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Tryptophan Fluorescence Reveals Structural Features of α-Synuclein Oligomers

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Oligomeric α-synuclein (αS) is considered to be the potential toxic species responsible for the onset and progression of Parkinson's disease, possibly through the disruption of lipid membranes. Although there is evidence that oligomers contain considerable amounts of secondary structure, more detailed data on the structural characteristics and how these mediate oligomer–lipid binding are critically lacking. This report is, to our knowledge, the first study that aimed to address the structure of oligomeric αS on a more detailed level. We have used tryptophan (Trp) fluorescence spectroscopy to gain insight into the structural features of oligomeric αS and the structural basis for oligomer–lipid interactions. Several single Trp mutants of αS were used to gain site-specific information about the microenvironments of monomeric αS, oligomeric αS and lipid-bound oligomeric αS. Acrylamide quenching and spectral analyses indicate that the Trp residues are considerably more solvent protected in the oligomeric form compared with the monomeric protein. In the oligomers, the negatively charged C-terminus was the most solvent exposed part of the protein. Upon lipid binding, a blue shift in fluorescence was observed for αS mutants where the Trp is located within the N-terminal region. These results suggest that, as in the case of monomeric αS, the N-terminus is critical in determining oligomer–lipid binding.

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Keywords: α-synuclein; oligomer; membrane; tryptophan; fluorescence

Introduction

The protein α-synuclein (αS) is commonly regarded as a possible causative factor in the pathogenesis of Parkinson's disease (PD). Three missense mutations in the αS gene[1–3] as well as locus duplication and triplication[4,5] have been found to cause rare familial variants of the disease. Additionally, aggregated αS is the main component of Lewy bodies, which are the intracellular inclusion bodies found in PD patients.[6] However, whether the aggregation of αS is related to its neurotoxicity or merely a sign of neuronal stress remains hotly debated and the mechanism by which αS causes neuronal degradation is still not known. It has been reported that Lewy body formation does not correlate with PD progression and severity.[7,8] Therefore, early intermediates in the aggregation of αS have been postulated as the toxic species.[9,10] These oligomeric intermediates have been found to be considerably toxic upon addition to cultured cells.[11,12] A possible mechanism of toxicity of αS oligomers is through the permeabilization of cellular membranes.[12,13] Oligomeric αS can permeabilize lipid vesicles and membranes, possibly through a pore-like mechanism.[14–16]

However, the topic of oligomer-induced toxicity remains controversial. Structural information about oligomeric species is critically lacking, and the structural basis for oligomer–lipid interaction and toxicity is not understood. In addition to its importance to toxicity, the structure of oligomeric intermediates is important for understanding the aggregation process. Aggregation of αS is believed to occur through a process of nucleation-dependent polymerization.[17] However, the nature of this nucleus and how it is formed are unknown. Initial

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Abbreviations used: αS, α-synuclein; PD, Parkinson's disease; Trp, tryptophan; WT, wild type; LUV, large unilamellar vesicle; NATA, *N*-acetyl-L-tryptophanamide; DOPS, 1,2-dioleoyl phosphatidylserine.

data show that oligomers are not randomly collapsed aggregates but contain significant amounts of secondary structure. Oligomers have been reported to contain considerable β-sheet structure,[18] although α-helical content has also been observed.[19] Several fundamental problems limit the determination of the structure of αS oligomers. Early intermediates can be unstable and in fast equilibrium with the monomeric protein.[11] Aggregation intermediates are transient in nature and disappear upon fibril formation.[20] Preparation of stable oligomers generally results in low yields, and thus only little material is available.[18] Finally, oligomers are possibly heterogeneous in size, structure and function.[11,21] Thus, determining the exact structure of all possible oligomers of αS remains a challenging task. However, the structure of the different oligomeric species and the fibrillar structure might be related since the driving force of aggregation is the same for all species. Therefore, establishing structural constraints for the αS oligomeric species should be feasible.

