Spectroscopic Investigation of the Intrinsically Disordered Protein Alpha-Synuclein \textit{in vitro} and in the Cell

Dissertation zur Erlangung des akademischen Grades eines Doktors der Naturwissenschaften (Dr. rer. nat.)

vorgelegt von

Julia Teresa Cattani

Universität Konstanz
Mathematisch-Naturwissenschaftliche Sektion
Fachbereich Chemie

Tag der mündlichen Prüfung: 01. Dezember 2017
1. Referent: Prof. Dr. Malte Drescher
2. Referent: Prof. Dr. Christine Peter
Table of Contents

1 Motivation

2 The intrinsically disordered protein alpha-Synuclein
  2.1 aS in solution .................................................. 3
  2.2 aS and membrane binding ...................................... 4
  2.3 The (patho)physiological role of aS .......................... 6
  2.4 aS disease mutants .............................................. 8
    2.4.1 Disease mutants in solution ............................ 8
    2.4.2 Interaction with membranes ............................. 8
    2.4.3 Aggregation behavior ..................................... 8
  2.5 aS aggregation .................................................. 9
  2.6 In-cell spectroscopy of aS ..................................... 10

3 Determining structure and dynamics of intrinsically disordered proteins (IDPs) 11
  3.1 Spectroscopic Techniques for IDP characterization ........ 11
  3.2 Circular dichroism (CD) spectroscopy ........................ 12
  3.3 Electron paramagnetic resonance (EPR) spectroscopy ....... 13
    3.3.1 Site-directed spin labeling .................................. 13
    3.3.2 Continuous wave (cw)-electron paramagnetic resonance (EPR) .... 14
    3.3.3 Pulsed EPR .................................................. 15
    3.3.4 In-cell EPR .................................................. 19

4 Study design
  4.1 From artificial systems into the cell .......................... 21
  4.2 Spin label strategy for EPR experiments ....................... 22
  4.3 Spin labeling positions for EPR spectroscopy experiments ... 22
  4.4 Influence of the spin label on aS characteristics .......... 23

5 Peptide fragments of aS in the presence of artificial membranes 25
  5.1 Peptide fragments of aS ........................................ 25
  5.2 Revealing the N-terminal membrane binding motifs of aS .... 26
  5.3 Conclusion ...................................................... 33

6 aS and the disease variants in the presence of artificial membranes 35
  6.1 Locally resolved binding of aS and its disease variants on artificial membranes ... 35
# Table of Contents

6.2 Preliminary Work ........................................ 36
   6.2.1 αS and disease variants on artificial membranes with varying charges .......................... 36
   6.2.2 αS and disease variants interacting with inner mitochondrial membrane mimicking LUVs .................................. 40
6.3 Improvement of cw-EPR spectral fits by using expanded fitting parameters ...... 42
6.4 CD spectroscopy of αS and disease variants on negatively charged membranes .. 44
6.5 Conclusion ............................................. 50

7 αS in the presence of biological membranes ........................................ 51
   7.1 αS binding onto yeast mitochondria ............................................. 51
   7.2 Locally resolved binding of wt-αS in the presence of yeast mitochondria ....................... 51
   7.3 Structural investigation of membrane bound αS ........................................... 56
   7.4 Conclusion ............................................. 62

8 αS and the disease variants in the cell .............................................. 63
   8.1 Room-temperature in-cell EPR of αS and its disease variants ........................................... 63
   8.2 The intracellular EPR signal ...................................................... 63
   8.3 Kinetics and signal shape ......................................................... 66
   8.4 Spatial distribution of the injected volume in the cell ...................................................... 68
   8.5 Microviscosity in oocytes of *Xenopus laevis* .................................................. 69
   8.6 Conformation of the oocyte-injected wt-αS mutants ................................................... 70
   8.7 Conformation of the oocyte-injected αS disease variants ............................................. 75
   8.8 Conclusion ............................................. 77

9 Details of Experiments and Analysis .................................................. 79
   9.1 Methods and Materials ............................................................ 79
      9.1.1 Protein expression of αS ...................................................... 79
      9.1.2 Site Directed Spin Labeling of αS .................................................. 79
      9.1.3 Peptide fragments of αS sequence .............................................. 80
      9.1.4 Preparation of protein fibrils ................................................. 80
      9.1.5 Atomic Force Microscopy ....................................................... 80
      9.1.6 Preparation of vesicles ......................................................... 81
      9.1.7 Sample preparation for cw-EPR with LUVs for conformational comparison ................. 81
      9.1.8 Dynamic light scattering (DLS) ................................................. 81
      9.1.9 Circular Dichroism Spectroscopy .............................................. 81
      9.1.10 Isolation of yeast mitochondria and control experiments .............................. 82
      9.1.11 Sample preparation of αS in the presence of isolated yeast mitochondria ................ 83
      9.1.12 Preparation of and microinjection into Xenopus laevis oocytes ......................... 83
      9.1.13 Continuous wave(cw)- EPR measurements ......................................... 84
      9.1.14 DEER EPR experiment .......................................................... 85
   9.2 Data analysis .............................................................. 85
      9.2.1 Analysis of CD spectra ......................................................... 85
      9.2.2 Data analysis of cw-EPR spectra .............................................. 86
| 9.2.3 | Data analysis of DEER EPR | 90 |
| 10 Summary | | 93 |
| 11 Zusammenfassung | | 95 |
| A Appendix | | 97 |
| References | | 115 |
| Abbreviations | | 137 |
| Published results | | 139 |
| Danksagung | | 141 |
CHAPTER 1

Motivation

The urge to contain the symptoms, to positively affect or reverse the course of a disease and even prevent diseases from occurring is a fundamental drive in today’s research of neurodegenerative diseases like Parkinson’s, Alzheimer’s or Huntington’s disease. In order to prevent the proteins in our own brain to start acting against our body, we need to know what triggers these proteins to stop function physiologically and to start to behave in a pathogenic way. A key in this task is to understand the exact physiological role of these proteins, which is after decades of research still not clear.

With proteins, function is closely related to the secondary and tertiary structure and the dynamics of the peptide chain. This is especially true for intrinsically disordered proteins (IDP), which are unstructured or coil-like in solution and adopt any specific secondary structure only when interacting with membranes or other macromolecules. Therefore, studying structure and dynamics of these proteins with spectroscopic techniques is a valuable contribution for solving the open questions about normal function of disease related intrinsically disordered proteins.

Alpha-Synuclein (aS) is an IDP related with the Parkinson’s disease. Aim of this work is to add a piece to the puzzle of the Parkinson protein alpha-Synuclein by getting new insights into the membrane binding behavior and learning more about the intracellular structure of aS and its disease variants. This is not an easy task, because sometimes aS does not behave or react as expected and is hard to handle. Thus, people name aS also “The Beast” or “Protein from Hell”, which is surely not as nice a name as “The Chameleon”.

Chapter 2

The intrinsically disordered protein alpha-Synuclein

The 140 amino acid (aa), intrinsically disordered protein (IDP) alpha-Synuclein (aS) comprises three characteristic regions within its primary sequence – the N-terminus, the NAC region and the C-terminus (see Figure 2.1) [1, 2]. It shows a wide range of different interaction behavior, dynamics and structures [3]. IDPs are characterized as a class of proteins, that lack a stable three dimensional structure in solution but adopt certain secondary and tertiary structures when interacting with their binding partners [4–6]. They are often associated with human diseases, in case of aS the Parkinson’s disease [7–9]. IDPs are involved in cell signaling and regulation [10]. The subsequent sections (Sections 2.1 to 2.6) will describe the different characteristics in more detail.

![Figure 2.1.: Schematic representation of aS protein domain structure.](image)

2.1. aS in solution

Like all IDPs, aS adopts a disordered, coil-like structure (see Figure 2.2) in solution [11]. However, several studies of aS in solution revealed the existence of transient structures and long-range interactions between different domains in the protein [12–14]. Dedmon et al. found long-range interactions between residues 30-100 in the central region and residues 120-140 in the negatively charged C-terminal part of aS by using spin-label NMR and ensemble molecular dynamics simulations [12]. The Blackledge group performed NMR experiments in search for a description of aS in solution [13]. They found two requirements: 1) consideration of random
2. The intrinsically disordered protein alpha-Synuclein

Figure 2.2.: Schematic picture of a possible disordered structure of aS in solution.

A combination of various NMR experiments was used by Bertoncini et al. in order to identify a highly hydrophobic part of the NAC region (amino acids 85-95) interacting with the C-terminal part (aa 110-130) [14]. Thus, aS has not a typical random coil structure in solution but a more complex behavior and a structure, that is more compact than expected for a random coil conformation [12].

2.2. aS and membrane binding

A central role in revealing the (patho)physiological function of aS is the investigation of its membrane binding behavior [15]. Figure 2.3 schematically depicts possible aS interactions with lipids in vivo.

In order to study aS upon membrane interaction and to avoid uncertainties of in vivo systems, at first studies with artificial, well controlled systems were performed. Numerous studies revealed that aS binds alpha-helically negatively charged membranes involving residues 1-100, the N-terminal part of aS and the NAC region (see Figure 2.1) [2, 17–23]. However, membrane binding is initiated in the N-terminal residues and the affinity is the highest for the N-terminus of aS [2, 23, 24]. The last 40 amino acids (the C-terminus) do not interact with membranes but were reported to bind metal ions [20, 25].

Ulmer et al. were able to extract a structure of aS bound to sodium dodecyl sulfate (SDS) micelles (see Figure 2.4) [20]. The revealed structure consisting of two antiparallel helices connected with a loop is in agreement with the findings of numerous other groups [26–28]. However, this horseshoe conformation with two helices was not the only conformation found for aS upon binding to membranes. Various studies found also an extended helix [22, 29, 30] and Robotta et al. even found a coexistence of both conformations [31]. The obtained structures of the various studies mostly depended on the used artificial membrane systems (bilayers, SDS micelles, small unilamellar vesicles (SUVs), large unilamellar vesicles (LUVs)). Whereas usage of micelles and SUVs resulted in a broken helix conformation, usage of LUVs gave either an extended helix or both conformations at once. These findings are consistent with studies that proved differences
2.2. αS and membrane binding

Taking a closer look at individual dynamics within the helix/helices, it was shown that despite αS being well embedded in the lipid bilayer there are still segments with shallower penetration and higher flexibility. Up to at least residue 90 [22, 23, 36, 37], the amino acids are mainly embedded in the lipid membrane and more tightly bound with exception of certain positions like residues 39, 48, 63, 75 and 90. Jao et al. determined the accessibilities of the extended conformation and found a superhelical twist within the helix [22]. They used electron paramagnetic resonance (EPR) spectroscopy, as well as circular dichroism (CD) spectroscopy and computational structural refinement based on the EPR data. The helix twist allows the lysine residues to interact with the membrane surface due to an orientation perpendicular to the helix axis. Pfeferkorn et al. could show that the first four amino acids are sufficient for membrane binding [38]. The group performed fluorescence and neutron reflectometry measurements of different
peptide fragments (the first 4, 6, 10 and 15 residues, respectively) from the N-terminal end of αS, where a tryptophan was introduced at residue 4.

On the one hand, the membrane influences the αS conformation. On the other hand, αS remodels the shape of the membrane and the lipid arrangement [39–45]. Madine et al. report protein causing clustering of acidic lipid-enriched and acidic lipid-deficient domains induced by lateral segregation [39]. Hähl et al. found out that αS insertion into a supported bilayer reduces the thickness of this bilayer [39]. For the pathophysiological role of αS its potential to introduce membrane curvature seems to be an essential part regarding vesicle interaction [46, 47]. Although these results help understanding the (patho)physiological role of αS, one has to keep in mind that these in vitro measurements simplify the real conditions and can hardly reflect the complexity of an in-cell environment.

In cells, αS was also found localized in and at mitochondria (a cell organelle, which consists of a double membrane) [48–52] as well as at the mitochondria-associated endoplasmic reticulum membranes (MAM) [53, 54]. An EPR study with αS binding to isolated mitochondria by Robotta et al. revealed that αS binds firstly, in an α-helically conformation and secondly, to the inner mitochondrial membrane [55]. In contrast, another study found αS not only on the inner membrane but also on the outer membrane, the cleft between both membranes, the cristae (folds in the inner membrane to enhance the surface of the inner membrane) and in the mitochondria matrix [56]. Banerjee et al. demonstrated that αS causes loss of mitochondrial transmembrane potential as well as a decrease in phosphorylation capacity [57]. Both findings hint at the disease related role and pathological behavior of αS also involving mitochondria (see Section 2.3).

2.3. The (patho)physiological role of αS

As mentioned before, αS counts to a class of IDPs that are all related to diseases, respectively [7] – in case of αS a neurodegenerative disease called Parkinson’s disease. Alpha-synuclein is present in the human brain’s cytosol in huge amounts of about 1% [16, 58] of the cytosolic proteins. But in spite of several decades of research the physiological function is still not clear [3]. Figure 2.5 depicts the proposed physiological conformations and functions of αS within the human body [59]. Several studies found a large fraction of αS in healthy cells as a soluble monomer [60–64]. In these studies either in-cell NMR spectroscopy was used or isolated αS was analyzed by polyacrylamide gel electrophoresis, mass spectrometry and CD spectroscopy. Both approaches can not exclude the simultaneous existence of fractions of membrane bound or oligomeric αS. There is also a theory that at least a small fraction forms an α-helically folded tetramer in the cytosol, which is in equilibrium with the monomer [65–67] and resists aggregation [65].

However, αS is also found associated with membranes at presynaptic terminals [59, 68, 69], involved in maintaining the distal pool of synaptic vesicles [70]. McLean et al. demonstrated a tight membrane interaction of αS in intact neuronal cell cultures [71]. Knock-out studies of αS in mice resulted in mice that were viable but exhibited minor neurological deficits [72]. Additionally, a reduced resistance of knock-out mice against a certain neurotoxin (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, MPTP) was found [73].

Furthermore, αS was identified to directly promote SNARE (soluble N-ethyl maleimide-sensitive-factor attachment receptor)-complex assembly through C-terminal interac-
2.3. The (patho)physiological role of αS

Figure 2.5.: Schematic depiction of the (patho)physiological role of αS. Graphic taken from [3].

Interaction with synaptobrevin-2 (one of the SNARE proteins) and N-terminal binding to phospholipids [74, 75]. The pathological side of αS is the involvement in the neurodegenerative Parkinson’s disease. The occurrence of dopaminergic loss and Lewy bodies, which consist of fibrillar inclusions of αS and can be found in the substantia nigra, are characteristic for Parkinson’s disease [76–78]. (Pore-like) oligomers and fibrils of αS are directly related with the pathological role of αS (see Figure 2.5) and are caused by aggregation of monomeric protein. Despite the fact that fibrils are found in the Lewy bodies, the toxic species of αS seems not to be the fibrils but the oligomers [79–81].

Another aspect of the disease role of αS, mentioned already in 2.2, is the interaction with mitochondria. Overexpression of αS and Parkinson’s disease influence the function and dynamics of mitochondria [82–85]. Alpha-synuclein can disturb the mitochondrial fusion and can cause fragmentation of mitochondria as shown by Kamp et al. [86] and Nakamura et al. [87]. The disease mutants A30P and A53T showed the same behavior, however affected mitochondria could be rescued by wt-αS in a study by Guardia-Laguarta et al. [53]. Another study showed that A53T influences the mitochondria only in an age-dependent manner [83]. In addition, αS inhibits the function of the mitochondrial complex I situated in the inner mitochondrial membrane [88], and increases mitochondrial autophagy [89, 90]. The endoplasmatic reticulum (ER) is closely related and connected with mitochondria via the mitochondria-associated endoplasmatic reticulum membranes (MAM). Therefore, perturbations in the endoplasmatic reticulum (ER) functions [91], called ER stress and found in PD patients [92], seems to be connected to the impairing of mitochondria by αS. Furthermore, increased αS levels block the ER to Golgi membrane trafficking [93, 94].
2.4. aS disease mutants

After finding accumulations of Parkinson’s disease in certain families, a sequence analysis in the aS gene [95] revealed a mutation that causes the substitution of residue 53 of aS from alanine to threonine (A53T) [96]. After this, more point mutations of aS were found. They either cause an early- or late-onset of this familial and inheritable form of Parkinson’s disease: A18T [97], A29S [97], A30P [98], E46K [99], H50Q [100, 101], G51D [102] and A53E [103]. Figure 2.6 shows a schematic representation of the aS sequence with the positions of the disease mutations, which are all situated in the N-terminal part of aS.

![Schematic representation of aS protein domain structure with positions of the disease mutations marked in red.](image)

2.4.1. Disease mutants in solution

An EPR study by Georgieva et al. showed that the transient structures observed for wild-type aS are not strongly influenced by the mutations A53T, E46K, A530P [104]. However, the Coskuner group performed molecular dynamics simulations along with thermodynamic calculations and found that theses transient structures disappear for A53T [105], are lessened for A30P [106] and increase for the E46K mutant [106].

2.4.2. Interaction with membranes

Not only the behavior in solution and the A30P, E46K, A53T mutations still can adopt broken helix conformation [104] but A30P has decreased affinity and a higher local disorder [104, 107]. E46K shows increased membrane binding, whereas A30P has a decreased affinity and the binding affinity of A53T is comparable or only slightly lessened to that of the wild-type [32, 105, 106, 106–109]. It was shown that A30P influences not only the membrane binding behavior around the point mutation but alters the membrane binding ability of the whole protein [106].

2.4.3. Aggregation behavior

The differences in the transient structures of the disease mutants are consistent with another study stating that altered site-specific microenvironment structures in the soluble form of the PD-associated mutants may attribute to their different aggregation propensities [110]. The point mutations A18T [116], A29S [116], A30P [112–114], E46K [117–119], A53E [111] and A53T [111–115, 120] show accelerated aggregation behavior compared to wt-aS, with A53T having a much
2.5. aS aggregation

Fibrillization of aS is a key feature of the Lewy bodies - the hallmark of Parkinson’s disease. Therefore, it is important to study the aggregation behavior of aS and the initiation of the misfolding. Studies proved the formation of endogenous inclusions after adding preformed fibrils of aS to cells that were (over)expressing aS [127–129]. Such preformed fibrils could act as seeds for aggregation in other cells after transmission took place [130–133]. Fibrilization is initiated in the central region of aS, the NAC region, although it is also involved in membrane interactions [1]. Different species during fibrillation can occur. Uversky et al. found a partially folded intermediate occurring during fibril formation [134], whereas Giehm et al. and Zijlstra et al. found oligomers consisting of ~30 monomers during the fibril formation [80, 135]. Lorenzen et al. further investigated this species and determined them as stable oligomers, which are not able to elongate fibrils any more.

Figure 2.7.: Schematic picture of aggregated aS forming β-sheets.

Several studies dealt with a connection between membranes and the formation of fibril seeds or direct aggregation. Lee et al. demonstrated a membrane bound aggregation of aS to membrane-associated oligomers [137]. Drescher et al. further described these aggregates to consist of at least two aS molecules in close contact that probably form larger aggregates with an ordered arrangement [138]. Alpha-synuclein disrupts lipid membranes while aggregating on the membrane surface [80, 139–141] and even forms porelike structures consisting of hexamers of aS [142]. Various studies also showed that aS oligomers can bind to vesicles and permeabilize the membrane depending on the lipid composition [143–146]. Chaudhary et al. proved that POPC/POPS vesicles can not be damaged by aS oligomers but pre-existing damages can be stabilized and expanded [147]. The aS peptide fragment 71-82 located in the NAC region of aS (see figure 2.1) was found to undergo irreversible self-aggregation in the presence of anionic vesicles [148]. Deleting this sequence in the full length aS protein leads to the loss of this larger effect than A30P [113]. H50Q was found to increase the overall aggregation propensity and strongly stabilize aS fibrils [121, 122].

Flagmeier et al. not only investigated the overall fibrillization but also differentiated between the rate of the fibril elongation, which showed no significant change for the mutants and the rates of the initiation of fibril production induced by lipid vesicles. Not only the fibril formation is influenced by the disease mutations, but also the fibril shapes. Van Raaij et al. revealed smaller diameters for fibrils of A30P and E46K compared to wt-aS [124]. The secondary structure consisting of β-strands and turns is unchanged in A30P fibrils [125] but altered in A53T fibrils [126] compared to wt-aS fibrils, respectively.
membrane-bound aggregation behavior and therefore the lipid membranes remain undisturbed [139].

The Subramaniam group focused on a certain region within the aa sequence, residues 52-55 (VATV) [149]. This linker within the KTKEGV motif proved to be important for the αS aggregation due to a slower aggregation of a delete mutant missing these four residues and this mutant decreasing the fibril formation rate of wt-αS. By use of two truncated variants, αS(1-108) and αS(1-124), the influence of the C-terminus on the aggregation behavior was studied by Hoyer et al. [115]. The partly or whole removal of the C-terminus lead to a significantly accelerated aggregation. Hoyer et al. concluded that, via formation of intramolecular contacts, the amino acids 109-140 regulate the aggregation of αS.

Uversky et al. investigated the influence of various metal ions on αS and found that some metals (e.g. Al\(^{3+}\), Fe\(^{3+}\), Co\(^{3+}\), Cu\(^{2+}\)) can enhance the rate of fibril formation [150]. In addition low concentrations of Al\(^{3+}\) even induced the forming of αS fibrils.

Other studies focused on the capability of not only helical αS but also αS that aggregates to remodel membranes [41, 141]. The structure of αS fibrils was determined to consist of β-strands and turn regions [151–153] as mentioned before in Section 2.4 and schematically depicted in Figure 2.7.

### 2.6. In-cell spectroscopy of αS

A combination of in-cell NMR and two EPR distance constraints revealed that ~90% ± 10% wt-αS is intrinsically disordered in the cell [62–64]. However, the Selenko group was only able to detect αS in solution by NMR experiments, and concluded that the absence of line broadening in the spectra ruled out any stable membrane interactions. They used electroporation of non-neuronal cells (A2780, HeLa) and neuronal cells (RCSN-3, B65, SK-N-SH) to introduce the either \(^{15}\)N isotope-enriched or spin-labeled αS [64]. Distance measurements of αS labeled at residues 42 and 122 as well as 24 and 122, respectively, revealed only a broad distance distribution as expected for unstructured αS.

