God gave me this illness to remind me that I’m not number One; he is.

/Muhammed Ali
THE INTRINSICALLY DISORDERED PROTEIN 
α-SYNUCLEIN AND ITS MEMBRANE 
INTERACTIONS STUDIED BY ELECTRON 
PARAMAGNETIC RESONANCE SPECTROSCOPY

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INTRODUCTION

The human protein α-synuclein (αS) is thought to be one of the key players in onset and progression of Parkinson disease (PD). This protein is a major component of the Lewy bodies, large intracellular inclusion bodies, which are a neuropathological hallmark of the disease. Furthermore, three point mutations in the gene coding of αS cause as rare familiar form of PD confirmed a causative role in pathogenesis.

αS links to various synucleinopathies and is typical model among the intrinsically disordered proteins (IDPs), a large fraction of eukaryotic proteins (> 30%), which possess little or no ordered structure under the physiological conditions. The protein displays remarkable conformational flexibility and, depending on the environment, can adopt variety of structurally distinct conformations. Owing to such behavior, αS has been called "protein-chameleon".

Although αS is strongly implicated in PD progression, the precise role of this protein is still unclear. The native function of αS is also poorly understood but it has been widely hypothesized that membrane interactions play a central role in the function and toxicity of αS.

Monitoring structure and dynamics of αS on a molecular level upon protein-membrane interactions remains a complex challenge. Site-directed spin labeling (SDSL) in combination with electron paramagnetic resonance (EPR) spectroscopy is a powerful tool to study the structural features of αS. Distance and mobility measurements are utilized, respectively.

In this work, we aim to monitor the interaction between monomeric αS and membranes using EPR spectroscopy methods in combination with SDSL approach. The major goal of the investigation is to examine membrane binding of αS in the presence of artificial and organellic membranes. Experiments using artificial membranes allow for addressing the following questions with respect to the mechanism of protein-membrane interactions:

- How do the membrane properties influence the binding process of αS?
- How do the αS disease point mutations affect the membrane binding?
- What are the conformational changes of αS upon protein-membrane interactions?

In order to contribute to understanding of the physiological function of αS it is essential to perform the experiments under more complex conditions.
conditions, e.g. to investigate αS interactions with organellic membranes such as mitochondria.

Determination of the structure of αS bound to membranes as well as its binding properties is expected to contribute to our understanding of the details of αS-membrane interactions and might shed light on both its physiological functions and its pathological role in PD.
2.1 INTRINSICALLY DISORDERED PROTEINS

Our view on proteins is deeply ingrained with the conviction that a well-defined three-dimensional structure is a prerequisite of understanding their function. The underlying paradigm is given as those: Amino Acid Sequence $\rightarrow$ 3 Dimensional Structure $\rightarrow$ Function [1]. A key point of this concept states that a protein can realize its biological function only upon folding into a unique, structured state, which represents a kinetically accessible and an energetically favorable conformation and is determined by its amino acid sequence. In recent surge of reports, however, it has been shown that the functional state for many proteins and protein domains is intrinsically unstructured. Also, it has been reported that IDPs comprise a large fraction of eukaryotic proteins; slightly more than 30% of the crystal structures in the Protein Data Bank (PDB) are completely devoid of disorder [2, 3].

An IDP is commonly defined as a protein that does not display a well-defined native structure when isolated in solution under near-physiological conditions [4]. The are notable differences between the amino acid sequences of IDPs and structured globular proteins. These dissimilarities appear in amino acid composition, sequence complexity, low mean hydrophobicity, aromaticity and high net charge [5]. It is evident that IDPs identified in vitro have a distinct amino acid composition, in that they are enriched in disorder-promoting amino acids (A, R, G, Q, S, P, E and K) and depleted in order-promoting, bulky hydrophobic (I, L, and V) and aromatic (W, Y, and F) amino acids [1].

Other manifestations of a distinct character of IDPs is that the proteins display remarkable conformational flexibility and often fold in a particular structure to achieve their function. The folding of these proteins can be triggered by interaction with other proteins, nucleic acids, membranes, or small molecules. Also changes in the protein environment can cause the folding. Despite being highly flexible and lacking stable secondary and tertiary structures, IDPs have particular biological functions. They are realized in regulation, recognition, and cell signaling for which binding to multiple partners and high-specificity interactions play a crucial role [6]. Probably because of their important roles in the cell, IDPs are often involved in diseases such as cancer and cardiovascular disease, they also appear to be involved with disproportionately high frequency in neurodegenerative diseases [7].
2.2 PARKINSON DISEASE AND α-SYNUCLEIN

PD is, besides dementia with Lewy bodies, Alzheimer’s disease, Down’s syndrome, multiple system atrophy, and neurogeneration with brain iron accumulation type 1, one of the most common synucleinopathies [8]. Synucleinopathies from a group of neurogenerative disorders characterized by fibrillary aggregates of the αS protein in the cytoplasm of selective populations of neurons and glia. Clinically, synucleinopathies are characterized by a chronic and progressive decline in motor, cognitive, behavioral, and autonomic functions, depending on the distributions of the lesions [8, 6].

PD is a progressive neurodegenerative disorder in the aging population, affecting more than 1% people over the age of 65 years, increasing to 4–5% in 85-year-olds [9] and cannot be predicted, and for which there is no cure [10]. The disease is characterized clinically by severe motor symptoms including uncontrollable resting tremor, muscular rigidity, impaired postural reflexes, and bradykinesia, which vary between patients [9, 11]. Pathologically PD is marked by dopamine neuron degeneration and depigmentation of the substantia nigra, a region in the brain which is important for movement. The disorder is also accompanied by neuron loss in the ventral tegmental area and locus coerules, but there is degeneration of other neurons as well [9, 12]. The main neuropathological feature of the disease consists of eosinophilic, round cytoplasmic inclusions in the form of Lewy bodies and Lewy neurites [13]. These inclusion bodies contain a large amount of αS, which suggests that this protein may play an important role in the pathogenesis of sporadic as well as familial disease [14].

2.3 PROPERTIES OF α-SYNUCLEIN

αS is a small cytosolic protein, which is highly expressed in the central nervous system and is concentrated in the presynaptic terminals representing 0.5-1% of the total cytosolic protein in the brain [15]. This protein links various synucleinopathies and is one of the canonical model systems among the IDPs. The primary structure of αS consists of 140 amino acids (Figure 2.1a) in which the following three domains can be distinguished (Figure 2.1b). The acidic N-terminal arm, residues 1-60, includes the sites of three familial PD mutations (indicated as orange arrows in Figure 2.1a) and contains four 11-amino acid, imperfect repeats with a highly conserved hexameric motif (KTKEGV) [16]. The middle non amyloid component (NAC) region [17, 18], residues 61-95, is the most hydrophobic region of the protein and is responsible for αS aggregation and β-sheet formation [19]. The C-terminal region, residues 96–140, is highly enriched in acidic residues and prolines and does not
acquire a defined secondary structure in solution, when αS is bound to membranes or when it forms amyloid fibrils [20, 21].

The conformational behavior of αS can strongly vary under different conditions and has been extensively analyzed [23]. This analysis proved that the structure of αS was extremely sensitive to its environment and could be easily modulated by a change in conditions. Therefore αS was named as protein chameleon [23, 24]. In solution, αS does not adopt any defined secondary structure and is considered to be natively unfolded [25]. Recent reports suggest that αS occurs in vivo as a helically-folded tetramer [26, 27], but these observations remain a matter of considerable debate [28]. However, in the presence of lipid membranes, αS adopts an α-helical secondary structure (residues 1-100) [29]. The intrinsic disorder and the presence of highly hydrophobic NAC region determine a strong aggregation propensity of αS. The aggregation of αS can be generally divided into three phases: oligomerization, fibril growth and saturation [30]. The aggregation pathway starting from αS monomers to the final fibrils deposited in the Lewy Bodies in plaques of PD patient brain is demonstrated in Figure 2.2.
Membrane interactions are believed to be essential for the function(s) of αS in a cell [31], because the protein is localized in close proximity to synaptic vesicles and it binds to the mitochondrial membrane [32]. There are mounting evidences ascribing a major role in maintenance of the synaptic vesicle reserve pool of the brain to the protein [11]. It has also been proposed that this protein is involved in lipid metabolism and transport [33, 34], vesicle docking at the membrane and exocytosis [35], lipid organization [36] and prevention of oxidation of unsaturated lipids [37]. Furthermore, αS-membrane interactions have been reported to play a role in PD pathology [31].

It has been shown that only the N-terminal residues take part in lipid binding [38], while the negatively charged C-terminus remains disordered [20]. The first part of the protein is taken up by imperfect 11-amino-acid repeats that bear the consensus sequence KTKEGV (Figure 2.1) [39], where individual repeats are separated by an inter-repeat region of five to eight amino acids. The N-terminal part of αS can be displayed as an amphipathic helix upon binding to membranes (Figure 2.3). In this conformation, these repeats generate an unbalanced distribution of polar and nonpolar residues that form separate hydrophobic and hydrophilic faces, a distribution well fitted for membrane binding. Upon binding to membranes, the two-thirds of the N-terminal sequence adopts a non-canonical conformation of a 11/3 periodicity (11 residues to complete three full turns) as opposed to the 18/5 periodicity of an ideal α-helix [40, 21]. In the αS 11/3 periodicity, the number of residues per turn is 3.67 and the rotation per residue is 98.18°, which allows for a better arrangement of the basic and acids residues to favor membrane binding [41].

Many structural studies have been performed and several different biophysical methods including circular dichroism (CD) spectroscopy [29, 42, 38], fluorescence spectroscopy [43], nuclear magnetic resonance (NMR)
Figure 2.3: An axial view of αS residues when displayed on an Edmundson helix wheel. Polar residues (i.e., S, E, and K) face the hydrophilic environment of the cytosol, whereas hydrophobic residues are buried in the acyl chains of the phospholipid bilayer. Positively charged lysine residues (K) separate the polar and hydrophobic domains and interact directly with the anionic surface of the phospholipid bilayer (adapted from [31]).

[44, 45, 46, 42, 47, 21, 20] and electron paramagnetic resonance (EPR)[48, 49, 40, 50, 51] spectroscopy have been used to analyze the conformation and the topology of membrane-bound αS.

*In vivo* the natural binding target of αS are synaptic vesicles, which surface topology *in vitro* is most closely mimicking by artificial lipid vesicles [47, 42, 44, 40, 46, 21]. The slow tumbling rate of intact phospholipid vesicles make the system unfeasible for direct studies using solution NMR methods. Therefore, most of the structural information available concerns studies with detergent micelles (SDS, sodium dodecyl sulfate) as membrane-mimetic environment, because their small size (∼5 nm) facilitates high-resolution structural analysis by NMR. The respective studies of SDS-bound αS [42, 46] have revealed the presence of so called horseshoe-like conformation, with two curved helices, with a break in the αS 38-44 region (Figure 2.4a) [47, 44, 46, 42]. The break was hypothesized to play a role in allowing the protein to bind to a range the surface curvatures. It is worth to mention, that in the recently report of Shvadchak and Subramaniam [52] it was suggested that the flexible break between the two helical domains of αS is close to residues 52-55.

The conformation of αS on lipid membranes, which would be more relevant physiologically, is still a subject of discussion. While there is a general consensus that αS adopts an amphipathic α-helical structure involving approximately the first 100 residues on artificial membranes, the debate remains regarding the exact nature of the interaction sites and arrangements of the helix (Figure 2.4). Some EPR reports suggest a broken-helix conformation [53, 51] similar to that of the micelle-bound protein, whereas others show an extended α-helix for residues 9-90 [40, 48, 50], a finding that is also supported by single molecule FRET (fluorescence
resonance energy transfer) measurements [54, 55]. However, it is likely that these differences can be accounted for by the fact that various membrane mimics (micelle, bicelle, and vesicles) and solution conditions (pH, salt, and temperature) were employed. Perhaps even more important than the aforementioned factors, is that experiments were performed at different lipid-to-protein ratios [56, 57] and lipid compositions [49, 57], a factor which has been shown to affect αS membrane binding modes. Though some questions remain, these results highlight the likely propensity for αS to undergo significant structural rearrangements under different conditions in solution [54, 55], an inherent trait which may be related to the function of αS in vivo.

2.5 α-SYNUCLEIN AND MITOCHONDRIAL DYSFUNCTION

Mitochondria have long attracted attention because of their role in human diseases [58]. They are essential for ATP production, i.e. the basic energy unit, and are susceptible to oxidative damage [59]. Mitochondria play a key role in cell survival through the regulation of programmed cell death, apoptosis, which may be important for cell death in PD. It has been demonstrated that mitochondria may be implicated in exocytosis at different steps, controlling recruitment and priming of synaptic vesicles [59]. Mitochondrial content is very high in presynaptic terminals, where mitochondrial function is critical for synaptic activity [60]. It was observed that the most vulnerable neurons in PD, dopaminergic neurons in the substantia nigra, contain a lower mitochondrial mass than other neurons, suggesting that a deficit in mitochondrial mass may contribute to vulnerability of these particular neurons to harmful conditions [61, 58, 62].

Mitochondria are organelles enclosed by a double membrane and are crucial for neuronal function and survival [63]. Mitochondria have two well-defined compartments, the matrix, surrounded by the inner membrane, and the intermembrane space, surrounded by the outer membrane [64, 65]. The inner mitochondrial membrane contains the protein...
complexes necessary for the electron transport chain. In order to create a large surface area for ATP production, the inner membrane is folded into numerous cristae [65, 63]. These cellular organelles produce most of the cell’s energy in the form of ATP by oxidative phosphorylation [66, 67].

Although the molecular mechanism underlying PD are unknown, considerable evidences support mitochondrial dysfunction and αS as two of the major contributions to this disease [69, 58, 67]. In a cell, αS can interact with different membrane surfaces like endoplasmic reticulum, synaptic plasma membrane, and vesicle fraction, but it has been observed that the protein is binding preferentially to mitochondria membranes (Figure 2.5) [70]. There are convincing evidences that the association of αS with mitochondria membranes are strongly connected with the presence of cardiolipin, which is enriched on both the outer and inner mitochondrial membranes [71, 72]. These fatty acids are unusual in their bicyclic nature, yielding a phospholipid with four, rather than two, fatty acid tails. Cardiolipins are implicated in a variety of critical processes such as mitochondrial fusion, protein complex stability [73], metabolite [73] and electron transport [74], and cell death [75].

The subcellular localization of αS in mitochondria varies across a range of in vitro and in vivo studies, depending on species, cell lines and variations in the intracellular pH [76, 77, 78, 79, 80, 81, 82, 83, 84, 85]. The protein was localized on the outer mitochondrial membranes [81,
77, 84, 85], while at other times, its fraction was detected on the inner membranes [76, 85, 80].

There are many consequences of αS interaction with mitochondria (Figure 2.5). There are a lot of examples in literature, that αS disrupts mitochondrial morphology. In cultured cells, increased synuclein levels produce fragmented mitochondria [84, 85, 86, 87, 88]. Also in vivo including in midbrain nigrostriatal dopamine (DA) neurons, αS alerts mitochondrial morphology [83, 85, 89]. Overexpression of αS can also selectively inhibit mitochondrial complex I activity [76, 80, 90, 91] and increase the number of mitochondria in autophagosomes in midbrain DA neurons [90].

On the other hand, several studies have demonstrated a protective role of αS against mitochondrial-mediated neuronal death [74, 92, 93, 94, 95, 96]. For example, it was shown that αS formed a covalent complex with anionic phospholipids and cytochrome c to prevent apoptosis [97]; mice lacking αS appear to be more resistant to mitochondrial toxin induced degeneration of dopaminergic neurons [95] and the overexpression of the protein led to protection of mice against neurodegeneration [96].
3.1 Site-Directed Spin Labeling Electron Paramagnetic Resonance Spectroscopy

In the field of structural biology and structural proteomics one of the most important challenges is the study of IDPs. IDPs lack a well-defined structure and display remarkable conformational flexibility. The structure of IDPs depends strongly of their environment, many IDPs do adopt more highly ordered conformation upon interaction with other cellular components. Especially for IDPs’ function it is important to know not only the protein structure, but also the dynamics and molecular mechanisms by which such protein and its cellular environment act together to accomplish these tasks.

Since the conventional high-resolution techniques such as X-ray crystallography and NMR spectroscopy are limited by technical difficulties to study complex IDPs assemblies, new biophysical characterization techniques are needed to advance the field. EPR spectroscopy of site-directed spin labeled biomolecules has emerged as a powerful method for studying the structure and conformational dynamics of IDPs under conditions relevant to their function [4, 24].

For site-directed spin labeling (SDSL), a spin-label side chain is introduced at a selected site via cysteine substitution mutagenesis followed by modification of the unique sulfhydryl group with a specific paramagnetic nitroxide spin labels (Figure 3.1). Nitoxides are stable free radicals of the general form ·O−NR1R2 where the unpaired electron, required for EPR detection, is (de-)localized on the N−O bond.

Among various spin labels utilized in literature, the spin label MTSL1 is the most used. Its advantages are sulfhydryl specificity, a small molecular volume and minimal effects on the protein structure [98, 99, 100] (Figure 3.1a). Alternatives to the methanethiosulfonate spin labels are maleimide-functionalized compounds, e.g. 3-maleimido-PROXYL2, which attach nitroxide spin labels to the thiol groups of cysteines [101] (Figure 3.1b). The advantage of the maleimide conjugation is its resistance towards reductive cleavage, which leads to immediate release of spin label side chains bound via disulfide bonds.