In order to overcome some of these challenges and to gain insights into oligomer structure and the structural basis for oligomer interaction with lipids, we have used tryptophan (Trp) fluorescence spectroscopy on stable purified αS oligomers. These oligomers occur during αS aggregation and have been previously used to study oligomer–lipid interactions.[18,22] Several single Trp mutants of αS were generated to assess the microenvironments of different regions of the protein in the monomeric, oligomeric and lipid-bound oligomeric states. Here, we show for the first time how individual monomers might be arranged in an oligomeric aggregate and what regions of the αS molecule mediate the lipid interaction in the oligomeric form. The results from acrylamide quenching experiments and spectral analyses indicate that, compared with the monomeric protein, the engineered Trp residues are more solvent protected in the oligomeric form. The negatively charged C-terminus was the most solvent exposed part of the oligomers. When lipid vesicles were added to oligomeric αS, a blue shift in fluorescence was observed for αS mutants where the Trp is located within the N-terminal region of residues 4–90. This observation indicates that, similar to the monomeric protein, the N-terminal part of the protein most likely mediates the lipid binding of αS oligomers.

Results

Since wild-type (WT) αS does not contain any Trp residues, replacing an amino acid in the αS sequence with a Trp results in site-specific information about the microenvironment. A number of mutants containing a single Trp substitution in different regions of the protein were prepared using site-directed mutagenesis (F4W, Y39W, A69W, A90W, A124W and A140W) (Fig. 1a). We performed aggregation experiments to check if the introduced mutations in αS affected fibril formation. All αS Trp mutants showed typical sigmoidal growth curves characteristic of fibril formation when monitored using a thioflavin-T fluorescence assay (Supplementary Fig. S1). Fibril formation was further confirmed by atomic force microscopy (Supplementary Fig. S2). Oligomeric mutant αS was prepared following the same protocol as that for WT αS. The resulting Trp-containing oligomers possessed properties similar to those prepared from WT αS. The oligomers induced membrane disruption when added to large unilamellar vesicles (LUVs) and did not induce a

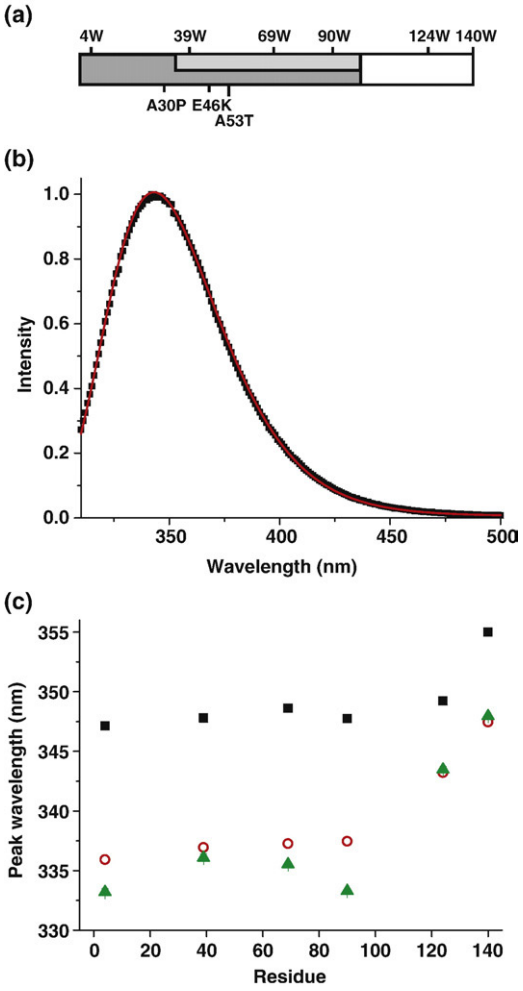

Fig. 1. Spectral analysis of the different αS Trp mutants. (a) Mutants containing a Trp residue in different regions of the protein were created. The N-terminal region around residues 1 to 100 (dark gray) are involved in lipid binding of monomeric αS, the region around residues 35 to 100 (light grey) make up the core of fibrillar αS, and the C-terminus comprising approximately residues 100 to 140 (white) remains unstructured under most conditions. The disease-related mutations are also shown in the figure. (b) Trp emission spectrum of oligomeric 124W αS. The spectrum is fitted with a log-normal function that estimates the peak emission wavelength and the spectral width at half maximum. (c) The peak wavelength of the Trp emission plotted for monomeric αS (squares), oligomeric αS (open circles) and lipid-bound oligomers (triangles) as a function of the position of the inserted Trp.

considerable increase in fluorescence in a thioflavin-T fluorescence assay (Supplementary Fig. S3).

