SVs. SVs are unique membranes as a result of their high cholesterol content (45%) and small diameter (0.03 microns). Both factors induce strain in the membrane leading to high fusion potential of the SV. For this research two SV mimics (simple and complex) were designed, utilizing a mass spectrometry study on SV membrane composition.[1] The simple SV mimic measured the effects of interacting head groups and cholesterol on membrane annealing in the presence of αS using a Carboxyfluorescein (CF) release assay .To further probe the question of conformational changes of αS in the presence of membrane Circular Dichroism (CD) monitored the secondary structure character. Both membrane annealing and a change in secondary structure were observed making it necessary to further investigate the relationship between protein and membrane with other methods. Using Differential Scanning Calorimetry (DSC) to monitor a lipid transition I hypothesized that αS has specificity for high curvature and complex composition of membrane. We tested this by varying liposome sizes and cholesterol content. Oppositely, the membranes impact on αS conformers was studied utilizing a DSC protein transition to see its effects. A conformational shift was found in the presence of complex SV mimic, showing αS's conformational specificity for this highly complex mimic. Due to this preference, a binding mechanism using the complex SV mimic needs to be studied. The mechanism of αS binding to membrane has the potential to shed light on the pathogenesis of αS in amyloid formation. Through Isothermal Titration Calorimetry (ITC) we will predict a simulated binding model for αS that will give more information about possible reasons for protein aggregation and benefit future studies on PD.

Introduction:

Parkinson's disease (PD) affects more than half a million Americans and it's estimated that the U.S. spends about 25 billion dollars a year treating and caring for patients with PD.[1] This vast number not only affects the people living with the disease, but their families and caregivers as well. As a result, research needs to be done on this currently incurable disease. Through investigation of patient's brains with PD, there is a high concentration of amyloid proteins present. Amyloids are an aggregation of transiently stable proteins, a common sign of not only PD but many neurodegenerative diseases.[2] The cause and mechanism of protein aggregation is unknown and can hold insight into treatments for many neurodegenerative diseases. From a brain autopsy of patients with PD the largest component in Lewy bodies, a type of amyloid associated with PD, is found to be the transiently stable protein α-Synuclein. This 140-amino acid long protein binds to the membrane and has a dual effect. The protein undergoes a structural change from being intrinsically disordered to a largely α-helical structure upon membrane binding. It also plays a role in lipid annealing within the membrane. This dual dependency makes the study of this protein very complex. αS is found in the presynaptic terminal of most neurons and is thought to play a role in synaptic vesicle (SV) trafficking and neurotransmitter release. The sensitivity of αS to membranes of high curvature and high cholesterol concentration was studied using Differential Scanning Calorimetry (DSC) by monitoring the lipid transition. Proteins role in lipid rearrangement was studied using CF release assay to monitor lipid annealing. We propose that SV membranes dictate αS binding affinity based on the curvature and/or cholesterol content of SVs and upon binding, αS decreases the SV membrane fusion potential by relieving the rigidity of SV membranes. On the other hand we want to track the membranes effects on available protein conformers, this can be done using CD to monitor protein secondary structure. To further investigate αS available conformers in

response to changing compositions of membrane DSC can be used to monitor the protein transition in the presence of membrane. Finally, using all of the information about α-Synuclein's complex relationship with SV membrane Isothermal Titration Calorimetry can be employed to propose a possible binding mechanism of αS. We propose that full-length αS in the presence of SV mimic will be able to undergo all of the possible conformers needed to bind membrane with high affinity, while the shortened αS fragment won't be able to find all possible conformations and will bind with lower affinity. This could be compared to possible truncations or mutations that occur in vivo that have been known to cause PD symptoms. A possible binding mechanism would give insight into PD pathogenesis and the cause of protein aggregation in the cells.

Materials/Methods:

Lipid Preparation

All lipids were purchased from Avanti Polar Lipids (Birmingham, Al.) and were used directly from the stock solution. Lipid samples without cholesterol were prepared by aliquoting appropriate amounts of lipids into a test tube and put under nitrogen until the chloroform solvent evaporated off. The sample was placed under pressure for 4 hours. After 4 hours it was taken off pressure, lyophilized, and put back under pressure for an additional 4 hours. The lipids were hydrated and extruded to appropriate sizes. Lipid samples containing cholesterol were prepared by aliquoting appropriate amounts of lipids into a 4:1 chloroform:methanol mixture. Rotating evaporation took place at a temperature of 55°C. The lipid samples were placed under pressure for 8 hours. After 8 hours the lipid sample was hydrated with the correct buffer and extruded to the correct liposome sizes.

