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### **Citation**

Kumar, P. (2017, June 27). *Parkinson's protein  $\alpha$ -synuclein : membrane interactions and fibril structure*. *Casimir PhD Series*. Retrieved from <https://hdl.handle.net/1887/50076>

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**Title:** Parkinson's protein  $\alpha$ -synuclein : membrane interactions and fibril structure

**Issue Date:** 2017-06-27

## **3 Membrane Binding of Parkinson's Protein $\alpha$ -Synuclein: Effect of Phosphorylation at Positions 87 and 129**

**Kumar P**, Schilderink N, Subramaniam V, Huber M. Membrane binding of Parkinson's protein  $\alpha$ -Synuclein: Effect of phosphorylation at positions 87 and 129 by the S to D mutation approach. *Israel Journal of Chemistry*, 2016; DOI: 10.1002/ijch.201600083

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### 3.1 Introduction

Parkinson's disease (1) is the second most spread neurodegenerative disease after the Alzheimer's disease (2). This disease is characterized by the formation of protein deposits such as Lewy bodies in the brain (3,4). The protein  $\alpha$ -Synuclein ( $\alpha$ S) constitutes the main component of these deposits (5–7). A number of post-translational modifications of  $\alpha$ S are present within the Lewy bodies in Parkinson's disease (PD) and related disorders (8,9). The major disease-associated post-translational modifications (PTMs) are phosphorylation (8,10), truncation, ubiquitination (11) and also oxidation (like nitration) (12). Phosphorylation is the most studied PTM among them.

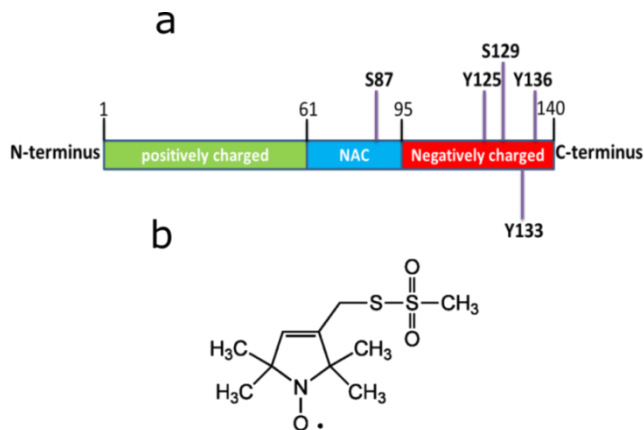


Figure 3.1. a. The most common phosphorylation sites in  $\alpha$ S. Given is the sequence number (in boldface) preceded by the residue (Y or S) that is phosphorylated. Also, the important regions of the protein are shown, indicated by sequence numbers at start and stop. Positively charged (green): Net positive charge of protein between residues 1 and 61, NAC – non-amyloid- $\beta$  component (blue), and negatively charged C-terminal part of the protein (red), from residue 95 onwards; b. chemical structure of the spin label MTSL, by which Cys is labelled.

The protein  $\alpha$ S has been found hyperphosphorylated in Lewy bodies and Lewy neurites (1,9,13). The role of phosphorylation of  $\alpha$ S in neurotoxicity is

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controversial. However, growing evidence suggests that phosphorylation could influence membrane/vesicle binding of  $\alpha$ S and its aggregation(8,14–17). Recent reviews summarize results of in vivo and in vitro studies and describe to which degree phosphorylation of  $\alpha$ S is linked to disease (18,19). The major phosphorylation sites of  $\alpha$ S are shown in Figure 3.1.

The phosphorylation sites Y125, S129, Y133, and Y136 are most discussed in the literature, for example S129 is highly phosphorylated in Lewy bodies. One more phosphorylation site is special, S87, since it distinguishes the human  $\alpha$ S sequence from that of mouse and rat (13). Also, a link between phosphorylation at site 87 and disease was discussed by Paleologou *et al.* (13).

Here we focus on the membrane-binding aspect of  $\alpha$ S phosphorylation in vitro at positions S87 and S129. Membrane-binding of  $\alpha$ S concerns an amphipathic helix spanning residues from 1-100 (20–22). The N-terminal half (residues 1-50) of the amphipathic helix is termed helix 1, and the other half (residues 51-100), helix 2. The affinity of  $\alpha$ S to membranes depends on the negative charge density ( $\rho$ ) of the membrane, where  $\rho$  represents the molar fraction of anionic lipids present in the membrane (23). Different binding properties were found for helix 1 and helix 2 (24).