A fluorescence study in HeLa cells by Roberti et al. revealed αS aggregates that appeared as amyloid aggregates of spheroidal morphology rather than Lewy bodies [154]. The timescale during which in cell aggregation occurred was 48 hours, much longer as in the study of Theillet et al..
3.1. Spectroscopic Techniques for IDP characterization

Knowing the structure of a protein is important for making functional predictions, for interpreting results obtained by characterization studies and to unravel protein ligand interactions. Crystallography and solution NMR are the classical approaches to solve and unravel protein structures [155]. Due to the absence of secondary structure elements of IDPS (other than random coil-like structure) in solution and a large conformational variety depending on the environment and their interactions with other macromolecules, there is the need to use other techniques to get structural and dynamical information about IDPs.

Techniques like circular dichroism (CD) spectroscopy [156–160], Infrared (IR) spectroscopy [161–163], EPR spectroscopy in combination with site-directed spin labeling (see Section 3.3.1) [22, 23, 164–171] and fluorescence measurements [35, 140, 154, 172–174] are useful tools for dynamical and structural as well as interaction investigations of IDPs. Infrared spectroscopy was used for example to investigate the functional activity of the IDP LEA7, which protects enzymes against dehydration [175]. A combination of IR spectroscopy and computational techniques is used to interpret the amide-I band for secondary structure analysis [163, 176].

The förster resonance energy transfer (FRET) is used for distance measurements by fluorescence spectroscopy to resolve protein structures and dynamics of IDPs [177–183]. However, this technique has limitations and drawbacks compared to EPR distance measurements, like bulkier and much larger labels and the presence of a background signal.

Standard solution NMR experiments are problematic with IDPs due to the non-existence of a defined structure in solution and the limited sensitivity. [184]. Despite these limitation NMR is a valuable tool to either study the structures [159, 185, 186] or the dynamics [187–190] of IDPs. Furthermore, NMR is used for in-cell measurements [63, 64, 191–196], although detection of especially large proteins or the interaction of proteins with other macromolecules or cellular components is challenging because of the slow tumbling of the proteins [184]. A combination of
3. Determining structure and dynamics of intrinsically disordered proteins (IDPs)

Several NMR experiments can be necessary to investigate IDPs [197].

Often a combination of several different spectroscopy techniques is needed to analyze the IDP of interest or to answer the research question [174, 198–202]. EPR along with (polarized) IR spectroscopy and CD spectroscopy was used to study aggregation processes and oligomers of αS [114, 148, 203, 204].

In the following sections CD spectroscopy and EPR spectroscopy will be further explained because they were the tools used to study αS in this work.

3.2. Circular dichroism (CD) spectroscopy

CD spectroscopy is a fast and easy to handle tool to study protein conformations. CD analyzes the difference (ellipticity θ) between the absorption of left- and right-handed circularly polarized light. Therefore, a spectrum is only detectable if the sample is chiral (optically active) which is the case with proteins and peptides. However, also other (macro)molecules like DNA, RNA and carbohydrates are optically active and can be analyzed for different forms or enantiomers [205]. The CD signal derived from proteins and peptides originates from the electronic transitions of the peptide backbone as well as from aromatic side chains [205]. With proteins and peptides the CD spectra reflect the amount of random coil, α-helical, β-sheet and β-turn content of the protein sample. The spectra can be quantitatively analyzed for their secondary structure contents [206].

![Figure 3.1.: CD spectra of poly-L-lysine for representative secondary structures. Graphic taken from reference [206].](image)

The alpha-helical content can be determined using either the mean residue molar ellipticity value at 222 nm [41, 207] or a software for determination of all secondary structure fractions by use of databases of known measured protein conformations (e.g. CDtool, CDPro, Dichroweb, BeStSel) [208–211]. All these methods require the knowledge of the exact protein concentration and the path lengths of the cuvettes [210, 212].
3.3. Electron paramagnetic resonance (EPR) spectroscopy

3.3.1. Site-directed spin labeling

In order to make a diamagnetic protein or other macromolecule detectable with EPR spectroscopy it can be modified with a radical probe, also called spin label. This procedure to introduce a spin label, which is typically a nitroxide [213, 214], into a protein is called site-directed spin labeling (SDSL) [215, 216]. The desired residue needs to be altered into a cysteine via mutagenesis while native cysteines need to be substituted with another amino acid, if the residue could be accessed by the spin label during the labeling reaction. Figure 3.2 shows three different nitroxide spin labels. MTSL ((1-Oxyl-2,2,5,5-tetramethylpyrroline-3-methyl)methanethiosulfonate) (a) is the most common nitroxide label used for SDSL [217–223]. However, the attachment of Proxyl (3-Maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxy) (b) is more stable against reducing environments due to the formation of a thioether bond with the cysteine [170, 224, 225]. Figure 3.3 depicts the labeling reactions for MTSL forming a disulfide bond (a) and Proxyl (b) forming a thioether bond. 4-Oxo-Tempo is a nitroxide label that undergoes no labeling reaction and is therefore used as contrast agent for EPR imaging [226, 227].

In cases where the classical SDSL approach can not be used, e.g. there are too many natural cysteines in the protein sequence or the natural cysteines can not be altered without harming the protein integrity, it is necessary to use different labels. Tyrosine can be used as an alternative labeling residue in combination with an isoindoline-based nitroxide [228]. Unnatural amino acids, e.g. TOAC (2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid) [229] and TOPP (3,3,5,5-tetra-methyl-2,6-dioxo-4-oxylpiperazin-1-yl)-l-phenylglycine) [230], which carry a nitroxide radical on their own and are more rigid than MTSL labeled cysteine, can be introduced during solid-phase peptide synthesis into the protein sequence. A drawback of this labeling technique is that peptide synthesis limits the size of the macromolecule. Kucher et al. are expressing GFP with an unnatural amino acid, that can be labeled with a nitroxide via click chemistry in the cell after biosynthesis [231] for use in in vivo studies [231]. An even more elegant way to introduce EPR active unnatural amino acids into a protein is via genetically encoding of the spin-labeled amino acid (aa) and protein labeling during the protein biosynthesis [232]. This approach is especially promising for in vivo EPR measurements, but has also the needs for labels that are stable against the reducing cell environment.

Other types of labels are bulkier than nitroxides, but show also higher stability against reduction. Gd$^{3+}$-chelates have a molar mass $M$ about 600 g mol$^{-1}$ in comparison to molar mass $M$ of a nitroxide of about 240 g mol$^{-1}$ [213, 233–237]. There are different tags used for chelating
3. Determining structure and dynamics of intrinsically disordered proteins (IDPs)

Figure 3.3.: Site-directed spin labeling reaction of a protein (orange) cysteine residue with MTSL (a) and Proxyl (b).

the Gd-label like DOTA (4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) [238], PyMTA (4-vinyl-PyMTA) [235, 239] or derivatives of DOTA [234, 236]. These labels are also attachable to the protein via a cysteine and are better suited for especially high-field EPR distance measurements compared to nitroxides [234, 240].

Another class of bulkier, but stable labels (compared to nitroxides) are carbon-centered triarylmethyl (trityl) radicals (molar mass $M = 1054–1280 \text{ g mol}^{-1}$) [241, 242]. Trityls are suitable for room temperature EPR distance measurements due to longer electron spin relaxation times [243]. Yet, these spin labels showed aggregation with membranes [244] and by now only distances up to 5 nm were detectable [242, 245].

3.3.2. Continuous wave (cw)-EPR

A spectrum of a spin-labeled macromolecule can often be described by the $g$ factor tensor of the spin label with $g = [g_x, g_y, g_z]$, the hyperfine coupling tensor $A$ with $A = [A_{xx}, A_{yy}, A_{zz}]$, the linewidth $l$ of the spectral lines and the rotational correlation time $\tau_R$ describing the re-orientation dynamics of the label with respect to the external magnetic field [246].

Figure 3.4 shows a standard nitroxide spectrum, consisting of three characteristic lines, with different $\tau_R$ values. The spectrum for $\tau_R = 10 \text{ ps}$ (blue) shows a fast motional spectrum of e.g. a free nitroxide label which depicts the isotropic borderline case, whereas the spectrum $\tau_R = 1 \text{ ns}$ (red) represents a spectrum of, for example, a spin labeled protein, with the first and third peak clearly decreased in amplitude and additionally the third peak clearly broadened. The spectrum for $\tau_R = 3 \text{ ns}$ shows slow tumbling of the nitroxide label caused e.g. by binding of the
labeled protein to a membrane. The rotational correlation time gives the rotational mobility of the spin label, e.g. attached to a protein. Therefore, changes of $\tau_R$ directly report on mobility changes of the spin label. The altered spin label mobility of a spin-labeled protein can be caused by changes in the protein dynamics or protein structure in the proximity of the labeled sites. The term dynamics implies any time-dependent changes in atomic coordinates of the protein, which is in general caused by the large ensemble of conformational transitions [247]. Thus, via observing changes of the spin label mobility at specific residues, it is possible to detect changes in the dynamics or the structure of an IDP [220, 248–252].

### 3.3.3. Pulsed EPR

The most common pulsed EPR experiment for determining distances between two spins labels is the four-pulse double electron-electron resonance (DEER) experiment [253–255]. It is commonly used to measure the distance distribution between two labeled position within one macromolecule [55, 252, 256, 257]. The transverse relaxation time is a crucial factor for pulsed EPR experiments and increases for nitrooxides and Gd$^{3+}$ drastically with lower temperatures, which is the main reason for measuring pulsed EPR at cryogenic temperatures. The distance is obtained as a distribution caused by the spin label rotamers (a representation of the conformational space of the spin label) [258] and conformational flexibility of the macromolecule. Distances between 1.8 to 10 nm are accessible [259, 260] due to limitations in signal-to-noise and limiting relaxation times of the spins for long distances. Shorter distances are accessible through the dipolar spectral line broadening of a cw-EPR spectrum [261].

The measured signal of a DEER experiment, the deer trace, contains the contribution from the magnetic dipole-dipole coupling $\omega_{dd}$ between two spins, which is inversely proportional to the cube of the distance (see Equation (3.1)). Figure 3.6 illustrates the interactions of two spins in an external magnetic field. The blue spin (spin$_{pump}$, pump spin) is flipped by 180° by a $\pi$-pulse at the pump frequency. As a consequence the local magnetic field of the blue spin is altered and therefore also the magnetic field at the location of the cyan spin (spin$_{obs}$, observer spin).
3. Determining structure and dynamics of intrinsically disordered proteins (IDPs)

Figure 3.5.: Pulse sequence and time delays used for performing a four pulse DEER.

![Pulse sequence and time delays](image)

Figure 3.6.: (a) Local magnetic field of spin\textsubscript{pump} with a spin\textsubscript{obs} near by. (b) Altered local magnetic field of spin\textsubscript{pump}, caused by inversion of the spin. The arrows indicate the orientation of the magnetic moment of the spins.

The cyan spin is observed at the observer frequency (see Figure 3.5). The inverted pump spin influences the echo intensity of the observer spin due to the dipolar coupling.

\[
\omega_{dd} = \frac{1}{r^3} \frac{\mu_0 g_1 g_2 \mu_B^2}{4\pi h} (3 \cdot \cos^2 \theta - 1)
\] (3.1)

The dipol-dipol interaction can be described by Equation (3.1), with \( r \), the distance between the two spins, the isotropic g values \( g_1 \) and \( g_2 = 2.0055 \), \( \mu_B \), the Bohr magneton and the dependence of the dipolar spectrum on the angle \( \theta \) between the magnetic field direction and the spin-spin vector. \( g_1 \) and \( g_2 \) are approximated by the isotropic value of 2.0055, respectively [262]. In frozen solutions with macroscopic isotropic systems the angle \( \theta \) is assumed to scale with \( \sin \theta \) for all orientations.

By alternating the time position of the pump pulse the echo can be detected in a time dependent manner. Therefore, the deer trace is the echo signal \( V(t) \) as a function of time, which contains a background factor \( B(t) \) derived from homogeneously distributed spins in space, that reflect no distances within the same macromolecule and the contribution of the form factor \( F(t) \) from interactions of the spins within the same macromolecule. After background correction, either by assuming a homogeneous background with dimensions depending on the sample conditions (in solution, bound to a surface) or by using an experimental background from a singly
labeled sample, the form factor is obtained, which can be analyzed for the contained distance distribution by using an analysis software [263].

In Figure 3.7 (a,b) DEER traces for a distance distribution with a small mean distance (3 nm) and a large width as well as for a distance distribution with a large mean distance (6 nm) and a small width (blue) are depicted. In black the fit for a three dimensional homogenous background decay, respectively, is shown. The form factor $F(t)$, obtained after background correction of the deer trace, is shown in (c) for both deer traces. The resulting distance distributions are depicted in (d). The shape of the form factor curve reflects the distance and the distribution. If the decay of the curve is fast, the distance contained is short, whereas strong oscillations represent a narrow distance distribution. The form factor decays to a non-zero value $1 - \Delta$ with $\Delta$ being the modulation depth that gives the mean number of spins per object [255] (see Figure 3.7 (c)).
3. Determining structure and dynamics of intrinsically disordered proteins (IDPs)

Figure 3.7.: (a-b) Simulated DEER traces with a three dimensional homogenous background decay (black) of a small mean distance distribution with a large width (red) as well as a large mean distance distribution with a small width (blue). (c) Form factor (DEER traces after correction for a three dimensional background) with the marked modulation depth $\Delta$. (d) Corresponding distance distributions.
3.3.4. In-cell EPR

The Goldfrab group performed the only in-cell EPR study on IDPs (aS), yet [64]. However, in-cell pulsed EPR studies on peptides [235], ubiquitin [238, 264], cytochrome P450 [242], GB1 (the immunoglobulin-binding domain of protein G) [265], the bacterial toxin colicin A [266] and DNA and RNA [267–269] were performed by various groups.

Dunkel et al. additionally performed cw-EPR measurements of colicin A in E.coli at room temperature by use of potassium ferricyanide to prevent label reduction.

All these studies demonstrate the application of EPR for in-cell measurements and show the potential of this technique for in-cell studies of IDPs.

The most critical points for in-cell EPR measurements are the label stability in the cell, the question of how to get the label into the cell as well as which cell types to use.

3.3.4.1. Suitable spin label for in-cell EPR

The crucial point of spin label stability in the cellular environment was already mentioned in Section 3.3.1. Kinetic analysis of the label stability proved that nitroxides are quite unstable in the cytosol of mammalian cells, because of their reduction to hydroxylamine [235, 265, 270]. Therefore, in-cell studies commonly use a Gd$^{3+}$-label performing DEER experiments at cryogenic temperatures [64, 235, 238, 268, 269].

Despite the limited nitroxide stability in the cell, there are examples of nitroxide labeling for in-cell measurements [264–266].

Additionally, the Schiemann group synthesized new trityl labels, which are stable against the reducing cell environment and suitable for in-cell measurements by five-pulse relaxation-induced dipolar modulation enhancement (RIDME) [271, 272], which is another pulsed EPR method.

3.3.4.2. Protein transduction into cells

If not using one of the in vivo labeling approaches presented in Subsection 3.3.1 [231, 232], one has to introduce the protein after labeling into the cell. The choice which cells and which transduction method to use are closely related, due to the fact that some methods require certain cell types.

The simplest method is by diffusion of the labeled protein through the cell membrane [266]. However, this technique is not feasible for all proteins, because of protein size or other factors that limit membrane permeability for a certain protein.

One method to make the cell membrane permeable for the labeled protein and diffusion into the cell easier, is introduction of an osmotic shock in the cell by hypotonic swelling [238, 273]. The morphology of the cell membrane has to be restored after incubation with the sample and a drawback of this method are possible changes in the cytoskeleton.

A different technique uses voltage to make the membrane permeable. By applying an electrical field around the cells for time periods varying between $\mu$s to ms, the cell membrane becomes permeable for the spin labeled sample [274, 275]. The permeabilization last several minutes, but sometimes readjusting the membrane to normal conditions can take up several hours [276]. It is a widely used technique and under controlled conditions it is even possible to control the amount
of up taken protein [64]. Alternatively, Ogino et al. used a toxin, streptolysin, for reversible pore-formation in HEK cells, transduction of a labeled protein and resealing of the membrane by Ca\(^{2+}\) for in-cell NMR measurements [277].

Other approaches aim at using the active transport ways of the cell for getting the sample into the cell cytosol. One approach is the use of cell-penetrating-peptides (CPPs) like the 11 aa protein transduction domain of the HIV protein TAT [278] and covalent attachment to the sample protein [279, 280]. The sample protein was successfully delivered to the cytosol of HeLa cells. In the cells protein, detached from the CPP was found, but it is not clear if this happens by either reduction of the disulfide anchor or by dissociation of the CPP from the protein [280].

Burks et al. demonstrated the use of EPR imaging probes, that are firstly encapsulated in liposomes and secondly by endocytosis (an active transport into the cell) and degradation by the cell released in the cells cytosol [281]. High spin concentrations of 150 mM nitroxide in the liposomes result in a quenched signal that is very weak. Release of the spin probes in the cell reduces the concentration due to the larger volume and results in a detectable dequenched nitroxide signal. The high spin label concentration is necessary, so that nitroxide, inside the intact liposomes, appears spectroscopically “dark” and only released spin probes, that can interact with the intracellular environment, give a detectable signal. This approach was demonstrated for use in EPR imaging, but it could be also used for active transport of spin labeled proteins through the cell membranes. However, one has to keep in mind that high protein concentrations as used in the study for the spin probes would most probably lead to aggregation of the proteins in the liposomes.

Another method for introducing the sample protein into the cell is by mechanically passing the membrane via microinjection of the labeled sample. HeLa cells were used for microinjection of fluorescence labeled aS in FRET microscopy studies [154, 282]. However, these cells are not suitable for in-cell EPR measurements with microinjection of the sample due to their small cell volume (~1 pL), which would require microinjection of several million cells for Q-band DEER experiments. In comparison, the Goldfarb group used ~2·10^6 electroporated human A2780 cells for their W-band DEER measurements [64]. In contrast, oocytes of *Xenopus laevis* are of perfect size for easy handling and microinjection of a relatively large sample volume (~50 nL) [268] and not more than three oocytes are needed for an pulsed in-cell EPR experiment at Q- or W-band (for X-band 50 oocytes are needed) [184, 235, 238, 264, 265, 267, 268]. These oocytes from the African clawed frog are a well established cell system not only for in-cell EPR, but also for in-cell NMR studies [184, 195, 283–290].

The transduction of proteins into cells via pore formation by use of CPPs and streptolysin were only used in in-cell NMR studies or magnetic resonance imaging, yet. However, transfer of the techniques for usage in in-cell EPR seems feasible.
CHAPTER 4

Study design

4.1. From artificial systems into the cell

In this work the focus is on giving new insights into dynamical and structural features of the intrinsically disordered protein alpha-Synclein. EPR spectroscopy techniques and CD spectroscopy were used as spectroscopic tools for this task. The systems that are used start with artificial membranes – LUVs that are easy to handle and well characterized – get more complicated by use of biological membranes – yeast mitochondria that consist of a complex biomembrane and describe a biological more relevant environment – and finally end with a highly complex system – the inside of a cell with numerous other organelles, macromolecules and biomembranes.

What happens if the first amino acids, essential for membrane binding, are deleted in the aS sequence? How do the point mutations A30P and A53T influence the helix formation of aS? Is the VATV motif (residues 52–55) only important for aggregation or does it also influence the binding and alpha-helical conformation of aS? CD spectra of different peptide fragments (including or missing characteristic motifs) of the N-terminus are used to characterize the alpha-helical binding onto negatively charged LUVs and to obtain a locally resolution. The spectra are fitted and the fractions of alpha-helix conformation for each peptide are determined and compared.

Is it possible to see the same influence of the disease mutants also for the full length protein? How is the membrane binding influenced by the charge density of the membrane. Are there differences in the N-terminal binding profile of the disease mutants compared to the wt-aS? Cw-EPR spectroscopy in combination with a side-scan over the whole aS sequence of full-length wt-aS and the disease variants A30P and A53T, in the presence of artificial membranes with different degrees of negative charge, is the tool used to answer these issues. The local binding behavior of aS and the aS disease variants is analyzed and compared.

How does the overall and the locally resolved binding behavior of aS change if we use biological membranes instead of artificial ones? To take the step from artificial membranes to more biologically relevant systems yeast mitochondria were used to investigate the binding dynamics as well as the bound structure of wild-type aS via cw-EPR spectroscopy at room temperature.
4. Study design

and DEER measurements.

Is it possible to detect intracellular membrane binding of aS and do the disease variants A30P and A53T behave differently as it is the case with artificial membrane systems? Is it possible to use the side-scan approach over the whole aS sequence in combination with cw-EPR spectroscopy at room temperature also in the cell? How stable is the Proxyl-label in the cell? Does the injected volume remain as a separated bubble in the cytosol or does the sample diffuse in the cell cytosol? Oocytes of *Xenopus laevis* are used as an in-cell mimicking environment and the samples are introduced *via* microinjection.

4.2. Spin label strategy for EPR experiments

4.3. Spin labeling positions for EPR spectroscopy experiments

![Micelle bound α-helix structure of aS with labeling positions marked in green (model PDB ID:1XQ8) [20].](image)

In order to perform EPR measurements on aS, we introduced a nitroxide spin label into aS. The spin label used in this study is 3-maleimido-proxyl (Proxyl) because of its higher stability in biological environment compared to MTSL [170, 224, 225]. A set of singly labeled aS, as well as the disease mutants A30P and A53T, at positions 9, 18, 27, 35, 41, 56, 69, 90 and 140, respectively, was prepared. The relative arrangement of the labels is depicted in Figure 4.1 and more schematic in Figure 4.2.

The labeled wt-aS samples are denoted aS9 with the label at position 9, aS18 with the label at position 18 and the rest accordingly. The labeled disease mutants are denoted A30P-9 with A30P carrying the label at position 9, A53T-18 with A53T labeled at position 18 for example.

Using this set of nine different spin labeling sites, respectively, a scan over the whole sequence of aS and the disease mutants with locally resolved binding characteristics is possible. For distance measurements shown in 7.3 the doubly labeled mutant aS9/27 was prepared.
4.4. Influence of the spin label on aS characteristics

Introducing a spin label via site-directed spin labeling (SDSL) for EPR measurements involves altering the amino acid sequence and introducing a probe molecule which is additionally attached to the aa chain. Thus, experiments are needed to check for conserved conformational behavior.

Alpha-Synuclein shows a random coil structure in solution whereas membrane binding to negatively charged membranes is observed. These two characteristics should be also observable with spin labeled samples of aS and the disease mutants A30P and A53T.

Therefore, CD spectra of exemplary labeling positions as well as the wt-proteins in aqueous buffered solution were recorded. Fig 4.3 shows the CD spectra of wt-aS, aS9, aS27, aS56 and aS90 (a), as well as the corresponding spectra for A30P (b) and A53T (c). Comparison of the spectral shape does not show significant changes upon labeling. The spectral shape suggests an intrinsically disorder of aS in buffer. There can be see small shifts of the wavelength comparing the spectra for the A30P samples. The cause of this shifts is not clear.