Carboxyfluorescein Release Assay

To monitor the effect of protein binding on membrane rearrangement LUVs were prepared containing CF dye. CF efflux was seen upon membrane rearrangement and monitored with excitation and emission wavelengths of 492 nm and 515 nm. The fluorescent data was monitored using a Fluorolog 3 double excitation and double emission monochromator (Horiba Jobin Yvon) in a 500 uL quartz cuvette. The experiments lasted over a period of 60 minutes using a temperature controlled water bath (Pharmacia Biotech Millitemp III). The buffer used for the samples had a composition of: 20 mM MOPS, 100 mM KCl and 0.02%NaN3 with 200 μM LUVs and 0.8 μM αS when protein was present. Triton X-100 was used as a detergent to destroy the membrane after each scan to reach the maximum efflux.

Differential Scanning Calorimetry-Liposome Transition

DSC was used to monitor the liposome transitions using a NanoDSC (TA Instruments, New Castle, DE) at a scan rate of 0.17 °C/min. The liposome samples used had a concentration of 10 mM of LUVs or SUVs. The equilibrium was found by putting the liposomes through transition over a temperature range of 5-55°C. Once equilibrium was found, αS was added to the sample to reach 250:1 [L]:[P] ratio. The proportions added were 18 μM aS and 6.67 mM liposome. These concentrations were confirmed using a Nanodrop Spectrometer (αS) and a phosphate assay (liposome).

Circular Dichroism Spectroscopy

αS was prepared for CD data collection. The CD experiments were done on a Jasco J-815 CD Spectrometer (Annapolis, MD) using a 0.1 cm quartz cuvette. The concentration of protein was 5-15μM αS and the concentration of liposomes were 1-5.6mM for all samples. Data points were collected in a range of 200-260 nm in 1 nm increments from -2°C to 60°C.

Differential Scanning Calorimetry-Protein Transition

A single αS DSC experiment was performed on a NanoDSC (TA Instruments, New Castle, DE) at a scan rate of 1°C/min. The sample contained 20 μM aS and 4 mM SV mimic liposome SUVs.

Results/Discussion:

Construction of physiological relevant SV mimics

Synaptic vesicles are highly unique membranes due to their complex composition and high curvature. To make an exact replica of physiological SVs would be difficult and costly. Not only is the membrane composition complex but it is necessary to keep in mind that SVs are covered in transmembrane proteins. So, in order to create mimics it is necessary to perform a study on the composition of a physiological SV. Using Mass Spectrometry it was found that SV membranes are composed of PC, PE, PI, PS, SM, cholesterol, and hexosylceramine.[3] To accurately simulate a physiological accurate binding membrane for αS, it is necessary to mimic the SV surface. If the composition of the liposome is not physiologically accurate we won't be able to conclude information about αS binding as a result of limited conformers or lipid interactions. From research it is known that αS interacts with the outer SV leaflet, and when fused together with the PM it binds to the inner leaflet of the PM. From the MS study it was found that SM and PC phospholipids were present in the outer leaflet of the PM, showing that αS doesn't bind to a leaflet with these lipids. To mimic the outer leaflet of the SV the composition included PE, PS, PI and cholesterol. This is laid out by lipid head groups in Table 1. From the MS analysis the differing tail length and saturation were taken into account to make the complex

mimic. A ratio of 55:45 phospholipid:cholesterol is also a unique characteristic of SVs and needs to be taken into account to create this mimic.

<u>Table 1:</u> Complex synaptic vesicle mimic composed of PS, PE, PI, and Cholesterol. The ratio of phospholipid to cholesterol is 55%: 45%.