There are three ways to generate protein constructs to study the effect of phosphorylation: a. to phosphorylate the respective residues enzymatically, which requires dedicated enzymes/ overexpression systems (25,26) and is reversible, b. by a semisynthetic approach in which a (phosphorylated) peptide is linked to the corresponding overexpressed protein (27), and c. by generating mutants whose side chains mimic the chemical properties of the phosphorylated state (negative charge) and size, sometimes referred to as pseudophosphorylation (28). Typically,

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serine (S) is replaced by aspartate (D) or glutamate (E) (13,17,29,30) to mimic phosphorylation and alanine (A) is used as reference for the non-phosphorylated state, especially for in vivo studies.

All three approaches have been used to study  $\alpha$ S-phosphorylation in vivo and in vitro, showing that in some cases, enzymatically phosphorylated  $\alpha$ S (P- $\alpha$ S) and pseudophosphorylated  $\alpha$ S behave differently (29,31). For example, enzymatic phosphorylation of  $\alpha$ S at S129 has been shown to have an inhibitory effect on  $\alpha$ S-aggregation, while pseudophosphorylation does not show such an effect (29). Apparently, the different behavior depends strongly on the properties probed and the environment  $\alpha$ S is exposed to. In the present study we focus on the phosphomimic approach with the S $\rightarrow$ D substitution to mimic phosphorylation, and investigate the constructs S87A or S129A : non-phosphorylated; S87D or S129D : phosphorylated.

We used large unilamellar vesicles (LUVs) (see appendix A) as membrane models with a 1:1 mixture of the lipids 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol) (POPG) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), generating a membrane with a charge density  $\rho = 0.5$ . Previous studies on model membranes showed that at high charge densities, i.e., above 0.8 – 0.9,  $\alpha$ S is fully bound to those membranes (23,24,32–34), revealing that the interaction is strong and dominated by electrostatics, which risks to mask the effects of phosphorylation. Additionally, such charge densities are non-physiological, so we avoided these high negative charge densities. At low charge densities ( $\rho \leq 0.2$ ), i.e., on neutral or weakly negatively charged membranes, binding is very low, resulting in a large fraction of unbound protein, which would also abolish any differential binding effect of phosphorylation. This made  $\rho = 0.5$  an optimum charge density to work at.

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To investigate membrane binding, we used spin label Electron Paramagnetic Resonance (EPR) spectroscopy. For spin labelling, the amino-acid residue at the sequence position of interest is replaced by a cysteine, which is reacted with a suitable functional group of the nitroxide spin label (see Fig. 1b), an approach introduced by the Hubbell group (35). In this way, a nitroxide, which contains an unpaired electron and is therefore EPR active, is covalently attached to the protein. Then the properties of the protein can be probed at the modified position by EPR. In the present study, we make use of the ability of EPR to detect the mobility of the spin label by room-temperature, continuous-wave (cw) EPR. Characteristic lineshapes of the spectra reveal the mobility of the spin label, with narrow lines corresponding to fast motion (i.e., rotational correlation times ( $\tau_r$ ) of several hundreds of ps) and broad lines to slow motion, in the ns-regime. In our particular case, slow motion of the spin label shows that the section of the protein to which the spin label is attached is bound to the membrane, whereas fast motion shows detachment of the protein from the membrane. The methodology described was introduced before and has proven valuable to determine the local binding of  $\alpha$ S to membranes (24,32–34).

The spin-labelled constructs are referred to as *SLposition $\alpha$ S/S87A(D)* or *SLposition $\alpha$ S/S129A(D)*, such that for example, *SL27 $\alpha$ S/S87D* is the construct with the spin label at position 27 and is the phosphorylated variant at position 87. We investigated several spin-label positions for each phosphorylation site, resulting in a total of nine constructs, summarized in Table 3.1.

In this work, we show how phosphorylation affects the binding of  $\alpha$ S to the membrane. It decreases the binding of  $\alpha$ S to the membrane when phosphorylated at the S87 position, whereas no effect is seen when phosphorylated at the S129 position. We also show that phosphorylation at position 87 does not detach the

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protein completely from the membrane, but rather causes local unbinding, which is particularly pronounced in the helix 2 region.

### 3.2 Materials and methods

#### 3.2.1 Protein expression and labelling

All  $\alpha$ S mutants were expressed in *Escherichia coli* strain BL21(DE3) using the pT7-7 expression plasmid and purified in the presence of 1 mM DTT as previously reported(36,37) Serine-87 is substituted either by Alanine (S87A, represents phosphorylation- inactive form) or by Aspartate (S87D, represents phosphomimic form). For labelling, a cysteine mutation was introduced at the desired residues.