There is a suite of SDSL techniques that are used to investigate structure and dynamics of macromolecules [102]. These include: spin label

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1 (1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl)-methanethiosulfonate
2 3-Maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxy
Figure 3.1: Site-directed spin labeling. Reaction of a) methanethiosulfonate spin label (MTSL) and b) 3-maleimide-PROXYL spin label with the sulfhydryl group of a cysteine side chain generating the disulfide-linked nitroxide side chain $R_1$ (see also Figure 3.8). The dot indicates the free electron in the $N-O$ bond of the spin label (adapted from [100]).

scanning to reveal secondary structure elements [103], lineshape analyses to probe conformational changes and protein-protein contacts [104], power saturation paramagnetic relaxation measurements [105] to investigate membrane protein topology [106], the accessibility of various sites and conformational changes [107], and double site-directed spin labeling to investigate distances between two sites [108, 109, 110].

In the current work, two various EPR techniques combined with SDSL have been used. With the aid of mobility (see 3.1.1) and distance (see 3.1.2) measurements, the binding and conformational properties of $\alpha$S have been studied.

3.1.1 Mobility measurements

Parameters that reflects spin label motion can be extracted from an EPR spectrum. Since rotational mobility is encoded in the EPR lineshape, this information is obtained by spectral simulation of an acquired spectrum.

Nitroxide spin labels are paramagnetic centers characterized by a $g$-tensor (Zeeman interaction) and hyperfine tensor $A$ (hyperfine interaction). The interaction between the magnetic moment of an unpaired electron and the magnetic moment of nitrogen $^{14}\text{N}$ nucleus with $I = 1$ is highly anisotropic and makes EPR spectra critically dependent on the mobility of the spin label. The mobility can be quantified by the rotational correlation time $\tau_r$, which corresponds to the typical time during which a molecule maintains its spatial orientation.
Figure 3.2: Set of various simulated EPR spectra, using EasySpin functions [111], of a nitroxide radical corresponding to different motional regimes characterized by various rotational correlation times A) $\tau_r = 1$ ps (garlic); B) $\tau_r = 1$ ns (garlic); C) $\tau_r = 10$ ns (chili); D) "rigid limit" spectrum (pepper).
A series of X-band EPR spectra corresponding to different rates of rotational motion are shown in Figure 3.2. The spectra were simulated by the EasySpin software [111], for various motional regimes, differing in their rotational correlation time $\tau_r$. When the nitroxide spin label is allowed to tumble rapidly in an isotropic way, magnetic interactions ($g$ and $A$ tensors) are completely averaged and the EPR spectrum displays three narrow lines of approximately equal height (spectrum A in Figure 3.2). As the motion slows down, the magnetic anisotropy is no longer totally averaged. It results in a differential broadening of lines in the spectrum, while line positions remain constant, as compared to rapid tumbling (spectrum B in Figure 3.2). This is so-called fast motional regime, valid until $\tau_r = 10^{-9}$ s. For values of $\tau_r > 10^{-9}$ s up to $10^{-6}$ s, the averaging of the magnetic interactions due to motion becomes less and less efficient, leading to further changes of the EPR spectrum (spectrum C in Figure 3.2). This range of $\tau_r$ values defines the intermediate motional regime. The spectrum D in Figure 3.2 corresponds to the slow motional regime ($\tau_r > 10^{-6}$ s), where the full effects of the anisotropy are observed. This spectrum, referred as "powder" or "rigid limit" one, is obtained for any nitroxide for a dilute powder or frozen solution [112, 113, 114].

When introduced into proteins, nitroxide spin labels give rise to EPR spectra, whose shape reflects the spin label mobility. Any change in the spin label environment resulting from either a physical or chemical event can then be identified from the change in the EPR signature. Notably, EPR spectra of spin-labeled proteins can be recorded at room temperature, thus providing information on the local environment of the spin label under conditions close to the physiological ones.

The mobility of a spin label attached to a protein is determined by a combination of the movement of the entire protein ($\tau_{prot}$) and different local movements ($\tau_{local}$) including local backbone fluctuations and internal dynamics of the spin-label side chain. Using appropriate conditions, it is generally possible to isolate these various sources of spin label dynamics [115, 116].

The mobility information can be extracted from EPR spectra by processing the data in a quantitative way, through simulation, which implies solving of the stochastic Liouville’s equations, an approach developed by Freed and coworkers [117, 118]. Besides the simulation method, semi-quantitative analyses can be used. They include measurements of EPR parameters that directly depend on the mobility of the spin label, like (i) the ratio between the peak-to-peak amplitude of the lateral and the central lines, arising from the different spin states of the nitroxide $^{14}$N nucleus [119], (ii) the peak-to-peak width of the central line or (iii) the outer line splitting $2A_{zz}$ [120, 112].

In 1996, Mchaourab and co-workers established the basis for the interpretation of the EPR lineshape of spin labels attached to proteins by investigating the relationship between the mobility of the nitroxide side
chain and the structure of T₄ lysozyme and showed that the mobility of the spin labels reflects backbone fluctuations, as well as the protein tertiary structure [121]. Since these pioneering studies, the importance of backbone fluctuations and of tertiary contacts on EPR spectra has been studied in more detail using a collection of nitroxide methanethiosulfonate reagents [122, 123].

3.1.2 Distance measurements between nitroxide spin labels

One of the most attractive, rapidly developing aspects of SDSL is the ability to perform distance measurements between two spin labels, either intramolecular distances between two labels in the same molecule or intermolecular distances between sites of different proteins (for detailed reviews, see [124, 125, 126, 127]). Distance measurements between two nitroxides can provide information on both protein structure and functional dynamics. The ability to monitor conformational changes within a large protein is based on changes in spin-spin interactions (as a consequence of changed distances between the two labeled sites). Notable application examples are determination of protein structures [128, 129], monitoring structural changes [130], and characterization of membrane protein dimerization or oligomerization within bilayers [131].

Three methods for measuring the dipolar coupling between electron spins have become increasing popular. In the conventional continuous wave (cw)-EPR approach, distances in the 8-20 Å range are derived from the analysis of line broadening of the spectrum (3-4 G) [108, 109]. Weaker dipolar interactions (0.1-3.5 G) between spins correspond to longer distances (20-80 Å) can be measured using double electron-electron resonance [132, 133, 134] and double quantum coherence [124, 135] methods. The third technique, relaxation enhancement of the nitroxide spin label by a paramagnetic metal, measures the T₁ relaxation time by saturation recovery or inversion recovery methods [125].

In the field of EPR distance measurements, double electron-electron resonance (DEER, also know as pulsed electron-electron double resonance (PELDOR)) is the most widely applied technique [132, 136, 137].

3.1.2.1 DEER

In this chapter the DEER experiment is introduced. Detailed explanations are given in several reviews [138, 139, 140, 141, 142] and text books [125, 143].

Distance determination in DEER relies on the dipole-dipole interaction between the magnetic moments μₐ and μₐ of two electron spins A and B, respectively. The interaction energy E between two spins in an external magnetic field is given by:

\[ E = -\mu_0 \frac{\mu_A \mu_B}{4\pi r^3} \left(1 - 3\cos^2 \theta_{AB}\right) \]  

(3.1)
Figure 3.3: Dipole-dipole coupling between two spins A and B. The local field at the position of spin A (left) induced by the presence of spin B (orange) is inverted by the $\pi$ pulse, thus the local field at the position of spin A (blue) is changed (right).

where $\theta_{AB}$ is the orientation angle of $\vec{r}$ with respect to the external magnetic field $\vec{B}_0$ (Figure 3.3).

For two electron spins interacting with each other and placed in the external magnetic field, the spin Hamiltonian consists of three terms:

$$\hat{H} = \hat{H}_Z + \hat{H}_J + \hat{H}_{dd},$$

(3.2)

where the first accounts for the Zeeman interaction of the electrons with the external magnetic field, the second term is the exchange interaction between two electrons, and the third term is the dipolar interaction. The Hamiltonian of the dipole-dipole ($dd$) interaction can be written as

$$\hat{H}_{dd} = \omega_{AB} \hat{S}_A \hat{S}_B,$$

(3.3)

where $\omega_{AB}$ describes the dipolar coupling frequency between the spins A and B, and is given by

$$\omega_{AB} = \beta_B^2 g_A g_B \frac{1}{h} \frac{1}{r^3} (3 \cos^2 \theta_{AB} - 1),$$

(3.4)

where $\beta_B$ is the Bohr magneton, $g_A$ and $g_B$ are the g factors of the respective electrons.

Distance measurements by DEER depend on the proportionality of the dipole-dipole coupling $\omega_{AB}$ to the inverse cube of the distance $r[143, 144]$. To extract the distance from the dipolar coupling, the orientation dependence has to be controlled. Therefore, the distance measurements are usually performed at low temperatures, where frozen ensemble of molecular conformers contributes to the total signal. In a frozen state, upon shock freezing in a glass forming solution, an isotropic orientation distribution is achieved. In this state, the detected dipolar spectrum consists of a superposition of dipolar spectra of all possible orientations of the spin vector $\vec{r}$ with respect to the external magnetic field, which can be described by a Pake pattern (Figure 3.4).

DEER enables measuring distance constraints in the range of 1.8 to 6 nm in membrane proteins[139, 140, 138] and up to 10 nm in deuterated
3.1 SDSL EPR Spectroscopy

Figure 3.4: Pake pattern representing the dipolar coupling between two spins. The $\theta_{AB}$ corresponds to the angle between the $\vec{r}$ and external magnetic field (adapted from [141]).

Figure 3.5: Optimum excitation positions and bandwidths in four-pulse DEER experiments on nitroxides in X-band. a) Echo-detected field sweep (EDFS) EPR spectrum (black, superposition of adsorption spectra of all orientations) with marked pump ($\nu_{\text{pump}}$) and observed position ($\nu_{\text{obs}}$) split by approximately 65-70 MHz. b) Excitation profiles of 32 ns pulse (blue) and 12 ns pulse (orange) at observer and pump frequencies, respectively.

proteins[145]. The upper distance limit obtained by DEER is restricted by the transverse relaxation time $T_2$, which strongly depends on temperature. It was shown that for nitroxides in aqueous solution $T = 50$ K is ideal for distance measurement by DEER[125].

In most biological applications, the paramagnetic species A and B are identical nitroxide labels. The definition to A and B spin is made from the EPR spectrum. At X-band frequencies, nitroxide spectra are dominated by the strong anisotropic $^{14}$N hyperfine coupling, that leads to a three-line spectrum with a hyperfine splitting of about 100 MHz. Therefore, spins A and B can be discriminated by their spectral position in EPR spectra (Figure 3.5a).
Figure 3.6: Pulse sequence of the four-pulse DEER experiment. Delays $\tau_1$ and $\tau_2$ are kept fixed, while the delay $t$ between the undetected first primary echo (dotted line) and the pump pulse is varied. The integrated echo intensity $V(t)$ is recorded.

A dead time free four-pulse DEER pattern[136] is depicted in Figure 3.6. At the frequency $\nu_{\text{obs}}$, which addresses spins A (observer spins), a Hahn-echo sequence is applied, which results in a refocused echo of the observer spins A. An additional microwave pulse at $\nu_{\text{pump}}$, affecting only the B spins (pumped spins), is applied at the variable time $t$. This so co-called pump pulse flips the B spins, that causes a change of the local magnetic field at the spins (Figure 3.3). The variation of the time $t$ of the pump pulse results in a modulation of the measured echo intensity of spin A. It is given by:

$$V(t) = 1 - \lambda [1 - \cos(\omega_{dd} t)]. \quad (3.5)$$

The obtained DEER signal depends on the value of the dipole-dipole interaction $\omega_{dd}$ between spins A and B, the t position of the pump pulse, and the modulation depth parameter $\lambda$ which is turn depends on the fraction of excited spins. For nitroxide labels the inversion efficiency with a 12 ns pump pulse at X-band is $\lambda \approx 0.5[125, 138]$.

The pump and observed pulses should be separated in a way that the broad pump pulse does not excite the observed spins directly. The optimal conditions in X-band for the nitroxides are met when the pump frequency ($\nu_{\text{pump}}$) is set to the central manifold of the echo-detected field sweep and the observer pulse frequency ($\nu_{\text{obs}}$) set to the low field manifold (Figure 3.5). Since the magnetic field is kept constant during the experiment, this is achieved by setting the $\nu_{\text{obs}}$ 65-70 MHz above $\nu_{\text{pump}}[136]$.

The theory underlying analysis of the DEER spectra is given below. The measured DEER signal (Figure 3.7a) is composed of two parts: the inter- ($B(t)$) and the intramolecular ($F(t)$) interactions of the electron spins:

$$V(t) = F(t)B(t). \quad (3.6)$$
Because in most cases only in the distance within a nanoobject, e.g., the spin-spin distance within a doubly labeled protein are an object of interest, the intramolecular contribution, defined as form factor \( F(t) \), should be separated from the intermolecular interaction. The so called background factor \( B(t) \) can be fitted to an exponential curve and subtracted from the experiment (Figure 3.7b). For a homogeneous distribution of labeled objects confined to \( D \) dimensions the background function is given by:

\[
B(t) = e^{-kt^{D/3}}, \tag{3.7}
\]

where \( k \) quantifies the density of the spins. Soluble biomacromolecules in glassy frozen solution are usually homogeneously distributed in three dimensions \( (D = 3) \). Membrane proteins bound to a lipid surface may be confined to \( D = 2 \) dimensions\[139]. The excluded volume which is occupied by the macromolecule itself might result in deviations from \( D = 2 \) or \( D = 3 \). For doubly-labeled samples, \( B(t) \) can be determined experimentally from singly labeled samples\[146].

The form factor is encoding not only the distance but also the distribution of distances in the measured system. Each single distance present in the sample can be transferred into a Pake pattern (Figure 3.4) by Fourier transformation. Computing the distance distribution from the Pake pattern or time-domain signal is an ill-posed problem. Small distortions in the DEER signal or Pake pattern such as noise, or deviations from the ideal Pake pattern due to orientation selection can have large effects on the distance distribution. For this reason, mathematical algorithms must be applied that stabilize the solution against noise-induced artifacts by requiring a certain smoothness of the distance distribution. Tikhonov regularization\[148] is the most widely used approach to solve this ill-posed problem. In this approach, the smoothing is applied in a way that allows for a compromise between the minimization of the mean square deviation of the theoretical and experimental form factors, and
the minimization of the roughness. This compromise is defined by the minimization of the target function:

$$G_\alpha(t) = \rho + \alpha \eta,$$

(3.8)

where $\rho$ is the mean square deviation between the theoretical and experimental form factors, $\eta$ is the roughness, and $\alpha$ is the regularization parameter. The optimal $\alpha$ which minimizes the target function is found by the L-curve criterion. The optimum $\alpha$ corresponds to the corner of the L-shaped curve. Tikhonov regularization involves a compromise, and if distance distributions involve both broad and narrow components, then it may not be the best approach. In these cases, more accurate distance distributions can be obtained by the superposition of several Gaussian peaks. The software package DeerAnalysis\cite{146, 149} enables evaluation of experimental DEER data according to theory outlined above.

A typical DEER distance measurement takes around 12-24 hours at protein concentrations of approximately 50-200 µM. For this reason the DEER experiment is rather expensive in terms of the measurement time. The sensitivity of DEER increases significantly if the measurement is performed at Q-band frequency (34 GHz) instead of commonly used X-band (9.5 GHz)\cite{150, 151, 152}.

3.1.3 Modeling

Although spin labeling is a well established biochemical procedure, it requires relatively much effort for engineering cystein point mutations, as well as expressing and labeling the protein. Trying to place spin labels into spatially restricted positions might lead to narrow distance distributions, but may cause local distortion of the protein structure or result in failure of the labeling. On the other hand, completely unrestricted positions will be easily labeled without affecting the protein structure. However, this may result in broad distributions of label conformations, hence poorly defined distance constraints. Therefore, it is advantageous to predict and model those sites and pairs of sites that can provide the most reliable information in accessibility and distances \cite{153, 154}.

The R1 chain commonly used in SDSL-EPR technique displays high conformational flexibility. The unique dynamic properties of this spin-label side chain R1, minimize disturbances of the native fold of the protein and can provide insight in structural information from the shape of its room temperature EPR spectrum. Nevertheless, the large conformational space accessible for the R1 side chain (Figure 3.8), have also an intrinsic limitation, due the rotatable bonds ($\chi_1 - \chi_5$) and a $\sim 7$ Å separation between the spin center at the the midpoint of the N – O bond and the C\text{\textalpha} of the protein. The five torsion angles lead to a very large number of possible positions of the unpaired electron spin density. This causes uncertainty in the interpretation of the experimental data in structural
terms. To deal with this problem, the rotamer library approach can be applied. This method allows for accurate prediction of the conformational distribution of a spin label and for simulating distance distributions between spin pairs in doubly mutated molecule.

Libraries of likely conformations of spin labels (rotamers) have been previously applied for explicit modeling of MTSL. An initial library of 62 rotamers [139] was expanded to 98 [155] and then to approximately 200 rotamers [153] in order to capture the allowable conformational space of the spin label. The rotamer libraries in the latter study established by Polyhach et al. [153] were derived from molecular dynamics calculations of spin label flexibility.

