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Alpha-Synuclein Disease Mutations Are Structurally Defective and Locally Affect Membrane Binding

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ABSTRACT: The intrinsically disordered human protein alpha-Synuclein (αS) has a prominent role in Parkinson's disease (PD) pathology. Several familial variants of αS are correlated with inherited PD. Disease mutations have been shown to have an impact on lipid membrane binding. Here, using electron paramagnetic resonance spectroscopy in combination with site-directed spin labeling, we show that familial PD-associated variants are structurally defective in membrane binding and alter the local binding properties of the protein.

Alpha-Synuclein (αS) is a 140-amino acid protein[1] belonging to the intrinsically disordered protein (IDP) family, a group of proteins that lack well-defined secondary structure in solution.[2] Upon macromolecular interaction, it undergoes conformational changes into different conformations; hence αS has been referred to as a "protein chameleon".[3] The primary structure of αS can be divided into distinct regions: a positively charged N-terminus (amino acids 1−95, which includes an amyloidogenic hydrophobic NAC region (amino-acids 60−95), and a highly negatively charged C-terminus (amino-acids 96−140). The N-terminal part (approximately residues 1−100) contains seven imperfect repeats of the KTKEGV motif that are able to form amphipathic helices that associate with membranes containing phospholipids,[4−11] such as synaptic vesicles or mitochondrial membranes.[12] αS binds alpha-helically to the inner mitochondrial membrane.[13]

Genetic studies have identified point mutations, A30P,[14] A53T[15] and E46K[16] as well as the recently discovered H50Q,[17] G51D[18,19] and A53E[20] mutations in the N-terminal region that are associated with familial forms of PD. The different αS variants have differing impact on the membrane binding properties and aggregate formation.[21] A53T, E46K and H50Q enhance mature fibril formation,[22,23] A30P enhances oligomer formation but retards mature fibril formation,[24,25] whereas G51D retards aggregation.[23,26] NMR data on micelle-bound αS suggest that the A30P disease mutant slightly destabilizes and alters the protein's helical structure around the site of the mutation.[27,28] E46K shows enhanced binding,[29,30] the A53T mutation appears to have little effect on the αS−lipid interaction,[27,28,30] but several reports indicate that the A30P mutation perturbs membrane binding in vitro[7] and in vivo.[4,31] The expression of both A30P and A53T αS makes the cells more vulnerable to oxidative stress[32,33] or dopamine toxicity.[34] Accordingly, the overall membrane binding properties of A30P and A53T have been studied extensively[4,7,29,35−37] using large unilamellar vesicle (LUVs) and artificial vesicles mimicking the inner mitochondrial membrane (IMM).[38]

More rapid oligomerization kinetics exhibited by the disease variants relative to the wild-type protein were reported.[24] It has been suggested that this might be the reason for the reduced overall binding affinity of the αS disease variants. Alternatively, the disease-related substitutions might locally distort the membrane binding properties resulting in a lower overall binding affinity. To address this question, we exploit electron paramagnetic resonance (EPR) spectroscopy in combination with site-directed spin labeling. This technique enables to focus on the local binding affinity around the attached spin label and leads to a more detailed view of the protein−membrane interactions of αS and two αS PD-associated variants, A53T and A30P.[39,40]

We prepared derivatives of A30P and A53T αS disease variants site-directedly spin labeled with 3-Maleimido-PROXYL at single cysteines introduced at positions 9, 18, 24, 27, 28, 32, 35, 41, 56, 68, 90 and 140, respectively. We analyzed the rotational mobility of the spin labels attached to αS by continuous wave (cw) EPR spectroscopy at X-band (~9.5 GHz) at room temperature. For a detailed study of membrane binding properties we have used LUVs made of mixtures of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) (POPG) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), resulting in lipid compositions with different surface charge densities ρ (ρ = [POPG]/([POPG] + [POPC])). Changes in the rotational mobility of the spin labels upon membrane binding allow monitoring the local degree of binding in the proximity of the labeled sites as a function of the membrane composition (see SI).