Complex SV mimic			
	FA1	**FA2**	**% Total Phospholipid**
PE Ratio			
3	16:00	18:01	38
2	18:00	18:01	35
1	18:00	22:06	13
PI Ratio			
1	18:01	18:01	0.5
2	16:00	18:01	1
1	18:00	20:04	0.5
PS Ratio			
1	18:00	22:06	12
2	18:00	18:01	10
Cholesterol	**-**	**-**	**45**

It has been found that αS preferentially binds acidic phospholipids such as PS and PE, so to show αS's interaction with these specific phospholipid head groups an even simpler mimic was created. The simplified mimic, whose composition is shown in Table 2, was designed to monitor specific head groups effects on protein binding and membrane annealing. This allows for the direct impact of protein binding to be observed with the two most abundant phospholipids found in SVs. The simple mimic will be used to monitor lipid transitions using DSC. The goal is to monitor lipid rearrangement which directly impacts membrane rigidity.

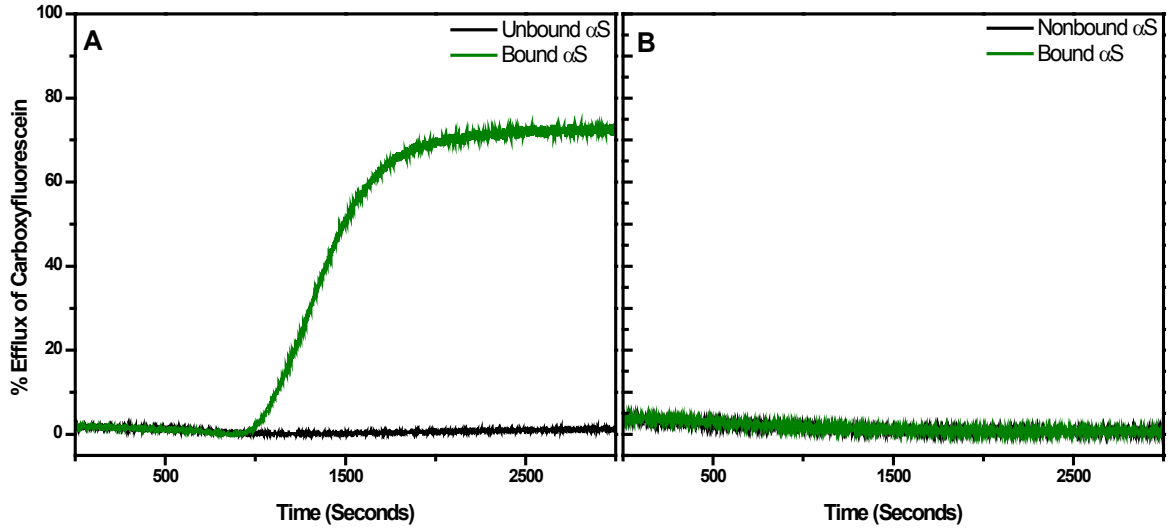
Table 2: Simple synaptic vesicle mimic composed of PE, PS, and cholesterol. The ratio of phospholipid to cholesterol is 55%:45%.

Simple SV mimic			
	FA1	**FA2**	**% Total Phospholipid**
POPE	16:00	18:01	38
SOPE	18:00	18:01	38
POPS	16:00	18:01	24
Cholesterol	-	-	45

αS's effects on membrane annealing as a result of binding

A CF release assay was used as a means to reveal if αS interacts with the membrane and rearranges the phospholipid head groups. It also was used as a mechanism to better understand the impact of cholesterol on liposome rearrangement. Cholesterol has many unique properties

that impacts both membrane annealing and protein binding when present in a membrane. Within a SV the amount of cholesterol is so high, 45%, that it plays an important role in the membrane-protein relationship. It induces rigidity in the membrane as a result of its strict composition and induces localized rafts within liposomes. The CF release assay monitored the transition of lipids from fluid to gel-like state upon cooling. The liposomes were prepared to have quenched CF molecules on the inside and upon lipid rearrangement some of these molecules escape and increase the fluorescence. In the absence of αS, no change in CF efflux was seen, Figure 1 (black). This suggests that the liposomes were ideally mixed and no rearrangement of phospholipids occurred. Upon the addition of αS a high percent efflux of CF was seen, Figure 1 (green). From this finding we can conclude that αS rearranges the phospholipids in the membrane. However, when cholesterol was added to the lipid mixture different results were seen. Both in the presence and absence of αS no CF release was seen. This shows the complexity that cholesterol adds to the lipid bilayer. Cholesterol itself has an ordering ability of lipids reducing the visible transition from fluid to gel-like state. It can physically sit within the membrane filling spaces between the phospholipids causing a more rigid state. It is important to note that this doesn't mean that αS doesn't have an impact on lipid annealing in liposome with cholesterol, but rather that the effects of cholesterol make the relationship more complex.