Figure 3.8: Conformational space of MTSL spin label attached to an protein. All flexible bonds within the R1 side chain are highlighted (adapted from [147]).
4.1 **SAMPLE PREPARATION**

4.1.1 **αS expression and purification**

All αS cysteine derivatives, wild-type αS as well as A30P and A53T disease variants were generously provided by Prof. Vinod Subramaniam’s group at the University of Twente (The Netherlands).

Wild-type αS does not contain any cysteine residues. The αS single-cysteine mutations at selected sites (9, 18, 27, 35, 41, 56, 69, 90 and 140) and double-cysteine mutations (9/69, 9/90, 18/69, 18/90, 27/56 and 9/27) were introduced using standard biochemical methods [159]. In order to perform site-directed spin labeling, αS mutants were expressed in Escherichia coli strain BL21(DE3) using the pT7-7 expression plasmid (courtesy of the Lansbury Laboratory, Harvard Medical School, Cambridge, MA) and subsequently purified in the presence of 1 mM DTT.

4.1.2 **Site-directed spin labeling**

Prior to labeling, αS cysteine mutant proteins were reduced with a six-fold molar excess (per cysteine) of DTT for 30 minutes at 293 K. Subsequently, samples were desalted with Pierce Zeba 2 mL desalting columns, followed by an immediate addition of a six-fold molar excess (per cysteine) of nitroxide spin label. For labeling MTSL [(1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl))-methanethiosulfonate] (Toronto Research Chemicals) and PROXYL [3-Maleimido-PROXYL] (Sigma-Aldrich) were used. The samples were incubated overnight in the dark at 293 K. Free label was removed using two additional desalting steps. Owning to the high reactivity of the label and the fact that the cysteine residues are freely accessible in the poorly folded structure, near quantitative labeling can be achieved under these conditions. The protein labeling efficiency was estimated to be > 90% from the double integral of cw-EPR spectra at 293 K. Protein concentrations were determined by measuring the absorbance at 275 nm using an extinction coefficient of 5600 M$^{-1}$ cm$^{-1}$ by NanoDrop 1000 spectrophotometer (Thermo Scientific). The measured protein concentrations were 150-250 µM. For experiments where higher protein concentrations were required, samples were concentrated using Amicon Ultra centrifugal filters (Millipore).

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1. The presented experiments and results contain data which has already been published. Chapter 5 and 6 are based on [156, 157, 158].
4.1.3 Liposome preparation

Negative charged 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1’-rac-glycerol) (POPG) and bovine heart cardiolipin (1’,3’-bis[1,2-dioleoyl-sn-glycero-3-phospho]-sn-glycerol; CL) as well as zwitterionic 1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphocholine (POPC) and 1-hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine (POPE) were purchased in chloroform solution, cholesterol (Ch) as powder from Avanti Polar Lipids (Birmingham, AL) and used without further purification. The various lipid compositions were prepared by mixing the chloroform dissolved lipids to specific ratios. To prepare large unilamellar vesicles (LUVs), a thin lipid film was formed by drying around 12.5 mg of lipid in a glass using a gentle stream of nitrogen gas. Trace amounts of solvent were removed by drying under vacuum overnight. The lipid film was then rehydrated in 10 mM Tris-HCl buffer, pH 7.4, to a final concentration of 100 mM lipid and incubated 30 min at 293 K. LUVs were prepared by repetitive extrusion through one layer of 100 nm polycarbonate film in a handheld extruder (Avanti Polar Lipids) above the lipid phase transition temperature. The average diameter of LUVs was about 100 nm, as measured by dynamic light scattering (Zetasizer Nano ZS; Malvern Instruments). The total phospholipid concentration was determined according to the protocol of Chen et al. [160]. Vesicles were used within 5 days of preparation but were found to be structurally stable for at least two weeks when stored at 4 °C.

4.1.4 Mitochondria isolation

Mitochondria isolation has been performed in Prof. Marcel Leist’s group by Hanne R. Gerding and PD Dr. Stefan Schildknecht at the Department of Biology at University of Konstanz.

Mitochondria were isolated from HEK 293 cells by a commercially available kit (Qproteome Mitochondria Isolation Kit, Qiagen, Hilden, Germany) and immediately used for sample preparation.

4.1.5 Sample preparation

4.1.5.1 αS with artificial membranes

αS solution in 10 mM pH 7.4 Tris-HCl buffer (protein concentration between 150-250 µM) was added to the liposome solution resulting in a protein:lipid-ratio of 1:250 and a final protein concentration of approximately 50 µM. The mixture was allowed to incubate for at least 30 minutes at 293 K before starting measurements.
4.1.5.2 \(\alpha S\) with mitochondria

For the mitochondria EPR experiments freshly isolated mitochondria were centrifuged (15,000 g; 8 min 4 °C), the pellet (10 µL) was mixed with 2 µL \(\alpha S9/27\) (2 mM) and incubated in the presence of 1xSigmaFAST protease inhibitor on ice for 20 min. Afterwards unbound \(\alpha S\) was removed by two to three sequential centrifugation steps (15,000 g; 8 min). After each centrifugation step, supernatant was discarded and the mitochondrial pellet was resuspended in new buffer containing 1xSigmaFAST protease inhibitor. The final protein concentration was approximately 6 µM.

To study the binding of \(\alpha S\) to mitochondria, \(\alpha S\) was added to 100 µL of freshly isolated mitochondria at varying concentrations. After 5 min incubation at 37 °C, unbound \(\alpha S\) was removed by centrifugation (20,000 g; 10 min). Mitochondrial pellets were washed two times with PBS. The final mitochondrial pellet was lysed and analyzed by Western blot. For the protease inhibitor time course experiment stock solutions of freshly isolated mitochondria either without protease inhibitor or with 1xprotease inhibitor provided by the Qproteome Mitochondria Isolation Kit (Qiagen) or with 1xSigmaFAST protease inhibitor (Sigma Aldrich) were prepared. 5 µg \(\alpha S\) were added to each stock solution and samples were incubated at 37 °C. 100 µL samples were taken from the stock solution at varying time points and analyzed by Western blot. Proteins were separated by 12% SDS gels, transferred onto nitrocellulose membranes (Amersham Biosciences, Buckinghamshire, UK) and blocked with 5% milk (w/v) in PBS-Tween (0.1%) for 1 h. Blots were incubated with monoclonal \(\alpha S\) antibody (1:1000) (BD Biosciences, Heidelberg, Germany) or a polyclonal rabbit antibody directed against the mitochondrial outer membrane protein TOM20 (Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C overnight. Horseradish-peroxidase conjugated secondary goat anti mouse IgG (Cayman Chemical, Michigan, USA), or donkey anti-rabbit (GE Healthcare, Buckinghamshire, UK) antibodies were used to visualize the primary proteins by incubation for 1 h at 293 K. Luminescence was detected and quantified by a FUSION SLTM system (Peqlab, Erlangen, Germany). The Western blot analysis was kindly performed by Hanne R. Gerding from the Biology Department at the University of Konstanz.

For cw-EPR experiments prepared samples were placed into 1 mm (outer diameter) capillaries (Ringcaps). For distance measurement 20% v/v glycerol was added, afterwards the samples were transferred into 3 mm (outer diameter) quartz tube (X-band experiments) or 1 mm (outer diameter) Q-band Bruker tube (Q-band experiments), shock frozen in liquid nitrogen and put into the spectrometer.
4.2 Measurement Parameters

4.2.1 Mobility measurements

All cw-EPR spectra of singly labeled αS in solution and in the presence of LUVs were recorded at 293 K on a MiniScope spectrometer (MS400, magnettech GmbH) equipped with a variable temperature unit (Temperature Controller TC-H02, magnettech GmbH). Samples were loaded into glass capillaries (outer diameter 1 mm) with typical sample volumes of 10 µL. Spectra were obtained with a modulation amplitude of 600 mG, microwave attenuation 12 dB and a sweep width of 150 G. The signal-to-noise ratio was improved by accumulation of 10 spectra featuring 60 s scan time each.

The cw-EPR experiments examined αS binding on mitochondria were performed at 293 K in X-band using a Bruker Elexsys E580 spectrometer equipped with a Super High-Q Cavity. Performing modulated field sweeps containing 1024 data points (sweep time 42 s) a modulation frequency of 100 kHz was used. Modulation amplitude, as well as time constant have been chosen so that the signal was not distorted. Typical values are 0.48 G and 40 ms, respectively. Spectrometer control was performed by the Bruker Xepr software.

4.2.2 Distance determination

All DEER experiments [136] were done at T = 50 K using a Bruker Elexsys E580 spectrometer (Bruker Biospin). For DEER experiments in X-band (9.4 GHz) the spectrometer was equipped with a split-ring MS3 resonator, the Q-band (34 GHz) experiments were done in EN5107D2 Q-band resonator. In both cases a helium gas flow system (CF935, Oxford Instruments) was used. The complete four-pulse, dead-time free DEER pulse sequence is given by: $\pi/2_{\text{obs}} - \tau_1 - \pi_{\text{obs}} - t - \pi_{\text{pump}} - (\tau_1 + \tau_2 - t) - \pi_{\text{obs}} - \tau_2 - \text{echo}$. The pump pulse (12 ns long $\pi$-pulse in X-band and 50 ns long in Q-band) was set to the maximum of the nitroxide spectrum. In X-band the observer frequency was increased by 70 MHz and in Q-band decreased by 45 MHz with respect to the pump frequency. The pump frequency in Q-band was adjusted in the center of the resonator mode and in X-band 35 MHz below the center of the resonator mode. Pulse lengths of the observer channel were of 16 and 32 ns length (X-band) and 36 and 72 ns (Q-band) for $\pi/2$- and $\pi$-pulses, respectively. All samples were measured at $\tau_2 = 1.5 - 4$ µs and $\tau_1 = 200$ µs. The X-band DEER time-traces for ten different $\tau_1$-values spaced by 8 ns started by $\tau_1 = 200$ ns were added to avoid artifacts from the proton nuclear modulations. Shot repetition time was set to 4 µs. Typical accumulation times per sample were 20 hours at X-band and 10-15 hours at Q-band.
4.2.3 Dynamic Light Scattering

Vesicle size and stability of LUVs of different lipid compositions and upon interaction with αS were studied by Dynamic Light Scattering (DLS). The DLS studies were performed at 298 K using a Malvern Zetasizer nano ZS spectrometer (Malvern Instruments Ltd), equipped with a 4 mW He-Ne laser (vertically polarized incident radiation of wavelength 633 nm) with the viscosity and refraction index set to those for water. Measurements were performed on freshly prepared LUVs and LUVs incubated for 1 hour with αS in protein lipid ratio of 1:250, where (∼2 µL) of sample was dispensed in 1 mL distilled water into a 1 cm polycarbonate cuvette.

4.2.4 Immunoelectron microscopy

Immunoelectron microscopy was carried out at the Electron Microscopy Center, University of Konstanz and performed by Dr. Joachim Hentschel and PD Dr. Stefan Schildknecht. αS (5 µg/mL) was added to the isolated mitochondria for 1 h. Unbound αS was removed by five sequential centrifugation steps (15,000 g; 3 min). After each centrifugation step, supernatant was discarded and the mitochondrial pellet was resuspended in new buffer. For general visualization of the mitochondrial population isolated, negative staining was applied. The mitochondrial suspension (0.5 µL) was added to a grid for 30 s, 0.5 µL of a 2% ammonium heptamolybdate solution was added for additional 30 s. Liquid was then removed by a paper towel, samples were dried and analyzed with a Zeiss Omega 912. For a more detailed analysis, freshly isolated mitochondria were fixed at 4°C for 15 min (2% glutaraldehyde, 3% formaldehyde in 0.1 M sodium-cacodyl buffer containing 10 mM CaCl₂, 10 mM MgCl₂, 90 mM sucrose). The samples were centrifuged at 15,000 g for 20 min, the pellet was washed in 100 mM sodium-cacodyl buffer and then treated with 2% Osmium-tetroxide for 1 h. Removal of water from the samples was conducted by the step-wise increase of ethanol concentrations. The samples were incubated in 30%, 50%, 70%, 90%, and finally in 100% ethanol for 30 min respectively. Following polymerization of the embedded sample, 70-100 nm sections were prepared. For contrast enhancement, 2% uranylacetate and 0.4% lead-acetate were used. For αS visualization, a monoclonal αS antibody (1:500) from Zymed Laboratories (Invitrogen, Darmstadt, Germany) and an anti-mouse colloidal gold conjugated secondary antibody were used.
4.3 DATA ANALYSIS

4.3.1 cw-EPR spectra

The cw-EPR spectra were analyzed using Matlab R2010a (The MatWorks, USA) and the toolbox EasySpin4.0.0 [111]. Varying simulation parameters, least-square fits to experimental data were performed. For simulations $A_{xx} = A_{yy} = 13$ MHz and $g = [g_x \ g_y \ g_z] = [2.00906 \ 2.00687 \ 2.003]$ [161] were chosen and kept constant. The parameter describing the local degree of binding (see chapter 5) was manually changed to test in which range acceptable simulations of the data were obtained to determine the error margins.

4.3.2 Distance determination

In order to analyze the data and extract the distance distributions the software package DEERAnalysis2011 has been used [141, 162, 144, 146]. Experimental background functions were derived from DEER traces of singly labeled $\alpha$S in solution, in the presence of investigated LUVs or mitochondria, respectively, and were used for background correction of the double mutant data. In order to derive the distance distribution by model free Tikhonov regularization [162], the regularization parameter was determined by analyzing the L-curve. The distance distributions were validated concerning the experimental noise (white noise of 0.003, level 1.0, trial number 5) and uncertainties in background correction (background start from 100 ns, trial number 11).

In order to derive the number of interacting spins per nano-object, i.e. to estimate the fraction of $\alpha$S in the horseshoe configuration, the calibrated modulation depths of the DEER curves have been analyzed [163, 164]. As a reference, the DEER trace of the corresponding $\alpha$S double mutant in solution, i.e. in the absence of the membrane, was used. Fractions in the order of 30% were obtained. Differences in that parameter between the mutants of 15% reflect the uncertainties in determining this parameter.

4.3.3 Modeling

The open-source package Multiscale Modeling of Macromolecules (MMM) [153] was used for modeling spin label conformations and predicting the distance distribution between $\alpha$S labeled sites. The rotamer library calculations for MTSL derived from the MD trajectories at 175 K were performed. In order to perform rotamer analysis of membrane bound $\alpha$S, a template of $\alpha$S in a hypothetical extended helix conformation was build with PyMOL software, in order to model the broken helix conformation, NMR data (PDB 1XQ8) [47] was used. The computed ro-
tamer distributions were used to calculate distance distribution between investigated pairs of spin.
5

INTERACTION OF α-SYNUCLEIN WITH ARTIFICIAL MEMBRANES

5.1 INTRODUCTION

The protein αS is considered to play a major role in the etiology of PD. Although the exact function of αS remains unclear, there is strong evidence that the biological function of αS is correlated with its binding to membranes [165, 31]. Therefore, the membrane interaction of αS has been the subject of intensive research over the past decade (see section 2.4). Monomeric αS binds to negatively charged membrane mimics such as large and small unilamellar vesicles (LUVs and SUVs, respectively) [166, 29, 43]. The membrane curvature and the lipid composition are of high importance for the membrane binding properties of αS [167, 43, 168, 169]. In the membrane binding process the N-terminal part of the protein, especially rich in lysine residues which attributed to the electrostatic interaction takes part [29].

Despite many investigations aimed to characterize the binding of αS to membranes, there is still a lack of understanding of the binding mode linking the properties of lipid membranes to αS insertion into these dynamic structures. Using SDSL in combination with EPR spectroscopy enables to study the local binding properties of αS on artificial membranes with different charge density (section 5.2.1.1). The local binding information gives a more differentiated picture of the affinity of αS to membranes.

In brains from patients with PD, αS is associated with the mitochondria membranes [32, 76]. It has been shown that the N-terminal part of the protein plays a crucial role in binding to mitochondria [76]. It has been reported that αS may associate with mitochondrial membranes in different ways [81, 84, 90, 80, 85], but the modes of binding with mitochondrial membranes have been poorly characterized. Mitochondria consist 23.5% of phospholipids by mass [170]. The outer mitochondrial membrane is built mostly of phosphatidyl choline (55.2%), and phosphatidyl ethanolamine (25.3%), while the major phospholipids of the inner mitochondrial membrane are phosphatidyl choline (44.5%), phosphatidyl ethanolamine (27.7%), and cardiolipin (21.5%). It has been observed that numerous mitochondrial proteins and processes require the presence of cardiolipin for their optimal function and any disturbance of the cardiolipin profile may result in mitochondrial dysfunction [171, 172]. In order to understand the αS interaction with mitochondria, experiments with model systems mimicking the inner as well as the outer mito-
chondrial membrane were performed and are described in section 5.2.1.2. Accordingly, the importance of cardiolipin in the αS-membrane interaction was examined.

The N-terminal part of αS, which is crucial for α-helix formation and lipid binding, hosts three independent missense mutations at the position 30, changing an alanine to proline (A30P) [173], at the position 53, changing an alanine to threonine (A53T) [174] and at the position 46, were glutamic acid is altered to lysine (E46K) [175]. These mutations have been genetically linked to familiar early-onset PD. It has been observed, that all three αS PD variants have an impact on the membrane binding properties and aggregate formation [176]. The effects of the two PD-linked mutations A30P and A53T on the lipid interactions of αS have been investigated by a number of groups with somewhat conflicting results. The A53T mutation appears to have a little affect on the αS lipid interaction [177], but several reports indicate that the A30P mutation decreases the extent of αS membrane binding in vitro [38, 178] and in vivo [179, 180]. Such an effect could directly influence the normal function of the protein.