In control experiments, we assessed whether insertion of the spin label itself affects the αS-conformation. Upon binding to negatively charged membranes, all tested samples showed CD spectra expected for α-helical structures[35,41] without spectral

differences between the spin labeled and unlabeled αS samples (Figure S3). In spite of these control experiments, the label might affect the membrane binding properties. Therefore, it is worth mentioning that the conclusions presented derive exclusively from comparing labeled wtαS with labeled A30P and A53T, to minimize any possible influence of the label on the interpretation.

Representative experimental EPR spectra of wtαS, A30P and A53T labeled at position 18 are shown in Figure 1. In the absence of LUVs, there is no significant difference between the measured spectra, which can be described by a spectrum S_A obtained by a one-component spectral simulation in the fast motion regime (Tables S1, S2). The obtained isotropic rotational correlation times (τ_r) are in the range of ~0.2−0.5 ns and indicate a high spin label mobility typical for unfolded proteins.[42,43]

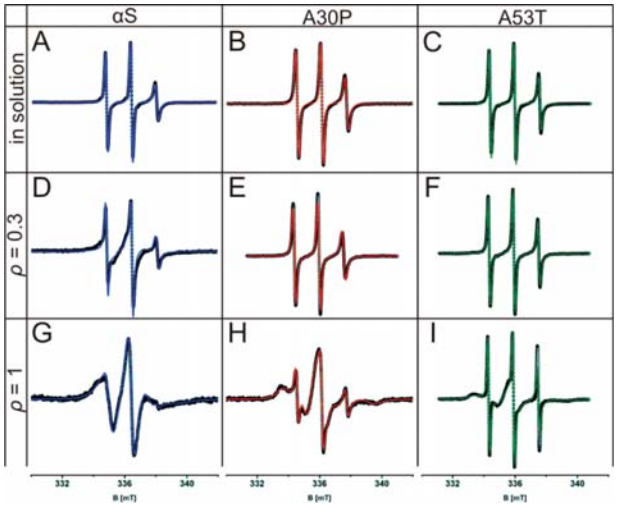

Figure 1. Representative experimental cw-EPR spectra (circles) and corresponding spectral simulations of wtαS (blue line), A30P (red line) and A53T (green line) labeled at position 18 in the absence (A, B and C) and the presence of LUVs (ρ = 0.3: D, E and F; ρ = 1: G, H and I). For full data set, see Figures S5−S33.

In the presence of LUVs with increasing ρ, i.e., with increasing content of negatively charged lipids, the spectra gradually change relative to the spectra in the absence of vesicles. Line broadening indicates a lower mobility of the spin label, which results from local binding close to the investigated spin label positions of a fraction b of the αS molecules to the LUVs. Each spectrum S in the presence of LUVs was consequently fitted with a superposition of two spectra S_A and S_B and the corresponding local binding parameter b was determined according to $S = (1 - b)S_A + bS_B$ (for full set of fit parameters, see Tables S1, S3 and S4). Most of the experimental spectra (>95% of the 200 experimental spectra) were well described by this simple model (using three free fitting parameters; other parameters were determined by individual experiments, see Figures S5−S34). For a small selection of experimental spectra (<5%), there are significant deviations between the fit and experimental data. This could imply that aSyn can adopt more states suggesting a more complex model. However, for these spectra, if we apply a two-state model with an increased number of free parameters, the fit to the experimental data improves (see SI for details and Figure S35). However, most importantly, the numerical results for the salient parameter b obtained by both fitting procedures are identical (within the error bars, see Table S7). To avoid the risk of overfitting the data, and to reduce computational overhead, we chose the simple fit procedure.

Figure 2 compares the local binding affinity b of wtαS, A30P and A53T labeled at position 18 for different POPG/POPC compositions. Generally, the degree of local binding b increases with increasing values of ρ. The local degree of binding for A30P and A53T differs from that for wtαS. Both disease variants analyzed start binding to the LUVs at higher POPG content than wtαS (For all results see Figure S1).