<u>Figure 1:</u> αS's effect on lipid rearrangement through a transition from fluid-like to gel-like state as characterized by CF release. Monitored in the absence of cholesterol (A) and presence of cholesterol (B).

To further study αS's ability to anneal lipids upon binding, a membrane was put through a transition from gel to fluid in the presence and absence of αS monitoring thermodynamic parameters by DSC. By thermally transitioning the membrane between two states the lipids will rearrange and find their most favorable position in the liposome. This transition was monitored with liposomes of two differing diameters to test for the specificity of curvature by αS (Figure 2). DSC thermograms give us a lot of valuable thermodynamic data. By integrating the heating thermogram, the area under the curve corresponds to the enthalpy (ΔH). The transition temperature (Tm) is the temperature at half the area under the curve. Using these two values the entropy (ΔS) of the liposome can also be determined, because at the Tm ΔG=0. Changing of these values relays valuable thermodynamic information about the effects of protein binding. Upon binding both liposomes, there is an increase in Tm (Table 3) showing the annealing of

lipids within the membrane and αS's ability to create localized concentrations of lipids that create favorable van der Waals interactions of the lipid tails, increasing the transition temperature. The peaks narrow in the presence of protein indicating a more ordered membrane. Upon transition αS was found to increase Tm while decreasing enthalpy and entropy (Table 3). This pattern was found in both LUVs and SUVs showing αS's role in stabilizing an ordered membrane, but making the curvatures impact on binding need to be studied in a different way. This is concluded based on the decrease in entropy, or increase in order of the lipid tails. It was found that αS binds to the membrane inducing a more ordered state in an already highly rigid membrane, this could play a role in SV fusion. With a more rigid membrane in the presence of αS, the propensity of fusion of the SV is much higher. This could make αS like a switch, that when bound, the SV becomes more likely to fuse with the plasma membrane.

Another unique characteristic of SVs is the high amount of cholesterol. The impact of cholesterol was also tested using DSC. However, this abundant amount of cholesterol led to no lipid transition being observable (Figure 3). Pairing this information with the information gained from the CF release assay, it can be concluded that cholesterol smooths the transition of liposomes making it unobservable using both CF and DSC (Table 4). This complex relationship doesn't allow us to jump to the conclusion that cholesterol leads to no transition, but rather the relationship of cholesterol in the membrane plays a complicated role in SVs and needs to be tested using a different method.

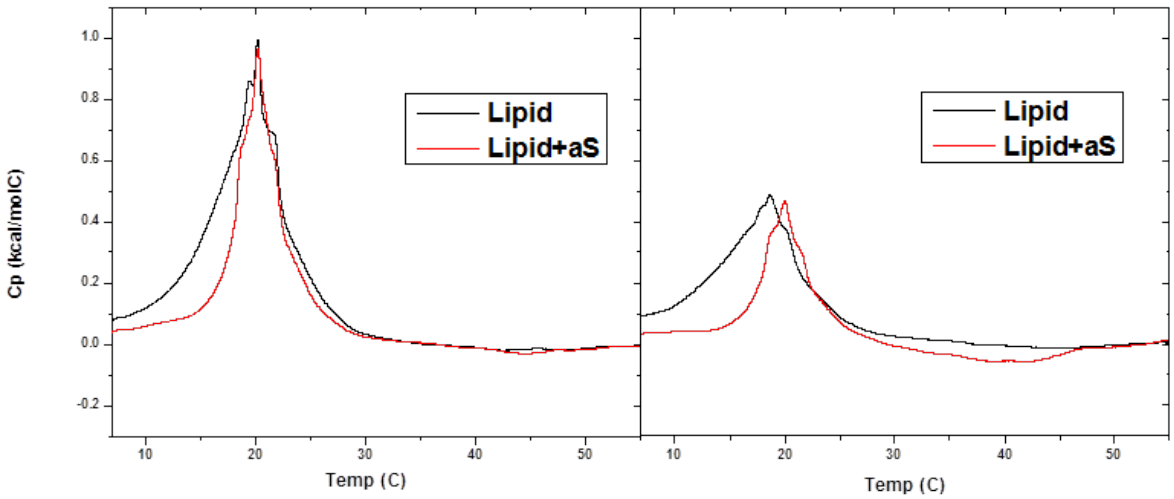


Figure 2: **Liposome transitions of 38:38:24 POPE:SOPE:POPS LUVs (left) and SUVs (right) in the presence (red) and absence (black) of bound αS.**