For investigating influence on the membrane binding affinity, CD-spectra of aS in the presence of negatively charged (POPG) LUVs were recorded. As Fig 4.4 shows is the spectral shape unaltered upon labeling. Thus, the label has no significant influence on the conformational behavior upon membrane binding. The spectra show a mainly alpha-helical conformation of the protein-lipid samples.
4. Study design

Figure 4.3.: CD spectra of wt-aS (a), A30P (b) and A53T (c) (black), as well as mutants singly labeled at residue 9 (red), 27 (blue), 56 (orange) and 90 green) in aqueous buffered solution. For concentration independent comparison of the spectral shape, normalized ellipticity values were used.

Figure 4.4.: CD spectra of wt-aS (a), A30P (b) and A53T (c) (black), as well as mutants singly labeled at residue 9 (red), 27 (blue), 56 (orange) and 90 green) in the presence of POPG LUVs. For concentration independent comparison of the spectral shape, normalized ellipticity values were used.
CHAPTER 5

Peptide fragments of αS in the presence of artificial membranes

5.1. Peptide fragments of αS

Figure 5.1.: Scheme of N-terminal peptide fragments of αS. For each fragment the amino acid (aa) sequence and the number of amino acids is depicted.
5. Peptide fragments of aS in the presence of artificial membranes

This chapter deals with the question, if peptide fragments taken from the whole aS sequence show differences in α-helix formation when interacting with membranes. As explained in Section 2.2 aS adopts an alpha-helical conformation when binding to negatively charged membranes [20, 22, 26–31]. Therefore, the detection of alpha-helical content can be used as an indicator for membrane binding and the amount of the alpha-helix content can be used as a measure of the membrane binding affinity. To get more detailed information about individual membrane binding motifs of the N-terminal part of aS, we used ten different peptide fragments of aS as shown in Figure 5.1. The peptides, termed P1-P10, represent characteristic regions and sequence motifs of aS, have these motifs missing or twice in their sequence or have certain residues altered by point mutations. P1 consists of the first 29 amino acids (aas), which show the highest membrane binding affinities of aS [23]. In contrast, in P2 ten aas of the P1 sequence are deleted. P3, P4, and P5 hold aas 38-44 (LYVGSKT), which is the motif where the bend of the two antiparallel aS helices [20, 26–28] is situated. Additionally, P4 has the disease related point mutation A30P implemented, whereas P5 is significantly shorter than P3 and P4. P6, P7, P9 and P10 all hold the VATV (52-55) motif, whereas it is missing in P8. Furthermore, P7 has the disease related point mutation A53T implemented, P9 has the VATV motif twice in a row and P10 is significantly longer than P6-P9. The alpha-helical content and thus the binding behavior of the peptides to the surfaces of negatively charged vesicles, POPG (1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol)) LUVs, was investigated by recording CD spectra of the samples. CD spectroscopy was chosen as the analyzing tool, because no labeling of the sample is required and direct analysis of the α-helical content of each peptide fragment is possible.

5.2. Revealing the N-terminal membrane binding motifs of aS

The peptide fragments P1-P10 were dissolved in ultrapure water (c (peptide)= 250 mM) and CD spectra were recorded. All spectra show the spectral form for a random coil conformation as expected for aS and aS fragments in solution (Fig. 5.2 (a)) [7]. Different intensities in ellipticity can result from variations of the concentration.

In order to study the membrane binding behavior of the peptides P1-P10, the peptides (mass concentration $\beta_i$ (peptide) = 0.2 mg/mL) were added to solutions of negatively charged LUVs (diameter of ~100 nm, mass concentration $\beta_i$ (LUVs) = 4 mg/mL). Figure 5.2 (b) shows the CD spectra of the peptides after 30 min incubation with the LUV solution. The ellipticity signal intensities vary again from sample to sample due to concentration differences. Analyzing the spectra qualitatively by comparing them by eye with a CD spectrum of an α-helix [206], three spectra show large deviations. Peptide 8 seems to undergo very weak to no membrane interaction. Peptide 5 shows the line shape of a β-sheet conformation [206]. The spectrum of Peptide 4 has a very broad line shape, hinting at the coexistence of various conformations in the sample [206]. The other peptide spectra in the presence of LUVs clearly show a dominant α-helical character. To be able to make more precise assertions, it is necessary to analyze the spectra quantitatively. As already mentioned in Section 3.2 standard analyzing methods are error-prone if protein concentrations and path length of cuvettes is not exactly known [206, 210, 212]. Alternatively, de Jongh et al. described an alternative strategy, where the spectral shape is fitted with Gaussian absorption bands obtained by deconvolution of reference spectra described in litera-
5.2. Revealing the N-terminal membrane binding motifs of aS

Figure 5.2.: (a) CD spectra of peptide P1-P10 solved in ultrapure water (mass concentration(peptide)= 250 mM). (b) CD spectra of peptide P1-P10 added to a solution of negatively charged POPG LUVs (mass concentration β_i (peptide) = 0.2 mg/mL, mass concentration β_i (LUVs) = 4 mg/mL). Spectra were recorded at 20 °C.
5. Peptide fragments of αS in the presence of artificial membranes

Figure 5.3.: Reconstructed CD spectra of the α-helix (black), the β-sheet (red) and random coil structure (blue). The spectra were obtained by summation of Gaussian absorption bands obtained by deconvolution of reference spectra from literature [291]. They also point out that differences in ellipticity values can result from absorption flattening [292] or side-chain contributions in the spectra.

Using the gaussian absorption bands described in de Jongh et al. one can reconstruct the spectra for a random coil, an α-helix and β-sheet protein conformation as depicted in Figure 5.3. de Jongh et al. described their strategy for oriented CD measurements, where the lipids, that induce alpha-helix formation of the protein, are fixed onto a surface. Thus, the α-helix bands are separated in a parallel and a perpendicular component. Combination of these two spectral components according to the paper and Equation (9.1) results into the isotropic distribution of helices. This approach can also be used for common CD spectroscopy with vesicles in solution by using the isotropic α-helix spectrum.

The spectra of the random coil, the alpha-helix as well as the β-sheet conformation were used for fitting the ratios of the different conformations in the CD spectra of P1-P10 in presence of POPG LUVs. Figure 5.4 gives the α-helical content of the CD spectra for P1-P10 in the presence of POPG LUVs. For exact ratio values (also of the other conformations) see Table A.1 in the Appendix. The original spectra and the according fits are shown in Figure 5.5.

The obtained values of the alpha-helix content result from either a equilibrium of membrane bound peptides and peptides in solution or only partial helix formation of the individual peptide molecule [23, 293]. The two different cases can occur simultaneously and not be distinguished by CD spectroscopy. However, it is still justifiable to compare the different peptides for their helix amounts and conclude on the role of the various N-terminal motifs.

P1 has a rather high alpha-helical content of about 70 %, which can be explained by its sequence taken out from the first 29 amino acids from the N-terminal αS region. It was shown that this region initializes the binding to membranes [23, 24] and acts as a kind of anchor [2]. Other studies with different αS peptide fragments from the N-terminal end came to similar results and found membrane binding affinities comparable to full length αS [38, 39]. Deleting residues out of this sequence results in a significant decrease in helix content to ~46 %) as it is the case with P2. Robotta et al. observed a similar effect of the αS full length delete mutant missing residues...
5.2. Revealing the N-terminal membrane binding motifs of aS

2-11 when interacting with mitochondrial membranes [23]. The fraction of \( \alpha \)-helix conformation for P3 is not significantly higher than for P2. P3 is slightly longer than P1, but it seems the first missing of the first 18 residues cause a clear loss in the membrane binding capacity and that the residues in the C-terminal direction can not bind as strong as these first 17 residues. Despite having way more amino acids compared to P2, the P3 alpha-helix amount is only slightly higher as for P2. This emphasizes again the influence of the first amino acids up to residue 18. P4 shows the effect of the A30P point mutation on the binding behavior because the helix fraction is nearly halved compared to P3. P5 has one of the lowest helical contents of all peptide fragments with only about 12%. This can for one thing based on the short sequence, but especially on the fact that this sequence includes the residues 38-44 (LYVGSKT) that form the break between the two helices in the broken helix conformation [20]. The two endings around the break seem not long enough to be able to undergo proper membrane binding. P6 has a very high fraction (~60%) of \( \alpha \)-helix content. Although it is shorter than P3 it has a better ability to bind to the lipid surface.

The characteristic feature in the sequence of P6 are the residues 52-55 (VATV). In the peptides P7, P8 and P9 this feature is altered, respectively. The sequence of P7 has the point mutation A53T and shows a reduction in the binding capability compared to P6. However, the point mutation A53T seems to alter the binding behavior not as strong as the A30P mutation, due to the smaller difference between P6 and P7 compared to the difference between P3 and P4. Deleting the VATV motif, as it is the case for P8, disturbs the helix formation very efficiently. This is in agreement with a study by Lokappa et al., who found a reduction in helix content from about 80% for wt-aS with POPG SUVs to only about 20% for an aS delete mutant missing residues 53-56 (ATVA) with POPG SUVs [294]. In contrast, repeat of this VATV sequence motif (as in P9) results in the highest helix fraction of all measured peptide fragments (~80%). P10, the longest

Figure 5.4.: Fractions of the alpha-helical conformation of the peptide fragments P1-P10 with error bars.

![Bar chart showing fractions of alpha-helical protein conformation for peptide fragments P1-P10](chart.png)
peptide (34 amino acids), shows also a high \(\alpha\)-helical content \(\sim 63\%\). However, the fraction is only slightly increased compared to P6 despite the length difference of 12 amino acids. Taken together the results for P6-P10, it seems the VATV motif is essential for membrane binding of the N-terminal end. Especially the results when deleting this motif is a strong evidence for that hypothesis. This finding is in contradiction to a study by Shvadchak and Subramaniam, who identify this VATV motif as important for fibril formation and postulate an alternative broken-helix model of aS with the break formed from this residues 52-55 [149].
5.2. Revealing the N-terminal membrane binding motifs of αS

Figure 5.5.: (continues on next page)
Figure 5.5.: CD spectra of aS peptide fragments P1-P10 (a-j) in the presence of POPG LUVs (black) with according fits (red).
5.3. Conclusion

In conclusion it was shown that aS peptide fragments, taken from the N-terminal part of the sequence, in combination with CD spectroscopy in the presence of membranes (LUVs) are a useful tool to reveal differences in the N-terminal membrane binding behavior. These peptides can help unravel new motifs in the aS sequence that influence drastically the characteristics of the whole protein. In this study it was confirmed that the first amino acids of the N-terminal part have a high membrane binding affinity as already shown by other studies [2, 23] with a specially high affinity of residues 1-18.

Furthermore, a characteristic motif of four amino acids (residues 52-55), so far only investigated for the influence on aggregation [149], was identified as an important sequence for the membrane binding of the final third of the N-terminus of aS. Both characteristics of the VATV motif seem to be no contradiction to each other in regards to the finding by Lee et al., who observed seeding of aggregation caused by nuclei originated from membrane-bound aS [137]. However, the membrane binding characteristic is probably related to the physiological role of aS, whereas the aggregation characteristic is part of the pathophysiological role of aS.

The disease mutants A30P and A53T also influenced the amount of alpha-helical content upon membrane interaction. Here A30P showed a larger negative effect than A53T, which is in agreement with other studies for A30P, but in the case of A53T a contradiction to other findings that found similar affinities as for the wild-type [107, 108]. The altered binding affinity in the presented study for A53T can be caused by usage of a different vesicle lipid composition compared to the other studies. Therefore, our result emphasizes the influence of lipid compositions on aS interactions, alpha-helix formation and thus binding behavior. On the one hand, our results reflect the importance of locally solving the N-terminal binding affinity of aS and comparison with the disease mutants. On the other hand comparing the presented results for the disease mutations with other studies shows evidently the influence of membrane compositions on the binding and shows the necessity to further investigate this finding by using vesicles with variations in the surface charge densities (see Chapter 6) or by using biologically relevant membrane systems like mitochondria (see Chapter 7).
CHAPTER 6

aS and the disease variants in the presence of artificial membranes

6.1. Locally resolved binding of aS and its disease variants on artificial membranes

This project was started by Marta Robotta and the preliminary work is reported in her dissertation [295]. The according subsections will be marked with the following symbol: ⋆. The sample preparation for the measurements as well as the figures and tables of these marked sections were made by Marta Robotta, too. The unmarked sections are new experiments or analysis performed by the author of this thesis.

For this study additional to the mutants already introduced in Section 4.2, three more singly labeled mutants for the A30P disease variant were used, A30P-24, A30P-28 and A30P-32 (see Figure 6.1). The additional labeling positions are situated near by the point mutation at residue 30 to give a more detailed resolution of the membrane binding behavior of A30P around its disease mutation. In contrast to the other studies shown in this study the wt-aS samples were labeled with MTSL and not with Proxyl.

Cw-EPR spectra at room-temperature in X-band in solution and in the presence of LUVs, made out of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1’-rac-glycerol) (POPG), were recorded. The LUVs have different charge densities $\rho$ with $\rho = [\text{POPG}]/([\text{POPC}]+[\text{POPG}])$. The spectra were fitted for the rotational correlation time $\tau_R$, which directly reports on the mobility of the protein, if compared to measurements in solution. For comparison CD spectra of non labeled samples (without the cysteine mutagenesis for spin labeling) of wt-aS, A30P and A53T with 100% negatively charged LUVs were recorded and analyzed for their alpha-helical content.
6. aS and the disease variants in the presence of artificial membranes

![Diagram of aS and disease variants](image)

Figure 6.1.: A30P mutants with three additional labeling positions, A30P-24, A30P-28 and hyphenationA30P-32.

6.2. Preliminary Work

6.2.1. aS and disease variants on artificial membranes with varying charges

Spectra for the labeling position 18 for wt-aS and the disease variants under three different conditions are shown in Figure 6.2. The spectra in solution (A-C) can be described by a one component (component \( S_A \)) fit with a fast rotational correlation time \( \tau_R \). The three spectra show no noteworthy differences between wt-aS, A30P and A53T in solution. Adding LUVs, with a fraction of negative charge (\( \rho = 0.3 \)), to the labeled samples results in spectra (D-I) that can be described by a two component fit.

Additionally to the free component \( S_A \) representing protein in solution there is also a second component of vesicle bound protein that can be described by a component \( S_B \) with a slow rotational mobility.

The spectra \( S \) in the presence of membranes (LUVs) can be describe with a superposition of the two different components as \( S = (1 - b)S_A + bS_B \) with \( b \), the fraction of the spin labeled sample described by \( S_B \), which is the membrane bound (around the labeling position) fraction. This fraction \( b \) can be therefore also interpreted as the local degree of binding for the labeled mutant [23].

The aS18 spectrum incubated with LUVs with \( \rho = 0.3 \) (Figure 6.2 (D)) is clearly altered by spectral line broadening compared to the spectrum in solution, whereas A30P-18 (E) and A53T-18 (F) mutants show no visible change. Yet, simulating the spectra one also gets a small membrane bound fraction for the disease mutants. All aS variants labeled at position 18 show a clear spectral change in the presence of LUVs with \( \rho = 1 \) (see Figure 6.2 (G-I)). Yet, the values of \( b \) obtained from the fits are quite diverse.

One way to illustrate the resulting differences in \( b \) for various \( \rho \) is to plot \( b \) against the \( \rho \) values for each labeling position (see Figure 6.3). In agreement with previous results [23], aS labeled at position 140 shows no interactions with membranes for this position. Therefore, the spectra can be described solely by the fast component \( S_A \) and the fraction \( b \) is zero. This behavior is also observable for the disease mutants ( see Figure 6.3 (h)).

Comparing the other labeling positions in Figure 6.3, as a general statement, the local degree of binding increases with increased fraction \( \rho \) of the negatively charged lipids. The disease variants show for the labeling positions, located at the N-terminus of aS, decreased local binding compared to wt-aS. Additionally, the binding of the disease variants onto the membrane starts
Figure 6.2.: Representative experimental cw-EPR spectra (circles) and corresponding spectral simulations of wt-aS (blue), A30P (red) and A53T (green). Spectra were recorded at 298 K, at 9.5 MHz, and a protein:lipid-ratio of 1:250, c(protein) \approx 50 \mu M.
at higher $\rho$ values than that of the wild-type. The dependence of $b$ on $\rho$ was parametrized using the function $f(\rho) = A/(1 + \exp(-k(\rho - I)))$, with $A$, the amplitude, $I$ the inflection point of the curve and $k$ the steepness of the fitted function, that gives sigmoidal curves as a result.

In order to put all the obtained data and values in one plot, the inflection point $I$ of the sigmoidal curves [296] was plotted against the labeling sites in Figure 6.4. The higher the value of $I$, the higher is the fraction of negative charge in the LUVs at which significant local membrane binding occurs.

A30P as well as A53T both show higher inflection points for all residues compared to wt-aS. This implies, that the overall membrane binding behavior is disturbed by the disease point mutations.

Furthermore, the point mutation in A30P seems to affect the binding behavior stronger than the point mutation in A53T. Both disease point mutations seem also to affect the local binding behavior. Especially around for the A30P variant a drastic decrease in binding is observable. For the A53T variant this effect is less pronounced.
Figure 6.3.: Comparison of the local degree of binding \(b\) for singly labeling at position (a) 9, (b) 18, (c) 35, (d) 41, (e) 56, (f) 69, (g) 90, and (h) 140 aS (blue), A30P (red) and A53T (green) in the presence LUVs made from POPG and POPC lipids compositions. The different charge density of LUVs were expressed by \(\rho\) value (\(\rho = \frac{\text{POPG}}{\text{POPG} + \text{POPC}}\)).
6. aS and the disease variants in the presence of artificial membranes

6.2.2. aS and disease variants interacting with inner mitochondrial membrane mimicking LUVs

As mentioned before (see Section 2.2), lipid composition and the lipid headgroups influence the binding behavior of aS [33–35]. For this reason, another series of measurements with a biologically more relevant lipid composition was performed. The LUVs contained POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), cardiolipin (CL) and cholesterol (Ch) in a molar ratio of 2:1.3:1:0.6, which reflects the composition of the inner mitochondrial membranes (IMMs) [297]. Cardiolipin carries two negative charges per lipid molecule and was identified as a key lipid, that helps aS targeting mitochondria [298].

Again cw-EPR spectra at room-temperature were recorded and fitted with a two component fit as shown in Section 6.2.1. One fast component $S_A$, with a high rotational mobility, and one slow component $S_B$, with a low rotational mobility and the fraction $b$. Figure 6.5 (a) shows for all three aS variants the local degree of binding $b$ plotted against each labeled residue. With all three aS variants the typical binding profile of aS can be observed, which shows tighter membrane binding till residue 56, and a decrease in binding for residues 69 and strongly for 90, which are situated in the NAC region. For all aS variants residue 140 undergoes no binding. Yet, A53T shows at position 18 a conspicuously low binding degree, which follows the general trend of the wild-type.

The overall binding affinity of the disease mutants is again (as with the POPG/POPC LUVs) clearly decreased in comparison to wt-aS. Again, A30P has a characteristically low degree of binding around the point mutation. Other than with the POPG/POPC

Figure 6.4.: Inflection points I of the sigmoidal curves for wt-aS without disease point mutation (blue), A30P (red) and A53T (green) at different labeled residues. The vertical lines mark the disease-related amino acid substitutions on residue 30 (A30P, red) and 53 (A53T, green). For aS140 in the study range $0<\rho<1$ no local binding was detected, therefore I cannot be determined.
6.2. Preliminary Work

Figure 6.5.: (a) Comparison of the local binding affinity $b$ of aS (blue), A30P (red) and A53T (green) at different labeled residues of aS in the presence of LUVs mimicking the IMM. (b) Local binding affinity $b$ of aS (blue), A30P (red) and A53T (green) at different labeled residues of aS in the presence of LUVs made of POPG/POPC of $\rho = 0.4$. The vertical lines mark the disease-related amino acid substitutions on residue 30 (A30P, red) and 53 (A53T, green).
LUVs this effect is not clearly detectable with A53T for the IMM LUVs. Although this effect is more clearly visible in the inflection point plot than single binding degree plots (compare Figure 6.4 with Figure 6.5 (b)). The net charge of the IMM mimicking LUVs is $\rho = 0.34$. Therefore, in Figure 6.5 (b) the local degree of binding on POPG/POPC LUVs with $\rho = 0.4$ is depicted as comparison. With the POPG/POPC composition the overall binding affinities are even lowered although the vesicles contain slightly more negative charges. This proves the relevance of cardiolipin for effective aS membrane binding.

6.3. Improvement of cw-EPR spectral fits by using expanded fitting parameters

The model used in Section 6.2.1 resulted mostly in only small deviations between the spectra and the fits. Yet, a small number of fitted spectra showed significant deviations between spectrum and fit like for example aS9 with the $\rho$ values 0.5 and 1, respectively.

This finding could indicate that the two component fitting model is not describing the system accurately enough and that additional states other than aS in solution and membrane bound aS do exist. Yet, increasing the number of fitting parameters of the component $S_B$ results in an improvement of the fit as shown in Figure 6.6 (for fitting parameters see Table 6.1).

In contrast to the simpler fits the linewidth was kept fixed at 0.13 mT and instead the line broadening upon binding is solely reflected in the rotational correlation time. The line broadening of EPR spectra can be described by solely increasing $\tau_R$, which makes variations of the linewidth unnecessary. Additionally, it is easier to compare the $\tau_R$ values, if all spectra have the same linewidth. Otherwise both parameters need to be considered for comparison, which is more complicated.

However, the numerical results for the value of fraction $b$ of the fits are unaltered within the errors (compare Table 6.2 and Table 6.1). Nevertheless, one has to be careful with using extended fitting parameters in respect to the question, if these additional parameters are still physically reasonable. Therefore, it was exemplarily shown that improvement of the fits is possible but to avert possible overfitting the simple fitting model shown in Section 6.2.1 and Section 6.2.2 was used.