The emission wavelength of Trp fluorescence is sensitive to the polarity of the environment. Spectral analysis of the Trp emission from proteins therefore reports on the properties of the microenvironment of the corresponding Trp residues. Solvent-exposed Trp residues show a red shift in the fluorescence compared with buried Trp residues in proteins. The Trp emission spectrum was recorded for the different mutants of αS in the monomeric and oligomeric forms. In addition, spectra of oligomers in the presence of 1,2-dioleoyl phosphatidylserine (DOPS) LUVs were recorded. We have previously reported that oligomeric αS can bind and permeabilize vesicles composed of DOPS.[14,22] The emission peak wavelengths were determined from the emission spectra (Fig. 1b) by fitting Eq. (1)[23] (see Materials and Methods). The resulting peak wavelengths for monomeric αS and oligomeric αS are plotted in Fig. 1c and summarized in Table 1 (see Supplementary Fig. S4 for the corresponding spectra). For monomeric αS, the Trp emission maximum wavelengths were between 347 and 349 nm for the 4W, 39W, 69W, 90W and 124W αS mutants and around 355 nm for 140W αS. Emission at these wavelengths is indicative of a relatively polar environment for all the monomeric Trp mutants, as can be expected for an intrinsically disordered protein. In the oligomeric form, the emission from Trp mutants 4W, 39W, 69W and 90W was considerably blue shifted with emission maxima of 336 to 338 nm. These residues are thus significantly less exposed to the solvent and buried in the aggregate. The blue shift was less pronounced for 124W αS and 140W αS, which showed emission maxima at 343 and 348 nm, respectively. It thus appears that the highly negatively charged C-terminus of the protein remains more solvent exposed and that approximately residue 4 to residue 90 make up the core of the oligomeric aggregate. When DOPS LUVs are added to the oligomers, the Trp emission spectra of the mutants 4W, 69W and 90W were even more blue shifted. This blue shift was less pronounced for 39W αS. The spectra of the 124W and 140W mutants remained unchanged. The blue shift upon lipid interaction indicates that these residues might embed in the hydrophobic core of the membrane or that the N-terminus changes its conformation upon membrane binding.[24]

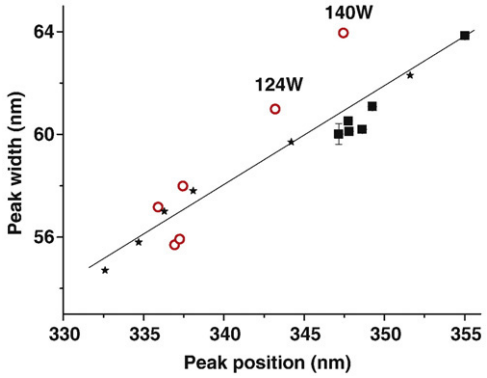

Fig. 2. Conformational heterogeneity of the different αS Trp mutants. The width of the emission spectrum is plotted as a function of the peak wavelength for monomeric αS (squares) and oligomeric αS (open circles). Data from the spectral analysis of NATA in solvents of different polarity (stars) and a linear fit through these points are also plotted. The error bars indicate the standard deviation ($n=2$).

In addition to the peak wavelength, the width of the emission spectrum also carries information. In homogeneous solvents, the width of the emission spectra depends on the emission wavelength.[23] In heterogeneous emitting systems, where different populations of Trp residues sense a different environment, spectral broadening does occur. This can be visualized by plotting the emission width as a function of peak wavelength. A broadened spectrum compared with the *N*-acetyl-L-tryptophanamide (NATA) emission in different solvents is a measure of emitter heterogeneity. As shown in Fig. 2, spectral broadening does not occur for monomeric αS. For the oligomeric protein, most mutants do not show signs of spectral broadening. Only the spectra of the 124W and 140W mutants show considerable deviations from NATA emission in pure solvents.