Table 3: **Thermodynamic parameters for 38:38:24 POPE:SOPE:POPS LUVs and SUVS in the presence and absence of bound αS.**

	Δ H (kcal/mol)	**Tm (°C)**	**ΔS (kcal/molK)**
LUVs	7.33	19.33	0.025
LUVs+aS	5.23	20.13	0.018
SUVs	4.65	17.88	0.016
SUVs+aS	3.12	19.75	0.011

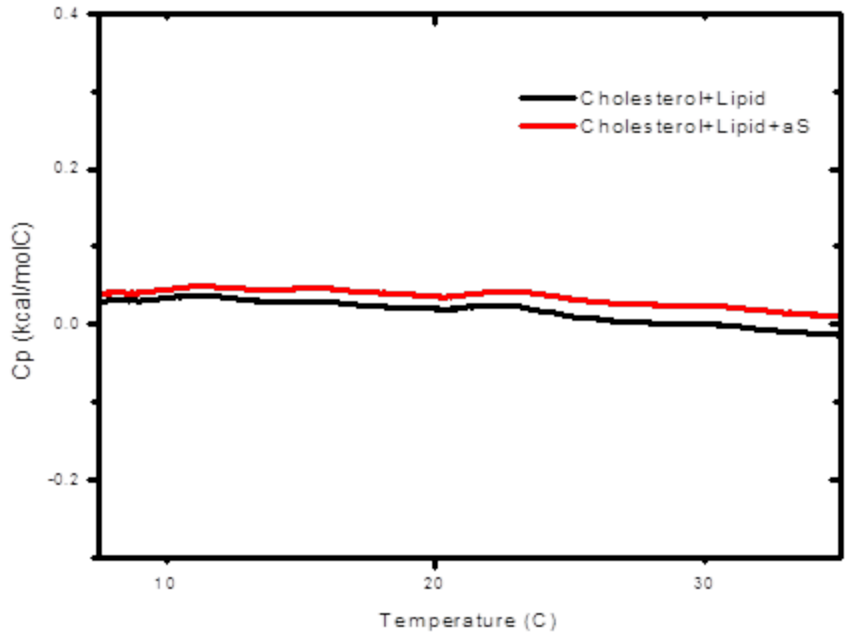


Figure 3: Liposome transitions of (38:38:24):45 (POPE:SOPE:POPS):Cholesterol LUVs in the presence (red) and absence (black) of bound αS.

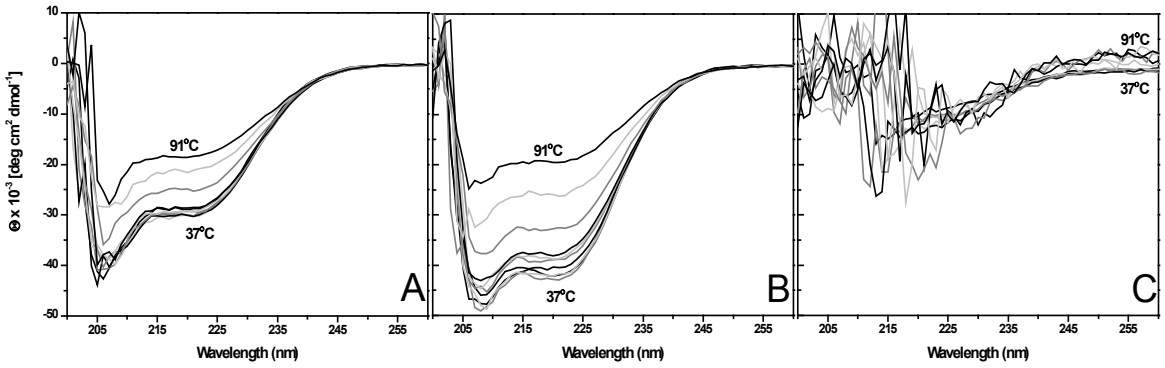
Table 4: Thermodynamic parameters for (38:38:24):45 (POPE:SOPE:POPS):Cholesterol LUVs in the presence and absence of bound αS.