In section 5.2.2, the binding properties of two PD disease variants of αS, namely A30P and A53T, are presented. The mobility measurements allowed to examine how the point mutation affects the binding of the N-terminal part of the protein to different charged membranes and those mimicking the mitochondrial membrane.

While αS is natively unfolded in solution [25], it is generally accepted that upon binding to membranes the protein adopts an amphipathic, α-helical structure involving residues 1-100 [29, 38, 181, 182, 40]. The exact arrangement of the helix on the membrane surface is however very controversial. The structure of micelle bound αS consists of two antiparallel α-helices with a short loop region in between them, revealed in the so called horseshoe-like model [42, 46] (Figure 2.4a). This model was also confirmed by EPR based techniques [183]. The relevance of this structure is likely limited because of the small size of the micelles, with a typical diameter of 5 nm [184], which may force the protein into a horseshoe conformation. The NMR method is limited in its capability to resolve the structure of the physiologically relevant membrane-bound αS, because of the slow tumbling rate of the object of interest. Different techniques, including EPR and single molecule spectroscopy, have been employed to determine the structure of membrane bound αS, confirming that the N-terminal part of the protein is involved in membrane binding. There is, however, ongoing debate about the conformation of the protein on the membrane. Some reports favoring a horseshoe structure [51, 42, 53], while others [21, 55, 46, 48, 50] report an extended helix conformation (Figure 2.4b), on vesicles, despite using similar experimental techniques and often with only slight variations in experimental conditions.
In section 5.2.3 EPR distance measurements (DEER, see section 3.1.2.1) were used to monitor distance distributions studying the structure of membrane bound αS. For the first time the results of the distance measurements clearly show that αS coexists in a superposition of the both horseshoe and extended conformation when bound on large vesicles.

5.2 RESULTS

5.2.1 Nonuniform binding affinity of the N-terminal part of αS

5.2.1.1 Binding properties of αS on LUVs made from POPG and POPC lipids

To characterize the protein-membrane interaction, nine αS derivatives containing single side chain R1 (see Figure 3.8) were generated by labeling specific cysteine residues introduced at positions 9, 18, 27, 35, 41, 56, 69, 90 and 140 (αS9, αS18, etc.) as indicated in Figure 5.1. For the labeling procedure the MTSL spin label was used (see section 3.1.1). The interactions of these labeled proteins with LUVs composed of different ratios of negatively charged POPG1 and zwitterionic POPC2 lipids at 293 K were studied. The rotational mobility was determined by cw-EPR spectroscopy in X-band, accompanied by spectral simulations performed with the Matlab based program EasySpin [185] (see section 3.1.1).

First, the EPR spectrum of singly labeled αS in the absence of LUVs was studied (Figure 5.2). It is described by a one-component spectral simulation $S_A$ in the fast motion regime, where the rotational correlation time $\tau_{r,A}$, the line width $\lambda_w$ and the hyperfine interaction $A_{zz}$ (Table 5.1) were fitted. The obtained fit parameters are consistent with the expectations for unstructured peptides in solution. The entire set of spectra of the nine studied singly labeled αS derivatives in solution as well as the simulated parameters are displayed in Figure A.4 and Table A.1 in Appendix A.

In the next step, the interaction of labeled αS with LUVs was studied. Varying the lipid compositions of the vesicle results in varying the surface charge density given by:

$$\rho = \frac{[\text{POPG}]}{[\text{POPG}] + [\text{POPC}]}.$$  \hfill (5.1)

The size of LUVs as well as their stability after addition of the protein in 1:250 (protein:lipid) molar ratio were checked with dynamic light scattering (DLS; Figure A.1 in Appendix A).

Representative spectra of singly labeled αS9 and αS90 upon interaction with differently charged LUVs are shown in Figure 5.3 and the entire set of spectra of the nine studied singly labeled samples in the presence of LUVs are displayed in Figures A.5–A.13 in Appendix A.

1 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-rac-(1’glycerol)
2 1-palmitoyl-2-oleoyl-sn-glicero-3-phosphocholine
Figure 5.1: Schematic representation of the spin labeled αS extended helix model. The orange dots mark the labeled residues.

Figure 5.2: Experimental spectra of representative singly labeled mutants (a) αS9 and (b) αS90 in the absence of LUVs (black circles) and their spectral simulation fit (red line).

The spectra of αS in the presence of LUVs can be described well by a superposition of two components featuring different rotational mobilities, a slow component $S_B$ and a fast component $S_A$:

$$S = (1 - b)S_A + bS_B.$$  

The simulation parameters obtained for αS in solution were taken as the fast component for $S_A$, while the rotational correlation time $\tau_{r,B}$ of $S_B$ and the fraction $b$ were fitted (Table 5.1).

The simulations of the EPR spectra obtained for all spin-labeled αS mutants in the presence of LUVs solely made of zwitterionic POPC lipids ($\rho = 0$, Figure 5.3a and 5.3b) show that the spectra contain only the fast component $S_A$ with parameters corresponding to those obtained for αS in solution. The finding suggests that there is no significant binding of αS to these uncharged membranes.

With an increasing POPG/POPC ratio the spectra of singly labeled mutants change gradually. Only the spectrum of αS140 remains the same irrespective of LUVs lipid composition (see Appendix A). The increase in fraction $b$ of the slow component $S_B$ for all mutants is reflected in line broadening (Figure 5.3c–f) relative to spectra of αS in solution (Figure 5.2) and in the presence of uncharged POPC LUVs (Figure 5.3 a,b). The
Figure 5.3: Representative experimental spectra (black circles) and corresponding fits of $\alpha_{S9}$ (red line) and $\alpha_{S90}$ (blue line) in the presence (a, b) of POPC ($\rho = 0$), (c, d) POPG/POPC ($\rho = 0.4$), and (e, f) POPG ($\rho = 1$) LUVs.
Table 5.1: Simulation parameters describing the rotational mobility for \( \alpha S_9 \) and \( \alpha S_{90} \) in solution and in the presence of LUVs$^\S$.

<table>
<thead>
<tr>
<th></th>
<th>( \tau_{r,A} ) [ns]</th>
<th>( \tau_{r,B} ) [ns]</th>
<th>( b )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \alpha S_9 ) in solution</td>
<td>0.44</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>( \alpha S_{90} ) in solution</td>
<td>0.36</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>( \alpha S_9 ) with LUVs (( \rho = 0 ))</td>
<td>0.44</td>
<td>-</td>
<td>0.00(2)</td>
</tr>
<tr>
<td>( \alpha S_{90} ) with LUVs (( \rho = 0 ))</td>
<td>0.36</td>
<td>-</td>
<td>0.00(2)</td>
</tr>
<tr>
<td>( \alpha S_9 ) with LUVs (( \rho = 0.4 ))</td>
<td>0.44</td>
<td>2.39</td>
<td>0.92(5)</td>
</tr>
<tr>
<td>( \alpha S_{90} ) with LUVs (( \rho = 0.4 ))</td>
<td>0.36</td>
<td>3.16</td>
<td>0.13(5)</td>
</tr>
<tr>
<td>( \alpha S_9 ) with LUVs (( \rho = 1 ))</td>
<td>0.44</td>
<td>2.56</td>
<td>0.99(2)</td>
</tr>
<tr>
<td>( \alpha S_{90} ) with LUVs (( \rho = 1 ))</td>
<td>0.36</td>
<td>2.45</td>
<td>0.80(5)</td>
</tr>
</tbody>
</table>

$^\S$ for values of \( g \), line width \( lw \) and hyperfine interaction \( A_{zz} \) see Table A.1 in Appendix A.

Spectral simulations for component \( S_B \) show that \( \tau_{r,B} \) is much smaller than the rotational correlation time of a LUVs (\( \tau_r \sim 0.1 \) ms), which was calculated from the Stokes-Einstein equation, given by:

\[
\tau_r = \frac{\eta V}{k_B T},
\]

where \( \eta \) is the viscosity of the solution, \( k_B \) is the Boltzmann constant, \( T \) is the absolute temperature and \( V \) is the volume of the species. Therefore, \( \tau_{r,B} \) rather reflects the residual mobility of the spin-label. Because the component \( S_B \) originates from spin-labels with reduced mobility and is observed only when LUVs are present, the restricted mobility of the spin-labels must stem from the interaction of \( \alpha S \) with the LUVs. Hence, the slow component \( S_B \) can be attributed to the labeled residue of \( \alpha S \) region, which is bound to the membrane. The \( b \) coefficient reflects the local degree of binding of the region of the protein.

A systematic study was performed to examine the influence of the surface charge density \( \rho \) of the LUVs on binding of \( \alpha S \). The local degree of binding was obtained for nine spin labeled \( \alpha S \) proteins. The results reported on interaction of the respective spin labeled regions of \( \alpha S \) with a membrane (Figure 5.4). For \( \alpha S_{140} \), the local degree of binding \( b \) is zero at all values of \( \rho \). For all other mutants, it was found that the degree of local binding \( b \) decreased with decreasing values of \( \rho \). From \( \rho = 1.0 \) to \( \rho = 0.3 \), the behavior is strongly dependent on the labeled region.

\[ \eta = 1.002 \text{ m}^{-1}\text{kg s}^{-1} \]
\[ k_B = 1.3806488 \times 10^{-23} \text{m}^2\text{kg s}^{-2} \text{K}^{-1} \]
\[ T = 293.15 \text{ K} \]
\[ V = 5.24 \times 10^{-22} \text{m}^3 \], the volume of a ball with a radius \( R = 50 \text{ nm} \)
Regions close to the N-terminus bind at lower membrane surface charge densities than the regions distal from the N-terminus. The differences in the dependence of $b$ on $\rho$ can be attributed to an effect of nonuniform binding affinity of $\alpha S$ to the membrane surface. For instance, the fraction $b$ of $\alpha S_9$ reflects the local binding affinity around residue 9, which starts to bind at $\rho \approx 0.1$, while the region around residue 90 does not bind until $\rho \approx 0.5$.

5.2.1.2 Interaction of $\alpha S$ with LUVs having lipid compositions similar to the inner and outer mitochondria membrane

The binding properties of $\alpha S$ in the presence of more physiologically relevant lipids were investigated. It has been reported that $\alpha S$ can interact with mitochondria membranes (see Introduction 5.1 and 2.5). In order to investigate how $\alpha S$ might interact with mitochondrial membranes, LUVs with a lipid composition mimicking the inner and outer mitochondrial membrane were prepared. The average diameter of the LUVs was determined by DLS and is $\sim 100$ nm. The LUV size and stability after protein addition were unchanged (Figure A.2 and A.3 in Appendix A).

First, the interaction of the nine previously investigated MTSL-singly labeled $\alpha S$ derivatives with LUVs having a molar POPC: POPE7: Cholesterol (Ch) ratio of 4.0:2.0:0.9, which corresponds to the composition of the outer mitochondrial membrane[170] were studied. No significant change in the line broadening of the experimental spectra (Figure 5.5a) in the presence of LUVs in comparison to the spectra of $\alpha S$ in solution were observed. The spectra in the presence of LUVs could be described

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7 1-hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine
well with a one component fits $S_A$, with the parameters obtained for $\alpha S$ derivatives measured in solution. This observation suggests no binding of the protein to this type of membrane.

LUVs with a molar ratio corresponding to the inner mitochondrial membrane (POPC:POPE:CL:Ch, 2:0:1:3:1:0:0:6)\(^8\) gave strikingly different results. In the presence of those LUVs, the experimental spectra of almost all mutants show drastic changes (Figure 5.5b). The spectral line broadening indicates a lower mobility of the spin label. Only the spectrum of $\alpha S_{140}$ remained unchanged upon addition of LUVs. The experimental spectra were fitted with two component fit (equation 5.2) and the fraction $b$ of the slow component $S_B$ was fitted.

The changes of the local degree of binding, which is described by the value $b$ as a function of the labeled position for the nine $\alpha S$ derivatives on LUVs are shown in Figure 5.6. It was observed that large regions of $\alpha S$ bind to LUVs mimicking the inner mitochondrial membrane but not to those mimicking the outer one. This result indicates the importance of CL in the binding process.

\[ 5.2.1.3 \quad \text{Significance of cardiolipin in the binding properties of } \alpha S \]

Since CL is mitochondria-specific, the binding of $\alpha S$ to LUVs containing different amounts of this phospholipid was investigated. EPR mobility measurements at 293 K were performed for the nine MTSL-labeled derivatives of $\alpha S$ in the presence of POPC/CL LUVs. Different amounts

\[ \text{cardiolipin} \]
of CL in the LUV composition resulting in different surface charge densities $\rho$, according to:

$$\rho = \frac{[\text{CL}]}{[\text{lipids}]}.$$

(5.4)

It has to be mentioned that CL has a dimeric structure consisting of two phosphatidyl residues connected by a glycerol bridge and four acyl chains, and therefore it can carry two negative charges. The structure of CL is shown in Appendix B.

The experimental spectra and corresponding simulations are shown in Figures A.16 and A.17 in Appendix A. The experimental spectra were fitted using a two component fit (equation 5.2). The fast component $S_A$ the raw experimental spectra of the respective singly labeled $\alpha$S mutants in solution was used. For the slow component $S_B$, the simulation parameters of the singly labeled mutants on CL LUVs ($\rho = 1$) were used, where a quantitative binding of the protein was detected. The only remaining parameter to be fitted is $b$, which reflects the degree of local binding in the proximity of different residues of $\alpha$S (for simulation parameters see Table A.2 in Appendix A). Figure 5.7 shows the changes of $b$, as a function of the labeled position. It can be observed that at low CL content up to $\rho = 0.1$ there is almost no binding at all investigated $\alpha$S residues. At $\rho = 0.2$, which corresponds roughly to the CL content of the inner mitochondrial membrane, a drastic increase of the local degree of binding of the N-terminal part of the protein is observed, while the C-terminal tail of the protein does not bind. These findings show that the CL is essential for the interaction between $\alpha$S and the inner mitochondrial membrane.
Figure 5.7: Local degree of binding in the proximity of different residues of αS in the presence of LUVs with different charge surface density (ρ) made from diverse CL/POPC compositions (ρ = 0 orange, ρ = 0.05 yellow, ρ = 0.1 cyan, ρ = 0.2 grey, ρ = 0.3 green, ρ = 0.5 blue, ρ = 0.7 red and ρ = 1 black).

and that the local binding affinity increases with the increasing ratio of CL.

5.2.2 Alteration of the local binding properties of αS by the αS Parkinson Disease Variants

The effect of the disease point mutations A30P and A53T on the local binding affinity of αS to different charged membranes was investigated. This enables a better insight into the influence of the two disease variants on the binding properties of the protein.

Mutants of the disease variants A30P and A53T were expressed containing a cysteine at position 9, 18, 27, 35, 41, 56, 68, 90 and 140 (αS\textsubscript{A30P}\textsubscript{9}, αS\textsubscript{A30P}\textsubscript{18}, αS\textsubscript{A53T}\textsubscript{9}, αS\textsubscript{A53T}\textsubscript{18}, etc.). These mutants were then labeled with PROXYL, for the labeling protocol see chapter 4. cw-EPR spectroscopic measurements (at 293 K) were utilized to study the local degree of binding of those samples and their interaction with different charged LUVs made from zwitterionic (POPC) and negatively charged (POPG) lipids.

The representative experimental spectra of αS, αS\textsubscript{A30P} and αS\textsubscript{A53T} labeled at position 9 (Figure 5.8a) and position 90 (Figure 5.8b) in the absence of LUVs are shown. There are no significant difference between the measured spectra, which narrow linewidth indicate a high spin label mobility. The spectra were fitted with a one component fit in a fast motion regime. The obtained isotropic rotational correlation times for those samples are in good agreement with the rotational correlation time of the spin label in unfolded proteins[186, 187]. The fit parameters are listed in Table A.4 in Appendix A.
Figure 5.8: Representative experimental cw-EPR spectra recorded at 292 K for αS (black), αS_{A30P} (red) and αS_{A53T} (blue) derivatives singly labeled at position 9 (a) and 90 (b) in the absence of LUVs.

In the presence of LUVs with the increasing amount of negatively charged lipids the spectra of αS as well as αS_{A30P} and αS_{A53T} gradually change their lineshape (Figure 5.9c, e and f). The line broadening relative to the spectra in the absence of vesicles indicates a lower mobility of the spin label, which results from a change in the local degree of binding for the investigated spin label positions.

The spectra in the presence of LUVs were fitted with a superposition of two components $S_A$ and $S_B$ and the local binding parameter $b$ was acquired (see equation 5.2). In the presence of LUVs made from POPC lipids ($\rho = 0$, Figure 5.9a and 5.9b) the spectra of the both disease mutants do not change compared to those obtained in solution without LUVs. The spectra contain only the fast component $S_A$ with parameters corresponding to those obtained in solution, what indicates no binding of αS_{A30P} and αS_{A53T} to the neutral charged membrane.