Spin labelling was done following the standard protocol, described briefly. Before starting labelling,  $\alpha$ S cysteine mutants were reduced with a six-fold molar excess per cysteine with DTT (1,4-dithio-D-threitol) for 30 min at room temperature. To remove DTT, samples were passed through a Pierce Zeba 5 ml desalting column. Immediately, a ten-fold molar excess of the MTSL spin label [(1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl)-methanethiosulfonate] was added (from a 25 mM stock in DMSO) and incubated for 1 h in the dark at room temperature. After this, free spin label was removed by using two additional desalting steps. Protein samples were applied onto Microcon YM-100 spin columns to remove any precipitated and/or oligomerised proteins and diluted in buffer (10 mM Tris-HCl, pH 7.4). Spin label concentrations were 2.5 mM at protein concentrations of 250  $\mu$ M. Owing to the high reactivity of the label and the fact that the cysteine residues are freely accessible in the poorly folded structure, near quantitative labelling can be achieved under these conditions(38). Samples were stored at -80 °C.



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### 3.2.2 Preparation of vesicles

All lipids were purchased from Avanti Polar Lipids, Inc. as chloroform solutions and were used without further purification. LUVs were prepared from 1 : 1 mixtures of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (POPG) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC). Lipids were mixed in the desired ratio and then chloroform was evaporated by dry nitrogen gas. The resulting lipid films were kept under vacuum overnight. Dried lipid films were hydrated with 10 mM Tris – HCl, pH 7.4 for 1 hour at 30 °C, and the resulting milky lipid suspensions were extruded through 100 nm pore size polycarbonated membranes using the mini extruder (catalogue no. 610000) from Avanti Polar Lipids. The size of the vesicles was determined by dynamic light scattering (DLS). The DLS-experiments were performed on a Zetasizer Nano-ZS (Malvern). We obtained vesicles with a homogeneous size distribution around diameter  $d = 100$  nm.

### 3.2.3 Sample Preparation

Spin-labelled  $\alpha$ S mutants were added from stock solutions (concentration between 150  $\mu$ M and 250  $\mu$ M) to the LUVs to obtain a lipid to protein ratio (L : P) of 250 : 1, and incubated for 30 min at room temperature before measuring. All samples were prepared and measured at least three times. All spin labelled  $\alpha$ S constructs used in this work are shown in Table 3.1.

### 3.2.4 Filtration experiments

To determine, whether  $\alpha$ S physically detaches from the membrane, we performed filtration experiments similar to those described in Drescher et. al (24). An  $\alpha$ S-vesicle solution, prepared as for the EPR experiments described above

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(sample preparation), was passed through a 100 kDa cut-off filter device (Amicon Ultra 100k), which retains the vesicles and thereby the membrane-bound  $\alpha$ S fraction, but is permeable for unbound  $\alpha$ S. The concentration of  $\alpha$ S in the filtrate is too low to measure directly, therefore the filtrate was concentrated using a 3 kDa cut-off filter device (Amicon Ultra 3k) and measured by EPR to determine the amount of  $\alpha$ S in the filtrate. The error in the final value, in the order of 20 %, derives largely from the errors in determining the volumes before and after the concentration step, and the error of the double integral procedure to determine the spin concentration by EPR.

Table 3.1. The  $\alpha$ S constructs used to study phosphorylation at position S87 and S129; SL denotes the spin-label.

<b>Spin label positions</b>	<b>S87A (non-phosphorylated)</b>	<b>S87D (phosphorylated)</b>
<b>SL27</b>	SL27 $\alpha$ S/S87A	SL27 $\alpha$ S/S87D
<b>SL56</b>	SL56 $\alpha$ S/S87A	SL56 $\alpha$ S/S87D
<b>SL63</b>	SL63 $\alpha$ S/S87A	SL63 $\alpha$ S/S87D
<b>SL69</b>	SL69 $\alpha$ S/S87A	SL69 $\alpha$ S/S87D
<b>SL76</b>	SL76 $\alpha$ S/S87A	SL76 $\alpha$ S/S87D
<b>SL90</b>	SL90 $\alpha$ S/S87A	SL90 $\alpha$ S/S87D
<b>Spin label positions</b>	<b>S129A (non-phosphorylated)</b>	<b>S129D (phosphorylated)</b>
<b>SL27</b>	SL27 $\alpha$ S/S129A	SL27 $\alpha$ S/S129D
<b>SL56</b>	SL56 $\alpha$ S/S129A	SL56 $\alpha$ S/S129D
<b>SL69</b>	SL69 $\alpha$ S/S129A	SL69 $\alpha$ S/S129D

### 3.2.5 Continuous wave-EPR experiments

The 9.7 GHz continuous-wave (cw) EPR measurements have been performed using an ELEXSYS E680 spectrometer (Bruker, Rheinstetten, Germany) with a Super high Q cavity (ER 4122 SHQE-W1/1108). Measurements were performed at

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20 °C, using 0.63 mW of microwave power, 100 kHz modulation frequency and a modulation amplitude of 0.1 mT. Total acquisition time for the EPR spectra was 20 minutes.