	Δ H (kcal/mol)	**Tm (°C)**	**ΔS (kcal/molK)**
LUVs	7.33	19.33	0.025
LUVs+aS	5.23	20.13	0.018
LUVs+Cholesterol	0.472	15.25	0.0016
LUVs+aS+Cholesterol	0.854	17.48	0.0029

Changes in available αS conformers as a result of changing liposome size and complexity

αS doesn't only impact membrane rearrangement, but membrane impacts the possible conformations αS can possess based on its composition and curvature. To monitor this relationship Circular Dichroism (CD) was used. αS is intrinsically disordered in vivo and upon

binding membrane is known to take on an α-helical secondary structure. Changes in composition and curvature of liposomes were tested to see their effects on available conformers of αS. Using a 60:40 POPC:POPS liposome mixture the effect of curvature was tested using LUVs and SUVs. Figure 4 A and B shows the α-helical character as measured by CD. In the presence of both types of liposomes α-helical character was observed. However, when the SUV liposomes were employed an enhanced α-helical character was observed. Just by changing the size of the liposome αS was able to possess different bound conformers. Comparing this data with a SV mimic liposome which more closely relates to the physiological environment αS would be exposed to, no observable secondary structure was seen. The CD signal was unreadable possibly due to the effect of light scattering from the complex composition of the mimic. This finding doesn't mean that there is no secondary structure change upon binding the SV mimic, but rather this change would have to be monitored using a different method that could measure the effects without the light scattering. The change of α-helical content with differing diameter sizes is very important and is one confirmation that the possible αS conformers is dependent and highly specific for the high curvature. This relates to the size of SV in vivo and thus shows evidence that αS relies on this high curvature for functionality.



<u>Figure 4</u>: CD spectra of three differing liposomes A) LUVs B) SUVs C) SV. Monitoring the transition in secondary structure of αS.

There is a complex relationship between the interaction of protein and membrane. αS shifts from intrinsically disordered to α-helical in the presence of specific membrane. From the CD data we found that there was increased α-helical character in the presence of similar curvature to SVs. However, in the presence of the complex mimic, most closely resembling SVs, a scattering of light led to an unobservable secondary structure shift. To further monitor the interaction a protein transition using DSC was performed (Figure 5). Interestingly enough a protein transition from folded to unfolded was found only in the presence of complex SV mimic with comparable composition and size as SVs, but not in the presence of a simple solution of POPC:POPS liposomes. This gives insight into the effects of cholesterol since it's the first experiment showing a transition in the presence of cholesterol. In all other experiments the cholesterol has scattered the signal. The complex mimic's effects on conformer formation of αS via DSC is extremely important. Using CD, a secondary structure shift was seen in the presence of simplified SV mimic of varying sized liposomes but no protein transition was seen using DSC in the presence of these same liposomes. On the other hand, no observable secondary structure transition was seen in the presence of complex SV mimic but using DSC it was the only membrane to support a protein transition. This interesting finding leads to the conclusion that the complex composition and high curvature of the complex SV mimic induces bound αS conformers that are needed to specifically interact with the membrane and cause a protein transition. Further showing αS's specificity for highly complex and curved membrane.