Figure 5.10 compares the local binding affinity in the proximity of the labeled positions of αS on different charged LUVs with αS PD variants αS_{A30P} and αS_{A53T} (entire set of experimental spectra with corresponding fit are shown in Figure A.18–A.35 and obtained fit parameters are listed in Table A.5 in Appendix A). Generally the degree of local binding $b$ increases with the increasing values of $\rho$. The investigated residues at the N-terminal part of the protein (labeled residues 9, 18, 27, 35, 41 and 56) bind at the lower membrane charge densities than the residues far from the N-terminus (labeled residues 69 and 90). Only the spectra of the protein labeled at position 140 do not change upon addition of LUVs. This is expected as residue 140 is the final residue of αS, located at the C-terminus of αS, a region that was shown[20, 21] not to interact with the lipid membranes. It can be observed that the local degree of binding for both disease variants differs from the investigated wild-type
Figure 5.9: Representative experimental cw-EPR spectra of $\alpha$S (black line), $\alpha$S$_{A30P}$ (red line) and $\alpha$S$_{A53T}$ (blue line) singly labeled at position 9 (left) and 90 (right) in the presence of POPC (a and b), POPG/POPC ($\rho = 0.4$) (c and d), and POPG (e and f) LUVs.
αS. Both analyzed disease variants start to bind to the LUVs at higher POPG content and αS\textsubscript{A53T} shows a slightly lower binding affinity to the membranes as αS\textsubscript{A30P}.

Figure 5.10 shows the degree of local binding affinity of the three investigated samples on LUVs with a charge density of ρ = 0.3. It was reported that this particular lipid composition reflects a physiologically relevant membrane composition\[188, 189, 71\]. For αS the local degree of binding b starts to decrease from the N-terminal part of the protein, where the region in the proximity of the labeled position 9 has b ∼ 90%. It can be observed that here the binding affinity of the both disease variants strongly differ from αS. The N-terminus of αS\textsubscript{A53T} shows a significantly smaller binding affinity of b ∼ 20 − 10%. In case of αS\textsubscript{A30P} the binding is almost zero, only the part in closer neighborhood to position 9 shows b ∼ 5%.
Figure 5.11: Comparison of the data obtained for $\alpha S$ (black), $\alpha S_{A30P}$ (red), and $\alpha S_{A53T}$ (blue). (a) Local binding affinity in the proximity of labeled residues on LUVs made from POPG and POPC lipids at $\rho = 0.3$ charge density; (b) Inflection points of the sigmoidal curves shown in Figure 5.10 at the investigated labeled residues.

Figure 5.11b systematizes the obtained results. Here, the inflection points of the sigmoidal curves[190] given by:

$$f(x) = \frac{a}{1 + be^{-kx}}$$

and shown in Figure 5.10 are analyzed. The inflection point is defined as:

$$x_0 = \frac{\ln b}{k}.$$  \hspace{1cm} (5.6)

For the $\alpha S$ (in black), the values rise smoothly starting from the N-terminal part of the protein. $\alpha S$ labeled at position 9 starts to binding at $\rho > 0.2$, while the binding affinity of the middle part of the protein (positions 18, 27, 35 and 41) oscillates around $\rho \approx 0.3$ and then starts to growing gradually until $\rho \approx 0.55$ for position 90. $\alpha S_{A30P}$ (in red) and $\alpha S_{A53T}$ (in blue) have in general common binding properties. Their N-terminus starts to binding first at $\rho > 0.45$, while the residues closer to the end of the helical region (residue 90) bind at $\rho \approx 0.75$. Two exception of the smoothly rise of the inflection point for the disease mutants can be observed. For $\alpha S_{A30P}$ in the proximity of position 27 and for $\alpha S_{A53T}$ at position 41 the inflection point is shifted to $\rho$-values significantly higher as for the other labeled positions in the middle part of the $\alpha$-helical region of the protein.

The local binding affinity of $\alpha S_{A30P}$ as well as $\alpha S_{A53T}$ disease variants on LUVs mimicking the outer mitochondrial membrane containing POPC: POPE: Ch of 4:2:0.9 molar ratio and inner mitochondrial membrane made from POPC: POPE: CL: Ch lipids in 2:1.3:1:0.6 molar ratio.
Figure 5.12: Comparison of the local binding affinity of αS (black), αS₃₀P (red) and αS₅₃T (blue) at investigated labeled positions in the presence of the mitochondrial inner membrane-like LUVs.

were analyzed. Representative spectra of αS₃₀P and αS₅₃T on outer mitochondrial-like membrane and containing the spin label at position 9 and 90 are shown in Figure A.15 and the obtained fitting parameters are listed in Table A.3 in Appendix A. On the outer membrane, the EPR spectra show no changes compared to the case without LUVs. This result indicates that no binding on the membrane takes place. In case of αS in presence of LUVs mimicking the inner mitochondrial membrane, the experimental spectra of almost all labeled samples change (Figure A.14 in Appendix A). The mobility of the spin label is reduced as indicated by the spectral line broadening. In line with previous results the spectra of labeled residue 140 remain unchanged only. The spectra were fitted with a two component fit and the local degree of binding was calculated (see Table A.3 in Appendix A).

Figure 5.12 sums up the results obtained for the proteins αS, αS₃₀P and αS₅₃T mixed with LUVs mimicking the inner mitochondrial membrane. It shows the obtained local degree of binding b at the examined label position. There is no difference between the two disease variants and αS at position 140. The local binding affinity of αS₃₀P and αS₅₃T disease mutants in the N-terminal part of the protein is similar and significantly a smaller in comparison to αS. It can also be observed that αS₃₀P shows smaller binding affinity to the membrane at the investigated positions than the αS₅₃T mutant.
To study the conformation of \( \alpha S \) on a quasi two-dimensional membrane surface, LUV (100 nm in diameter) of negatively charged lipids POPG were used. It has been shown in section 5.2.1.1 that \( \alpha S \) binds most effectively to negatively charged membranes consistent with the exposure of positively charged amino acid residues in the helical section of the N-terminus of \( \alpha S \). The POPG LUVs are stable upon addition of \( \alpha S \) (Figure A.1 in Appendix A) and cw-EPR on singly labeled \( \alpha S \) showed that, under the conditions used here, quantitative binding is achieved (see Figure 5.3e and Table 5.1). Furthermore, no evidence for aggregates or oligomers, such as those observed for \( \alpha S \) on POPG SUV’s[191], was found under the employed conditions.

In order to monitor the structure of membrane bound \( \alpha S \) a set of double cysteine mutants was specifically labeled with MTSL (see section 3.1). The following spin labeled proteins were obtained and designated \( \alpha S_{9/69} \) (two spin labels attached at positions 9 and 69), \( \alpha S_{9/90}, \alpha S_{18/69}, \alpha S_{18/90}, \) and \( \alpha S_{27/56} \) as indicated in Figure 5.13. Distances between the spin labels were measured by DEER (see section 3.1.2.1), which allowed to access distances between 1.5 nm and 8 nm[133]. Distances below 1.5 nm were excluded by cw-EPR at 120 K for all double mutants (Figure A.36 in Appendix A).

First, \( \alpha S_{9/69}, \alpha S_{9/90}, \alpha S_{18/69}, \) and \( \alpha S_{18/90} \) on LUVs were studied by DEER. The obtained DEER time traces (shown in Figure A.37 in Appendix A) were background corrected to separated the intamolecular contribution from the intermolecular interaction within a nanoob-

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9 In this section some results of my Master thesis are reported to give an appropriate context.
Figure 5.14: (a) DEER traces after background correction (thin lines) of four double-cysteine mutants bound to POPG LUVs. The corresponding fit (thick lines) based on distance distributions simulated by the Gaussian fit model. (b) Respective Gaussian-like distance distributions.

ject. The single spin labeled αS9 in the presence of LUVs was measured to derive the background function experimentally. Both sample preparation procedure and experimental parameters were kept the same as for the double spin labeled αS. The resulted dipolar evolution curve is shown in Figure A.38 in Appendix A. Its decay corresponded to a background model of a homogeneous two-dimensional distribution, which was therefore used for background correction of the double mutant data (Figure 5.14a). The distance distributions for this double labeled αS samples can be accurately described by a single Gaussian fit (Figure 5.14b). The corresponding parameters are given in Table 5.2. The width of the distribution results from the flexibility of the spin-label linker as well as from the conformational flexibility of the protein itself. The latter is reflected by increasing width with increasing distance. The distances obtained for these four mutants are in good agreement with the NMR structure, i.e. the horseshoe model. The extended conformation should give distances larger than 8 nm, which will be not detectable in this experiment.

From the modulation depth of the DEER response the number of interacting spins can be determined. Taking into account the labeling efficiency and the fact that under our conditions no intermolecular interactions are observed the number of interacting spins is expected to be two, i.e. two labels per molecule. The reduced modulation depth found for these double mutants indicates that only a fraction of membrane bound αS contributes to the DEER signal (∼ 30% ± 15%). This finding suggests the the missing fraction exhibits distances which are not accessible by DEER, i.e. are larger that 8 nm. Those large distances are expected for the investigated double mutants when αS adopts an extended helix as already proposed for membrane bound αS[21, 55, 46, 48, 50]. In summary these findings suggested that there is a coexistence of different structures.
In order to verify the hypothesis that αS coexists in the horseshoe as well as in the extended helix conformations, double mutant αS27/56 with the labels attached close to the potential kink (residues 38-44) in the protein was designed. This mutant should allow for detecting the label-label distance in both conformations. The expected distances are 2.5 nm for the horseshoe (derived from the PDB data set 1XQ8[47]) and 4.4 nm (assuming an ideal continuous α-helix), respectively. The result of the corresponding DEER experiment is shown in Figure 5.15.

In contrast to the other mutants, the DEER time trace (Figure 5.15a) for the αS27/56 mutant cannot be described by a single Gaussian. A model-free analysis using Tikhonov-regularization results in the distance distribution depicted in Figure 5.15b (blue line), which clearly consists of two components (see Figure A.39b in Appendix A for L-curve). This model-free distance distribution was fitted using two Gaussians (Figure 5.15b, orange line) and both contributions were quantified: contribution I: distance $d_I = 4.3 \text{ nm}$, width $w_I = 1.4 \text{ nm}$, fraction $a = 80\%$ and contribution II: $d_{II} = 2.8 \text{ nm}$, $w_{II} = 1.0 \text{ nm}$, $b = 20\%$, respectively. Deriving the distance distribution again, now preselecting for two Gaussians, we found contribution I: distance $d_I = 4.3 \text{ nm}$, width $w_I = 0.95 \text{ nm}$, fraction $a = 80\%$ and contribution II: $d_{II} = 2.7 \text{ nm}$, $w_{II} = 0.55 \text{ nm}$, $b = 20\%$ (see Table 5.2 and Figure A.40 in Appendix A). The full quantitative agreement of these distances derived by two strategies confirms that the analysis is model independent. The Gaussian centered at shorter distance agrees well with the expected distance of 2.5 nm for the horseshoe conformation derived from the NMR structure (PDB 1XQ8[47]) while Gaussian centered at the longer distance is consistent with the 4.4 nm expected for an extended α-helix. This can only be explained if horseshoe and extended forms coexist under the membrane conditions employed in the present study.
Table 5.2: Parameters of Gaussian distance distributions for doubly labeled αS derivatives bound to POPG LUVs obtained by DEER and distances expected from NMR structure[47] and model of extended helix.

<table>
<thead>
<tr>
<th>Cys mutant</th>
<th>Distance [nm]</th>
<th>FWHM&lt;sup&gt;c&lt;/sup&gt; of distribution [nm]</th>
<th>Fraction of αS contributing to distance</th>
<th>Horseshoe model: C_β-C_β distance [nm]</th>
<th>Extended helix model: C_β distance&lt;sup&gt;a&lt;/sup&gt; [nm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>9/69</td>
<td>3.7±0.2</td>
<td>1.8±0.2</td>
<td>2.7</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>9/90</td>
<td>3.5±0.1</td>
<td>1.6±0.2</td>
<td>2.1</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>18/69</td>
<td>2.7±0.1</td>
<td>0.95±0.05</td>
<td>2.8</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>18/90</td>
<td>3.3±0.1</td>
<td>1.4±0.2</td>
<td>3.7</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>27/56&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.7±0.1</td>
<td>0.55±0.05</td>
<td>20±5%</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.3±0.1</td>
<td>0.95±0.05</td>
<td>80±5%</td>
<td>4.9</td>
<td></td>
</tr>
<tr>
<td>27/56&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.8±0.1</td>
<td>0.2±0.03</td>
<td>15±5%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.1±0.1</td>
<td>0.2±0.05</td>
<td>85±5%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> derived from simple extended model, assumed a continuous 11/3 periodicity helix

<sup>b</sup> Fit with two-Gaussians model (see experimental details)

<sup>c</sup> Full Width at Half Maximum

<sup>d</sup> Q-band DEER experiment
The distance measurement of αS27/56 in the presence of POPG LUVs has also been performed in Q-band in order to gain sensitivity. The corresponding DEER time trace before and after background correction, the L-curve as well as the distance distribution obtained by Tikhonov regularization are shown in Figure 5.16.

The distance distribution obtained in Q-band experiment clearly consists of two components. The model-free distance distribution was fitted using two Gaussians (Figure 5.17). The shorter distance (blue dashed line) as well as the longer distance (orange dashed line) are in a very good agreement with the distance distribution measured in X-band frequency. Also fractions of αS contributing to both distances fit those obtained in X-band experiment (Table 5.2).

Aiming to check the validity of the two obtained experimental distances for αS27/56, the MMM software[154] was applied and distance distributions between those investigated sites of αS, using the MTSL rotamer library was derived from the corresponding 175 K MD trajectory and compared with the experimental DEER data. The NMR structure of human micelle-bound αS (PDB 1XQ8)[47] was used as a template for the horseshoe-like αS conformation and a simple elongated helix model prepared with PyMOL software[192] was used for the extended helix form. Figure 5.18 shows that the theoretical distance distribution for horse-shoe αS form (in red) fits perfectly the shorter measured distance and the simulated MMM distances for a extended helix model (in blue) also is in a good agreement with the longer experimental distance obtained for αS27/56 double mutant (black line).
Figure 5.16: Q-band DEER data for αS27/56 in the presence of LUVs made from POPG lipids. (a) DEER time trace (black) and experimental intramolecular background decay (red); (b) Experimental DEER trace after background correction (black) fitted with Tikhonov regularization (red); (c) L-curve corresponding to DEER trace in (b) for α = 0.001, 0.01, 0.1, 1, 10, 100, 1000, 10000 and 100000, respectively. The optimal α-value can be determined unambiguously as α = 1000 (displayed in red) and was used for deriving the distance distribution shown in (d). (d) Model-free distance distribution derived by Tikhonov regularization (black solid line) with lower (black dotted line) and upper error estimates (black dashed line).
Figure 5.17: Q-band distance distribution of αS27/56 in the presence of POPG LUVs obtained by Tikhonov regularization (black solid line) fitted with two Gaussians (blue and orange dashed lines). Asterisks mark artifacts due to Tikhonov regularization (see Figure 5.16d).

Figure 5.18: Validation of the distance distribution obtained for doubly labeled αS27/56. The theoretical distance distributions between αS labeled sites 27 and 56 for the horseshoe-like model (red dashed line), and for the extended helix model (blue dashed line) was predicted by MTSL rotamer library using MMM software[154]. The presented distance distribution of αS27/56 (black solid line) were obtained by DEER experiment performed in Q-band. Asterisks mark artifacts due to Tikhonov regularization (see Figure 5.16d). The simulated distributions are normalized to the maxima of corresponding distance distributions obtained by Tikhonov regularization.
5.3 DISCUSSION

The main goal of the present study was to characterize αS binding properties and conformation features on artificial membranes. It was reported that the presence of unsaturated acids in the membrane composition increases the binding of αS on the neutral as well as the charged membranes [169]. It is known, that not only the membrane charge has a very strong influence on αS affinity [193, 49], but also the membrane curvature [194]. As a model system, LUVs made from different anionic POPG and zwitterionic POPC lipid compositions have been chosen. LUVs are 100 nm in diameter and are stable upon the interaction with αS, in opposite to the SUVs, which membrane structure and stability can be affected by αS interaction, leading to membrane leakage [50, 157].

Our data reveal a marked preference by αS for binding to negatively charged phospholipids (Figure 5.3 and 5.4), what is consistent with a number of published studies [195, 43, 196, 197]. This effect can be explained by electrostatic forces between the rich in lysine residues N-terminal part of the protein. In agreement with earlier studies [38, 20, 42, 46], it was found that membrane interaction is mediated by the N-terminal part of the protein, which contains seven imperfect repeats. In contrast, the C-terminal region of the protein remains unstructured even in the presence of membranes.

EPR spectroscopy employed in this work enables to look precisely at the binding in the proximity of the marked residue and provided a more differentiated view of the interaction of αS with the membranes than a global binding affinity measurements. The EPR results not only imply that the N-terminal part of αS could initiated the binding of these protein to the membranes, but also suggest that at lower surface charge densities the binding affinity of the regions closer to the N-terminal region of the protein is stronger that the regions distal from the N-terminus.

On small SDS micelles, αS is in an α-helical conformation resulting in a formation of two elongated helices interrupted by a loop region containing residues 38-44 [42, 46]. The mobility measurements data on positions 35 and 41, do not show an increased local binding affinity in comparison to the residues in the α-helical region, indicating that the both sites occurred in the α-helical ordered region. This is in good agreement with another study: αS adopts an uninterrupted N-terminal α-helix on artificial membranes [40, 198]. However, it has to be mentioned, that the very recent paper of Shvadchak et al. suggests [52] presence of a short flexible break close to residues 52-55 between two helical domains of αS. In this report it has been also suggested that αS adopts a helical conformation only in the repeat region and that residues 89-97 located after the last 11-amino acid repeat are membrane-immersed but are not in the helical conformation.
αS can efficiently discriminate membranes according to their physical properties, what suggests a high degree of selectivity in its binding and ability to migrate between membranes of different compositions. These αS properties play an important role in the proper localization as well as function of the protein in a cellular environment, e.g. on mitochondria.