### 3.2.5.1 Simulation of cw-EPR spectra

Spectral simulations were performed using Matlab (7.11.0.584, Natick, Massachusetts, U.S.A) and the EasySpin package(39). For all simulations, the following spectral parameters were used:  $g = [2.00906, 2.00687, 2.00300]$  (40), the hyperfine tensor parameters  $A_{xx} = A_{yy} = 13$  MHz, and  $A_{zz}$  was varied (see Table 3.2). Usually a superposition of more than one component was required to simulate the spectra. The parameters were manually changed to check in which range acceptable simulations of the experimental spectra were obtained to determine the error margins. To simulate spectra of  $\alpha$ S bound to membranes, the  $\tau_r$  of the fastest component was kept at the  $\tau_r$  value of the spectra of the respective protein construct in the absence of vesicles.

## 3.3 Results

We investigate the binding of phosphorylation variants of  $\alpha$ S at positions 87 and 129 to LUVs of 100 nm diameter. The LUVs are composed of a 1:1 mixture of POPG and POPC, generating a membrane of charge density  $\rho = 0.5$ . We first describe the results of phosphorylation at position 87, then at 129.

Figure 3.2 shows the spectra of the spin labelled constructs probing phosphorylation at position 87 in the presence of LUVs (for complete list of constructs, see Table 3.1). In this set, helix 1 is probed in the middle, at residue 27, helix 2 is probed at five positions starting from position 56 and terminating in 90. Figure 3.22a shows the spectra of  $\alpha$ S in the non-phosphorylated and Figure 3.2b

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in the phosphorylated form. Spectra in Figure 3.2a differ from those in Figure 3.2b, most notably, each spectrum in Figure 3.2b has narrower lines than its counterpart in Figure 3.2a. As described in the introduction, narrow lines derive from spin-labels that are rotating fast. As discussed in more detail below, fast rotation shows that the section of the protein to which the spin label is attached is not bound to the membrane. More detailed information was obtained by spectral simulation of the experimental spectra, which yields the parameters of mobility of the spin label, the rotational correlation time ( $\tau_r$ ) and, in the case of multicomponent spectra, the amount by which each fraction contributes. These parameters are given in Table 3.2. In Fig. 2c, an example of a simulation is shown. Three fractions are visible, the fast, the slow and the immobile component, which have increasingly large linewidths. The individual components add up to give the experimental spectrum. Table 3.2 reveals that all but two spectra consist of a superposition of two components, the fast and slow components, except for the SL56 $\alpha$ S/S87A variant, which in addition has a third, the immobile component, and the SL90 $\alpha$ S/S87A and SL90 $\alpha$ S/S87D variants, which have only one component, the fast component. Each component reflects a part of the protein population: The fast fraction is due to protein in which the region around the site that is spin labelled is not attached to the membrane, whereas the slow and immobilized fractions are due to sections bound to the membrane. The amount by which each component contributes to the spectra (Table 3.2, columns four and six) reflects the fraction of protein contributing to each component. The correlation times can be determined to several tens of ps, in the case of the fast fraction, and several hundreds of ps for the slow fraction (see Table 3.2). The contribution of the fast component of  $\alpha$ S in the non-phosphorylated form is smaller than in the phosphorylated form for each probing position. The opposite is the case for the

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contribution of the slow components. Both these trends reveal that phosphorylation reduces membrane binding.

To illustrate the effect of phosphorylation at position 87, Figure 3.3 shows a plot of the amount of the fast fraction for phosphorylation at position 87 as a function of the sequence number at which mobility is probed. For all monitoring positions, the amount of mobile fraction is larger in the phosphorylated variant. At monitoring positions 27 and 56, the amount of mobile fractions of non-phosphorylated  $\alpha S$  is below 10 %, which indicates strong binding, but at later positions (helix 2) the amount of fast fractions increases to 70 % indicating the loosening of the helix 2 of  $\alpha S$ , when it is non-phosphorylated, in agreement with previous findings for wt  $\alpha S$  (24). For the phosphorylated  $\alpha S$ , the amount of the mobile fraction is higher than in the non-phosphorylated form for all positions monitored, enhancing the tendency for local unbinding in helix 2 until, at position 90, the bound fraction is so low that it becomes undetectable.