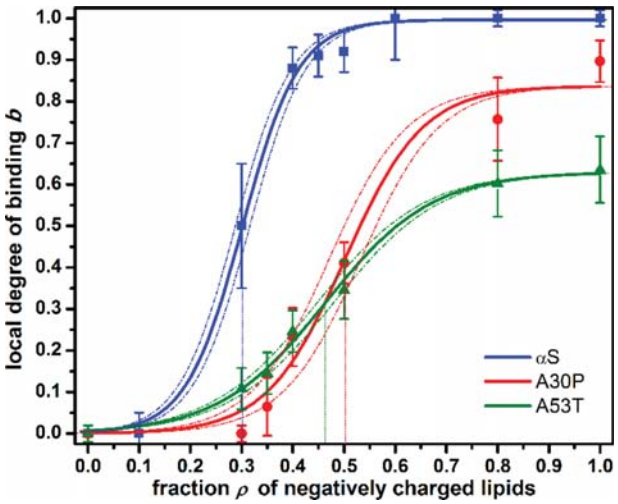

Figure 2. Parameter b reflects the degree of local binding in the proximity of the labeled residue. Here, we show data for protein labeled at residue 18 of wtαS (blue), A30P (red) and A53T (green). The vertical lines mark the inflection point I of the sigmoidal curves, which were used to parametrize $b(\rho)$. The dotted lines indicate deviating sigmoidal curves with I varied within the error bars determined by the fit.

To parametrize the dependence of b on ρ, we used sigmoidal curves (Figure 2) of the form $f(\rho) = A/(1 + \exp(-k(\rho - I)))$, where A is the amplitude, I the inflection point and k the steepness of the fitted function (parameters see Table S6).

For an overview of the entire set of investigated samples, the inflection point I of the sigmoidal curves[44] is plotted as a function of the labeled positions studied in Figure 3. Note that a low I value represents significant local binding affinity b already at low membrane charge density ρ. For wtαS, the behavior of I reflects that αS's membrane binding is triggered by its N-terminus.[40] For instance, the region around residue 9 features significant local binding affinity for ρ > 0.2, whereas local binding of the middle part of the protein (approximate residues between 18 and 41) requires at least $\rho \approx 0.3$. The region around residue 90 requires at least $\rho \approx 0.55$ for local binding. There is a tendency that the N-terminus is tighter bound than the C-terminus. A30P and A53T show in general similar binding properties. Although we have focused here on the local binding affinities, the curves suggest qualitatively a reduced overall binding to membranes that is qualitatively consistent with the reduced α-helical content determined by CD spectroscopy (reduction of ~30%, Figure S3d). The N-termini bind for ρ > 0.45, whereas the residues closer to the end of the helical region (residue 90) require at least $\rho \approx 0.75$ for

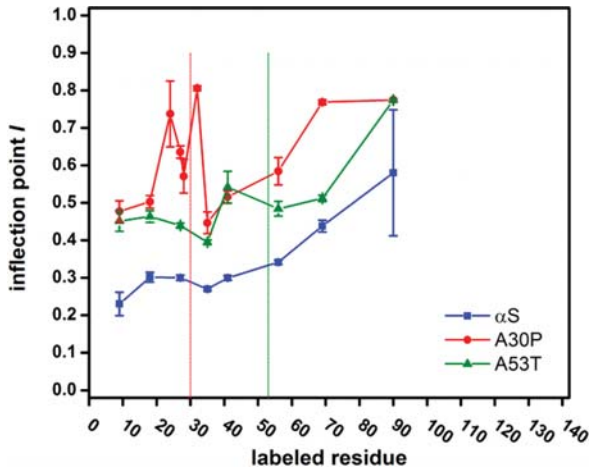

Figure 3. Inflection points I of the sigmoidal curves for wtαS without disease point mutation (blue), A30P (red) and A53T (green) at different labeled residues. The vertical lines mark the disease-related amino acid substitutions on residue 30 (A30P, red) and 53 (A53T, green). For αS140 in the study range $0 < \rho < 1$, no local binding was detected, therefore I cannot be determined.