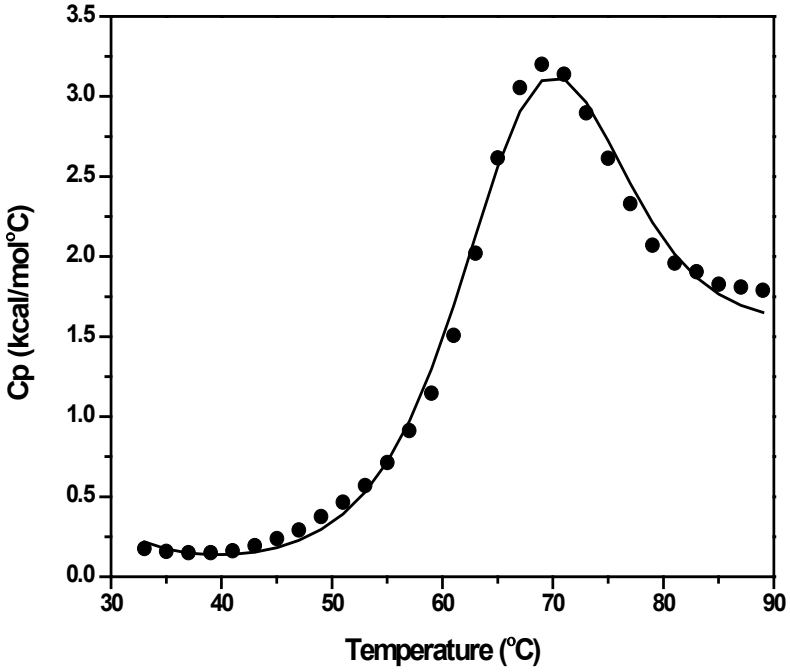


Figure 5: DSC thermogram of αS in the presence of complex synaptic vesicle mimic with physiologically relevant cholesterol composition and high curvature.

αS's interaction and specificity for physiologically relevant SV mimics can lead to a possible binding mechanism

From these previous findings it's important to note αS has bound conformers only accessible when subjected to complex SV mimics. Knowing this it's important to study a binding mechanism in the presence of this complex SV mimic. Using Isothermal Titration Calorimetry I hypothesize that that full-length αS in the presence of SV mimic will be able to undergo all of the possible conformers needed to bind membrane with high affinity, while any shortened αS fragment won't be able to find all possible conformations and will bind with lower affinity. In preparation for this experiment, the starting parameters must be found in order to ensure a

measurable heat of binding and saturation. The goal of this project is to use various resources in order to predict the appropriate starting concentrations of protein and lipid for an α-S ITC scan. The complex composition of SV needs to be taken into account to accurately visualize αS binding to the outer leaflet. From our previous CD and DSC scans we found that αS under goes a conformational change from intrinsically disordered to α-helical in the presence of membrane. It has also been found that αS preferentially binds acidic phospholipids which will need to be taken into account during our calculations. The approximate SA of αS was found to be 15 nm^2 and the approximate SA of a phospholipid head groups is 0.5nm^2.[4] From these SA values we find that ~30 lipids will bind 1αS giving us N=30.

Using an approximation of 1kcal/mol free energy being given off upon a K/R residue binding a PS head group an association constant of $2.42x10^{11}$ M^{-1} was found, which is extremely high. This is assuming αS is intrinsically disordered and all K residues are exposed, which we know is not the case from our CD scans. Thus, this thought was put aside and a known αS ITC run was studied. From the Beyer paper, a N and Ka value was found for a fragment containing residues 6-115 and POPC/POPG liposomes.[5] Their Ka value was then used compared to a ratio of their PG % to our PS % and an approximate Ka value for our liposome was found to be $1.1x10^6$ M^{-1}. Using this Ka value and converting it into a Kd, the concentration of initial protein was estimated as 30x Kd and was found to be 27.27μM αS. The initial concentration of lipid was found to be 15*N*[P]. Using the N value calculated from the SA's a starting concentration of 15 mM lipid was found. These values are approximations and could be much different as a result of αS having a higher affinity for PS head groups and could bind more head groups than calculated. Comparing these values to the Beyer paper it was found that a starting concentration of 8.5 μM αS gives off a quantifiable amount of heat and as a result our starting protein concentration can

be scaled down to 10 μM α-S.[5] To ensure saturation however the lipid concentration will remain 15mM. This decision was based on the fact that the Beyer paper used a much higher concentration of liposome (45mM) but PS head groups will bind with higher affinity and the N value could be different than the N value calculated or the N value in the Beyer paper as a result of the complex lipid mimic.

From research, calculations, and conceptual thinking I conclude that an ITC run will be performed using 10 μM full-length αS and 15 mM SV mimic. Based on this first run we will adjust parameters in order to predict a binding model for αS.

Conclusion:

Overall, αS has a complex relationship with interacting membrane. It was found to play an ordering role in lipid annealing. This would lead to an increase in rigidity of the membrane in an already highly rigid system. It was also found that αS undergoes a secondary structure change to increased α-helicity character in the presence of highly curved membrane. And, a protein transition was only seen when αS interacts with a complex SV mimic. We can conclude that αS has a high specificity for complex composition and high curvature, physiologically relating to SV in vivo. All of these studies can be joined in order to develop a possible binding mechanism of αS with SV which could shed light on the cause of protein aggregation in PD.

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