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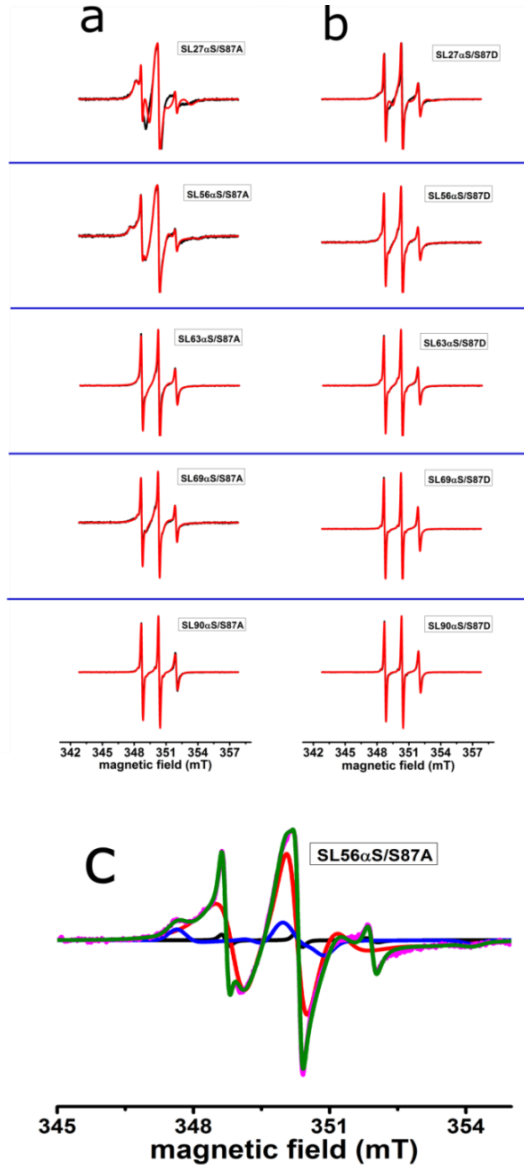


Figure 3.2. Effect of phosphorylation at position 87 on  $\alpha$ S-binding to LUVs: Room temperature, EPR spectra of spin-labelled  $\alpha$ S constructs (for nomenclature see Table 3.1) with LUVs of a 1:1 mixture of POPG and POPC; a. non-phosphorylated b. phosphorylated form. Black line: experiment, red line: simulation. c. Decomposition of EPR spectrum into components shown for SL56 $\alpha$ S/S87A. The fast (black), slow (red) and immobilized (blue) components are shown as well as the added simulation (green) and the experimental spectrum (pink).

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Table 3.2. Effect of phosphorylation of  $\alpha$ S at position 87 (S87A/D): Parameters describing the mobility of the spin label in the EPR spectra;  $\tau_R$ , rotation correlation time of the spin label,  $A_{zz}$ , the hyperfine splitting along the Z-direction.

$\alpha$ S spin-label positions	components contributing to spectra	S87A (non-phosphorylated)			S87D (phosphorylated)		
		$\tau_R$ (ns)	contribution (%)	$A_{zz}$ (MHz)	$\tau_R$ (ns)	Contribution (%)	$A_{zz}$ (MHz)
SL 27	fast	$0.4 \pm 0.03$	$6 \pm 0.4$	110	$0.4 \pm 0.02$	$32 \pm 2$	110
	slow	$8.5 \pm 0.2$	$94 \pm 0.4$	85	$9.3 \pm 0.65$	$67 \pm 2$	83
	immobile	na	na	na	na	na	na
SL 56	fast	$0.4 \pm 0.02$	$6 \pm 0.2$	110	$0.4 \pm 0.03$	$34 \pm 3$	110
	slow	$3.2 \pm 0.07$	$78 \pm 1.2$	102	$3.1 \pm 0.4$	$63 \pm 3$	102
	immobile	>50	$16 \pm 1$	91	na	na	na
SL 63	fast	$0.35 \pm 0.04$	$29 \pm 2$	110	$0.4 \pm 0.03$	$51 \pm 5$	110
	slow	$2.6 \pm 0.3$	$70 \pm 2$	105	$2.5 \pm 0.6$	$44 \pm 5$	108
	immobile	na	na	na	na	na	na
SL 69	fast	$0.3 \pm 0.02$	$23 \pm 2$	110	$0.3 \pm 0.02$	$75 \pm 9$	110
	slow	$2.5 \pm 0.2$	$75 \pm 2$	110	$2.5 \pm 1.2$	$20 \pm 9$	110
	immobile	na	na	na	na	na	na

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SL 76	fast	0.4 ± 0.04	42 ± 5	110	0.4 ± 0.02	79 ± 8	110
	slow	3.5 ± 0.8	57 ± 5	110	3.5 ± 3.2	16 ± 8	110
	immobile	na	na	na	na	na	na
SL 90	fast	0.4 ± 0.04	70 ± 10	110	0.3 ± 0.03	100* ± 8	110
	slow	2.5 ± 1.3	24 ± 10	110	na	na	na
	immobile	na	na	na	na	na	na

na: A component seen in other spectra, but not required to obtain a good simulation of the experimental spectrum in question, revealing that the rotational correlation time of the spin label does not contain contributions on the time scale of the component in question (for details see text and Fig. 2). For error determination see Materials and methods.\*including 4.5 % contribution of spin label with natural abundance of  $^{13}\text{C}$ .