<table>
<thead>
<tr>
<th>$\rho$</th>
<th>$g_x$</th>
<th>$g_y$</th>
<th>$g_z$</th>
<th>$A_{xx}$ [MHz]</th>
<th>$A_{yy}$ [MHz]</th>
<th>$A_{zz}$ [MHz]</th>
<th>$\tau_R$ [ns]</th>
<th>fraction $b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>2.0056</td>
<td>2.0087</td>
<td>2.0043</td>
<td>33.98</td>
<td>8.38</td>
<td>85.05</td>
<td>4.41</td>
<td>0.97 ±0.03</td>
</tr>
<tr>
<td>1</td>
<td>2.0058</td>
<td>2.00896</td>
<td>2.0044</td>
<td>32.43</td>
<td>10.74</td>
<td>87.92</td>
<td>5.31</td>
<td>0.99 ±0.03</td>
</tr>
</tbody>
</table>

Table 6.1.: Simulation parameters (rotational correlation time $\tau_R$, hyperfine interactions and g tensors, fraction $b$) of spectral component $S_B$ of aS9 in the presence of LUVs with different surface charge density ($\rho = [\text{POPG}]/([\text{POPG}]+[\text{POPC}])$) with extended fitting parameters. The linewidth was 0.13 mT and taken as fixed.
6.3. Improvement of cw-EPR spectral fits by using expanded fitting parameters

Figure 6.6.: Experimental spectra (black), corresponding restricted model fits (red line) and extended model fits (blue line) of aS9 in the presence of LUVs with different surface charge density ($\rho = \frac{[POPG]}{([POPG] + [POPC])}$). For the restricted model $A_{zz}$, rotational correlation time $\tau_R$ and fraction $b$ of the slow component $S_B$ were fitted. For the extended model additionally the parameters $g_x, g_y, g_z, A_{xx}$ and $A_{yy}$ of component $S_B$ were fitted (see Table 6.1).

<table>
<thead>
<tr>
<th>$\rho$</th>
<th>$\tau_R$ [ns]</th>
<th>fraction $b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>2.15</td>
<td>0.92 ± 0.05</td>
</tr>
<tr>
<td>1</td>
<td>2.56</td>
<td>1.00 ± 0.04</td>
</tr>
</tbody>
</table>
6. aS and the disease variants in the presence of artificial membranes

6.4. CD spectroscopy of aS and disease variants on negatively charged membranes

In order to compare and validate the results derived from the EPR spectra (by using spin labeled samples), CD spectra of wild-type aS, A30P and A53T (without altered residues for labeling) in the presence of LUVs, solely made of POPG, were recorded to determine the amount of the alpha-helical fraction in the samples, respectively.

Figure 6.8 shows the results of four individual measurements of wt-aS, A30P and A53T with POPG LUVs in a protein:lipid ratio of 1:40, which is significantly lower than the ratio used for the EPR measurements (protein:lipid ratio 1:250), but in the same range of ratios used in other CD spectroscopy studies of aS [39, 107, 293]. DLS measurements of the vesicles before and after incubation with wt-aS were performed as a control experiment and shown in Figure 6.7. The vesicles have a diameter of ~100 nm, which stays unperturbed upon aS addition. This indicates that the protein:lipid ratio is high enough and aS does not disrupt the double membrane of the LUVs.

The standard deviation of the four identical measurements is quite high, especially for A30P, caused by probably large concentration differences, temperature fluctuations or varying values of the demountable cuvette pathlength (caused by different amounts of solution between the two cuvette glass slides). In order to circumvent the concentration dependency and the high standard deviation of the ellipticity value, a better way to analyze the spectra is to use the gaussian absorption band spectra of the secondary structure components [291] for fitting as demonstrated already in Chapter 5. Figure 6.8 depicts the mean values of the helix fraction for wt-aS, A30P and A53T and Figures 6.9 to 6.11 shows the experimental and simulated CD spectra for wt-aS, A30P and A53T for each of the four measurements. The values of the fractions of the alpha-helical content for each fit can be found in Table 6.3. For a complete overview of the values for all components see in the Appendix Tables A.2 to A.4. The alpha-helix fraction values obtained from the CD spectral fitting can be directly compared with the binding affinities determined form the cw-EPR data due to the fact that helical conformation when binding to membranes [20, 22, 26–31]. In agreement with the binding affinity wt-aS shows the highest alpha-helical content. In contrast, the mean values of the fitted helical content results in a reduction of ~30 % for A30P and a reduction of ~20 % for A53T which is also in good agreement with the EPR spectra analysis (see Figure 6.4).
6.4. CD spectroscopy of αS and disease variants on negatively charged membranes

Figure 6.7.: (a) DLS data of POPG LUVs in Tris-HCl buffer after extrusion. (b) DLS data of POPG LUVs after incubation with wt-αS. Three independent measurements are shown (blue, green, red). The particle size distribution is depicted of logarithmically spaced size classes on the X-axis with relative percentage of particles in each size class on the Y-axis. The vesicles have a diameter of ~100 nm which stays unperturbed upon αS addition.

Table 6.3.: Alpha-helical content of wt-αS, A30P and A53T in the presence of POPG LUVs. The values were obtained by simulating the spectral shape each secondary structure element as described by de Jongh et al. [291]. For a complete overview of the values for all components see Tables A.2 to A.4. The errors of the mean values are obtained by considering the errors for each measurement and taking the average.

<table>
<thead>
<tr>
<th></th>
<th>wt-αS</th>
<th>A30P</th>
<th>A53T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measurement 1</td>
<td>0.85 ± 0.05</td>
<td>0.61 ± 0.05</td>
<td>0.65 ± 0.05</td>
</tr>
<tr>
<td>Measurement 2</td>
<td>0.85 ± 0.05</td>
<td>0.55 ± 0.05</td>
<td>0.65 ± 0.05</td>
</tr>
<tr>
<td>Measurement 3</td>
<td>0.85 ± 0.05</td>
<td>0.60 ± 0.05</td>
<td>0.70 ± 0.05</td>
</tr>
<tr>
<td>Measurement 4</td>
<td>0.78 ± 0.05</td>
<td>0.60 ± 0.05</td>
<td>0.67 ± 0.05</td>
</tr>
<tr>
<td>Mean value</td>
<td>0.83 ± 0.05</td>
<td>0.59 ± 0.05</td>
<td>0.67 ± 0.05</td>
</tr>
</tbody>
</table>
Figure 6.8.: CD spectra of four individual measurements of wt-aS (a), A30P (b) and A53T (c) in the presence of POPG LUVs. (d) Mean value of the alpha-helix fraction obtained by simulations shown in Figures 6.9 to 6.11 of wt-aS, A30P and A53T in the presence of POPG LUVs. For exact values see Table 6.3.
Figure 6.9.: CD spectra of four individual measurements of wt-aS (solid lines) with fits (dotted lines), respectively. For fitting values of alpha-helical fraction see Table 6.3.
Figure 6.10.: CD spectra of four individual measurements of A30P (solid lines) with fits (dotted lines), respectively. For fitting values of alpha-helical fraction see Table 6.3.
Figure 6.11.: CD spectra of four individual measurements of A53T (solid lines) with fits (dotted lines), respectively. For fitting values of alpha-helical fraction see Table 6.3.
6. aS and the disease variants in the presence of artificial membranes

6.5. Conclusion

In this study we used a site-scan over the whole aS sequence via SDSL cw-EPR spectroscopy and sample series of POPG/POPC LUVs with different $\rho$. Strong local effects of the disease related point mutations on the binding affinity were revealed. The neighboring residues of the A30P point mutation show a drastic decrease in membrane binding affinity. For the point mutation A53T a similar, but dampened effect on the binding was identified for the neighboring residues. Additionally, over the whole N-terminus the binding of the disease variants of aS is not uniform, which was shown before for wt-aS [22, 23].

Furthermore, cw-EPR spectroscopy proved that disease related point mutations reduce the overall binding affinity of aS. In contradiction to older studies that only showed this effect for A30P, the overall binding of A53T was decreased compared to wt-aS as well [107, 108]. Our finding is in agreement with a negative effect of both point mutations on binding behavior of aS already shown for the peptide fragments of aS in Chapter 5.

The overall binding affinity of the disease mutants was additionally detected by CD spectroscopy measurements of unlabeled wt-aS and aS variants in the presence of POPG LUVs. The results were compared to the results obtained by the EPR data and were found to fit regarding the relative values. However, CD spectroscopy is the better tool to determine the overall binding in a quantitative way.

Moreover, the same effects of the disease variants on the binding behavior were detected for biologically more relevant membranes like IMM mimicking LUVs with the characteristic lipid cardiolipin having a positive influence on the binding behavior of aS and the disease variants.

Additionally, an improved fit strategy was developed.

The overall reduced binding is quite similar for both disease mutants. Thus, it seems that the different strength of the local effects for the disease mutants mainly influences the characteristic behavior of A30P and A53T, which is quite different [32, 104–109].

The findings of this study are in contrast to other studies [108, 299], which were not able to detect a notable difference between the binding behavior of aS and A53T, which is again (compare Section 5.3) probably caused by use of other lipid compositions of the vesicles.

Overall, the presented study shows the drastic influence a disease mutation of a single residue can have on the characteristic properties of the whole protein sequence. Membrane binding of aS is highly influenced by the charge density of the membranes and the lipid composition, with the biological cardiolipin playing an essential role for the affinity and even initializing of membrane binding [298].
aS in the presence of biological membranes

7.1. aS binding onto yeast mitochondria

The previous chapters give new insights about the N-terminal binding characteristics in the presence of artificial membranes but the presented studies can hardly reflect the aS binding behavior under more physiological conditions with membranes, which for example are covered by or hold other proteins or exhibit ion channels. Therefore this chapter analyzes aS binding behavior in the presence of a biologically more relevant membrane system, namely isolated mitochondria.

As described in Sections 2.2 and 2.3, the interaction of aS with mitochondria plays an important role, not only for the physiological, but also for the disease related side of aS. In order to better understand both sides one has to know and comprehend the physiological interplay first. Therefore, it is important to know more about the binding behavior of aS in the presence of mitochondria.

Cw-EPR experiments at 293 K with wt-aS labeled at residues 9,18,27, 35, 41, 56, 69, 90 and 140, respectively, in the presence of isolated yeast mitochondria were performed. The cw-EPR spectra can be analyzed by using the same approach as demonstrated before in Chapter 6. Changes in the dynamics and structure upon mitochondria addition, namely the local degree of membrane binding, in close proximity of the labeled residue can be obtained and related to the labeling position.

Additionally, for one doubly labeled mutant, aS9/27, a DEER experiment is performed to verify the alpha-helical structure of mitochondria bound aS.

7.2. Locally resolved binding of wt-aS in the presence of yeast mitochondria

Cw-EPR spectra of the singly labeled aS mutants in SEM buffer are recorded and fitted with a one component fit describing the parameters of a component $S_A$ with a fast rotational correlation
time, according to the fitting model used in Chapter 6.

The spectra with according fits are depicted in Figure 7.1 and fitting parameters are listed in Section 7.2. The g-tensor was determined for aS9 in aqueous buffered solution and taken for all other fits. In contrast to the spectra in solution shown in Chapter 6 a different buffer (SEM buffer instead of Tris-HCL buffer) and temperature (293 K instead of 298 K) was used. All spectra in solution reflect a typical fast motion spectrum with a fast rotational correlation time $\tau_R$ faster than 0.6 ns.

The aS mutants (c ≈ 1 mM) were incubated under shaking with isolated yeast mitochondria for 3 min at 37°C. Excessive protein was washed away, resulting in concentrations about 60 µM, and cw-EPR spectra of the samples were recorded. The spectra, depicted in Figure 7.2 with according fits, show fluctuations in the SNR due to varying labeling efficiencies, which are difficult to quantify due to problems with an exact protein concentration determination (compare aS140 and aS9).

The spectra for the labeling positions 9, 18, 27, 35, 41 and 56 show typical spectral shapes of a dominating slow component. Characteristic are the additional broad peak in the low field at around 332 mT, the distinct broadening of the middle peak and an additional, very weak and broad peak at 338 mT. However, the spectrum of aS27 shows clearly a lower amount of the slow component, because the additional low field peak is barely visible. In contrast, the spectra for aS69, aS90 and aS140 in the presence of mitochondria have characteristic fast motion shape. Yet, the spectrum of aS140 is very noisy and has a baseline, which shows wavelike fluctuations and appears for unknown reasons, or some kind of broad background signal of unknown origins, e.g. a contamination. This makes an exact analysis of this spectrum error-prone and is reflected in the extremely high error for the fraction $b$. However, studies found no binding between residue 140 and membranes [20, 23] and therefore the binding is set as zero for this residue. Smaller fluctuations of the baseline are also observed for the spectra of aS18 and aS35 with mitochondria. Yet, the clear spectral broadening and fitting is unperturbed, as comparison with the spectral shape and values for fraction $b$ of e.g. the spectrum of aS41 proves.

The spectra were fitted with a two component fit using the free, fast component $S_A$ and the bound, slow component $S_B$ according to the fitting model used in Chapter 6. Fitting parameters as well as the fraction $b$ according to $S = (1-b)S_A + bS_B$ of the bound component $S_B$ for each fit are listed in Section 7.2.

In order to illustrate the local differences of the membrane bound aS, the fraction $b$, which is equivalent to the local degree of binding, is plotted against the labeled residues (see Figure 7.3). The locally resolved binding profile shows fluctuations in binding for different residues in the N-terminal region of aS. Conspicuously, residue 27 shows the lowest binding of the N-terminal labeling sites, which was already recognizable in the shape of the spectrum. The binding factor decreases with residues 69 and 90, situated in the NAC region. These observations are in agreement with binding studies performed on artificial membranes [22, 23]. Furthermore, the overall binding is decreased compared to a study by Robotta et al. [55] performed on LUVs mimicking the inner mitochondrial membrane. This finding can be explained by the fact, that numerous other proteins are bound to the mitochondria membrane as well. Therefore, limited free space on the mitochondrial membrane reduces the overall membrane binding of aS.
7.2. Locally resolved binding of wt-aS in the presence of yeast mitochondria

Figure 7.1.: Cw-EPR spectra of wt-aS labeled at various positions (a)-(i) in aqueous buffered solution (black) with according fits (blue) obtained by a one component model.
Figure 7.2: Cw-EPR spectra of wt-aS labeled at various positions (a)-(i) in the presence of mitochondria (black) with according fits (red) obtained by a two component model.
Table 7.1: Simulation parameters (hyperfine coupling constants $A_{xx} = A_{yy}$ and $A_{zz}$ and the rotational correlation time $\tau_r$) for the fast component $S_A$ and the slow component $S_B$. The fraction $b$ of the spectral component $S_B$ of aS mutants bound to yeast mitochondria is given as well. The g-tensor is set as $[g_x, g_y, g_z] = [2.00906, 2.00687, 2.003]$ and the linewidth is kept fixed as 0.13 mT for all fits.

<table>
<thead>
<tr>
<th></th>
<th>$A_{xx}$ (MHz) = $A_{yy}$ (MHz)</th>
<th>$A_{zz}$ (MHz)</th>
<th>$\tau_r$ (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>aS9</td>
<td>15</td>
<td>103</td>
<td>0.56</td>
</tr>
<tr>
<td>aS18</td>
<td>8</td>
<td>98</td>
<td>0.43</td>
</tr>
<tr>
<td>aS27</td>
<td>3</td>
<td>101</td>
<td>0.37</td>
</tr>
<tr>
<td>aS35</td>
<td>2</td>
<td>103</td>
<td>0.46</td>
</tr>
<tr>
<td>aS41</td>
<td>12</td>
<td>83</td>
<td>0.47</td>
</tr>
<tr>
<td>aS56</td>
<td>13</td>
<td>90</td>
<td>0.44</td>
</tr>
<tr>
<td>aS69</td>
<td>10</td>
<td>87</td>
<td>0.24</td>
</tr>
<tr>
<td>aS90</td>
<td>22</td>
<td>80</td>
<td>0.50</td>
</tr>
<tr>
<td>aS140§</td>
<td>11</td>
<td>114</td>
<td>0.16</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>$A_{xx}$ (MHz) = $A_{yy}$ (MHz)</th>
<th>$A_{zz}$ (MHz)</th>
<th>$\tau_r$ (ns)</th>
<th>Fraction $b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>aS9</td>
<td>18</td>
<td>92</td>
<td>10.7</td>
<td>0.85±0.05</td>
</tr>
<tr>
<td>aS18</td>
<td>9</td>
<td>87</td>
<td>6.8</td>
<td>0.87±0.05</td>
</tr>
<tr>
<td>aS27</td>
<td>9</td>
<td>82</td>
<td>6.4</td>
<td>0.65±0.07</td>
</tr>
<tr>
<td>aS35</td>
<td>13</td>
<td>76</td>
<td>10.0</td>
<td>0.79±0.1</td>
</tr>
<tr>
<td>aS41</td>
<td>10</td>
<td>76</td>
<td>12.6</td>
<td>0.78±0.05</td>
</tr>
<tr>
<td>aS56</td>
<td>12</td>
<td>82</td>
<td>7.5</td>
<td>0.83±0.05</td>
</tr>
<tr>
<td>aS69</td>
<td>10</td>
<td>79</td>
<td>2.8</td>
<td>0.47±0.15</td>
</tr>
<tr>
<td>aS90</td>
<td>18</td>
<td>71</td>
<td>16.9</td>
<td>0.29±0.2</td>
</tr>
<tr>
<td>aS140§</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0±0.8</td>
</tr>
</tbody>
</table>

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

7.2. Locally resolved binding of wt-aS in the presence of yeast mitochondria
Figure 7.3.: Local degree of binding of wt-aS in the presence of mitochondria at different labeled residues (9, 18, 27, 35, 41, 56, 69, 90, 140) of aS.

7.3. Structural investigation of membrane bound aS

The structure of aS upon binding to yeast mitochondria was studied by using the doubly labeled mutant aS9/27. Firstly binding of aS9/27 to the mitochondria was checked by recording a cw-EPR spectrum at room-temperature at X-band. A comparison of the spectra for aS9/27 in aqueous buffered solution compared to aS9/27 in the presence of mitochondria (see Figure 7.4) clearly shows that the spectrum is altered in the mitochondria bound aS9/27 sample. The middle peak shows significant broadening and an additional high-field as well as a low-field peak are prominent in the spectrum.

DEER experiments of the singly labeled samples of aS9 and aS27 with mitochondria used in Section 7.2 were performed, in order to determine an experimental background trace for the

Figure 7.4.: Cw-EPR spectra at room temperature of aS9/27 in aqueous buffered solution (blue) and aS9/27 upon binding onto mitochondria (black).
7.3. Structural investigation of membrane bound aS

Therefore, the samples were directly shock frozen in liquid nitrogen after the cw-EPR experiment and addition of 20% glycerol. The recorded DEER traces were added up in the Xepr software of the spectrometer. DEER traces of singly labeled sample contain no distance information but only background, which can be described by the following formula with $c_B$ the concentration of B spins, the instantaneous diffusion strength $K_B$, the time $t$ and the dimension $D$ of the homogenous distribution of the B spins [300]:

$$B(t) = \exp(-c_B K_B t^D/3)$$  \hspace{1cm} (7.1)

The obtained trace of aS9 + aS27 both in the presence of mitochondria was fitted using the implied function in the DeerAnalysis software and analyzed for its homogenous background dimension, which gave the value 1.8, via the DeerAnalysis software [263]. Figure 7.5 shows the deer traces of aS9, aS27 and aS9 + aS27. This procedure is especially advantageous, if the background traces are significantly shorter recorded than the double mutant DEER trace. due to differences in sample quality (labeling efficiencies) or the relaxation times.

In order to measure the distance distribution between the residues 9 and 27 and to deduce the structure of aS in the presence of mitochondria DEER measurements of aS9/27 bound to the mitochondria as well as with aS9/27 in aqueous buffered solution at 50 K were performed. The DEER traces of aS9/27 bound to the mitochondria as well as with aS9/27 in aqueous buffered solution were recorded with 12 h accumulations. Figure 7.6 (a) shows the DEER traces of aS9/27 in solution compared to aS9/27 upon mitochondrial binding. A comparison of the form factors (after background correction, see Figure 7.6 (b)) shows a significant loss in modulation depth and decreased signal-to-noise ratio for the mitochondrial sample caused by the complex biological system.

Figure 7.6 (c) shows the distance distribution for both form factor curves obtained via Tikhonov regularization. Additionally, the simulated distance for a aS9/27 mutant with Proxyl label and alpha-helical conformation is depicted.

The distance distribution for aS9/27 in solution is quite broad but still narrower than the expected broad distance distribution over the whole distance range for an IDP, that adopts an random coil-like structure solution [11]. However, it can be explained by the presence of transient structures found for aS [12], which cause the maximum of the distribution to be at a shorter distance compared to the helix simulation or the experimentally obtained distance for aS bound onto mitochondria. The width as well as the maximum of the distance distribution for mitochondria bound to aS9/27 are in very good agreement with the 1XQ8 helix model of aS [20]. This proves that aS9/27 binds alpha-helically to the mitochondrial membrane, which results in a much narrower distribution compared to aS9/27 in buffered aqueous solution. The MMM software [258] uses the rotamer approach, that considers the remaining flexibility of the spin label caused by the linker that binds the label to the protein and displays these rotamers graphically as shown in Figure 7.6 (d). This results in a distribution in the measured distance despite a fixed helix of aS, This flexibility by using the rotamer approach and MMM software as shown in Figure 7.6 (d).

Figure 7.7 shows the L curves with the optimal regularization parameter $\alpha$ for the model free Tikhonov regularization according to the L-curve criterion [255]. The optimal $\alpha$ is displayed by
Figure 7.5.: DEER traces of (a) aS9, (b) aS27 upon binding to mitochondria, respectively. c) The DEER trace obtained by summation of the two DEER traces was used for determining the experimental background dimension (1.8), which is needed for background correction of the double mutant aS9/27 bound to mitochondria.
the software via automatic L curve corner recognition and represents the corner of the L curve, which is defined by the minimum square norm of the second derivative of the distance distribution $P(r)$ and the minimum mean square deviation among all regularization parameters for which Tikhonov regularization was performed [263]. The regularization parameter gives the quantification of a compromise between the resolution and smoothness of the distance distribution.