The subcellular localization of αS in mitochondria is still unclear. In our study the inner and outer mitochondria membrane composition were mimicked to test the local binding affinity of αS to those artificial membranes. Figure 5.6 indicates that large regions of αS bind to LUVs mimicking the inner mitochondrial membrane but not to those mimicking the outer one, which lacks CL. The localization of αS on the inner membrane corresponds to the previous study on a translocation of the protein across membranes [199, 200, 201]. It was proposed that αS can translocate the plasma membrane and that in this process a critical role might play the N-terminal part of the protein containing the motifs important for the intracellular delivery of the protein [202, 22].

Since only the inner membrane contains CL, these observations stress on the role CL plays in the binding of αS. Cardiolipins are important molecules within the mitochondria and play key role in many mitochondrial processes found to be deficient in PD models, a like mitochondrial fusion, metabolite transport and protein complex stability [73, 203]. It was observed that loss of cardiolipin results in loss of mitochondrial membrane potential [204, 205] and cardiolipin requisite for proper electron transfer [206]. In summary, these phospholipids with four, rather than two, fatty acid tails are integral to many mitochondrial processes found to be deficient in models of PD.

Cardiolipin therefore proves to be an interesting target for αS toxicity. Because these phospholipids are characteristic component of the mitochondria membrane, a role of CL in the binding properties of αS was investigated, in which amounts of those lipids in the LUVs composition was gradually changed. The binding affinity study of nine singly labeled αS showed that the local binding affinity increase with the increasing CL concentration (Figure 5.7). Interestingly, a drastic jump of the αS binding occurs at the CL composition (ρ = 0.2) close to those in the inner mitochondria membrane, which serves as a direct confirmation of the importance of those phospholipids in the αS-mitochondria interaction.

αS is linked to early-onset PD by three point mutations, A30P, A53T and the later described E46K [173, 174, 175]. The point mutations are located in the N-amino-terminal repeats, the same region that binds to membranes. Therefore their influence on the binding affinity of αS to differential charged lipids membranes was investigated using SDSL in combination with EPR spectroscopy. This technique enables to study not only the overall binding affinity of the protein to the membranes, but also the local binding affinity around the attached spin label, what can leads to a more detailed view of the protein-membrane interactions.
αS in its monomeric state is known to bind to negatively charged membranes [20, 181, 40, 38, 182, 47] and the binding is triggered by the N-terminal part of the protein [156]. Our observations of A30P and A53T αS membrane interactions revealed that both mutants significantly perturb the local binding affinity of the protein to lipid vesicles (Figure 5.10 and 5.11). In comparison to A30P and A53T disease variants, a wild type αS shows a significantly higher binding affinity of the spin labels attached to the N-terminal region of the protein.

At physiologically relevant lipid composition (ρ = 0.3, Figure 5.11a) and vesicles mimicking inner mitochondrial membrane (Figure 5.12) the local binding affinity of both disease mutants is substantially smaller than for wild-type protein. Especially A30P shows drastically reduced binding in comparison to the A53T and the wild type αS (Figure 5.11a). Our results are consistent with the data reported in [180].

The data showing the inflection point in the dependency of the investigated labeled position (Figure 5.11b) suggested that the A30P mutation may slightly destabilize or alter the protein’s binding affinity around the site of mutation. This is reflected by the greatly reduced local binding affinity monitored by spin label attached to position 26, placed in close neighborhood of this disease mutation. A similar effect is observed for the A53T data, where the binding affinity considerably decreases in the proximity to the point mutation at the labeled position 41, but note worthy not for 56.

The A30P variant is less effective in binding (Figure 5.11), probably because of disruption of the N-terminal helical structure by the addition of proline [45]. It has been previously reported by others [178, 166, 38, 180] that the A30P has a lower affinity for lipid vesicles compared to the wild-type protein. It was also shown in an NMR study on micelle bound αS, the A30P disease mutant slightly destabilize and alter the protein’s helical structure around the site of mutation [177].

The results obtained for A53T disease variant show that also these point mutation has an influence of the binding properties of αS. The mutant is less effective in binding to membranes than the wild type protein (Figure 5.11). This may be a consequence of the more rapid oligomerization kinetics exhibited by the mutant relative to the wild-type protein [207]. An alternative possibility is that A53T mutant is structurally defective in binding to the membrane by having threonine at position 53 instead of alanine. Alanine is one of the strong α-helix forming residues whereas threonine is indifferent in α-helical propensity [208]. Considering the correlation between the α-helix formation of αS and membrane binding, the A53T point mutation may have a negative effect on the α-helix formation, resulting in reduced membrane binding. The binding of A53T mutant αS to phospholipids determined by CD spectroscopy was quantitatively identical to the wild-type protein [209]. This may be
due to the inability of the spectroscopy technique to quantitative small differences in lipid binding.

Another important issue addressed in the work was the structural characterization of membrane-bound αS. It is well established that αS is unfolded in solution and undergoes a conformational change into a helical structure upon interaction with membranes, where the 100 N-terminal residues of αS adopt an α-helical conformation, whereas the 40 C-terminal residues remain disordered [56]. However, the arrangement of the helical region of αS upon binding to the membrane surface is still controversial.

To answer the question, DEER experiments on a set of doubly labeled αS probes in the presence of POPG LUVs were performed. The advantage of the DEER method is yielding background-free signals, not being limited in complex size, and giving access to distance distributions. These features are essential for the results. Our data (Figure 5.15) demonstrate for the first time that αS coexist in a mixture of both horseshoe and extended helix forms on a quasi two-dimensional membranes surface. The fraction of the horseshoe conformation is significantly smaller (~ 20%) than that of the extended conformation, revealing a preference for the extended form.

Our results successfully integrate the findings of many diverse biophysical αS studies, mentioned in the Introduction. The protein is very sensitive to the exact experimental conditions and it is reasonable to infer that only small changes are needed to tip the balance between the horseshoe and extended helix forms. Therefore, different lipid compositions, vesicle sizes, or even subtle differences in preparation protocols may easily shift the equilibrium, providing a rationale for the different results found in previous studies [21, 51, 42, 53, 55, 46, 48, 50].

Lipid membranes are dynamic structures. It has been postulated that a bilayer remodeling can be a consequence of the interaction between lipid surface and proteins [210]. Recent paper by Mizuno et al. [211] reported that αS can induce membrane curvature and that it can convert large phospholipid vesicles into bilayer tubes and cylindrical micelles. On those membrane surfaces αS was found in an extended helix conformation. It was suggested that at higher αS concentration the cylindrical long micelles can be disarranged and highly curved and smaller rounded structures can be formed, on which αS adopts the horseshoe-like conformation. This report may explain the coexistence of the both αS conformations found in our study and also confirmed in [212].

The present study illustrates the large range of different structures that can be investigated by EPR spectroscopy. Besides studying the structural basis for the putative physiological functions of αS, SDSL in combination with EPR spectroscopy could also be used to investigate the conformational changes involved in the transition from unfolded or helical synuclein into oligomers or aggregates. It has been shown, that
membrane interaction of αS can facilitate the formation of such toxic species [213, 214]. Thus, the molecular understanding of the membrane-bound form, examine in this work might prove to be an important starting point for the understanding of the misfolding process that occurs in PD.
6.1 INTRODUCTION

One of the challenges in elucidating the structure and conformation of αS is to characterize the protein in its physiological environment. Since EPR spectroscopy in combination with site-directed spin-labeling is virtually background free it can be used to study αS properties not only on artificial membranes but even in a complex, physiological relevant environment, like on mitochondria from human cells (see section 2.5).

Since a long time it has been considered that αS and mitochondria are key players in the PD pathology and that the binding of αS to mitochondria is crucial [68, 215]. At present, it is still unclear how αS affects mitochondria. αS may associate with the mitochondrial membranes in different ways, but until now only a small subfraction of αS interacting with mitochondrial membranes has been poorly characterized [76].

In this chapter site-directed spin-labeling in combination with EPR spectroscopy supported by biochemical analysis and (immunogold) electron microscopy has been used to study the binding properties of αS. The question of the relevant structure of αS on mitochondria has been addressed with the DEER technique (section 3.1.2.1). The results indicate that the N-terminal part of the protein is crucial for binding and αS in the mitochondria bound state is in the α-helical conformation.

6.2 RESULTS

6.2.1 Binding affinity study of αS to isolated mitochondria

The αS conformational properties in a physiologically relevant environment were studied on mitochondria isolated from human embryonic kidney 293 (HEK293) cells, kindly provides by the Leist’s group from the Department of Biology, University of Konstanz. These cells are commonly used in αS studies [216, 217], because of many neuronal characteristics [218].

The purity of the mitochondrial fraction and stability of cellular organelles upon interaction with αS was proven with electron microscopy analysis. Figure 6.1a shows electron microscopy images of the negatively stained mitochondria, indicating that the isolated mitochondria fraction is homogeneous and does not contain other cellular components. In the next step, the isolated mitochondria were incubated with αS (1 µg/mL)
Figure 6.1: Immunoelectron microscopy of isolated mitochondria. (a) Overview of mitochondria isolated from HEK 293 cells. (b) A single mitochondrion treated and stained with αS (indicated by arrows). Experiment performed by Dr. Joachim Hentschel and PD Dr. Stefan Schildknecht in the Electron Microscopy Center, University of Konstanz.

for 1 h and washed five times to remove unbound αS. Binding was then visualized by staining with a monoclonal anti-αS antibody for 3 h at 293 K, the grids were then rinsed three times and incubated with a colloidal gold-conjugated secondary antibody (Figure 6.1b). The gold nanoparticles conjugated with the secondary antibody appear as black dots and indicate binding of αS to mitochondria.

The N-terminal part of αS plays a key role in the binding process of the protein, as it was in detail investigated and described in the previously chapter. It was shown that differences in lipid composition or membrane fluidity may result in different αS binding behavior [219]. Therefore the importance of the first part of the N-terminal region of the protein in membrane binding in a more physiological relevant system was verified. An αS variant lacking amino acids 2-11 was expressed and the binding of αS wild-type (αSwt) and αSΔ2−11 to isolated mitochondria using sodium dodecyl sulfate-polyacrylamide gel electrophoresis experiments were performed. Here, the outer mitochondrial membrane protein TOM20 was used as loading control. The results are shown in Figure 6.2. The band intensity at 17 kDa [220] of mitochondria bound αSwt increases with an increasing αSwt concentration in the range of 0.125-5 μg/mM αS. The observation that αSΔ2−11 does not bind to native mitochondria indicates the importance of N-terminal region for binding properties of αS, that is to say, the localized interactions are crucial for membrane binding under cellular conditions.

Quantitative binding studies of αS to mitochondria were performed by cw-EPR experiments at 293 K. Figure 6.3 shows the corresponding EPR binding experiments of singly labeled αS9 and αS27, and double labeled αS9/27 on mitochondria isolated from HEK293 cells. The spectra of αS in the presence of mitochondria (Figures 6.3b, 6.3d and 6.3f) clearly differs from that without mitochondria (Figures 6.3a, 6.3c and 6.3e). The spectra were simulated and the obtained spectral parameters are listed in Table 6.1. The spectral simulations indicate that under these
Figure 6.2: Binding study of $\alpha S_{\text{wt}}$ and $\alpha S_{\Delta 2-11}$ on mitochondria. Isolated mitochondria corresponding to a total protein concentration of 1 mg/mL were incubated with $\alpha S_{\text{wt}}$ and $\alpha S_{\Delta 2-11}$ at different concentrations. Quantitative data were obtained from three independent experiments and expressed as means ± the standard deviation (performed in the Leist’s group from the Department of Biology, University of Konstanz).

conditions approximately 68% of the protein molecules bind to the mitochondria.

It is worth to mention that the EPR binding study of $\alpha S$ to mitochondria was technically possible because of employing of protease inhibitor (see Figure A.41 in Appendix A). It was observed that during incubation of $\alpha S$ with mitochondria without the use of a protease inhibitor, complete and rapid degradation of $\alpha S$ occurs. The optimization of the incubation protocol allowing the binding measurements was performed by Hanne Gerding from the Department of Biology, University of Konstanz.

6.2.2 $\alpha$-helical conformation of $\alpha S$ bound to mitochondria

In order to unravel the conformation of $\alpha S$ bound to mitochondria, the doubly labeled $\alpha S$ mutant $\alpha S_{9/27}$ was studied. These labeling positions were chosen because they are in the N-terminal part of $\alpha S$ which possesses a high local binding affinity according to results presented in chapter 5. Figure 6.4a shows the visualization of the possible spin label orientations with respect to the protein molecule using the rotamer library approach (see section 3.1.3). The two labeled positions are expected to be in the same helix regardless of whether a single extended helix or a two-segment helix is formed [50, 47] and are pointing outwards into the aqueous environment with respect to the NMR structure [47]. The predicted distance distribution in Figure 6.4b is in very good agreement with the experimental distance distribution obtained for the double mutant on negatively charged artificial membranes, were $\alpha S$ is bound in $\alpha$-helical form [29, 38].

To monitor the conformation of $\alpha S$ bound to mitochondria EPR distance measurements were performed (Figure 6.5a). First, the distances of $\alpha S_{9/27}$ in solution (blue) as well as in the presence of artificial mem-
Figure 6.3: Experimental spectra (black circles) and corresponding fits of (a, b) $\alpha$S9, (c, d) $\alpha$S27 and (e, f) $\alpha$S9/27, in absence (a, c, e blue line) and in the presence of mitochondria (b, d, f red line). The obtained fit parameters are listed in Table 6.1.
Table 6.1: Parameters of spectral simulation of $\alpha S_9$, $\alpha S_{27}$ and $\alpha S_9/27$ free in solution and in the presence of mitochondria. Hyperfine interaction $A_{zz}$, rotational correlation time $\tau_r$, line width $lw$ and fraction $b$ of mitochondria bound state.

<table>
<thead>
<tr>
<th></th>
<th>$A_{zz}$ [MHz]</th>
<th>$\tau_r$ [ns]</th>
<th>$lw$ [mT]</th>
<th>$b$ $^\S$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha S_9$ in solution</td>
<td>107.58</td>
<td>0.35</td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td>$\alpha S_9$ on mitochondria</td>
<td>104.55</td>
<td>12.75</td>
<td>0.39</td>
<td>70%±5%</td>
</tr>
<tr>
<td>$\alpha S_{27}$ in solution</td>
<td>110.16</td>
<td>0.43</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td>$\alpha S_{27}$ on mitochondria</td>
<td>104.00</td>
<td>10.37</td>
<td>0.40</td>
<td>60%±5%</td>
</tr>
<tr>
<td>$\alpha S_9/27$ in solution</td>
<td>108.08</td>
<td>0.62</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td>$\alpha S_9/27$ on mitochondria</td>
<td>99</td>
<td>9.12</td>
<td>0.47</td>
<td>75%±5%</td>
</tr>
</tbody>
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$^\S$ The spectra of spin-labeled $\alpha S$ in the presence of mitochondria were fitted with two component fit. For the fast component, which reflects the unbound fraction of the protein the parameters obtained for corresponding spin-labeled $\alpha S$ in solution were taken and the $A_{zz}$, $\tau_r$, $lw$ and $b$ were fitted.

Figure 6.4: (a) Visualization of the rotamer approach used to theoretically predict (b) the distance distribution for $\alpha S_9/27$ in its membrane bound state. Purple balls show positions of the electron spins scaled according to populations of the respective rotamers. For the visualization and distance distribution simulation the NMR structure of human micelle-bound $\alpha S$ (PDB 1XQ8) was taken as a template. The asterisk marks artifact in the distance distributions due to Tikhonov regularization.
Figure 6.5: (a) Experimental DEER data for $\alpha$S9/27 after background correction in solution (blue thin line), in the presence of POPG LUVs (green thin line), and in the presence of mitochondria (red thin line) as well as corresponding fits (thick lines). (b) Corresponding distance distributions obtained by Tikhonov regularization (see section 3.1.2.1) of $\alpha$S9/27 in its intrinsically disordered state (blue dotted line), in its $\alpha$-helical state (green dotted line) and in the presence of mitochondria (red circles). The latter can be described by a superposition (black solid line) of the experimental distance distributions obtained for $\alpha$S9/27 in solution and in the presence of POPG LUVs. The asterisk marks artifact in the distance distributions due to Tikhonov regularization, for raw DEER data and the L-curve see Figure A.42-A.44 in Appendix A.

Branes made from POPG lipids (green) were measured. In the binding affinity study described in chapter 5 (Figure 5.4), it was shown that a quantitative binding of $\alpha$S occurs on the POPG LUVs. Figure 6.5b shows the corresponding distance distributions for $\alpha$S9/27 in its disordered state (blue dotted line) as well as in its alpha-helical state bound to artificial POPG membranes (green dotted line). The distance distribution obtained for $\alpha$S9/27 in the presence of POPG LUVs is in full agreement with the theoretically predicted distance distribution (Figure 6.4b) based on the NMR-structure [47].