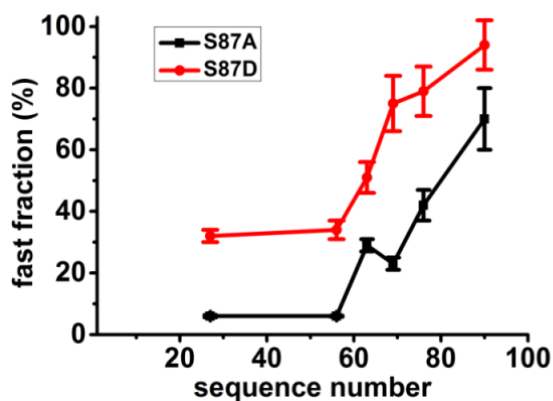


Figure 3.3. Local unbinding effect of phosphorylation at position 87: Amount of fast fraction in  $\alpha\text{S}$  87 A, D mutants in the presence of LUVs as a function of the sequence number. black: non-phosphorylated ( $\alpha\text{S87A}$ ), red: phosphorylated ( $\alpha\text{S87D}$ ) (see Table 3.2 for values), the lines connecting the points are guides to the eye.

To determine if the phosphorylation reduces the overall membrane affinity of  $\alpha\text{S}$ , i.e., if  $\alpha\text{S}$  detaches completely from the membrane, resulting in  $\alpha\text{S}$  protein that is



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free in solution (physical unbinding) we separated the unbound fraction of  $\alpha$ S from the membrane-bound fraction, by filtrating the sample through a filter that retains the vesicles and  $\alpha$ S bound to them. The amount of physically unbound protein in the filtrate is then determined by EPR, as described in Drescher et al. (24) (for details see Materials and methods). The amount of unbound  $\alpha$ S is given in Table 3.3 and is below 16 % for all constructs. Thus the amount of physically unbound  $\alpha$ S is significantly lower than the amount of the fast fraction measured by EPR (see Table 3.2), showing that the local unbinding far out-weighs any physical unbinding. The percentages in Table 3.3 for spin label positions 27 and 56 are slightly lower than for the other positions. Given that the differences are just outside the error margins of the procedure, we cannot draw conclusions.

Table 3.3. Physical unbinding of  $\alpha$ S S87D from the membrane. Results of filtration experiments (for details, see Materials and methods and also Results).

mutants	$\alpha$ S unbound fraction (%)
SL27 $\alpha$ S/S87D	5.9 $\pm$ 2.0
SL56 $\alpha$ S/S87D	5.2 $\pm$ 1.0
SL69 $\alpha$ S/S87D	15.1 $\pm$ 3.0
SL90 $\alpha$ S/S87D	13.6 $\pm$ 3.0

For phosphorylation at position 129, Figure 3.4 shows the superposition of the spectra of non-phosphorylated and phosphorylated variants for three spin label positions (see Table 3.1). In contrast to phosphorylation at position 87, A and D variants at position 129 have similar spectra, obviating the need for detailed spectral analysis. Apparently, phosphorylation has a much smaller influence at position 129 than at position 87.

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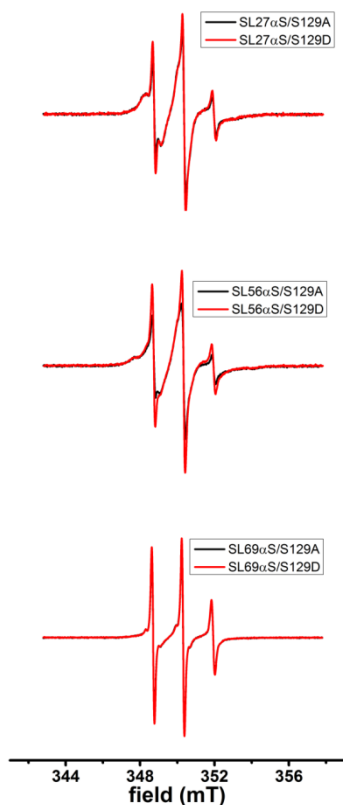


Figure 3.4. Effect of phosphorylation at position 129 on  $\alpha$ S-binding to LUVs: Room temperature, EPR spectra of spin-labelled  $\alpha$ S constructs (for nomenclature see Table 3.1) with LUVs of a 1:1 mixture of POPG and POPC; Superposition of non-phosphorylated (black line) with phosphorylated EPR spectra (red line), normalized by their double integral value.

### 3.4 Discussion

We have investigated how membrane binding of  $\alpha$ S depends on the phosphorylation state of positions 87 and 129. Membrane binding is detected locally, via the mobility of spin labels attached to specific positions in the protein. An increased spin-label mobility shows that the protein detaches from the membrane around the position probed.