For both obtained distance curves a validation using the implemented tool in the DeerAnalysis software [263] was performed (see Figure 7.7). As a result for both measurements (in solution and with mitochondria) the validation shows the correctness of the obtained distance distribution. For aS9/27 in buffer the validation shows that the distances appearing above 4.5 nm is a measurement artifact.
Figure 7.6.: (a) DEER traces of aS9/27 in aqueous buffered solution (blue) and upon mitochondrial binding (black) with background fit (red), respectively. (b) Form factor of aS9/27 in solution (blue) and upon mitochondrial binding (black) with fitting of the curve. (c) Distance distribution obtained from Form factor using Tikhonov regularization for aS9/27 in solution (blue), with mitochondria (black) and simulated distance using MMM software [258] (dashed, red). (d) Rotamers of the Proxyl spin label attached to residues 9 and 27 of aS (in an alphahelical conformation, model 1XQ8 PDB database [20]), which show the possible orientations of the label relative to the protein. The green spheres show the possible positions of the nitroxide radical and are scaled for the population.
7.3. Structural investigation of membrane bound aS

Figure 7.7.: (a) L curve of the Tikhonov regularization for aS9/27 in aqueous buffered solution with chosen $\alpha$ parameter (2876.771) marked in red. (b) Validation of the distance distribution obtained by Tikhonov regularization for aS9/27 in aqueous buffered solution. The obtained distance distribution (blue, solid line), the mean value - 2× standard deviation (blue, dotted line) and the mean value + 2× standard deviation (blue, dashed line) are displayed. (c) L curve of the Tikhonov regularization for aS9/27 bound onto mitochondria with chosen $\alpha$ parameter (489.3205) marked in red. (d) Validation of the distance distribution obtained by Tikhonov regularization for aS9/27 bound onto mitochondria. The obtained distance distribution (black, solid line), the mean value - 2× standard deviation (black, dotted line) and the mean value + 2× standard deviation (black, dashed line) are displayed.
7.4. Conclusion

The binding dynamics and structure of aS onto biologically relevant membranes – namely yeast mitochondria – was investigated. The combination of cw-EPR spectroscopy and a site-scan over the whole aS sequence using nine different labeling sites, allowed to get a locally resolved binding profile of aS upon mitochondrial binding, which shows a decreased overall binding affinity compared to binding to artificial membranes [23, 55]. However, the overall binding affinity is quite high and higher than expected in regards to the mitochondrial membrane system, that is fully packed with proteins [301]. Robotta et al. showed that aS binds to the inner mitochondrial membrane that contains cardiolipin [55]. Cardiolipin triggers the aS-binding onto mitochondrial membranes [298], which can explain the only slightly decreased membrane binding affinity compared to the artificial systems used in Chapter 6.

The obtained binding profile shows the strong binding of the N-terminal part of the protein and the lowered binding affinity in the NAC region, which is both in agreement with results from studies using artificial systems [22, 23].

Furthermore, by use of a doubly labeled aS mutant for a DEER experiment, it was shown, that aS adopts an alpha-helical conformation upon binding to yeast mitochondria, which is in agreement with the aS conformation found in the presence of HEK mitochondria [55]. The experimentally obtained distance distribution fits the simulation perfectly in width and maximum of the distance.
aS and the disease variants in the cell

8.1. Room-temperature in-cell EPR of aS and its disease variants

What structures of aS occur if the system is more complex and instead of using artificial membranes or isolated mitochondria aS and its variants A30P and A53T are transduced into cells? In order to set the results obtained from the studies of aS in the presence of artificial membranes and mitochondria in a more physiological context, we performed a structural investigation of intracellular aS. Nine singly labeled mutants of wt-aS, A30P and A53T, respectively, were used for a systematic site-scan along the protein sequence (as before with the artificial membranes) to investigate the intracellular structures of aS and its disease variants at room-temperature. Oocytes of *Xenopus laevis* in stage V/VI were chosen as a system that mimics the *in vivo* environment of aS. These oocytes are a well established system in biology, but also for in-cell spectroscopy (NMR and EPR) [184, 195, 267, 268, 284, 290]. Due to their relatively large size (volume ~1 µL) they are easy to handle and the protein sample can be transferred into the cells via microinjection. Microinjecting the protein sample allows to control the amount of protein that is introduced and to transduce the spin-labeled protein rapidly into the cell.

8.2. The intracellular EPR signal

Microinjection of the labeled sample has influence on the lifetime of the oocytes. Oocytes that are first morphologically intact, show serious leakage of cytosol, complete disruption of the cell membrane or apoptosis with timespans exceeding 3 h incubation at 20 °C, as is shown in Fig 8.1. In comparison injection of a buffered solution of D₂O results in a cell stability up to 12 h [235]. However, the shortened lifetime of the oocytes after microinjection seems not to be specific to aS. Injection of a model compound (oligo(phenylene ethynylene) with two Gd-spin labels also results in short lifetimes of about 3 h [235]. To ensure intact cells in the EPR samples, the experiment times in the presented study were kept to 60 min.

To ensure intact cells in the EPR samples, the experiment times in the presented study were kept to 60 min. 3 to 6 h after detecting the in-cell EPR signal an additional EPR measurement
Figure 8.1.: Micrographs of Xenopus laevis oocytes in the EPR test-tube after incubation for 10 min (left), 3 h (center), 6 h (right) after microinjection of 50 nL of a 1 mM solution of spin-labeled aS.

was performed in order to get the background signal of the oocytes after the spin label signal has vanished due to the reductive cell environment. This step is necessary due to endogenous paramagnetic species that are present in the oocytes [235] and that superpose the spin label signal.

Figure 8.2 shows that the peaks in the cw-EPR spectra of the free Proxyl label (c = 2.9 mM) injected into oocytes and the oocyte background signal (oocytes injected with Tris-HCl buffer) occur at similar values in the magnetic field. Note, due to the high Proxyl-concentration used the oocyte background appears smaller than in the oocyte samples with injected protein. Especially the middle peak of the Proxyl spectrum is unsymmetrically affected by the background signal. Therefore, for each injected protein sample the background signal is recorded individually, smoothed using a Savitzky-Golay filter and subtracted from the according intracellular aS spectrum. In Figure 8.3 exemplarily the background signal of A53T-35, injected into oocytes, before and after smoothing, is depicted. There is still some nitroxide signal left, which is observed in some other background spectra as well, caused by not complete reduction of the nitroxide at the measurement point of time.

The original spectra, the background spectra and the background-corrected spectra of each mutant are shown in the Appendix in Figure A.7 – Figure A.9. Differences in the signal-to-noise ratios (SNRs) are caused by position dependent labeling efficiencies (~70 %) and fluctuations in the protein concentration.

For each intracellular measurement the SNR is plotted against the number of accumulated slices. The maximum of the curve gives the value for the accumulations that results in the best SNR Figure 8.4 for each in-cell measurement as shown exemplarily in Figure 8.4 for A30P-56 injected into oocytes. Table 8.1 gives the number of accumulated slices for each aS sample and the according plots can be found in Figures A.10 to A.12 in the Appendix. Overall, the SNR is limited because of the injected aS concentrations. These are set to result in an intracellular concentration (~50 µM) which is in the order of magnitude of physiological aS concentrations in the cytosol [69].
8.2. The intracellular EPR signal

Figure 8.2.: Cw-EPR spectrum of Proxyl (black) and Tris-HCl buffer (orange) injected into oocytes. The orange spectrum shows the oocyte background positioned relative to the nitroxide signal in black.

Figure 8.3.: Background signal of A53T-35 injected into oocytes before (orange) and after smoothing (blue) using a Savitzky-Golay filter.

Table 8.1.: Number of slices for each measurement to accumulate for getting the optimal SNR ratio.

<table>
<thead>
<tr>
<th>Labeling position</th>
<th>wt-aS</th>
<th>A30P</th>
<th>A53T</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>8</td>
<td>14</td>
<td>19</td>
</tr>
<tr>
<td>18</td>
<td>19</td>
<td>19</td>
<td>23</td>
</tr>
<tr>
<td>27</td>
<td>12</td>
<td>12</td>
<td>26</td>
</tr>
<tr>
<td>35</td>
<td>19</td>
<td>18</td>
<td>28</td>
</tr>
<tr>
<td>41</td>
<td>25</td>
<td>14</td>
<td>24</td>
</tr>
<tr>
<td>56</td>
<td>11</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>69</td>
<td>15</td>
<td>34</td>
<td>15</td>
</tr>
<tr>
<td>90</td>
<td>19</td>
<td>24</td>
<td>11</td>
</tr>
<tr>
<td>140</td>
<td>15</td>
<td>25</td>
<td>15</td>
</tr>
</tbody>
</table>
8. aS and the disease variants in the cell

Figure 8.4.: Signal-to-Noise-Ratio (SNR) of A30P-56 in oocytes of the exemplary accumulated cw-EPR spectrum slices to determine the maximum value of the SNR and thus, the number of slices to accumulate.

8.3. Kinetics and signal shape

The cell environment reduces the nitroxide [270, 302] and the EPR signal decreases to zero over time as exemplarily shown in Figure 8.5 for the mutant A30P-41, injected into oocytes, with a half-life of $17.6 \pm 1.7$ min at room-temperature (293 K). The decay curves of all mutants (see Figures A.1 to A.3) give a broad range of half-life values listed in Table 8.2. For the mutant aS18 it was not possible to get a reasonable decay curve and therefore half-life value from the data. This can be explained with very small EPR signal intensity for this mutant. Huge signal jumps in the decay curve of mutant aS35 are also caused by the small EPR signal intensity. The difference in labeling efficiency and label concentration of the samples makes a direct correlation between the Table 8.1 and Table 8.2 not possible.

There are no characteristic differences between the values for the wt-aS and the disease mutants. However, comparing the different labeling positions, the half-life values of the mutants from the N-terminal end show lower half-lives than the ones from the NAC region. Additionally, the C-terminus has a slightly higher half-life, than the N-terminus. These findings hint at a permanent intracellular structure of aS and the disease variants, which makes residues from the NAC region less exposed to the cytosol than residues from the N- or C-terminus.

Taking a closer look at the spectral shape at the beginning and at the end of the cw-EPR measurement (see exemplarily Figure 8.6) reveals that small changes in the spectral shape during the recording time can not be ruled out. However, comparing the slopes of the signals between the first and second peak the spectrum form the start has a larger slope than the spectrum from the end of the measurement. Yet, the smaller this slope is the larger the rotational correlation time.
8.3. Kinetics and signal shape

Table 8.2: Half-life values given in minutes, determined from exponential fits of the signal decay curves (Figures A.1 to A.3) from cw-EPR spectra of αS, A30P and A53T mutants injected into oocytes.

<table>
<thead>
<tr>
<th>Labeling position</th>
<th>wt-aS</th>
<th>A30P</th>
<th>A53T</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>7.0 ± 0.9</td>
<td>7.2 ± 1.1</td>
<td>9.5 ± 2.0</td>
</tr>
<tr>
<td>18</td>
<td>-</td>
<td>10.8 ± 0.8</td>
<td>8.5 ± 2.3</td>
</tr>
<tr>
<td>27</td>
<td>7.2 ± 0.5</td>
<td>7.3 ± 0.5</td>
<td>14.7 ± 3.1</td>
</tr>
<tr>
<td>35</td>
<td>6.0 ± 2.7</td>
<td>9.9 ± 0.8</td>
<td>12.4 ± 1.1</td>
</tr>
<tr>
<td>41</td>
<td>11.7 ± 0.4</td>
<td>17.6 ± 1.7</td>
<td>15.1 ± 0.7</td>
</tr>
<tr>
<td>56</td>
<td>8.9 ± 1.0</td>
<td>8.5 ± 0.5</td>
<td>11.2 ± 0.6</td>
</tr>
<tr>
<td>69</td>
<td>13.0 ± 2.9</td>
<td>16.9 ± 1.7</td>
<td>12.1 ± 1.0</td>
</tr>
<tr>
<td>90</td>
<td>10.7 ± 2.1</td>
<td>12.8 ± 0.9</td>
<td>6.6 ± 0.2</td>
</tr>
<tr>
<td>140</td>
<td>9.1 ± 1.3</td>
<td>9.0 ± 1.0</td>
<td>8.6 ± 0.4</td>
</tr>
</tbody>
</table>

Figure 8.5: Time dependent signal intensity (amplitude of the middle nitroxide peak) of spin-labeled αS upon microinjection into oocytes for A30P-41. The signal decays with a half-life of 17.6 ± 1.7 min.

Figure 8.6: Normalized (by maximum) cw-EPR spectra of A53T-56 injected into oocytes accumulated in the period measured 12-20 min (black) and 51-62 min (blue) after injection, respectively.
8. aS and the disease variants in the cell

\( \tau_R \) [23]. This means at least a faction of the injected aS sample gets more mobile in this example during the recording time and changes from a more structured state into a more unordered state. Comparing the time dependent spectral shape of other samples, this effect is either not present Figure A.4 (e), Figure A.5 (b), (g), (h), Figure A.6 (b), (f), (i) or not detectable due to a bad SNR e.g. Figure A.4 (a)-(d) and (f)-(i). However, we are mainly interested in the ordered structural states aS and its variants adopt in the cell and a good SNR plays a crucial role for analyzing the in-cell spectra for these structure elements. Therefore, we only analyze the slices from the beginning of the recording time that result in the best SNR. Figures A.4 to A.6 shows the complete set of spectra that compare the accumulated slices of the beginning with the accumulated slices of the end of the recording according to Table 9.5.

8.4. Spatial distribution of the injected volume in the cell

The spatial distribution of the oocyte-injected sample A30P-41 was exemplarily calculated by determining the locally concentration via double electron-electron resonance (DEER) measurements (see Figure 8.7 for the DEER traces and Section 9.2.3.2 for the analysis). Taking the reduction of the spin label into account, the concentration of the injected A30P-41 sample was diluted by factor 3.6, which corresponds to a volume increase by factor 3.6 after 15 min of incubation. Therefore, the protein sample does not remain in the injected buffer volume but is spreading into the cytosol of the oocytes. Just before submission of these results a study by Lawless et al. was published that also showed via DEER measurements and determining local concentrations, that 30 min is a sufficient time for a small injected protein (~60 kDa) to diffuse into the oocyte cytosol [265].

![DEER traces](image)

Figure 8.7.: Four pulse DEER-traces of A30P-41 in oocytes (black) and Proxyl in Tris-HCl (20 vol% glycerin) (as reference) (blue) and corresponding fits (dashed red lines). Concentration of Proxyl sample (reference sample) 414 \( \mu \)M, local concentration of A30P-41 165 \( \mu \)M.
8.5. Microviscosity in oocytes of *Xenopus laevis*

Albeit one may expect intuitively a high microviscosity for the cell cytosol due to the molecular crowded environment, measurements could not prove this intuition [303]. One has to keep in mind that there needs to be a way for small macromolecules relevant for the metabolism to get fast to their targets or reaction partners. Therefore, the viscosity on this microscale needs to be low enough that these processes can happen on a small time scale.

Viscosity measurements using different cell types and techniques revealed values from 1.1 cP to 10 cP compared to the viscosity of 1 cP for water [303]. In human kidney cells the microviscosity inside the cytosol as well as the fluid-phase viscosity near the cell plasma membrane was found to be like that of bulk water [304, 305].

In this study the spin label 4-Oxo-Tempo (Tempone) was used as a tool to investigate the viscosity of the oocyte cytosol. The intracellular cw-EPR spectrum of injected Tempone was compared to the spectrum of Tempone recorded in aqueous buffered solution (Figure 8.8 (a)). There is no detectable change in the signal shape between the two spectra, which points to similar micro-viscosities in both media. Ye *et al.* determined by NMR spectroscopy the microviscosity in oocytes of *Xenopus laevis* to increase only by a factor of 1.2 compared to water [289]. A spectral simulation of the Tempone spectrum in buffer with an increased rotational correlation time $\tau_R$ by factor 1.2 according to the increased viscosity in cells shows no detectable spectral changes (Figure 8.8 (b)).

The C-terminal part of aS does neither interact with membranes [20] nor is involved in aggre-
8. aS and the disease variants in the cell

Figure 8.9.: Cw-EPR spectrum of aS140 in aqueous buffered solution (green) and injected into oocytes (black).

gation of the protein [306]. Figure 8.9 shows that the spectral shapes of both measurements are the same. Thus, comparing the spectrum of aS140 in aqueous buffered solution with the in-cell spectrum of aS140 additionally proves again the negligible changes in micro-viscosity between buffered aqueous solution and the cell cytosol.

8.6. Conformation of the oocyte-injected wt-aS mutants

Three different basic structures, besides numerous states in between, for intracellular aS are possible: random coil-like conformation for non-interacting aS, \( \alpha \)-helix conformation for membrane bound aS or \( \beta \)-sheet conformation for aggregated aS. Therefore, we took two characteristic labeling positions (aS9 and aS69) and obtained typical spectra for these three conformations under controlled conditions (see Figure 8.10). The spectra of non-interacting aS were obtained by using a aS samples in buffered aqueous solution (c (protein) = 250 \( \mu \)M), whereas for the spectra of membrane bound aS the samples in aqueous buffered solution were incubated for 30 min at room-temperature with a solution of POPG LUVs (protein:lipid-ratio 1:250; c (protein) = 50 \( \mu \)M). The aS samples, made out of aS9 and aS69 mixed with unlabeled aS, respectively, for the spectra of aggregated aS were incubated for two weeks at 37\(^\circ\)C under shaking to form fibrils of aS, that were sonicated directly before recording the spectra to obtain short preformed fibrils. Residue 9 of aS (in the N-terminus) was chosen as a typical residue with strong spectral changes upon membrane binding, whereas aS69 (in the NAC region) shows a stronger change upon aggregation than aS9.

The conformations of the aS samples, used for recording the spectra in Figure 8.10, were cross-checked by using CD spectroscopy and atomic force microscopy (AFM). CD spectra for random coil structure of aS9 and aS69 in solution can be found included in Figure 4.3 (a) in Section 4.3. Alpha-helical bound aS9 and aS69 in the presence of POPG LUVs, respectively, can be found included in Figure 4.4 (a) in Section 4.3. CD spectra and AFM images of sonicated fibrils with \( \beta \)-sheet conformation can be found in Figure 8.13 and Figures 8.11 to 8.12. The spectra for the
8.6. Conformation of the oocyte-injected wt-aS mutants

![Experimental cw-EPR spectra](image)

Figure 8.10.: Experimental cw-EPR spectra (dotted lines) at 293 K of aS9 (a) and aS69 (b) unstructured in buffered aqueous solution (cyan), alpha-helical upon membrane binding to POPG LUVs (orange) and upon aggregation (magenta). Corresponding spectral simulations are shown as solid lines.

unstructured aS in solution, in the presence of LUVs and of aggregated aS, which are normalized for the number of spins using the double integral, are clearly distinguishable from each other. Therefore, despite only determining protein dynamics with cw-EPR, it is possible to directly conclude which structure the intracellular aS adopts.

In order to analyze changes of the structure of aS upon injection into the cell, the intracellular spectra of each labeling position was compared to the spectra in solution, respectively (Figure 8.14). There is no significant deviation detectable between the in-cell spectra and the spectra in buffer, which indicates that microinjected aS remain unstructured in the cell.

The SNR of the intracellular spectra is quite low compared to the spectra in solution. There is the possibility that signals of membrane bound or aggregated aS are hidden in the spectra due to the higher noise level.

Thus, Figure 8.10 shows also simulations of the control spectra. Parameters of the simulations can be found in Tables A.5 to A.9. The spectra for the aggregated aS were described according to Iurascu et al. [307] with a four component simulation. The spectra in solution and upon interaction with POPG LUVs were simulated as described in Robotta et al. [23]. These spectral descriptions were used to perform a quantitative analysis of the intracellular spectra. Cw-EPR spectra for membrane bound aS at labeling position 9 with different ratios of intrinsically disordered aS as well as for aggregated aS at labeling position 69 with different ratios of intrinsically disordered aS were simulated (see Figure 8.15). The simulations reveal a possible presence of a small fraction (<15%) of membrane bound aS or aggregated aS.
8. aS and the disease variants in the cell

Figure 8.11.: Two dimensional (a) and 3D (b) representations of AFM images of sonicated aS fibrils (wtaS:aS9 = 20:1) on a mica substrate.

Figure 8.12.: Two dimensional (a) and 3D (b) representations of AFM images of sonicated aS fibrils (wtaS:aS69 = 20:1) on a mica substrate.

Figure 8.13.: CD spectra of sonicated aS fibrils (wtaS:aS9 = 20:1) in black and sonicated aS fibrils (wtaS:aS69 = 20:1) in red in Tris-HCl (10 mM, pH 7.4, 100 µM EDTA, 150 mM NaCl) show β-sheet conformation of fibrillized aS.
8.6. Conformation of the oocyte-injected wt-aS mutants

Figure 8.14.: Cw-EPR spectra of aS labeled at various positions (a)-(i) in aqueous buffered solution (green) and injected into oocytes (after background correction, black).
Figure 8.15.: (a) Experimental spectrum of aS9 injected into oocytes upon background correction (black) and spectral simulation of different ratios of the monomeric, intrinsically disordered spectral component $S_A$ and the alpha-helically membrane bound spectral component $S_B$. (b) Experimental spectrum of aS69 injected into oocytes upon background correction (black) and spectral simulation of different ratios of a monomeric, intrinsically disordered spectral component $S_A$ and a fibril component $S_F$. 
8.7. Conformation of the oocyte-injected aS disease variants

Analyzing the spectral shape of the intracellular spectra for the disease mutants A30P and A53T and comparing them to the spectral shape of the spectra recorded in aqueous buffered solution, respectively, again shows no significant difference between the spectra (see Figures A.13 and A.14 in the Appendix). Furthermore, comparison of wt-aS, A30P and A53T for each labeling position reveals no difference between the mutant spectra compared to the wild-type spectra (Figure 8.16) of aS. Again, it is possible that at least a small fraction of the disease mutant samples (up to ~15%) are either aggregated or membrane bound. Other than this, there are no conformational changes observable, although one has to keep in mind the limited recording time of the intracellular spectra due to the signal reduction inside the cell. Because there is no drastic difference between the in-cell spectra of wt-aS and the disease mutants, it seems, that local conformational effects, as shown in Chapter 6 and by other groups [108, 120, 123] are more relevant for the inheritable form of PD. This may also include the kinetics of fibril formation [120, 123].
8. aS and the disease variants in the cell

Figure 8.16.: Cw-EPR spectra of aS (black), A30P (red) and A53T (blue) with various labeling positions (a)-(i) injected into oocytes.
8.8. Conclusion

In conclusion this study presents a systematic spin-labeling site-scan [22] over nine-labeling sites in combination with microinjection into oocytes of *Xenopus laevis* and in-cell cw-EPR spectroscopy at room-temperature. The use of cw-EPR with singly labeled mutants results in spectra with good SNR considering the reducing cellular environment. The injected sample diffuses after injection and is found after 15 min in a volume, increased by factor 3.6, compared to the injection volume. Commonly, rather bulky Gd-labels [237, 238] are used for in-cell EPR distance measurements using DEER. For DEER measurements it is essential to have molecules that are doubly labeled which requires immediate shock-freezing of cell samples or stable labels that are not reduced in the cellular environment. Cw-EPR requires only one (intact) label per protein, which allows to use a small nitroxide label (molar mass $M = 237.27 \text{ g mol}^{-1}$). Additionally, the use of nitroxides as label for structure studies of aS is well established [22, 23].