The distance distribution obtained for $\alpha$S9/27 in the presence of mitochondria was obtained using a model-free Tikhonov regularization [148] (see Figure 6.5b, in red). This distance distribution can be described by a superposition (black solid line) of both the distance distribution of intrinsically disordered $\alpha$S9/27 and of the distance distribution of $\alpha$-helically membrane bound $\alpha$S9/27. The fraction of the latter (69%) corresponds quite well to the bound fraction as determined independently by the mobility measurements (Figure 6.3). We thus conclude that $\alpha$S binds $\alpha$-helically to mitochondria.
The goal of this chapter is to deepen knowledge of binding properties of \(\alpha S\) in a biological environment and to study the conformation of mitochondria-bound \(\alpha S\). Mitochondria are membranous organelles that have been suggested as cellular targets for \(\alpha S\) neurotoxicity [58, 67, 69]. However, the localization as well as the structural arrangement of \(\alpha S\) on mitochondria are still poorly characterized.

The number of N-terminal amphipatic repeats modulates the propensity of \(\alpha S\) to adopt an \(\alpha\)-helical conformation \textit{in vitro} [221]. The immunogold electron microscopy showed a high purity of the mitochondria isolated fraction and intact mitochondria upon binding of \(\alpha S\) (Figure 6.1). A biochemical analysis of \(\alpha S\) lacking amino acid 2-11 revealed that the N-terminal part of the protein is crucial for binding to native membranes (Figure 6.2). This findings are in agreement with data, demonstrating that the \(\alpha S_{\Delta 2-11}\) variant drastically reduced \(\alpha S\) toxicity in yeast and deletion of \(\alpha S\) N-terminal amino acids decreased \(\alpha\)-helical propensity and vesicle binding ability \textit{in vitro} [22].

To characterize the sub-localization of \(\alpha S\) experiments with artificial membranes, mimicking the inner and outer membrane of mitochondria, were shown in the previous chapter 5. The results showed that the protein is likely binding to the inner membrane, which contains cardiolipin (Figure 5.6). Devi et al. [76] reported that mitochondria-localized \(\alpha S\) is predominantly associated with inner mitochondria membrane in the human system, but also a small fraction of the protein was found on the outer mitochondria membrane of human brain. Simultaneously it has been suggested that \(\alpha S\) can get across the plasma membrane and in its translocation a critical role play the N-terminal part of the protein [202]. These results supported the findings on isolated mitochondria indicating the importance of the N-terminal of \(\alpha S\) in the interaction with biological membranes shown here.

EPR spectroscopy in combination with SDSL enables revealing the conformational properties of \(\alpha S\) on human mitochondria. Mobility measurements show that under the employed experimental conditions approximately 68% of the protein is bound to the organellic membrane surface (Figure 6.3). To reveal the conformation of \(\alpha S\) on the investigated system DEER experiments were performed. Analysis of the obtained distance distributions of doubly labeled \(\alpha S_{9/27}\) on isolated mitochondria, supported by distance measurements of \(\alpha S_{9/27}\) in solution and in the presence of negatively charged LUVs (Figure 6.5), enables the conclusion that the \(\alpha\)-helical binding mode that is reported for \(\alpha S\) interacting with artificial membranes also holds true in the complex environment of intact mitochondria.

Herein, for the first time, EPR distance measurements of \(\alpha S\) on a protein bound to an organelle are shown. \(\alpha S\) bound to human mitochondria
adopts an α-helical conformation (Figure 6.5). This can open a new field in structural characterization of IDPs in its physiological relevant environment and might also contribute to understanding the pathological role of αS in the mitochondrial dysfunction in PD.
Due to the conformational flexibility of IDPs, it is very challenging to study the structure and dynamics of these proteins by using traditional analytic tools. X-ray crystallography and NMR spectroscopy offer atomic-level information of the examined object, but have limitations associated with the size, dynamics, and polymorphism complexity of IDPs in membrane-systems.

EPR spectroscopy in combination with SDSL is an alternative approach for accessing local structural and dynamic information of IDPs like αS by means of probing dynamics and coupling of stable nitroxide radicals, attached to biomolecular sites of interest. Its unique benefit is that a measurement is not intrinsically limited by size or complexity of protein and its environment. Continuous wave (cw) EPR line shape analysis can track conformational changes of protein segments and their interactions with membranes and at the same time the local structure of the protein with respect to the lipid bilayer surface can be determined. A pulsed dipolar EPR method, namely the DEER technique, provides structural information in various biological environments, by measurements of distance distributions, like mitochondria studied in this work.

In this work, various EPR techniques were employed to characterize the membrane interaction of αS, one of the canonical systems among the IDPs and at the same time the main player in PD progression. Although the exact function of the protein is unknown, it has been suggested that the interaction of αS with membranes plays a role not only in the normal function of the protein but also in the pathology of PD.

In Chapter 5, αS on artificial membranes is characterized. The EPR results do not only imply that the N-terminal part of the αS could initiate the binding of the protein to the membranes, but also suggests that, at lower surface charge densities, the binding affinity of the regions closer to the N-terminal region of the protein is stronger than the regions distal from the N-terminus. In addition, the binding properties of two αS disease variants, A30P and A53T, were examined, indicating that the point mutation significantly perturb the local binding affinity of the protein to lipid vesicles. The study using LUVs with lipid compositions mimicking the inner and outer mitochondria membrane showed that αS binds to membranes mimicking the inner mitochondria membrane only, most likely due to the CL content. The distance measurements experiment with a set of doubly spin labeled αS probes demonstrated, for the first time, that αS coexist in a mixture of both horseshoe and extended helix forms on a quasi two-dimensional membranes surface.
Chapter 6 describes the binding and conformational properties of αS in a physiological relevant system. In this study, isolated mitochondria from human HEK293 cells were used. The examination reveals that the binding of αS on mitochondria is triggered by the N-terminal part of the protein. With the aid of EPR distance measurements, it was shown for the first time that αS bound to human mitochondria adopts an α-helical conformation.

The results presented in this thesis show that SDSL EPR has developed as a powerful tool in order to study structure and dynamics of bio-macromolecules, like the intrinsically disordered protein αS. The results may contribute significantly to the efforts to determine the details and structural consequences of αS-membrane interactions and may to help to understand its physiological as well as its pathological role in PD.
ZUSAMMENFASSUNG

Aufgrund der konformationellen Flexibilität der intrinsisch ungeordnete Proteine (Intrinsically Disordered Proteins, IDPs) stellt es eine große Herausforderung dar, Struktur und Dynamik dieser Proteine mit traditionellen analytischen Verfahren zu studieren. So ermöglichen die Röntgenstrukturanalyse und Kernspinresonanz-Spektroskopie es zwar, Informationen auf atomarer Ebene zu erlangen, jedoch weisen sie Limitierungen in Bezug auf Größe, Dynamik, Polymorphismus und Komplexität der IDPs in Membran-Systemen auf.


αS ist eines der kanonischen Systeme unter den IDPs und gleichzeitig um einen Hauptakteur der Parkinsonschen Krankheit (Parkinson disease, PD). Obwohl die exakte Funktion des Proteins noch unbekannt ist, wurde vorgeschlagen, dass die Wechselwirkung von αS mit Membranen nicht nur in der physiologisch Funktionsweise des Proteins eine Rolle spielt, sondern auch in der Pathologie der PD.

In Kapitel 5 wird αS auf künstlichen Membranen charakterisiert. Die Ergebnisse implizieren nicht nur, dass der N-terminale Teil der αS die Bindung des Proteins an die Membran initiiert könnte, sondern zeigen auch auf, dass die Bindungssaffinität der N-terminal Regionen, stärker ist als die der eher C-terminal Regionen. Zusätzlich wurden die Bindungseigenschaften zweier αS-Mutationen, A30P und A53T, untersucht, was darauf hinwies, dass diese Punktmutation signifikante Störungen der lokalen Bindungssaffinität hervorrief. Es konnte beobachtet werden, dass αS an Membranen bindet, die die innere Membran der Mitochondrien imitieren, nicht jedoch solche, die die äußere Membran imitieren, höchstwahrscheinlich aufgrund des CL Gehalts. Zum ersten Mal wurde gezeigt, dass αS in einer Mischung aus Hufeisen und ausgestreckte helikaler Form auf einer quasi zweidimensionalen Membranoberfläche bindet.

Die Ergebnisse, die in dieser Arbeit präsentiert wurden, zeigen, dass mit SDSL EPR die Struktur und Dynamik des intrinsisch ungeordneten Proteins αS aufgeklärt werden kann. Die Ergebnisse werden dazu beitragen, die Details und strukturellen Konsequenzen von αS-Membran-Wechselwirkungen zu verstehen und könnte damit helfen, seine normale sowie pathologische Rolle in PD zu verstehen.
Figure A.1: DLS data of POPG LUVs before (black) and after (red) addition of αS (protein:lipid ratio 1:250). The particle size distribution consisting of a number of logarithmically spaced size classes on the X-axis with the relative percentage of particles in each size based on the volume they occupy on the Y-axis.

Figure A.2: DLS data of LUVs mimicking the inner mitochondrial membrane (POPC/POPE/CL/Cl in 2:1.3:1:06 molar ratio) before (black) and after (red) addition of αS (protein:lipid ratio 1:250). The particle size distribution consisting of a number of logarithmically spaced size classes on the X-axis with the relative percentage of particles in each size based on the volume they occupy on the Y-axis.
Figure A.3: DLS data of LUVs mimicking the outer mitochondrial membrane (POPC/POPE/Cl in 4:2:0.9 molar ratio) before (black) and after (red) addition of αS (protein:lipid ratio 1:250). The particle size distribution consisting of a number of logarithmically spaced size classes on the X-axis with the relative percentage of particles in each size based on the volume they occupy on the Y-axis.

Figure A.4: Experimental spectra (circles) and corresponding fits (red line) of the nine investigated αS derivatives labeled with MTSL in solution.
Figure A.5: Experimental spectra (circles) and corresponding fits (red line) of the αS9 in the presence of LUVs with different surface charge density ($\rho = [\text{POPG}]/([\text{POPG}] + [\text{POPC}])$).
Figure A.6: Experimental spectra (circles) and corresponding fits (red line) of the αS18 in the presence of LUVs with different surface charge density ($\rho = [\text{POPG}]/([\text{POPG}] + [\text{POPC}])$).
Figure A.7: Experimental spectra (circles) and corresponding fits (red line) of the $\alpha$S27 in the presence of LUVs with different surface charge density ($\rho = [\text{POPG}]/([\text{POPG}]+[\text{POPC}])$).
Figure A.8: Experimental spectra (circles) and corresponding fits (red line) of the αS35 in the presence of LUVs with different surface charge density ($\rho = [\text{POPG}]/(\text{[POPG]} + \text{[POPC]})$).
Figure A.9: Experimental spectra (circles) and corresponding fits (red line) of the \( \alpha_{S41} \) in the presence of LUVs with different surface charge density \( \rho = [\text{POPG}] / ([\text{POPG}] + [\text{POPC}]) \).

Figure A.10: Experimental spectra (circles) and corresponding fits (red line) of the \( \alpha_{S56} \) in the presence of LUVs with different surface charge density \( \rho = [\text{POPG}] / ([\text{POPG}] + [\text{POPC}]) \).
Figure A.11: Experimental spectra (circles) and corresponding fits (red line) of the αS69 in the presence of LUVs with different surface charge density ($\rho = |\text{POPG}|/(|\text{POPG}| + |\text{POPC}|)$).
Figure A.12: Experimental spectra (circles) and corresponding fits (red line) of the \( \alpha S_{90} \) in the presence of LUVs with different surface charge density (\( \rho = [\text{POPG}]/([\text{POPG}] + [\text{POPC}]). \)
Figure A.13: Experimental spectra (circles) and corresponding fits (red line) of the αS140 in the presence of LUVs with different surface charge density ($\rho = [\text{POPG}]/([\text{POPG}] + [\text{POPC}])$. 

(a) $\rho = 0$   (b) $\rho = 0.1$   (c) $\rho = 0.2$   (d) $\rho = 0.3$

(e) $\rho = 0.4$   (f) $\rho = 0.45$   (g) $\rho = 0.5$   (h) $\rho = 0.8$

(i) $\rho = 1$
Figure A.14: Experimental spectra (circles) and corresponding fits (red line) of spin-labeled αS, A30P and A53T in the presence of LUVs imitating the inner mitochondrial membrane made from POPC/POPE/CL/Cl lipid composition in 2:1.3:1:0.6 molar ratio.
Figure A.15: Representative experimental spectra (circles) and corresponding fits (red line) of spin-labeled at positions 9 and 90 αS, A30P and A53T in the presence of LUVs imitating the outer mitochondrial membrane made from POPC/POPE/Cl lipid composition in 4:2:0.9 molar ratio.
Figure A.16: Experimental spectra (circles) and corresponding spectral simulations (red line) of spin-labeled αS single cysteine mutants in the presence of LUVs containing different cardiolipin (CL) concentration resulting in different charge surface density ρ = [CL]/([CL] + [POPC]).
Figure A.17: Experimental spectra (circles) and corresponding spectral simulations (red line) of spin-labeled αS single cysteine mutants in the presence of LUVs containing different cardiolipin (CL) concentration resulting in different charge surface density $\rho = [\text{CL}]/([\text{CL}] + [\text{POPC}])$. 

αS9

αS18

αS27

αS35

αS41

αS56

αS69

αS90

αS140

$\rho = 0.2$  $\rho = 0.1$  $\rho = 0.05$
Figure A.18: Experimental spectra (circles) and corresponding fits (red line) of the \( \alpha_{59} \text{A30P} \) in solution (a) and in the presence of LUVs with different surface charge density \( \rho = [\text{POPG}]/([\text{POPG}]+[\text{POPC}]) \) (b-h).

Figure A.19: Experimental spectra (circles) and corresponding fits (red line) of the \( \alpha_{518} \text{A30P} \) in solution (a) and in the presence of LUVs with different surface charge density \( \rho = [\text{POPG}]/([\text{POPG}]+[\text{POPC}]) \) (b-h).
Figure A.20: Experimental spectra (circles) and corresponding fits (red line) of the αS27 A30P in solution (a) and in the presence of LUVs with different surface charge density ($\rho = \frac{[POPG]}{([POPG] + [POPC])}$) (b-h).

Figure A.21: Experimental spectra (circles) and corresponding fits (red line) of the αS35 A30P in solution (a) and in the presence of LUVs with different surface charge density ($\rho = \frac{[POPG]}{([POPG] + [POPC])}$) (b-h).
Figure A.22: Experimental spectra (*circles*) and corresponding fits (*red line*) of the $\alpha_{S41 \ A30}$ in solution (a) and in the presence of LUVs with different surface charge density ($\rho = [\text{POPG}]/([\text{POPG}] + [\text{POPC}])$) (b-h).

Figure A.23: Experimental spectra (*circles*) and corresponding fits (*red line*) of the $\alpha_{S56 \ A30}$ in solution (a) and in the presence of LUVs with different surface charge density ($\rho = [\text{POPG}]/([\text{POPG}] + [\text{POPC}])$) (b-h).
Figure A.24: Experimental spectra (circles) and corresponding fits (red line) of the \( \alpha_{S69} A_{30}P \) in solution (a) and in the presence of LUVs with different surface charge density (\( \rho = \frac{[POPG]}{([POPG] + [POPC])} \)) (b-g).

Figure A.25: Experimental spectra (circles) and corresponding fits (red line) of the \( \alpha_{S90} A_{30}P \) in solution (a) and in the presence of LUVs with different surface charge density (\( \rho = \frac{[POPG]}{([POPG] + [POPC])} \)) (b-h).
Figure A.26: Experimental spectra (circles) and corresponding fits (red line) of the \( \alpha S_{140} A_{30} \) in solution (a) and in the presence of LUVs with different surface charge density \( (\rho = [\text{POPG}]/([\text{POPG}] + [\text{POPC}])) \) (b-h).

Figure A.27: Experimental spectra (circles) and corresponding fits (red line) of the \( \alpha S_{9} A_{53} T \) in solution (a) and in the presence of LUVs with different surface charge density \( (\rho = [\text{POPG}]/([\text{POPG}] + [\text{POPC}])) \) (b-h).
Figure A.28: Experimental spectra (circles) and corresponding fits (red line) of the \(\alpha S_{18}A_{53}T\) in solution (a) and in the presence of LUVs with different surface charge density \((\rho = [\text{POPG}] / ([\text{POPG}] + [\text{POPC}])\) (b-h).

Figure A.29: Experimental spectra (circles) and corresponding fits (red line) of the \(\alpha S_{27}A_{53}T\) in solution (a) and in the presence of LUVs with different surface charge density \((\rho = [\text{POPG}] / ([\text{POPG}] + [\text{POPC}])\) (b-h).
Figure A.30: Experimental spectra (circles) and corresponding fits (red line) of the $\alpha S_{35} A_{53} T$ in solution (a) and in the presence of LUVs with different surface charge density ($\rho = [POPG]/([POPG] + [POPC])$) (b-h).

Figure A.31: Experimental spectra (circles) and corresponding fits (red line) of the $\alpha S_{41} A_{53} T$ in solution (a) and in the presence of LUVs with different surface charge density ($\rho = [POPG]/([POPG] + [POPC])$) (b-h).
Figure A.32: Experimental spectra (circles) and corresponding fits (red line) of the αS69A53T in solution (a) and in the presence of LUVs with different surface charge density ($\rho = [POPG]/([POPG] + [POPC])$) (b-h).