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The membrane composition was chosen to be conducive to intermediate binding with a charge density of  $\rho = 0.5$ , to avoid: a) dominant electrostatic effects observed at higher charge densities, where they cause strong, undifferentiated binding and are non-physiological, b) overall unbinding observed at low charge densities (23,24,32–34), as described in the introduction. The membrane was offered in the form of LUVs of a diameter of 100 nm. We mimic phosphorylation by the phosphorylation-mutation approach, replacing S by D, an approach used before (13,17,29,30) (for details, see introduction). Although some studies showed that biochemically phosphorylated  $\alpha$ S can have different properties than phosphorylation mimics (29,31), the latter constructs provide a robust system to study phosphorylation effects in vitro, explaining their popularity.

Under the conditions of our study, phosphorylation at position 129 has no noticeable effect on membrane binding, whereas 87 has, similar to what was observed by other techniques in the past (13). In the following, we will first discuss the influence of phosphorylation at position 87 on  $\alpha$ S-membrane binding, and then compare the results obtained on both phosphorylation sites to previous findings in the literature.

When position 87 is phosphorylated, membrane binding is reduced relative to the non-phosphorylated case. An almost the same reduction of the binding is observed at positions 27 and 56 in the helix 1 region, see Figure 3.3. Similar to wild type  $\alpha$ S (24), also in the S87A variants, helix 2 has a lower membrane affinity than helix 1. Phosphorylation enhances this trend, up to the point that at probing position 90, the bound fraction becomes so low that it is undetectable. Complete, detachment of the phosphorylated protein from the membrane does not play a role: as seen in Table 3.3, the physically unbound fraction is below 16 % for all constructs. To place this into perspective, the amount of physically unbound  $\alpha$ S is

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maximally one third of the amount of fast fraction determined from EPR, showing that the majority of the fraction, seen by EPR, derives from protein that is attached to the membrane, presumably at the residues preceding the probed sequence position, e.g. for sample SL27/ $\alpha$ S87P, residues 27 and below.

Fluctuations in the amount of fast fraction (Table 3.2, SL 63, non-phosphorylated (SL63/S87A) has a larger amount of fast fraction than SL 69) and a larger amount of physically unbound  $\alpha$ S for SL positions in helix 2 (Table 3.3), could indicate an influence of the spin label on  $\alpha$ S-membrane binding. If such an effect is present, it never exceeds a contribution of 10 %, and therefore is not relevant for the conclusions drawn.

Overall we find that phosphorylation at position 87 decreases the membrane affinity of  $\alpha$ S, more for helix 2. This effect is fully consistent with the change in the charge caused by the conversion of S $\rightarrow$ D or by phosphorylation: A negative charge in the helix 2 will weaken the electrostatic interaction with the negatively charged membrane surface as it counteracts the effect of several lysines (Lys;K) in the  $\alpha$ S sequence from residues 1-100. Reduced membrane binding of S87E and P-S87 has been reported before, e.g. (13) (41).

Reduced membrane binding affects the entire protein, but is most pronounced in the helix 2 region, and may selectively influence the behavior of helix 2. Some models propose that the physiological function of  $\alpha$ S involves vesicle fusion events in which helix 1 and helix 2 interact with different types of membranes (42). We therefore speculate that phosphorylation at position 87 could be used to tune how  $\alpha$ S operates in vesicle trafficking.

For the  $\alpha$ S129 A/D variants, the difference in mobility of the spin label for phosphorylated and non-phosphorylated forms is minute, showing that under the

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membrane conditions employed here, phosphorylation at this site does not affect membrane binding. The C-terminus of  $\alpha$ S is already negatively charged and was not found to interact with the membrane in previous studies (20,21,24,38,43), which is fully consistent with the lack of changes in membrane-binding observed in the present study upon phosphorylation at position 129.

The results of the present study suggest that phosphorylation at position 87 tunes those functions of  $\alpha$ S that involve membrane binding and vesicle interaction, whereas phosphorylation at position 129 acts on other aspects of  $\alpha$ S in the organism. Previously (13) several possibilities of how phosphorylation at position 129 could affect  $\alpha$ S in vivo behavior are described and the study of Kosten et.al (44) shows that the phosphorylation at position 129 depends on the phosphorylation state of position 125, suggesting a complex interplay of posttranslational modifications in the C-terminus.

Most of the current research is focused on phosphorylation at position 129, and the phosphorylation degree at this position is related to disease effects, as reviewed in (45). In agreement with our results, several studies show that  $\alpha$ S phosphorylation at 129 has no or little effect on membrane binding, see for example (28). However, several studies find an influence of phosphorylation at 129 on the aggregation of  $\alpha$ S (28,29,46) and on membrane binding of  $\alpha$ S aggregates (46) suggesting that in vivo effects are linked to aggregation-sensitive processes.