Our study shows the potential of in-cell EPR to perform strategic site-scans along protein sequences to reveal their intracellular dynamics and structure without the need for oxidizing agents, which were used in other in-cell EPR studies to hinder reduction of the spin label in the cell [265, 266].

In summary, the findings of this study firstly, support the results by other groups [62–64] for wt-aS and secondly, demonstrate that the disease mutants A30P and A53T are also mainly disordered in the cell and therefore show the same intracellular behavior as wt-aS.

Furthermore, comparing the half-life values of the intracellular signal decay, shows the largest values for the NAC region mutants, the smallest values for the N-terminal aS mutants. Together with the finding of mainly disordered aS in the cell these findings hint at the presence of transient structures of the intrinsically disordered conformation [12], where the NAC region interacts with the C-terminus of aS.

No obviously detectable, spontaneous fibril formation occurred in the presented in-cell study. One reason could be the intracellular transient structures that protect the NAC region of aS against aggregation. Another reason could be the lack of preformed fibrils or other aggregated aS in the cells, which act as seeds and were found to be necessary for initiating fibril formation in transgenic mice [127]. However, in an organism, where PD occurred on its own, the pathological side and aggregation of aS is surely triggered by something else.

For all three aS variants the existence of a small fraction (<15%) of alpha-helical aS, aggregated aS or otherwise structured aS can not be excluded. Taking the different characteristics in binding and aggregation behavior of the aS variants in relation, it seems that this potential small fraction is the biologically more relevant fraction for the PD disease.
All chemicals, if not otherwise stated, were purchased by Sigma Aldrich Co. LLC. Mil-Q water was generated by using a Milli-Q Academic Ultrapure Water System (Merck KGaA, Darmstadt Germany).

9.1. Methods and Materials

9.1.1. Protein expression of αS (performed by Nathalie Schilderink in the research group of Prof. Vinod Subramaniam at the University of Twente)

Wild-type (wt) αS, A30P and A53T do not contain any native cysteine residues. Wild-type-αS, A30P and A53T cysteine-mutations at sites 9, 18, 27, 35, 41, 56, 69, 90, 140 have been introduced using standard biochemical methods as described in Van Raaij et al. [124] αS mutants were expressed in Escherichia coli strain BL21(DE3) using the pT7-7 expression plasmid (courtesy of the Landsbury Laboratory, Harvard Medical School, Cambridge, MA). The protein was purified using 1 mM dithiothreitol (DTT), kept in Tris-HCl buffer (10 mM, pH 7.4) at a concentration of 250 µM and was stored at -80°C.

9.1.2. Site Directed Spin Labeling of αS

Prior to site-directed spin labeling, the protein was reduced with a 6x molar excess of -SH groups (DTT) for 30 minutes at room temperature. Afterwards DTT was removed with Pierce Zeba 2 mL desalting columns. As washing buffer for the desalting columns Tris-HCl buffer (10 mM, pH 7.4, 50 mM NaCl) was used. Afterwards, a 3x molar excess of 3-maleimido-PROXYL spin label (3-Maleimido-2,2,5,5-tetramethyl-1-pyrrolidinloxy, Proxyl) or MTSL ((1-Oxyl-2,2,5,5-tetramethylpyrroline-3- methyl)methanethiosulfonate) (for αS samples used in Chapter 6) was immediately added and the protein solution was incubated for two hours in the dark at room temperature. Subsequently, free label was removed by using two additional desalting steps using the desalting columns and Tris-HCl buffer (10 mM, pH 7.4) for washing of the columns. The
buffer volume of the protein solution was reduced in order to achieve final protein concentrations of about 1 mM. Therefore, the protein solution was put onto vivaspin 2 10 kDa molecular weight cut-off (MWCO) filters and the volume was reduced by a factor 4 via centrifugation at 4 °C and 3500 g. Between the washing and final preparation steps the samples were kept on ice to minimize aggregation and stored at -80 °C afterwards. The labeling efficiency was experimentally determined by measuring the spin label concentration via cw-EPR and determining the protein concentration using a Nanodrop 1000 spectrophotometer (Thermo Scientific), the absorbance intensity at 276 nm and the molar extinction coefficient $\epsilon = 5745$. The obtained labeling efficiency is in the range of 60-100%.

### 9.1.3. Peptide fragments of aS sequence

Ten different peptide fragments (with varying masses) of aS (lyophilized, trifluoroacetic acid salt; Biosyntan, Berlin) were solved in different amounts of Milli-Q water as listed in Table 9.1 to achieve a protein concentration of 250 µM.

### 9.1.4. Preparation of protein fibrils

To get fibrils of aS, aggregation reactions were set up with 100 µM aS (diamagnetic ratio wt:aS9 = 20:1 and wt:aS69 = 20:1) in Tris-HCl (10 mM, pH 7.4, 100 µM ethylenediaminetetraacetic acid (EDTA), 150 mM NaCl) with a volume of 100 µL following a slightly altered protocol than Sidhu et al. [308]. The solution was incubated at 37 °C shaking with 1000 rpm for two weeks. For preparation of sonicated fibrils the aggregated stock solution was sonicated for 10 min using an Elmasonic S10H bath sonicator (Elma, Singen).

### 9.1.5. Atomic Force Microscopy (recorded with the help of Philipp Graus in the group of Johannes Boneberg at the University of Konstanz)

AFM samples were prepared by adsorbing 20 µL of the sonicated fibril solution on a freshly cleaved mica plate (Muscovite mica, V-1 quality, EMS) for 4 min and washing five to six times.
with 100 µL Milli-Q water and drying under a gentle stream of nitrogen gas. AFM images were recorded on a Multimode Microscope (Bruker Biospin, Karlsruhe) in soft tapping mode with an Otespa-R3 tip (Bruker). All images were acquired with a resolution of 1024 x 1024 pixels per image. Images were analyzed by Gwyddion 2.46 data analysis software [309].

9.1.6. Preparation of vesicles

Anionic POPG [1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1’-rac-glycerol)] was purchased in chloroform solution from Avanti Polar Lipids (Birmingham, AL). 500 µL lipid stock solution of POPG were put under a gentle stream of nitrogen to remove the chloroform. The resulting lipids films were placed under vacuum overnight and then rehydrated in a volume of 300 µM of Tris-HCl buffer (10 mM, pH 7.4) to a final concentration of around 40 mM lipid and incubated 30 min at room temperature. LUVs were prepared by repetitive extrusion through one layer of 100 nm polycarbonate film in a handheld extruder (Avanti, Polar Lipids). The average diameter of LUVs was about 100 nm, as measured by dynamic light scattering. The total phospholipid concentration was determined according to the protocol of Chen et al. [310]. 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. For preparation of the LUVs, used in Chapter 5 with the peptide fragments of aS, was done with MiliQ water instead of Tris-buffer.

9.1.7. Sample preparation for cw-EPR with LUVs for conformational comparison

Wild-type aS labeled at residues 9 and 69 in Tris-HCl buffer (10 mM, pH 7.4, protein concentration between 150-250 µM) was added to the POPG vesicle solution, respectively, 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 room-temperature before starting the measurements. By use of a syringe (with a long thin needle) the LUV protein mixture was transferred into a glass capillary (ringcaps, Hirschmann Laborgeräte) that had been sealed at one side by heating the glass. Following this step, the capillary was placed in a 3 mm EPR guiding tube (Bruker Biospin), inserted into the spectrometer resonator and the cw EPR measurement was started.

9.1.8. Dynamic light scattering (DLS)

Vesicle size and stability of LUVs of different lipid compositions and upon interaction with aS were studied by DLS. The DLS measurements were performed at 25 °C 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). 2 µL lipid solution was dispensed in 1 mL Tris-HCl buffer (10 mM, pH 7.4) into a 1 cm polycarbonate cuvette.

9.1.9. Circular Dichroism Spectroscopy

CD measurements were performed on a Jasco J815 spectropolarimeter (Jasco Analytical Instruments) at 20 °C in 0.5 mm demountable cuvettes (Hellma, Forest Hills, NY) and a sample volume
9. Details of Experiments and Analysis

of 100 µL. Spectra were recorded over the range of 180 to 260 nm, with a step resolution of 0.5 nm, a bandwidth of 1 nm and a scan speed of 50 nm/min in continuous mode. Using two measurement channels, the CD absorption signal and the corresponding photomultiplier voltage (HT) were measured simultaneously. In the samples for protein and peptide monomers in solution 0.2 mg/mL was used. For the fibril samples 0.43 mg/mL was used due to lower absorption values for smaller protein amounts.

For the samples in the presence of vesicles 0.2 mg/mL protein or peptide was incubated with 4 mg/mL LUVs. The protein samples were measured in Tris-HCl buffer (10 mM, pH 7.4) whereas the peptide fragments were measured in ultrapure water. Five scans per sample were averaged for the monomeric protein and peptide samples with or without vesicles.

For the aS fibril spectra 10 scans were averaged.

9.1.10. Isolation of yeast mitochondria and control experiments (performed in the research group of Thomas Becker at the University of Freiburg)

The *Saccharomyces cerevisiae* (YPH499) wild-type cells were grown to an OD$_{600}$ of 1 in yeast extract-peptone-glycerol (YPG) medium (1% (w/v) yeast extract, 2% (w/v) bacto peptone und 3% (v/v) glycerol) at 30 °C. Mitochondria were isolated by differential centrifugation as described in Wenz et al. [311]. Cells were harvested by centrifugation at 2500 g, washed with distilled water and incubated in 2 mL of Tris buffer (0.1 M, pH 9.4) and 10 µM DTT per gram wet weight of cells for 20 min at 24 °C. Cells were reisolated and washed with zymolyase buffer (1.2 M sorbitol and K$_2$HPO$_4$ (20 mM, pH 7.4)). Cells were incubated with 4 mg Zymolyase (Seikagaku) per gram cell pellet in 7 mL per gram cell pellet zymolyase buffer for 40 min at 24 °C to digest the cell walls. After an additional washing step with zymolyase buffer, the spheroblasts were homogenized on ice in homogenization buffer (0.6 M sorbitol, Tris buffer (10 mM, pH 7.4), 1 mM EDTA, 1 mM PMSF, and 0.2% [wt/vol] BSA) with a glass potter. Cell debris was removed by centrifugation at 2500 g, and mitochondria were pelleted at 17000 g. The mitochondrial pellet was washed with SEM buffer (250 mM sucrose, 1 mM EDTA, and 10 mM MOPS/KOH, pH 7.2) and resuspended in SEM buffer. The protein concentration was adjusted to 10 mg/ml. The mitochondria were shock frozen in liquid nitrogen and stored at -80 °C until usage.

To control the integrity of the mitochondrial outer membrane proteinase K was added to isolated mitochondria. The protection of intermembrane-space exposed proteins towards the added protease revealed the intactness of the outer membrane as described by Horvath et al. [312] with the sample protein phosphatidylserine decarboxylase 1 (Psd1).

The membrane potential was determined as described by Böttinger et al. [313] by the uptake of the fluorescence dye DiSC3 3,3’-dipropylthiadicarbocyanine iodide (DiSC3) in isolated mitochondria in reaction buffer (0.6 M sorbitol, 0.1% (w/v) bovine serum albumin, 10 mM MgCl$_2$, 0.5 mM EDTA, K$_2$ (20 mM, pH 7.2)). The uptake of the fluorescence dye depends on the inner membrane potential and results in quenching of the fluorescence signal that was measured at a wavelength of 670 nm (excitation wave length 622 nm).
9.1.11. Sample preparation of aS in the presence of isolated yeast mitochondria

Protein solutions (c(protein) ≈ 1 mM) of singly labeled aS mutants (positions 9, 18, 27, 35, 41, 56, 69, 90, 140) and the double mutant aS9/27 in Tris-HCl buffer (10 mM, pH 7.4), respectively, was added to 30 µL mitochondria in SEM-buffer (pH 7.2) and incubated for three minutes at 37 °C and 450 rpm. These ideal amounts of aS and mitochondria, that result in the highest fraction of bound aS, were determined by Hanne Gerding in the research group of Marcel Leist and adopted. To remove unbound protein the solution was centrifuged twice at 4 °C and 8000 g and removing the supernatant and washing with 20 µL SEM-buffer in between. Finally, the pellet was solved in 6 µL SEM-buffer. By use of a syringe (with a long thin needle) the mitochondria protein mixture was transferred into a glass capillary (ringcaps, Hirschmann Laborgeräte) that had been sealed at one side by heating the glass. Following this step, the capillary was placed in a 3 mm EPR guiding tube (Bruker Biospin), inserted into the spectrometer resonator and the cw-EPR measurement was started.

Samples of aS9 with mitochondria, aS27 with mitochondria, and aS9/27 with mitochondria were used for successive DEER experiments. Therefore, these samples were recollected by using a syringe (with a long thin needle). 20 vol% glycerol was added, the sample solution was transferred into a Q-band sample tube (diameter 1 mm) using the same syringe.

9.1.12. Preparation of and microinjection into Xenopus laevis oocytes

The Xenopus laevis oocytes on stage V/VI (purchased from EcoCyte Bioscience, Caustrop-Rauxel) were kept in MBS (modified Barth’s saline, 1x:88 mM NaCl, 1 mM KCl, 1 mM MgSO4, 5 mM HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl] ethanesulfonic acid), 2.5 mM NaHCO3, 0.7 mM CaCl2·H2O) at 18 °C. The protein samples used in this study were the singly labeled mutants (residues 9, 18, 27, 35, 41, 56, 69, 90 and 140) of wt-aS, A30P and A53T. Additionally also Tris-HCl buffer (10 mM, pH 7.4), 4-Oxo-Tempo (4 mM in Tris-HCl buffer (10 mM, pH 7.4), Proxyl (2.9 mM, Tris-HCl buffer (10 mM, pH 7.4) were injected, respectively, as control experiments. Prior to the microinjection, about ten oocytes were prepared on a home-made poly(tetrafluoroethylene) holder in MBS (1x) and visually controlled before usage. Only oocytes with a normal circular shape and no signs of cell membrane damage as well as apoptosis were used. 50.6 nL of the 1 mM protein solution in Tris-HCl buffer (10 mM, pH 7.4) was microinjected into the oocytes using a Nanoject II automatic nanoliter injector with fitting micromanipulator MM33 (Drummond, Broomall, PA). Subsequently, the oocytes were washed carefully with MBS (1x).

Five microinjected oocytes were then collected cautiously in a Q-band tube (quartz glass, 1 mm inner diameter, purchased from Bruker) via slightly negative pressure on one end of the tube. The oocytes were visually inspected directly afterwards as well as after the EPR measurement using a Stemi 2000-C binocular microscope mounted with an AxiaCam ERC 5s camera (Zeiss, Oberkochen). Oocytes with disrupted cell membranes, large amounts of leaked cytosol or light spots of apoptosis at the dark animal pole are considered as destroyed and not further usable. After removing supernatant MBS using a syringe (with a long thin needle) the sample tube was sealed at the top with a capillary tube sealing compound (Cha seal, Kimble Chase Life Science and Research Products, LLC) to guarantee a fixed sample position within the tube.
The tube was placed in a 3 mm EPR guiding tube (Bruker Biospin), inserted into the spectrometer resonator and the cw-EPR measurements were started immediately (about 10 minutes after microinjection).

For a DEER experiment 50.6 nL of A30P-41 with an initial spin concentration of 1.12 mM was injected into three oocytes (volume 1 μL), respectively. The sample was incubated for 15 minutes and shock-frozen for the DEER measurement.

### 9.1.13. Continuous wave (cw)- EPR measurements

#### 9.1.13.1. Measurements for the study shown in Chapter 7

For preparing the samples in aqueous buffered solution, 1 μL of the concentrated αS samples in Tris-buffer (obtained as described in Section 9.1.1) was diluted in 1 μL SEM buffer.

All cw-EPR spectra were recorded in X-band (9.5 GHz microwave frequency) using an Elexsys E580 spectrometer equipped with a Super High-Q cavity probe head (both Bruker) at 293 K. The temperature was controlled using an ESR900 helium gas flow system (Oxford Instruments). We performed modulated field sweeps, with a sweep width of 150 G, containing 4096 data points (sweep time 167.77 s) at a modulation frequency of 100 kHz. The modulation amplitude was 0.6 G and the time constant 40.96 ms.

The protein samples in aqueous buffered solution were accumulated for 10 scans. The protein samples with mitochondria were accumulated for 30-50 scans, depending on the EPR signal.

#### 9.1.13.2. Measurements for study shown in Chapter 8

For preparing the samples in aqueous buffered solution, 1 μL of the concentrated αS samples in Tris-buffer (obtained as described in Section 9.1.1) was diluted in 1 μL Tris-buffer.

All cw-EPR spectra were recorded in X-band (9.5 GHz microwave frequency) using an Elexsys E580 spectrometer equipped with a Super High-Q cavity probe head (both Bruker) at 293 K. The temperature was controlled using an ESR900 helium gas flow system (Oxford Instruments). We performed modulated field sweeps, with a sweep width of 200 G, containing 2048 data points (sweep time 83.89 s) at a modulation frequency of 100 kHz. The modulation amplitude was 0.6 G and the time constant 40.96 ms to get an optimal SNR, but no distortion of the signal.

For the 4-Oxo-Tempo samples, a modulation amplitude 0.5 G, a time constant 20.48 ms and a sweep width of 160 G were chosen and 4096 data points were recorded (sweep time 83.89 s).

The spectra were recorded with a time delay of 100 ms between each scan for the in-cell samples. This resulted in a time resolved measurement signal. After about 30-60 min the nitroxide signal had disappeared due to the reductive cell environment and the measurement was stopped. After 3-6 h an additional measurement was started to determine the background signal of the oocytes. 20 to 30 scans were accumulated and used as background signal. Spectrometer control was performed by the Bruker Xepr software. The reference spectra were recorded as follows: Spectra in Tris-HCl buffer (10 mM, pH 7.4) were recorded with 5-10 scans for each sample. Spectra in the presence of POPG LUVs were recorded with 10 scans for each sample. For the αS fibril samples also a time delay of 100 ms was chosen and about 600 scans were recorded.
9.1.14. DEER EPR experiment

DEER experiments were performed with the following samples: aS9/27 in solution, aS9 in the presence of mitochondria, aS27 in the presence of mitochondria, oocyte-injected A30P-41, Proxyl in Tris-HCl bulk solution (20 vol% glycerol, as a reference for the in-cell A30P-41 sample for determining the local concentration).

The DEER measurements were performed in Q-band at 34 GHz using an Elexsys E580 spectrometer equipped with an EN 5107D2 Q-band EPR probe head (both Bruker Biospin) and a 10 W MW power solid state amplifier (HBH Microwave GmbH) at 50 K. The temperature was controlled with a CF935 helium gas flow system (Oxford Instruments). The four-pulse, dead-time free DEER 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 echo amplitude is observed as a function of time \(t\) starting with \(t = 280\) ns. The pump pulse (typically values 20-40 ns corresponding to a \(\pi\)-pulse) was set to the maximum of the nitroxide spectrum (at a typical resonance frequency of 33.859 GHz) and the observer pulse was set 50 MHz higher; \(\pi\) pulses at observer frequency were of around 40-60 ns with \(\pi/2\) pulses for observer being half as long.

The samples were measured at \(\tau_1 = 400\) ns and a shot repetition times between 3-5.5 ms depending on the sample system. The values for \(\tau_2\) were 2100–3800 ns also depending on the sample system.

9.2. Data analysis

9.2.1. Analysis of CD spectra

Baseline-correction, subtraction of the background spectrum and smoothing (Savitzky-Golay method with a convolution width of 5) was used for processing the raw data. The background spectra were either recorded for ultrapure water, Tris-HCl buffer (10 mM, pH 7.4) or LUVs in Tris-HCl buffer (10 mM, pH 7.4), depending on the sample system. The spectra were cut off with HT values above 550 V. This was the case for aqueous solutions with protein or peptides at 190 nm and for protein or peptide solutions in the presence of LUVs at 200 nm. Above a HT value of 550 V the absorption signal gets very noisy and unreliable.

Data were normalized by the peak area from 250 to 200 nm (for samples with vesicles) and to 190 nm (for samples without vesicles), in both cases with no base and adding the part under the baseline, using a function implemented in the SpectraManager 2 software (Jasco Analytical Instruments).

Fitting of CD spectra was performed manually using Excel 2016 (Microsoft office professional plus 2016, Microsoft, USA) the Gaussian absorption bands, that describe the typically secondary structure types and were obtained by de Jongh et al. by deconvolution of reference spectra [291]. The descriptions of the absorption bands by de Jongh et al. are listed in Table 9.2.

The absorption bands for \(\alpha\)-helix were described as \(\theta_{\text{parallel}}\), which is the summation of bands 4-5 which absorb light parallel to the helix axis, and \(\theta_{\text{perp}}\), the summation of bands 1-3, which absorb light parallel to the helix axis. In a case with no fixed orientation (as for protein bound to LUVs) the two components can be added up using the following equation with \(\theta_v\) representing
Table 9.2: Parameters describing CD spectroscopy reference spectra from literature of the four secondary structure elements by gaussian absorption bands \( (\lambda e^{-((\lambda - \lambda_0)^2)/\Delta^2}) \) obtained by de Jongh et al. [291].