Quenching of Trp fluorescence by added solutes is another powerful method to measure the accessibility of Trp residues.[25] Since we have observed in earlier work that the membrane interaction of oligomeric αS is dependent on the salt concentration,[14] we have chosen acrylamide as a quenching agent instead of iodide ions. The Trp fluorescence quenching by acrylamide is mainly a

Table 1. Summary of the results from the spectral analyses and quenching experiments

	Monomer			Oligomer			Oligomer + LUVs		
Mutant	λ (nm)	K (M^{-1})	V (M^{-1})	λ (nm)	K (M^{-1})	V (M^{-1})	λ (nm)	K (M^{-1})	V (M^{-1})
4W	347.2±0.07	9.8±1.4	2.0±0.3	335.9±0.08	3.8±0.07	0.8±0.02	333.2±0.32	3.0±0.1	0.3±0.1
39W	347.8±0.03	19.3±1.2	1.4±0.3	337.0±0.03	3.3±0.02	0.7±0.01	336.1±0.33	3.5±0.02	—
69W	348.6±0.20	11.5±1.1	0.6±0.1	337.3±0.03	4.3±0.2	0.5±0.06	335.5±0.16	3.3±0.02	0.1±0.06
90W	347.8±0.09	14.2±1.2	0.8±0.2	337.5±0.01	3.9±0.2	0.6±0.07	333.3±0.09	3.1±0.2	0.1 ±0.01
124W	349.2±0.13	10.2±0.4	0.8±0.1	343.2±0.04	6.0±0.2	0.5±0.04	343.5±0.26	6.7±0.2	—
140W	355.0±0.05	19.3±1.2	1.4±0.3	347.5±0.05	8.6±0.2	0.4±0.04	347.9±0.05	9.3±1.0	—

Peak wavelengths for different Trp mutants and the results of fitting Eq. (2) to the Stern–Volmer plots (Supplementary Fig. S4) generated from fluorescence quenching by acrylamide. Data are the average value and standard deviation from duplicate experiments.

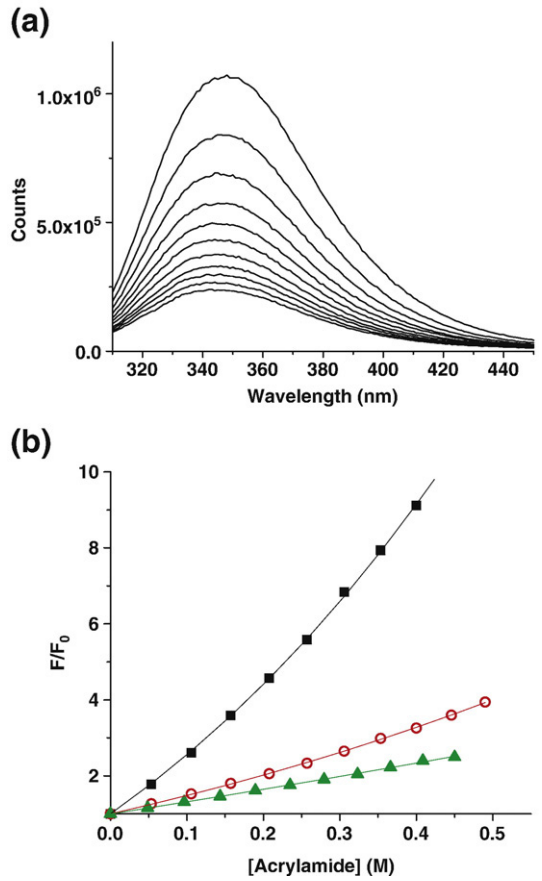

Fig. 3. Example of a typical quenching experiment. (a) Quenching of Trp emission of oligomeric 90W αS by addition of increasing amounts of acrylamide. (b) Stern–Volmer plots of monomeric 90W αS (squares), oligomeric 90W αS (open circles) and lipid-bound oligomeric 90W αS (triangles). The data were fitted using the modified Stern–Volmer equation (Eq. (2)).