In conclusion, the large spectrum of phosphorylation effects on  $\alpha$ S in vivo and in vitro (13–16,19,28–31,41,45–54) furnishes the need for isolating the different factors that can be modulated by  $\alpha$ S phosphorylation in vitro. The present study gives one such example, where we show that in vitro-phosphorylation mimics at

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position 87 (S87D) reduce  $\alpha$ S-membrane binding in a local, sequence dependent manner, whereas the same modification at position 129 (S129D) has no influence on membrane binding. We expect that this approach provides a foothold to interpret the challenging in vivo physiological and pathological functions of  $\alpha$ S.

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## Appendix A to Chapter 3

In chapter 3, the effect of phosphorylation of  $\alpha$ -Synuclein ( $\alpha$ S) on binding to large unilamellar vesicles (LUVs) was studied, here we investigate the interaction of phosphorylated and non-phosphorylated  $\alpha$ S with small unilamellar vesicles (SUVs) with the same lipid compositions as used for LUVs, i.e., POPG : POPC = 1 : 1 (charge density  $\rho = 0.5$ ). We describe the binding of S87 variants of  $\alpha$ S at three probing positions (27, 56 and 69) by spin-labeled electron paramagnetic resonance (EPR) spectroscopy.

SUVs were prepared as described in chapter 2. The size of the vesicles was determined by dynamic light scattering (DLS). The DLS-experiments were performed on a Zetasizer Nano-ZS (Malvern). We obtained vesicles with a homogeneous size distribution with a diameter of approximately  $d = 50$  nm.

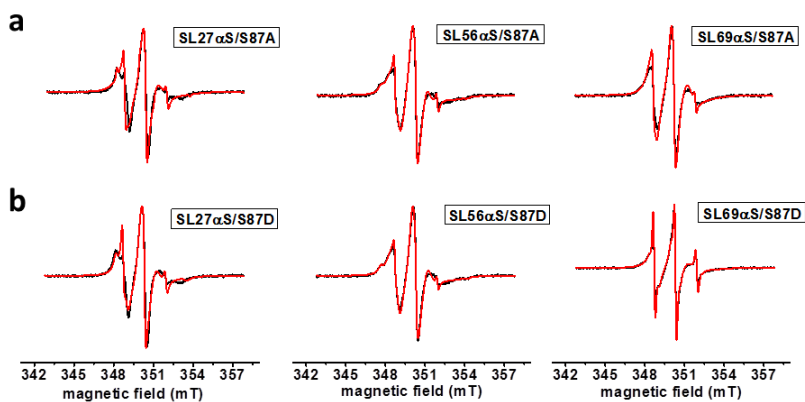


Figure A1. Effect of phosphorylation at position 87 on  $\alpha$ S-binding to SUVs: Room temperature, EPR spectra of spin-labeled  $\alpha$ S constructs (SL27, 56, and 69) with SUVs of a 1:1 mixture of POPG and POPC; a. non-phosphorylated b. phosphorylated form. Black line: experiment, red line: simulation.

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Figure A1 shows the spectra of spin-labeled constructs of  $\alpha$ S probing the effect of phosphorylation at position 87 in the presence of SUVs. In this set, helix 1 is probed in the middle, at residue 27, helix 2 is probed at positions 56 and 69.

Table A1. Effect of phosphorylation of  $\alpha$ S at position 87 (S87A/D): Parameters describing the mobility of the spin label in the EPR spectra;  $\tau_r$  rotation correlation time of the spin label

$\alpha$ S spin label positions	components contributing in simulations	S87A (non-phosphorylated)		S87D (phosphorylated)	
		$\tau_r$ (ns)	contribution (%)	$\tau_r$ (ns)	contribution (%)
SL 27	fast	0.4	5	0.4	5
	slow	2.5	90	2.8	93
	immobile	>50	5	>50	2
SL 56	fast	0.48	2	0.44	1
	slow	3.2	91	2.9	90
	immobile	>50	6	>50	8
SL 69	fast	0.3	2	0.3	14
	slow	2.2	98	2.5	86
	immobile	na	na	na	na

for error see table 2

Figure A1a shows the spectra of  $\alpha$ S in the non-phosphorylated and Figure A1b in the phosphorylated form. In contrast to what we observe with LUVs, the spectra of  $\alpha$ S27 and  $\alpha$ S56 in the non-phosphorylated case (Figure A1a) are similar to those of the phosphorylated case (Figure A1b). The spectrum of  $\alpha$ S69 in the phosphorylated case shows that the lines are narrower compared to its

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non-phosphorylated counterpart. This is also evident from the simulation parameters shown in Table A1. Overall,  $\alpha S$  shows stronger binding to SUVs than to LUVs (chapter 3) for the non-phosphorylated and phosphorylated variants, which is masking the effect of phosphorylation seen on LUVs.