<table>
<thead>
<tr>
<th>Band</th>
<th>( \lambda ) (x ( 10^{-3} )) mdeg cm(^2) dmol(^{-1} )</th>
<th>( \Delta ) (nm)</th>
<th>( \lambda_0 ) (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-29.55</td>
<td>7.16</td>
<td>220.48</td>
</tr>
<tr>
<td>2</td>
<td>-63.58</td>
<td>8.99</td>
<td>207.70</td>
</tr>
<tr>
<td>( \alpha )-helix 3</td>
<td>95.65</td>
<td>7.63</td>
<td>190.44</td>
</tr>
<tr>
<td>4</td>
<td>-69.40</td>
<td>8.99</td>
<td>227.70</td>
</tr>
<tr>
<td>5(^8)</td>
<td>6.09</td>
<td>9.02</td>
<td>188.50</td>
</tr>
<tr>
<td>( \beta )-strand 1</td>
<td>-18.55</td>
<td>14.29</td>
<td>216.45</td>
</tr>
<tr>
<td>2</td>
<td>29.89</td>
<td>8.85</td>
<td>197.77</td>
</tr>
<tr>
<td>3</td>
<td>12.78</td>
<td>5.29</td>
<td>191.42</td>
</tr>
<tr>
<td>1</td>
<td>19.28</td>
<td>8.92</td>
<td>223.17</td>
</tr>
<tr>
<td>( \beta )-turn 1</td>
<td>35.90</td>
<td>6.68</td>
<td>199.37</td>
</tr>
<tr>
<td>3</td>
<td>-83.58</td>
<td>8.13</td>
<td>190.64</td>
</tr>
<tr>
<td>1</td>
<td>3.88</td>
<td>9.12</td>
<td>217.85</td>
</tr>
<tr>
<td>( \text{random coil} ) 2</td>
<td>-40.15</td>
<td>8.22</td>
<td>197.54</td>
</tr>
<tr>
<td>3</td>
<td>-8.91</td>
<td>4.22</td>
<td>189.55</td>
</tr>
</tbody>
</table>

\(^8\) Helix band 5 was described by \( A(2[(\lambda - \lambda_0)(\lambda_0/\Delta^2) + 1])e^{-(\lambda - \lambda_0)^2/\Delta^2} \) according to Tinoco [314].

the ellipticity in an isotropically distributed sample:

\[
\theta_V = \frac{\theta_{\parallel} + 2\theta_{\perp}}{3}
\]  

(9.1)

The \( \beta \)-strand, the \( \beta \)-turn and the random coil spectrum is described by summation of three bands, respectively. The hereby obtained spectra of a random coil, a \( \alpha \)-helix and \( \beta \)-sheet are depicted in Figure 5.3. The descriptions of the structure types were used to fit the experimental spectra, by summation of all structure type spectra and varying the fractions of the different components. Therefore, the simulated and the experimental spectra were normalized by the ellipticity value at 222 nm. The value for the \( \beta \)-turn fraction was in all simulations zero and is therefore not listed in Table A.1 and Tables A.2 to A.4. Errors for the fraction of \( \alpha \)-helical protein were obtained by systematically varying the value to determine in which range acceptable simulations of the data were achieved. The values at which the spectra of the simulation and the fit started to differ clearly by eye was taken as lower and upper error margins (typical values are 0.05 – 0.07).

9.2.2. Data analysis of cw-EPR spectra

9.2.2.1. Analysis of spectra shown in Chapter 6 by an extended fitting model

The spectra were analyzed using Matlab R2016b (The MatWorks, Inc. Natrick, USA) and the toolbox EasySpin 5.1.8 [315]. Varying simulation parameters, least-squares fits to experimental data were performed using esfit and the EasySpin function chili.
Table 9.3.: Root-mean-square deviation (RMSD)-values of the extended model two component fits of aS9 in the presence of LUVs with different charge densities ($\rho = \frac{[\text{POPG}]}{([\text{POPG}] + [\text{POPC}])}$) with assumed upper and lower borders.

<table>
<thead>
<tr>
<th>$\rho$</th>
<th>RMSD</th>
<th>upper and lower borders for RMSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.00928</td>
<td>±0.00173</td>
</tr>
<tr>
<td>ρ = 1</td>
<td>0.00984</td>
<td>±0.00213</td>
</tr>
</tbody>
</table>

The expanded fitting strategy was exemplarily shown for aS9 in the presence of LUVs with $\rho = 0.5$ and $\rho = 1$, respectively.

The spectra of aS in buffer were described by the fast component $S_A$. For the simulation of $S_A$ the following parameters were used: $A_{xx} = A_{yy} = 13$ MHz and $g = [g_x, g_y, g_z] = [2.00906, 2.00687, 2.003]$ [316] were taken as fixed. The linewidth $l_w$ and $\tau_R$ were fitted. The obtained values of $S_A$ were used for the fast component of the two component fit of aS in the presence of LUVs with different charge densities ($\rho = \frac{[\text{POPG}]}{([\text{POPG}] + [\text{POPC}])}$) according to $S = (1 - b)S_A + bS_B$ with a second component $S_B$ (bound, slow component). The component $S_B$ was fitted using an extended model, where the linewidth was kept fixed (the same value as for component $S_A$) and the hyperfine interactions ($A_{xx}, A_{yy}, A_{zz}$) and g tensors $(g_x, g_y, g_z)$ were fitted along $\tau_R$ (see Table 6.1). Errors for the parameter $b$ were also determined by systematically varying its value to test in which range acceptable simulations of the data were obtained. Table 9.3 gives the root-mean-square deviation (RMSD)-values for the expanded two component fits of aS9 in the presence of LUVs with $\rho = 0.5$ and $\rho = 1$, respectively, with the upper and lower boarder taken for determining the error of fraction $b$.

9.2.2.2. Analysis of spectra shown in Chapter 7

The spectra were baseline-corrected with a 2nd order polynomial over the whole measurement range via the included tool in the spectrometer Xepr software (Bruker Biospin). The spectra were analyzed using Matlab R2016b (The MatWorks, Inc. Natrick, USA) and the toolbox EasySpin 5.1.8 [315]. Varying simulation parameters, least-squares fits to experimental data were performed using the EasyS pin esfit and the EasySpin function chili. The raw spectra of the aS samples with mitochondria were quite noisy, but contained more data points than needed. Therefore, the spectral points were reduced from 4096 to 820 by using the reduction tool (in the processing panel of the spectrometer Xepr software (Bruker Biospin) with 5 as value for the number of points), which resulted in smoothed spectra with 10 measurement points from peak to peak, which gives sufficient resolution. The spectra of aS in SEM buffer were described by the fast component $S_A$. The g-tensor $[g_x, g_y, g_z]$, the hyperfine coupling constants $A_{xx} = A_{yy}$ and $A_{zz}$ and the rotational correlation time $\tau_R$ were fitted. The g-tensor for aS9 in SEM buffer was taken as $[g_x, g_y, g_z] = [2.00906, 2.00687, 2.003]$ [23] and was used as fixed value for all cw-EPR spectra fitted in this study. Therefore, the magnetic field (B) values of the other samples were adjusted.
Table 9.4: Root-mean-square deviation (RMSD)-values of the two component fits of wt-aS mutants in the presence of mitochondria with chosen upper and lower borders.

<table>
<thead>
<tr>
<th></th>
<th>RMSD</th>
<th>upper and lower borders for RMSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>aS9</td>
<td>0.04801</td>
<td>± 0.00353</td>
</tr>
<tr>
<td>aS18</td>
<td>0.04717</td>
<td>±0.00436</td>
</tr>
<tr>
<td>aS27</td>
<td>0.02362</td>
<td>±0.00393</td>
</tr>
<tr>
<td>aS35</td>
<td>0.06093</td>
<td>±0.00551</td>
</tr>
<tr>
<td>aS41</td>
<td>0.04353</td>
<td>±0.00641</td>
</tr>
<tr>
<td>aS56</td>
<td>0.03321</td>
<td>±0.00577</td>
</tr>
<tr>
<td>aS69</td>
<td>0.02955</td>
<td>±0.00268</td>
</tr>
<tr>
<td>aS90</td>
<td>0.02890</td>
<td>±0.00425</td>
</tr>
<tr>
<td>aS140</td>
<td>0.07599</td>
<td>±0.00823</td>
</tr>
</tbody>
</table>

manually in the esfit least-squares fitting to fit the g-tensor \( g = \nu / B \). The obtained value of \( \tau_R \) was used for the two component fit for aS mutants in the presence of mitochondria with the free, fast component \( S_A \) and the bound, slow component \( S_B \) according to the fitting model used in Chapter 6. Fitting parameters as well as the fraction \( b \) according to \( S = (1 - b)S_A + bS_B \) [23] of the bound component are listed for each fit in Section 7.2. Errors for the parameter \( b \) were determined by systematically varying its value to test in which range acceptable simulations of the data were obtained. Table 9.4 gives the root-mean-square deviation (RMSD)-values for all spectral two component fits of wt-aS mutants in the presence of mitochondria with the upper and lower boarder taken for determining the error of fraction \( b \).

9.2.2.3. Analysis of Cw-EPR spectra shown in Chapter 8

All measured scans were baseline-corrected with a 2nd order polynomial over the whole measurement range via the included tool in the spectrometer Xepr software (Bruker Biospin). Analysis of the spectra was performed using Matlab R2016b (The MatWorks, Inc. Natrick, MA) and the toolbox EasySpin 5.1.8 [315]. For the aS fibril spectra all scans were accumulated because no signal decay was observed.

Accumulation of the intracellular signal

For the in-cell aS spectra, the optimal number of scans to accumulate was determined by optimizing the resulting signal-to-noise ratio (SNR) of the middle peak amplitude Therefore, the SNR was plotted against the number of accumulated slices. The position of the maximum of the graph gives the number of slices, that need to be accumulated, to achieve the best SNR (see exemplarily Figure 8.4).

Analysis of oocyte background signal

Caused by the oocyte background signal, a background correction of the in-cell spectra is needed. The background spectra were adjusted for the magnetic field \( (B) \) position of the middle peak of the nitroxide signal, which was necessary due to deviations of the spectrometer frequency, using the relation between the magnetic field \( B \), the measurement frequency \( \nu \) and the
9.2. Data analysis

Table 9.5: Accumulated slices of the intracellular spectra accumulated for spectral comparison.

<table>
<thead>
<tr>
<th>Labeling position</th>
<th>wt-aS</th>
<th>A30P</th>
<th>A53T</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>16-21</td>
<td>15-20</td>
<td>20-27</td>
</tr>
<tr>
<td>18</td>
<td>15-20</td>
<td>25-30</td>
<td>20-25</td>
</tr>
<tr>
<td>27</td>
<td>20-25</td>
<td>25-30</td>
<td>25-30</td>
</tr>
<tr>
<td>35</td>
<td>20-26</td>
<td>25-35</td>
<td>25-32</td>
</tr>
<tr>
<td>41</td>
<td>25-35</td>
<td>25-35</td>
<td>25-35</td>
</tr>
<tr>
<td>56</td>
<td>15-25</td>
<td>25-35</td>
<td>30-39</td>
</tr>
<tr>
<td>69</td>
<td>20-30</td>
<td>30-40</td>
<td>25-30</td>
</tr>
<tr>
<td>90</td>
<td>20-27</td>
<td>25-30</td>
<td>20-25</td>
</tr>
<tr>
<td>140</td>
<td>18-25</td>
<td>30-40</td>
<td>20-25</td>
</tr>
</tbody>
</table>

g-tensor (of the in-cell spectrum): \( B = \nu / g \). The background was smoothed using a Savitzky-Golay filter (exemplarily shown for A53T35 in Figure 8.3). The modified background spectrum was subtracted from the accumulated spectrum of the labeled aS variants injected into oocytes (see Figure A.7 – Figure A.9). In some background spectra there is still some nitroxide signal left because the time between the actual measurement and the background measurement was not long enough and the nitroxide is not fully reduced, yet.

**Kinetics of the nitroxide reduction in the cell**

The signal intensity (amplitude of the middle peak) was plotted against time and fitted with an exponential decay function using Origin (OriginLab, Northhampton, MA, USA) (see Figures A.1 to A.3). The half-life values of the fits are listed in Table 8.2.

**Spectral line shape of the in-cell signal**

The signal shapes of the samples injected into oocytes were analyzed by plotting the accumulated slices 1-10 (expection: aS9 1-8, A30P-56 1-9, A53T-56 1-7) and the accumulated slices according to Table 9.5.

**Microviscosity comparison**

The spin label 4-Oxo-Tempone was used to analyze the intracellular viscosity. The spectrum of 4-Oxo-Tempo in buffer was simulated using the EasySpin function *garlic*. The simulations were done with 0.046 mT linewidth and g-tensor = \([g_x, g_y, g_z] = [2.0268, 2.0033, 1.9897] \). \( A_{xx} = A_{yy} \) and \( A_{zz} \) and the rotational correlation time \( \tau_R \) were fitted for 4-Oxo-Tempo in buffer. A similar simulation with an increased rotational correlation time \( \tau_R \) (by factor 1.2) assuming an increase in viscosity by factor 1.2 was done to mimic the viscosity increase in the cell. Simulation parameters are listed in Table A.10.

**Fitting of reference spectra for conformational comparison**

The EasySpin function *chili* was used for all fits and simulations and a fixed linewidth of 0.13 mT. The spectra of aS9 and aS69 in Tris-HCl buffer (10 mM, pH 7.4) were fitted using a one component fit and the g-tensor \([g_x, g_y, g_z] = [2.0105, 2.0055, 2.0029] \), other parameters are shown in Table A.5. The spectra of aS9 and aS69 bound on POPG LUVs were fitted using a fit of two
9. Details of Experiments and Analysis

components in analogy to Section 9.2.2.1. The parameters of the fast component $S_A$ were taken from the fit of the individual measurements of $aS9$ and $S69$ in buffer (see Table A.5), respectively. The fitting parameters for the slow component $S_B$ are listed in Table A.6 for $aS9$ with LUVs and in Table A.7 for $aS69$ with LUVs.

The spectra of $aS9$ fibrils and of $as69$ fibrils were simulated using a four component fit [307] (a fast (monomeric) component $S_C$ and the slow oligomeric and fibril components $S_D$, $S_E$ and $S_G$). $A_{xx} = A_{yy}$ and $A_{zz}$ and the rotational correlation time $\tau_R$ were fitted for all components. This approach is not based on a physical model of the rotational behavior of the spin label in the fibril, which corresponds very likely to a more complicated motional model. The presented parameter set is very unlikely to fit a spectrum taken at high frequency if this will be done. However, for parameterizing the experimental data for later use in the spectral simulations shown in Figure 8.15 (b) the approach is well suited. The obtained fiting parameters are shown in Table A.8 for $aS9$ fibrils and in Table A.9 for $aS69$ fibrils.

A spectral simulation with varying ratios (values for fraction $b$) of $S_A$ (intrinsically disordered) and $S_B$ (membrane bound) for comparison with the experimental data of $aS9$ in oocytes was performed an plotted in Figure 8.15 (a).

Another spectral simulation with different ratios of the component $S_A$ and the fibril components $S_D$, $S_E$ and $S_G$, taken together in an overall fibril component $S_F$, where all obtained fitting parameters and ratios were kept, was done to simulate aggregated $aS69$. The obtained simulated spectra as well as the intracellular $aS69$ spectrum are depicted in Figure 8.15 (b).

9.2.3. Data analysis of DEER EPR

Data analysis of the DEER traces was performed using the DeerAnalysis_2016 software package [263] and the MMM_2015.2 software [258].

9.2.3.1. Analysis of the data shown in Chapter 7

Deer traces of $aS9$ and $aS27$ in the presence of mitochondria, respectively, were added up using the spectrometer Xepr software (Bruker Biospin). The dimension value 1.8 of the experimental background for the doubly labeled sample was obtained by fitting the dimension of the deer trace $aS9 + aS27$ bound to mitochondria. Therefore the background start was set to zero. The DEER trace of $aS9/27$ in Tris-buffer was analyzed by setting the background dimension as 3 (an assumption for a spin-labeled protein in solution). The DEER trace of mitochondria-bound $aS9/27$ was analyzed by setting the background dimension to 1.8 (obtained from the single mutant traces). For both traces the distance distribution was obtained by Tikhonov regularization. The validation tool (with all optional parameters (see Table 9.6) was used for both measurements to validate the obtained distance distribution and resulted in a RMSD of 0.003555 for $aS9/27$ in solution and a RMSD of 0.007745 for $aS9/27$ bound to mitochondria. The white noise level was set to level 1.0 with one trial.
### Table 9.6: Parameters and ranges and trial numbers used for Tikhonov validation.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Min.</th>
<th>Max</th>
<th>Trial number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Background start</td>
<td>240</td>
<td>1000</td>
<td>11</td>
</tr>
<tr>
<td>Background dim.</td>
<td>1.80</td>
<td>2.40</td>
<td>3</td>
</tr>
<tr>
<td>Background density</td>
<td>0.3</td>
<td>1.2</td>
<td>3</td>
</tr>
<tr>
<td>Modulation depth</td>
<td>0.100</td>
<td>0.350</td>
<td>3</td>
</tr>
</tbody>
</table>

#### 9.2.3.2. Analysis of the data shown in Chapter 8 for spatial distribution of aS in the oocytes

The DEER is used to determine the spatial distribution of aS upon microinjection. 50.6 nL of A30P-41 with an initial spin concentration of 1.12 mM was injected into three oocytes (volume 1 µL, respectively. The sample was incubated for 15 minutes and shock-frozen for the EPR measurement. Proxyl in Tris-HCl bulk solution (20 vol% Glycerol) was used as reference. For determining the concentration of the reference sample, a cw-EPR spectrum was measured using an EMXnano spectrometer (Bruker Biospin). A modulated field sweep containing 3333 data points (sweep time 84.72 s) at a modulation frequency of 100 kHz, 0.6 G modulation amplitude and a time constant of 20.48 ms at room temperature was performed. Spectrometer control and quantitative analysis were performed by the Bruker Xenon software. Using the spin calculation tool, included in the software, the concentration of the reference sample was determined to be 414 µM. The corresponding DEER traces of A30P-41 and for the (shock frozen) reference sample are shown in Figure 8.7. The data were analyzed following the description in the Deer-Analysis2013b manual section 10.6.7. The local spin concentration of A30P-41 was determined to be 165 µM 15 min after incubation. Taking the intracellular lifetime of 17 min into account (Table 8.2) the dilution to 165 µM (local spin concentration) of A30P-41 results into an expansion of the injected volume to a volume of about 182 nL in the cell, which results in an expansion by factor 3.6 after 15 minutes of incubation before shock-freezing the sample:

\[
\begin{align*}
  c(\text{injected Proxyl}) &= 1.12 \text{ mM} \\
  c(\text{Proxyl after 15 min in the cell}) &= 594 \mu\text{M}
\end{align*}
\]
Intracellular volume after 15 min

\[
= \frac{(\text{injected volume}) \cdot c(\text{Proxyl after 15 min in the cell})}{c(\text{local, obtained from DEER measurement})}
\]

\[
= \frac{50.6 \text{ nL} \cdot 594 \mu \text{M}}{165 \mu \text{M}}
\]

\[
= 182.16 \text{ nL}
\]

Volume increase compared to injected volume 182.16 nL : 50.6 nL = 3.6
CHAPTER 10

Summary

In this work, the dynamics and the structure of the intrinsically disordered protein alpha-Synuclein (aS) were investigated.

CD spectroscopy of sequence fragments of aS, in the presence of negatively charged LUVs, revealed differences in the binding affinities within the N-terminal part of aS. The importance of the first 18 residues of aS for adopting an alpha-helical conformation was detected, which is in agreement with other studies.

Furthermore, the motif VATV (residues 52-55), so far only known for its importance in aggregation of aS, was also proven as important for membrane binding, which was not considered before.

A negative effect of the disease related point mutation A30P on the binding affinity was observed by use of a fragment containing the mutation and comparing the helix fraction with a peptide missing this mutation. This result is in agreement with older studies. Additionally, a dampened negative effect (compared to A30P) was also observed for the A53T disease mutation, which is in contrast to other studies with A53T.

A novel simulation approach was used to analyse a site-scan of the whole aS sequence combined with cw-EPR at room-temperature. This completed a study performed by Marta Robotta [295].

Altogether, in this study the negative influences of the disease variants A30P and A53T was shown to affect the overall binding affinity of aS with A30P having the largest effect. These findings were confirmed by CD spectroscopy experiments. Furthermore, the disease point mutations also affected the locally binding behavior around the mutation site. The findings were also shown for aS and its variants with biologically more relevant vesicles made of lipids, that mimic the inner mitochondrial membrane.

In order to take the step from artificial systems to physiologically more relevant systems, the site-scan approach with cw-EPR was used to study the binding behavior of wt-aS onto isolated yeast mitochondria. The results showed a typical binding profile with fluctuations for the binding of the N-terminal residues of aS as well as a decrease for the residues situated in the NAC region.
Additionally, the structure of the membrane bound aS was investigated by performing a DEER experiment with a mitochondria bound aS mutant, which was doubly labeled at residues situated in the N-terminal end of aS, residues 9 and 27. The obtained distance distribution was compared to a simulated distance distribution of this mutant with alpha-helical conformation under respect of spin label flexibility. The experiment is in perfect conformity with the simulation regarding maximum of the obtained distance and width of the distribution. Therefore, it was concluded that aS binds alpha-helically onto the membrane surface of yeast mitochondria.

In the final study the in-cell dynamics and conformation were investigated by using cw-EPR spectroscopy at room temperature and microinjection of *Xenopus laevis* oocytes. Again the site-scan with the various labeling sites was used not only for the aS wild-type but also the disease variants A30P and A53T.

The in-cell signal was analyzed for stability in the cell, for conserving of the spectral shape and for the spectral motional regime. In-cell diffusion was monitored by pulsed EPR measurements.

The in-cell signal showed an exponential decay with varying half-life values depending on the labeling positions. Comparison of the obtained half-life values hinted at the existence of transient structures of the intracellular aS Residues at the N-terminal end show rather short half-lives, whereas the C-terminal residues have larger half-life values. The residues in the NAC region have the longest half-lives, which hints at the existence of transient structures [12] of aS and the disease mutants also in the cell.

The cw-EPR signal of wt-aS, A30P and A53T was compared to the signal obtained from measurements in buffered aqueous solutions, respectively, and no differences in the shape were found. Therefore, it was concluded that the wild-type as well as the disease variants remain intrinsically disordered upon microinjection into oocytes. The in-cell signal showed an exponential decay with varying half-life values depending on the labeling positions. The residues in the NAC region have the longest half-lives, which hints at the existence of transient structures [12] of aS and the disease mutants also in the cell. Due to a low SNR resulting from the reducing intracellular environment, it is possible that a small fraction (< 15 %) of alpha-helical or aggregated aS exists.

The approach demonstrates the potential of in-cell EPR to study proteins, especially IDPs, via site-scan at room-temperature to determine their intracellular dynamics and structures.
Zusammenfassung

In dieser Arbeit wurden sowohl die Dynamiken als auch die Struktur des intrinsisch ungeordneten Proteins Alpha-Synuclein, welches bei der Parkinson-Krankheit eine Rolle spielt, untersucht.

Mittels CD-Spektroskopie wurden anhand von Alpha-Synuclein Fragmenten und negativ geladenen Vesikeln, Unterschiede in der Bindungsaffinität des N-terminalen Endes nachgewiesen. Es wurde gezeigt, dass die ersten 18 Aminosäuren wichtig für die Membrananbindung von Alpha-Synuclein sind, was in Übereinstimmung mit älteren Studien ist.