Figure A.33: Experimental spectra (circles) and corresponding fits (red line) of the αS69A53T in solution (a) and in the presence of LUVs with different surface charge density ($\rho = [POPG]/([POPG] + [POPC])$) (b-h).
Figure A.34: Experimental spectra (*circles*) and corresponding fits (*red line*) of the αS90 A53T in solution (a) and in the presence of LUVs with different surface charge density ($\rho = [\text{POPG}]/([\text{POPG}] + [\text{POPC}])$) (b-h).

Figure A.35: Experimental spectra (*circles*) and corresponding fits (*red line*) of the αS140 A53T in solution (a) and in the presence of LUVs with different surface charge density ($\rho = [\text{POPG}]/([\text{POPG}] + [\text{POPC}])$) (b-h).
Figure A.36: cw-EPR spectra at 120K of doubly labeled αS with POPG LUVs containing 25% spin labeled αS and 75% unlabeled wild-type αS in protein-lipid ratio of 1/250. The singly labeled diamagnetically diluted αS9 was chosen as a standard to normalized intensities and positions of the spectra using the xepr Bruker software.

(a) αS9/69  
(b) αS9/90  
(c) αS18/69  
(d) αS18/90

Figure A.37: The experimental X-band DEER traces before background correction of the doubly labeled αS cysteine mutants in the presence of POPG LUVs. The background fit is shown in red.
Figure A.38: DEER trace for singly labeled αS9 bound to POPG LUVs (black) and fit of a homogeneous two-dimensional background model (red).

Figure A.39: (a) DEER time trace of αS27/56 bound to POPG LUVs (black) together with the fitted intramolecular background decay (red); (b) L-curve relating to DEER trace of αS27/56 in (a) for \( \alpha = 1, 10, 100, 1000, 10000, \) and 100000, respectively. The optimal \( \alpha \)-value can be determined unambiguously as \( \alpha = 1000 \) (displayed in red) and was used for deriving the distance distribution shown in Figure 5.15b.
Figure A.40: (a) DEER trace of αS27/56 bound to POPG LUVs after background correction: data (black), fit assuming a distance distribution consisting of two Gaussians (red) and obtained by Tikhonov-regulization (blue). (b) Comparison of distance distributions derived by two Gaussians fit (red) and Tihkonov regularization (blue). The data obtained for Tihkonov regularization were already presented in Figure 5.15.

Figure A.41: Selective degradation of αS can be prevented with the use of SigmaFAST protease inhibitor. Western blots showing time course of degradation of αS on mitochondria in the absence (left) and in the presence of protease inhibitor (right) compared to the outer mitochondrial membrane protein TOM20, which is not degraded under these conditions and hence serves as a suitable loading control (performed by Hanne Gerding).
Figure A.42: DEER data of doubly labeled αS9/27 in solution. (a) DEER time trace (black) and experimental intermolecular background decay (red); (b) Experimental DEER trace after background correction (black) fitted with Tikhonov regularization (red); (c) L-curve relating to DEER trace in b) for α=1, 10, 100, 1000, 10000 and 100000, respectively. The optimal α-value can be determined unambiguously as α=1000 (displayed in red) and was used for deriving the distance distribution shown in: (d) model free distance distribution derived by Tikhonov regularization (blue solid line) with lower (blue dotted line) and upper error estimate (blue dashed line).
Supporting Information

Figure A.43: DEER data of doubly labeled $\alpha$S9/27 in the presence of LUVs made from POPG lipids. (a) DEER time trace (black) and experimental intermolecular background decay (red); (b) Experimental DEER trace after background correction (black) fitted with Tikhonov regularization (red); (c) L-curve relating to DEER trace in b) for $\alpha=1, 10, 100, 1000, 10000$ and 100000, respectively. The optimal $\alpha$-value can be determined unambiguously as $\alpha=1000$ (displayed in red) and was used for deriving the distance distribution shown in: (d) model free distance distribution derived by Tikhonov regularization (green solid line) with lower (green dotted line) and upper error estimate (green dashed line).
Figure A.44: DEER data of doubly labeled αS9/27 on mitochondria. (a) DEER time trace (black) and experimental intermolecular background decay (red); (b) Experimental DEER trace after background correction (black) fitted with Tikhonov regularization (red); (c) L-curve relating to DEER trace in b) for α=1, 10, 100, 1000, 10000 and 100000, respectively. The optimal α-value can be determined unambiguously as α=1000 (displayed in red) and was used for deriving the distance distribution shown in: (d) model free distance distribution derived by Tikhonov regularization (black solid line) with lower (black dotted line) and upper error estimate (black dashed line).
Table A.1: Simulation parameters of spin-labeled $\alpha S$ single cysteine mutants in solution and in the presence of LUVs showed in Figure A.4–A.13. The spectra in solution were simulated with the fast component $S_A$ in which the EasySpin function garlic and $A_{zz} = 111.185$ MHz were used. The spectra in the presence of LUVs were simulated with two component fit, where for the fast component $S_A$ the fit parameters obtained for the single mutant in solution was taken and the rotational correlation time $\tau_r$ and fraction $b$ of the spectral slow component $S_B$ were simulated using the EasySpin function chili. For $S_B$ the $A_{zz}$ was fitted and resulted in $A_{zz} = 100 \pm 6$ MHz. For all simulations $A_{xx} = A_{yy} = 13$ MHz and $g = [g_x \ g_y \ g_z] = [2.00906 \ 2.00687 \ 2.003]$ were chosen.

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§ Was fitted only with the fast component $S_A$. 

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Table A.2: Simulation parameters of spectral component $S_B$ describing the rotational mobility for spin-labeled $\alpha S$ single cysteine mutants in the presence of LUVs containing different cardiolipin compositions ($\rho = [CL]/([CL] + [POPC])$). Rotational correlation time $\tau_r$, hyperfine interaction $A_{zz}$, and fraction $b$ of the slow component $S_B$. For the fast component $S_A$ the raw experimental data of the respectively single cysteine mutants measured in solution shown in Figure A.4 were taken. The parameter $b$ was systematically varied to test in which range acceptable simulations of the data were obtained to determine the error margins.

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$\S$ All spectra of $\alpha S140$ and in the presence of LUVs $\rho = 0$ were well described only with the fast component $S_A$. 

Table A.3: Fraction $b$ of spectral component $S_B$ for spin-labeled $\alpha$S, A30P and A53T disease variants in the presence of LUVs mimicking the inner and outer mitochondrial membrane. Rotational correlation time $\tau_r$ and hyperfine interaction $A_{zz}$ of the slow component $S_B$ obtained on CL LUVs ($\rho = 1$) and for the fast component $S_A$ the raw experimental data of the respectively single cysteine mutants measured in solution were taken. The parameter $b$ was systematically varied to test in which range acceptable simulations of the data were obtained to determine the error margins.

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<td>0.01±0.01</td>
<td>0.00±0.04</td>
</tr>
<tr>
<td>$\alpha$S140$^\S$</td>
<td>0.01±0.02</td>
<td>0.01±0.01</td>
<td>0.00±0.02</td>
</tr>
</tbody>
</table>

$^\S$ All spectra of $\alpha$S140 and in the presence of LUVs mimicking the outer mitochondrial membrane were well described only with the fast component $S_A$. 

$^{\text{April 16, 2015, at 16:27}}$
Table A.4: Simulation parameters of spectral component $S_A$ describing the rotational mobility for singly spin-labeled A30P and A53T in solution. Rotational correlation time $\tau_r$, hyperfine interaction $A_{zz}$ and linewidth $lw$.

<table>
<thead>
<tr>
<th></th>
<th>$A_{zz}$ [MHz]</th>
<th>$lw$ [mT]</th>
<th>$\tau_r$ [ns]</th>
</tr>
</thead>
<tbody>
<tr>
<td>A30P</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\alpha_{S9}$</td>
<td>107.93</td>
<td>0.19</td>
<td>0.39</td>
</tr>
<tr>
<td>$\alpha_{S18}$</td>
<td>108.42</td>
<td>0.19</td>
<td>0.36</td>
</tr>
<tr>
<td>$\alpha_{S27}$</td>
<td>108.14</td>
<td>0.13</td>
<td>0.45</td>
</tr>
<tr>
<td>$\alpha_{S35}$</td>
<td>108.49</td>
<td>0.18</td>
<td>0.46</td>
</tr>
<tr>
<td>$\alpha_{S41}$</td>
<td>108.30</td>
<td>0.19</td>
<td>0.44</td>
</tr>
<tr>
<td>$\alpha_{S56}$</td>
<td>108.22</td>
<td>0.19</td>
<td>0.37</td>
</tr>
<tr>
<td>$\alpha_{S69}$</td>
<td>108.06</td>
<td>0.14</td>
<td>0.36</td>
</tr>
<tr>
<td>$\alpha_{S90}$</td>
<td>108.31</td>
<td>0.19</td>
<td>0.32</td>
</tr>
<tr>
<td>$\alpha_{S140}$</td>
<td>108.48</td>
<td>0.19</td>
<td>0.16</td>
</tr>
<tr>
<td>A53T</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\alpha_{S9}$</td>
<td>107.89</td>
<td>0.14</td>
<td>0.49</td>
</tr>
<tr>
<td>$\alpha_{S18}$</td>
<td>108.02</td>
<td>0.14</td>
<td>0.27</td>
</tr>
<tr>
<td>$\alpha_{S27}$</td>
<td>108.18</td>
<td>0.14</td>
<td>0.48</td>
</tr>
<tr>
<td>$\alpha_{S35}$</td>
<td>108.21</td>
<td>0.13</td>
<td>0.54</td>
</tr>
<tr>
<td>$\alpha_{S41}$</td>
<td>108.08</td>
<td>0.14</td>
<td>0.52</td>
</tr>
<tr>
<td>$\alpha_{S56}$</td>
<td>108.40</td>
<td>0.14</td>
<td>0.36</td>
</tr>
<tr>
<td>$\alpha_{S69}$</td>
<td>108.18</td>
<td>0.14</td>
<td>0.29</td>
</tr>
<tr>
<td>$\alpha_{S90}$</td>
<td>108.12</td>
<td>0.14</td>
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</tr>
<tr>
<td>$\alpha_{S140}$</td>
<td>108.54</td>
<td>0.14</td>
<td>0.21</td>
</tr>
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</table>
Table A.5: Simulation parameters of spectral component $S_B$ describing the rotational mobility for spin-labeled $\alpha S$ single cysteine mutants in the presence of LUVs made from different POPG/POPC compositions ($\rho = [\text{POPG}]/([\text{POPG}] + [\text{POPC}])$). Rotational correlation time $\tau_r$, hyperfine interaction $A_{zz}$ and fraction $b$ of the slow component $S_B$. For the fast component $S_A$ the raw experimental data of the respectively single cysteine mutants measured in solution were taken. The parameter $b$ was systematically varied to test in which range acceptable simulations of the data were obtained to determine the error margins.

<table>
<thead>
<tr>
<th>A30P</th>
<th>$\rho = 1$</th>
<th>$\rho = 0.8$</th>
<th>$\rho = 0.5$</th>
<th>$\rho = 0.4$</th>
<th>$\rho = 0.35$</th>
<th>$\rho = 0.3$</th>
<th>$\rho = 0.8$</th>
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<tbody>
<tr>
<td>$\alpha S_9$</td>
<td>98.35</td>
<td>5.18</td>
<td>0.89±0.84</td>
<td>0.8±0.1</td>
<td>0.45±0.05</td>
<td>0.4±0.3</td>
<td>0.2±0.1</td>
</tr>
<tr>
<td>$\alpha S_{18}$</td>
<td>96.74</td>
<td>6.79</td>
<td>0.90±0.05</td>
<td>0.76±0.1</td>
<td>0.41±0.05</td>
<td>0.23±0.07</td>
<td>0.07±0.07</td>
</tr>
<tr>
<td>$\alpha S_{27}$</td>
<td>98.80</td>
<td>4.70</td>
<td>0.82±0.05</td>
<td>0.73±0.07</td>
<td>0.13±0.05</td>
<td>0.00±0.1</td>
<td>0.02±0.08</td>
</tr>
<tr>
<td>$\alpha S_{35}$</td>
<td>100.92</td>
<td>3.99</td>
<td>0.91±0.06</td>
<td>0.87±0.09</td>
<td>0.53±0.07</td>
<td>0.33±0.05</td>
<td>0.25±0.07</td>
</tr>
<tr>
<td>$\alpha S_{41}$</td>
<td>96.70</td>
<td>6.08</td>
<td>0.9±0.1</td>
<td>0.82±0.07</td>
<td>0.37±0.05</td>
<td>0.21±0.07</td>
<td>0.04±0.05</td>
</tr>
<tr>
<td>$\alpha S_{56}$</td>
<td>94.03</td>
<td>6.79</td>
<td>0.91±0.07</td>
<td>0.80±0.05</td>
<td>0.26±0.08</td>
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<td>0.00±0.06</td>
</tr>
<tr>
<td>$\alpha S_{69}$</td>
<td>96.68</td>
<td>6.15</td>
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<td>0.73±0.12</td>
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<td>0.00±0.06</td>
</tr>
<tr>
<td>$\alpha S_{90}$</td>
<td>104.92</td>
<td>3.16</td>
<td>0.9±0.1</td>
<td>0.7±0.1</td>
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<td>0.00±0.05</td>
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</tr>
<tr>
<td>$\alpha S_{140}$</td>
<td>-</td>
<td>-</td>
<td>0.00±0.02</td>
<td>0.00±0.02</td>
<td>0.00±0.02</td>
<td>0.00±0.02</td>
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Table A.5: (continued)

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<th>$\alpha$518</th>
<th>$\alpha$527</th>
<th>$\alpha$535</th>
<th>$\alpha$541</th>
<th>$\alpha$556</th>
<th>$\alpha$569</th>
<th>$\alpha$590</th>
<th>$\alpha$5140$^\dagger$</th>
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<tr>
<td>A53T</td>
<td>$\rho = 1$</td>
<td>$\rho = 0.8$</td>
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<td>$\rho = 0.4$</td>
<td>$\rho = 0.35$</td>
<td>$\rho = 0.3$</td>
<td>$\rho = 0$</td>
<td></td>
</tr>
<tr>
<td>$S_B$</td>
<td>$A_{zz}$ [MHz]</td>
<td>$\tau_\tau$ [ns]</td>
<td>$b$</td>
<td>$S_B$</td>
<td>$A_{zz}$ [MHz]</td>
<td>$\tau_\tau$ [ns]</td>
<td>$b$</td>
<td>$S_B$</td>
</tr>
<tr>
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<td>-------------</td>
<td>-------------</td>
<td>-------------</td>
<td>-------------</td>
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</tr>
<tr>
<td>99.48</td>
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<td>0.9±0.1</td>
<td>0.52±0.08</td>
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<td>0.31±0.05</td>
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<td>0.25±0.05</td>
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<td>0.93±0.08</td>
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<td>0.87±0.07</td>
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<td>0.81±0.08</td>
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<td>0.16±0.02</td>
<td>0.03±0.02</td>
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<tr>
<td>94.78</td>
<td>7.20</td>
<td>0.73±0.03</td>
<td>0.73±0.03</td>
<td>0.32±0.05</td>
<td>0.14±0.1</td>
<td>0.03±0.05</td>
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<td>0.00±0.02</td>
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</tr>
<tr>
<td>-</td>
<td>-</td>
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<td>0.00±0.02</td>
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<td>0.00±0.02</td>
<td>0.00±0.02</td>
<td>0.00±0.02</td>
<td>0.00±0.02</td>
</tr>
</tbody>
</table>

$^\dagger$ All spectra of $\alpha$5140 and in the presence of LUVs $\rho = 0$ were well described only with the fast component $S_A$. 
PHOSPHOLIPID STRUCTURES

CL

POPE

POPC

POPG
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>αS</td>
<td>α-Synuclein</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>Ch</td>
<td>cholesterol</td>
</tr>
<tr>
<td>CL</td>
<td>cardiolipin</td>
</tr>
<tr>
<td>cw</td>
<td>continuous wave</td>
</tr>
<tr>
<td>DEER</td>
<td>double electron-electron resonance</td>
</tr>
<tr>
<td>DLS</td>
<td>dynamic light scattering</td>
</tr>
<tr>
<td>EDFS</td>
<td>echo-detected field sweep</td>
</tr>
<tr>
<td>EPR</td>
<td>electron paramagnetic resonance</td>
</tr>
<tr>
<td>FRET</td>
<td>fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>HEK293</td>
<td>human embryonic kidney 293</td>
</tr>
<tr>
<td>IDP</td>
<td>intrinsically disordered protein</td>
</tr>
<tr>
<td>LUV</td>
<td>large unilamellar vesicle</td>
</tr>
<tr>
<td>MD</td>
<td>molecular dynamics</td>
</tr>
<tr>
<td>MMM</td>
<td>multiscale modeling of macromolecules</td>
</tr>
<tr>
<td>MTSL</td>
<td>(1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl)-methane-thiosulfonate</td>
</tr>
<tr>
<td>NAC</td>
<td>non amyloid component</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson disease</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein Data Bank</td>
</tr>
<tr>
<td>POPC</td>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>POPE</td>
<td>1-hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
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<td>-------------</td>
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<td>POPG</td>
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<td>PROXYL</td>
<td>3-Maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxy</td>
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<tr>
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<td>sodium dodecyl sulfate</td>
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<tr>
<td>SDSL</td>
<td>site-directed spin labeling</td>
</tr>
<tr>
<td>SUV</td>
<td>small unilamellar vesicle</td>
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<tr>
<td>wt</td>
<td>wild-type</td>
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