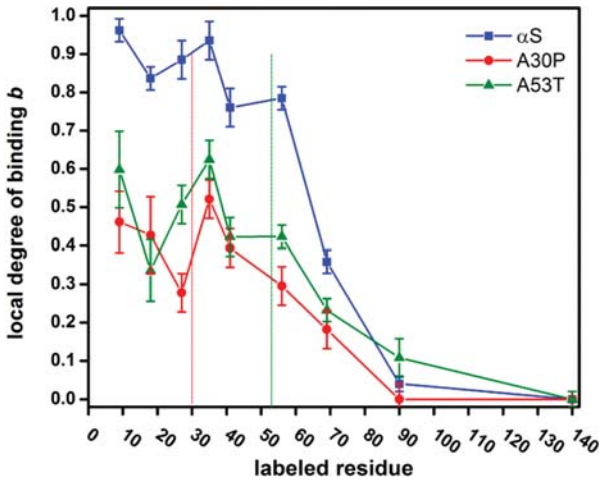

Figure 4. Comparison of the local binding affinity b of αS (blue), A30P (red) and A53T (green) at different labeled residues of αS in the presence of LUVs mimicking the IMM. Full set of fit parameters is shown in Table S5.

binding. However, in addition to the general increase of I for A30P and A53T compared to wtαS, local changes of I along the protein sequence are evident. For A53T the effect is rather subtle, although a local maximum of I at residue 41 can be identified. For A30P, local effects are much more pronounced. Residues 24, 27, 28 and 32 flanking the disease substitution feature increased I values indicating reduced local binding affinity.

Because lipid composition or membrane fluidity has an impact on the binding properties of αS,[45] we investigated whether distortion of local binding affinity due to disease mutations can also be observed on a more biological relevant membrane system, namely LUVs mimicking IMM. Previous reports have shown that αS binds to membranes mimicking the inner but not the outer mitochondrial membrane.[13] Therefore, we analyzed the local binding affinity on LUVs mimicking the IMM made from POPC/1-hexadecanoyl-2-(9*Z*-octadecenoyl)-*sn*-glycero-3-phosphoethanolmine (POPE)/ cardiolipin (CL)/ cholesterol (Ch) lipids in 2:1.3:1:0.6 molar ratio (experimental spectra with spectral simulations are shown in Figure S34). Figure 4 shows the local degree of binding b for wtαS, A30P and A53T in the presence of IMM-mimicking LUVs. The local degree of binding in the N-terminal part is in the same range $b \approx 0.3-0.6$ for A30P and A53T, but is significantly smaller than for wtαS ($b \approx 0.75-0.98$), again reflecting a reduced overall binding affinity of the disease mutants.

Again, also for αS interacting with IMM, we observe a decrease of the local binding affinity close to the point mutations A53T but in particular close to A30P. To compare the results on IMM with LUVs made of POPG/POPC lipids, see Figure S2.

In this work, the local membrane binding properties of the two αS disease mutations A30P and A53T were investigated using site-directed spin labeling in combination with EPR spectroscopy allowing determining the local binding affinity to membranes. In a recent study,[40] it was shown that the N-terminal repeats of wild-type αS undergo a nonuniform binding to membranes. Our current results suggest a nonuniform binding affinity of the N-terminus of A30P and A53T αS disease mutants, too. However, our study also revealed that both point A30P and A53T αS mutations significantly perturb the local binding affinity of αS to phospholipid membranes (Figures 2 and S1).

The data (Figure 3) suggest that the A53T mutation slightly destabilizes the protein's binding affinity in proximity of the site of the substitution. This is reflected by the reduced local binding affinity near the disease mutation, i.e., at the labeled position 41. A much stronger, but similar effect is observed for the A30P data, where the local binding affinity considerably decreases in the region flanking the point mutation. The effect is also pronounced on membranes mimicking the inner mitochondrial membrane.

In summary, we have shown that the disease-related variants studied, in which an alanine residue (one of the strong α-helix forming residues[46]) is replaced either by threonine (A53T) or proline (A30P), are structurally defective in binding to the membrane resulting in reduced overall membrane binding.

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Notes

The authors declare no competing financial interest.

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