Darüber hinaus wurde herausgefunden, dass die Sequenzabfolge der Aminosäuren 52-55 (VATV) ebenfalls für die Membrananbindung sehr wichtig ist. Bisher war diese Sequenz lediglich als entscheidend für die Aggregation von Alpha-Synuclein betrachtet worden.


Für EPR-Messungen ist es nötig, das Protein mit Spinsonden zu markieren. Es wurde mittels CD-Spektroskopie gezeigt, dass das anbringen einer Spinsonde an Seitenketten des Proteins, das Membrananbindungsverhalten von Alpha-Synuclein nicht beeinflusst.


Insgesamt wurde in dieser Studie gezeigt, dass die Punktmutationen A30P und A53T die

Gleiche Resultate wurden auch für die Anbindung an biologisch relevantere Membranen durch die Verwendung von Vesikeln, die die innere mitochondriale Membran nachahmen, gefunden.


In einer abschließenden Studie wurde die Dynamik und Struktur von Alpha-Synuclein und den Punktmationen bei Raumtemperatur in Zellen untersucht. Dazu wurde das Protein in Oozyten des afrikanischen Krallenfroschs (Xenopus laevis) mikroinjiziert. Dabei wurden wieder die neun verschiedene Markierungspositionen verwendet. Das intrazelluläre Proteinsignal wurde auf Stabilität, auf Erhalt der spektralen Form hin untersucht.


Die Spektren der intrazellulären Proteinproben wurden mit Spektren verglichen, die von Proteinproben in wässrigen Pufferlösungen stammen. Beide Spektren waren im Rahmen des Rauschens des intrazellulären Spektrums identisch, was die Folgerung zulässt, dass die Alpha-Synuclein-Proben (sowohl Wildtyp als auch Punktmutanten) nicht nur in Lösung sondern auch in der Zelle intrinsisch ungeordnet vorliegen.

Durch das schlechte Signal-zu-Rausch-Verhältnis der intrazellulären Spektren, das durch die Reduktion des Spinmarkers verursacht wird, kann das Vorhandensein eines kleinen membrangebundenen oder aggregierten Anteils von bis zu 15% nicht ausgeschlossen werden. Der gezeigte Ansatz zeigt das Potenzial von intrazellulärer EPR-Spektroskopie in Kombination mit Scans über die gesamte Proteinsequenz zur Untersuchung von intrinsisch ungeordneten Proteinen, um deren Dynamiken und Strukturen in Zellen bei Raumtemperatur zu bestimmen.
Appendix

Table A.1: Secondary structure content of Peptides P1-P10 in the presence of POPG LUVs. The values were obtained by simulating the spectral shape of each secondary structure element as described by de Jongh et al. [291]. The value for the \(\beta\)-turn fraction was in all simulations zero and is therefore not listed in the table. The error values of the alpha-helical component were obtained as described in Section 9.2.1

<table>
<thead>
<tr>
<th></th>
<th>(\alpha)-helix</th>
<th>(\beta)-sheet</th>
<th>random coil</th>
<th>error of alpha-helical component</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>0.7</td>
<td>0.3</td>
<td>-</td>
<td>0.1</td>
</tr>
<tr>
<td>P2</td>
<td>0.46</td>
<td>0.2</td>
<td>0.34</td>
<td>0.1</td>
</tr>
<tr>
<td>P3</td>
<td>0.48</td>
<td>0.49</td>
<td>0.03</td>
<td>0.1</td>
</tr>
<tr>
<td>P4</td>
<td>0.25</td>
<td>0.43</td>
<td>0.32</td>
<td>0.05</td>
</tr>
<tr>
<td>P5</td>
<td>0.12</td>
<td>0.88</td>
<td>-</td>
<td>0.06</td>
</tr>
<tr>
<td>P6</td>
<td>0.6</td>
<td>0.25</td>
<td>0.15</td>
<td>0.05</td>
</tr>
<tr>
<td>P7</td>
<td>0.52</td>
<td>0.34</td>
<td>0.14</td>
<td>0.05</td>
</tr>
<tr>
<td>P8</td>
<td>0.115</td>
<td>0.235</td>
<td>0.65</td>
<td>0.1</td>
</tr>
<tr>
<td>P9</td>
<td>0.8</td>
<td>0.2</td>
<td>-</td>
<td>0.05</td>
</tr>
<tr>
<td>P10</td>
<td>0.63</td>
<td>0.34</td>
<td>0.03</td>
<td>0.05</td>
</tr>
</tbody>
</table>
### Table A.2.

Secondary structure content of wt-aS in the presence of POPG LUVs. The values were obtained by simulating the spectral shape of each secondary structure element as described by de Jongh et al. [291]. The value for the β-turn fraction was in all simulations zero and is therefore not listed in the table.

<table>
<thead>
<tr>
<th></th>
<th>α-helix</th>
<th>β-sheet</th>
<th>random coil</th>
<th>error in alpha-helix component</th>
</tr>
</thead>
<tbody>
<tr>
<td>measurement 1</td>
<td>0.80</td>
<td>0.20</td>
<td>-</td>
<td>0.05</td>
</tr>
<tr>
<td>measurement 2</td>
<td>0.85</td>
<td>0.15</td>
<td>-</td>
<td>0.05</td>
</tr>
<tr>
<td>measurement 3</td>
<td>0.85</td>
<td>0.15</td>
<td>-</td>
<td>0.05</td>
</tr>
<tr>
<td>measurement 4</td>
<td>0.78</td>
<td>0.22</td>
<td>-</td>
<td>0.05</td>
</tr>
</tbody>
</table>

### Table A.3.

Secondary structure content of A30P in the presence of POPG LUVs. The values were obtained by simulating the spectral shape of each secondary structure element as described by de Jongh et al. [291]. The value for the β-turn fraction was in all simulations zero and is therefore not listed in the table.

<table>
<thead>
<tr>
<th></th>
<th>α-helix</th>
<th>β-sheet</th>
<th>random coil</th>
<th>error in alpha-helix component</th>
</tr>
</thead>
<tbody>
<tr>
<td>measurement 1</td>
<td>0.61</td>
<td>0.26</td>
<td>0.13</td>
<td>0.05</td>
</tr>
<tr>
<td>measurement 2</td>
<td>0.55</td>
<td>0.30</td>
<td>0.15</td>
<td>0.05</td>
</tr>
<tr>
<td>measurement 3</td>
<td>0.60</td>
<td>0.23</td>
<td>0.17</td>
<td>0.05</td>
</tr>
<tr>
<td>measurement 4</td>
<td>0.60</td>
<td>0.22</td>
<td>0.18</td>
<td>0.05</td>
</tr>
</tbody>
</table>

### Table A.4.

Secondary structure content of A53T in the presence of POPG LUVs. The values were obtained by simulating the spectral shape of each secondary structure element as described by de Jongh et al. [291]. The value for the β-turn fraction was in all simulations zero and is therefore not listed in the table.

<table>
<thead>
<tr>
<th></th>
<th>α-helix</th>
<th>β-sheet</th>
<th>random coil</th>
<th>error in alpha-helix component</th>
</tr>
</thead>
<tbody>
<tr>
<td>measurement 1</td>
<td>0.65</td>
<td>0.25</td>
<td>0.10</td>
<td>0.05</td>
</tr>
<tr>
<td>measurement 2</td>
<td>0.65</td>
<td>0.20</td>
<td>0.1</td>
<td>0.05</td>
</tr>
<tr>
<td>measurement 3</td>
<td>0.7</td>
<td>0.2</td>
<td>0.1</td>
<td>0.05</td>
</tr>
<tr>
<td>measurement 4</td>
<td>0.67</td>
<td>0.33</td>
<td>-</td>
<td>0.05</td>
</tr>
</tbody>
</table>
Figure A.1.: Time-dependent signal intensities (amplitudes of the middle peaks) of the aS mutants injected into oocytes.
Figure A.2.: Time-dependent signal intensities (amplitudes of the middle peaks) of the A30P mutants injected into oocytes.
Figure A.3.: Time-dependent signal intensities (amplitudes of the middle peaks) of the A53T mutants injected into oocytes.
Figure A.4.: Normalized (by maximum) cw-EPR spectra of wt-aS mutants injected into oocytes of Xenopus laevis slices accumulated at the beginning of the recording and at the end, respectively. Table 9.5 lists which slices were accumulated.
Figure A.5.: Normalized (by maximum) cw-EPR spectra of A30P mutants injected into oocytes of *Xenopus laevis* slices accumulated at the beginning of the recording and at the end, respectively. Table 9.5 lists which slices were accumulated.
A. Appendix

Figure A.6.: Normalized (by maximum) cw-EPR spectra of A53T mutants injected into oocytes of *Xenopus laevis* slices accumulated at the beginning of the recording and at the end, respectively. Table 9.5 lists which slices were accumulated.
Figure A.7.: Cw-EPR spectra of wt-aS samples injected into oocytes of Xenopus laevis (black), smoothed oocytes background (light blue) and background-corrected spectra (red).
Figure A.8.: Cw-EPR spectra of A30P samples injected into oocytes of Xenopus laevis (black), smoothed oocytes background (light blue) and background-corrected spectra (red).
Figure A.9.: Cw-EPR spectra of A53T samples injected into oocytes of Xenopus laevis (black), smoothed oocytes background (light blue) and background-corrected spectra (red).
Figure A.10.: Signal-to-Noise-Ratio (SNR) of cw-EPR spectrum slices for aS mutants to determine the maximum value of the SNR and thus the number of slices to accumulate.
Figure A.11.: Signal-to-Noise-Ratio (SNR) of cw-EPR spectrum slices for A30P mutants to determine the maximum value of the SNR and thus the number of slices to accumulate.
A. Appendix

Figure A.12.: Signal-to-Noise-Ratio (SNR) of cw-EPR spectrum slices for A53T mutants to determine the maximum value of the SNR and thus the number of slices to accumulate.
Figure A.13.: Cw-EPR spectra of the A30P variant at various positions (a)-(i) in aqueous buffered solution (green) and injected into oocytes (after background correction, black).
Figure A.14.: Cw-EPR spectra of the A53T variant at various positions (a)-(i) in aqueous buffered solution (green) and injected into oocytes (after background correction, black).
Table A.5.: Simulation parameters of aS9 and aS69 in aqueous buffered solution, simulation and experimental data are shown in Figure 8.10. Simulations were performed using EasySpin function chilli. Linewidth was 0.13 mT for both simulations. The g-Tensor was \([g_x \ g_y \ g_z] = [2.0105 \ 2.0055 \ 2.0029]\).

<table>
<thead>
<tr>
<th>14N Hyperfine Coupling (MHz)</th>
<th>Rotational correlation time (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A_{xx} = A_{yy})</td>
<td>(A_{zz})</td>
</tr>
<tr>
<td>aS9</td>
<td>13</td>
</tr>
<tr>
<td>aS69</td>
<td>5</td>
</tr>
</tbody>
</table>

Table A.6.: Simulation parameters of the fast component \(S_A\) and the slow component \(S_B\) of aS9 in the presence of POPG LUs. Simulation and experimental data are shown in Figure 8.10. Linewidth was 0.13 mT for both simulation components.

<table>
<thead>
<tr>
<th>14N Hyperfine Coupling (MHz)</th>
<th>Rotational correlation time (ns)</th>
<th>Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A_{xx} = A_{yy})</td>
<td>(A_{zz})</td>
<td>(\tau_R)</td>
</tr>
<tr>
<td>(S_A)</td>
<td>13</td>
<td>111</td>
</tr>
<tr>
<td>(S_B)</td>
<td>11</td>
<td>111</td>
</tr>
</tbody>
</table>

Table A.7.: Simulation parameters of the fast component \(S_A\) and the slow component \(S_B\) of aS69 in the presence of POPG LUs. Simulation and experimental data are shown in Figure 8.10. Linewidth was 0.13 mT for both simulation components.

<table>
<thead>
<tr>
<th>14N Hyperfine Coupling (MHz)</th>
<th>Rotational correlation time (ns)</th>
<th>Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A_{xx} = A_{yy})</td>
<td>(A_{zz})</td>
<td>(\tau_R)</td>
</tr>
<tr>
<td>(S_A)</td>
<td>5</td>
<td>127</td>
</tr>
<tr>
<td>(S_B)</td>
<td>14</td>
<td>105</td>
</tr>
</tbody>
</table>

Table A.8.: Simulation parameters of aS9 fibril fragments using a four component fit [307]. Simulation and spectrum are shown in Figure 8.10. Linewidth was 0.13 mT for all simulation components.

<table>
<thead>
<tr>
<th>14N Hyperfine Coupling (MHz)</th>
<th>Rotational correlation time (ns)</th>
<th>Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A_{xx} = A_{yy})</td>
<td>(A_{zz})</td>
<td>(\tau_R)</td>
</tr>
<tr>
<td>(S_C)</td>
<td>30</td>
<td>88</td>
</tr>
<tr>
<td>(S_D)</td>
<td>17</td>
<td>100</td>
</tr>
<tr>
<td>(S_E)</td>
<td>16</td>
<td>96</td>
</tr>
<tr>
<td>(S_G)</td>
<td>10</td>
<td>85</td>
</tr>
</tbody>
</table>
Table A.9.: Simulation parameters of aS69 fibril fragments using a four component fit [307]. Simulation and spectrum are shown in Figure 8.10. Linewidth was 0.13 mT for all simulation components.

<table>
<thead>
<tr>
<th></th>
<th>$^{14}$N Hyperfine Coupling (MHz)</th>
<th>Rotational correlation time (ns)</th>
<th>Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$A_{xx} = A_{yy}$</td>
<td>$A_{zz}$</td>
<td>$\tau_R$</td>
</tr>
<tr>
<td>$S_C$</td>
<td>24</td>
<td>90</td>
<td>0.51</td>
</tr>
<tr>
<td>$S_D$</td>
<td>31</td>
<td>72</td>
<td>0.79</td>
</tr>
<tr>
<td>$S_E$</td>
<td>25</td>
<td>78</td>
<td>0.85</td>
</tr>
<tr>
<td>$S_G$</td>
<td>22</td>
<td>114</td>
<td>7.02</td>
</tr>
</tbody>
</table>

Table A.10.: Parameters for simulating 4-Oxo-Tempo in aqueous buffered solution and spectral simulation parameters for a changed intracellular viscosity ($= \tau_R$) by factor 1.2. Linewidth for both simulations was 0.046 mT and g-tensor = $[g_x \ g_y \ g_z] = [2.0268 \ 2.0033 \ 1.9897]$. Spectrum and simulations are shown in Figure 8.8.

<table>
<thead>
<tr>
<th></th>
<th>$^{14}$N Hyperfine Coupling (MHz)</th>
<th>Rotational correlation time (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$A_{xx} = A_{yy}$</td>
<td>$A_{zz}$</td>
</tr>
<tr>
<td>4-Oxo-Tempo in buffer</td>
<td>18</td>
<td>98</td>
</tr>
<tr>
<td>4-Oxo-Tempo with changed viscosity</td>
<td>18</td>
<td>98</td>
</tr>
</tbody>
</table>
References


References


References


References


References


References


References


<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>AFM</td>
<td>atomic force microscopy</td>
</tr>
<tr>
<td>aS</td>
<td>alpha-Synuclein</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>cw</td>
<td>continous 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>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminoetraacetic acid</td>
</tr>
<tr>
<td>EPR</td>
<td>electron paramagnetic resonance</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmatric reticulum</td>
</tr>
<tr>
<td>FRET</td>
<td>förster resonance energy transfer</td>
</tr>
<tr>
<td>IR</td>
<td>infrared spectroscopy</td>
</tr>
<tr>
<td>LUV</td>
<td>large unilamellar vesicle</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>MTSL</td>
<td>(1-Oxyl-2,2,5,5-tetramethylpyrrole-3- methyl)methanethiosulfonate</td>
</tr>
<tr>
<td>PD</td>
<td>parkinson’s disease</td>
</tr>
<tr>
<td>POPC</td>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>POPG</td>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1’-rac-glycerol)</td>
</tr>
<tr>
<td>Proxyl</td>
<td>3-Maleimido-2,2,5,5-tetramethyl-1-pyrrolidinloxy</td>
</tr>
</tbody>
</table>
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDSL</td>
<td>site-directed spin labeling</td>
</tr>
<tr>
<td>SNR</td>
<td>signal-to-noise ratio</td>
</tr>
<tr>
<td>SUV</td>
<td>small unilamellar vesicle</td>
</tr>
<tr>
<td>Tempone</td>
<td>4-Oxo-Tempo, 4-Oxo-2,2,6,6-tetramethyl-1-piperidinyloxy</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>wt</td>
<td>wild-type</td>
</tr>
</tbody>
</table>
Published results


* These authors contributed equally.
Danksagung

Zum Schluss möchte ich mich bei einigen Menschen bedanken.

Zuallererst bedanke ich mich bei meinem Doktorvater Malte (Drescher). Danke, dass du mich vor vier Jahren in deine Arbeitsgruppe aufgenommen und mir das Thema Alpha-Synuclein gegeben hast, auch wenn „das Thema der Doktorarbeit eigentlich nicht so wichtig“ ist und „es [...] mehr auf die Leute [ankommt]“, damit man „jeden morgen gerne in die Arbeit kommt“. Ich kann dir da nur zustimmen und kann sagen, dass ich meistens gerne in die Arbeit ging, aber das Thema war trotzdem auch noch spannend. Danke für die gute Betreuung dieser Arbeit.

Christine Peter möchte ich für die Übernahme des Zweitgutachtens danken. Danke Marcel Leist für die Übernahme des Prüfungsvorsitzes der mündlichen Prüfung.


Bei der Konstanz Research School Chemical Biology (KoRS-CB) möchte ich mich für das exzellente Kursprogramm (gerade was transferable skills angeht), die Förderung von Wissensaustausch mit anderen Doktoranden (besonders über die einzelnen Disziplinen hinweg) und die jährlichen Retreat bedanken. Danke auch meinem Thesis-Komitee, Karin Hauser und Alexander Bürkle für stets neue Anregungen und kritisches Hinterfragen in den Treffen.

Danke dem SFB969 für die Finanzierung meines Projektes und die Finanzierung von Reisen, um an zahlreichen Workshops und Konferenzen teilzunehmen zu können.

Danke an die AG Leist (Hanne Gerding, Stefan Schildknecht, Christiaan Karreman und Marcel Leist) für zahlreiche Alpha-Synuclein Diskussionen und die Vermittlung der Mitochondrien-Proben.

Danke an Thomas Becker von der Universität Freiburg für die Bereitstellung von Hefe-Mitochondrien.

Danke an das RSC ESR Group Committee für die Verleihung eines Posterpreises bei der 50th RSC ESR Conference in Oxford 2017.

Danke dem Organisationskomitee der 7th EFEPR Summer School in Berlin, dass ich an der Summer School teilnehmen durfte und danke dem SPP 1601 Programm für ein Travel Stipend zur Summer School.
Vielen lieben Dank an Marta Kolesko (geborene Robotta). Danke, dass du mir all dein Wissen über Alpha-Synuclein vermittelt hast, stets hilfbereit bei Problemen warst, für das gemeinsame Paper und für das gemeinsame Schwimmen im Schwaketenbad (als es noch stand), die vielen Privatgespräche als Pause von der Arbeit und deine Freundschaft.

Danke an Juliana Martins für ihren Beitrag zum JACS-Paper.

Danke Christian (Hintze), dass du dir die Zeit genommen hast, mir das Messen an den Elexsys-Spektrometern beizubringen, Hilfestellung bei Matlab-Fragen gegeben hast und sonst immer auch hilfbereit bei Problemen warst.

Vielen Dank Andreas Groß, dass du mir gezeigt hast, wie man Oozyten mikroinjiziert und für Hilfe bei Spektrometerproblemen.

Danke an Juliane Stehle, Lara Williams, Christopher Dietrich, Laura Knörr und Anandi Kugele, die im Rahmen von Mitarbeiterpraktika und Hiwi-Jobs an dieser Arbeit mitgewirkt haben.

Vielen Dank an Artem Fedoseev für die simple, aber geniale Idee, die Kapillaren mit den Ooozyten von oben zu verschließen, damit der Kit kein Störsignal gibt.

Andreas Heim möchte ich fürs Einschleußen in die TFA danken, damit ich dort die Spitzen fürs Mikroinjizieren ziehen konnte.

Vielen Dank an Philipp Graus für die große Hilfe mit den AFM-Aufnahmen.

Danke Patrick Roser für gemeinsame CD Messungen mit den Peptiden in Konstanz und auch in Karlsruhe am KIT, für die Verbreitung von guter Laune und fürs Korrekturlesen.

Danke an Anne Ulrich, Jochen Bürck, Bianca Posselt und Siegmar Roth vom KIT in Karlsruhe für die Möglichkeit am KIT Messungen durchzuführen, die nette Betreuung sowie hilfreiche Tips zur Datenauswertung.

Danke Theresa Braun fürs Korrekturlesen und Beantwortung von Bio-Fragen.

Danke Martin Spitzbarth für unzählige Male, die du mir mit Latex aber besonders mit Matlab geholfen hast und das, obwohl du Matlab hasst.

Danke an Dennis Bücker fürs überaus kritische Korrekturlesen und für deine Freundschaft.

Sabrina Weickert möchte ich ganz herzlich für die gemeinsame Zeit, die zahlreichen gemeinsamen Lach-Flashs, die in Tränen endeten, ein stets offenes Ohr für fachliche als auch private Dinge angeht und die Freundschaft danken.

Danke an die ganze AG Drescher für die freundliche Atmosphäre, die kleinen privaten Gespräche in der Kaffee- oder Mensapause, viel Kuchen, die stets vorhandene Hilfsbereitschaft was wissenschaftliche Fragen und Probleme angeht und insgesamt für die schöne Zeit. Ihr werdet mir fehlen!

Danke meinen Freunden für die Ablenkung vom Doktoranden-Alltag.

Vielen lieben Dank meinen Geschwistern Sonja und Stefan Cattani, sowie meinem Schwager Thorsten Naserke für ein stets offenes Ohr, Ermütigungen und Ratschläge zur rechten Zeit. Danke Stefan für deinen Humor, der einen stets zum Lachen bringt.

Vielen lieben Dank meinem Freund Enrico Kolb für dein Verständnis, wenn ich mal wieder gestresst war, deine Unterstützung und so vieles mehr.

Ich danke von Herzen meinen Eltern Hildegard und Peter Cattani für die jahrelange Unterstützung, die Aufmunterungen und Ermütigungen in schwierigen und stressigen Zeiten und zahlreiche Spielenachtstage oder -abende bei meinen Besuchen daheim.