collisional effect and is thus dependent on the concentration of quencher and the solvent accessibility of the Trp residue. Figure 3a shows an example of a typical quenching experiment in which a spectrum is recorded after each addition of acrylamide. Stern–Volmer plots were generated from these spectra (Fig. 3b). For monomeric αS and oligomeric αS, the Stern–Volmer plots showed an upward curvature. Therefore, these were fitted with a modified form of the Stern–Volmer equation (Eq. (2) that also accounts for static quenching.[25] The quenching experiments were performed for all αS Trp mutants. The results of fitting the Stern–Volmer plots are summarized in Table 1 (the corresponding plots are shown in Supplementary Fig. S5). Since the curvature of the Stern–Volmer plots might also be caused by a heterogeneity of the position of the Trp residue in the oligomer, the Stern–Volmer constants derived from the fitting procedure were not directly used for comparison of the different Trp mutants. The fit results were rather used to calculate the quenching ratio at 0.5 M acrylamide as a general parameter of accessibility (Fig. 4) to allow an unbiased comparison among the different Trp mutants. For monomeric αS, the Trp fluorescence can be easily quenched, especially at the N- and C-termini of the protein (4W and 140W). In the oligomeric form, the Trp residues in the mutants 4W, 39W, 69W and 90 W are considerably more protected, which is in agreement with the observed blue shift in emission. Compared with monomeric αS, the oligomers from the 124W and 140W Trp mutants were more protected from quenching by acrylamide. However, consistent with the view from the spectral analysis that the C-terminus of αS is solvent exposed, the oligomers from the 124W and 140W mutants showed the highest Stern–Volmer constants. The addition of DOPS LUVs to the oligomers generally led to a small reduction in the accessibility of the Trp residue. Pronounced differences between the different regions of the protein were not observed.

To compare the structural properties of oligomers with those of fibrils, we also performed Trp fluorescence spectroscopy and quenching experiments on fibrils prepared from the different Trp mutants. However, the results were generally of poor quality. Around 5% to 10% of soluble αS (as assayed by the optical absorbance of the supernatant after centrifugation) was still present after 1 week of aggregation. In addition, fibril-containing solutions were turbid and larger particles sank to the bottom of the cuvette. However, the results from the 69W mutant highlight that the residues in the hydrophobic amyloidogenic part of the protein are buried (Fig. 5). The Trp emission was highly protected from quenching by acrylamide. Furthermore, the emission peak wavelength was shifted to around 324 nm. In the densely packed amyloid core of the fibrils, the residues are thus

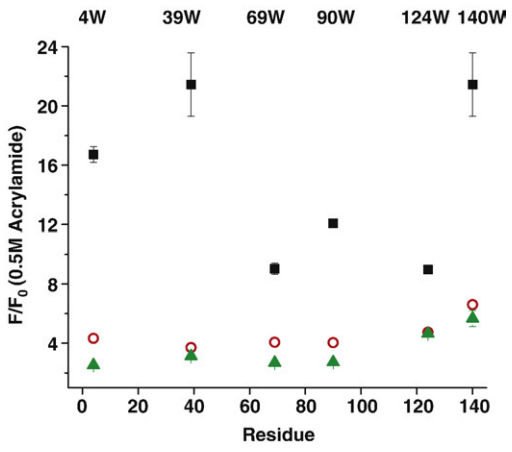

Fig. 4. A comparison of the accessibility of the different Trp mutants to acrylamide. The calculated quenching ratio at 0.5 M acrylamide from the fit results (Table 1) is plotted as a function of the position of the inserted Trp for monomeric αS (squares), oligomeric αS (open circles) and lipid-bound oligomeric αS (triangles). The error bars indicate the standard deviation ($n=2$).

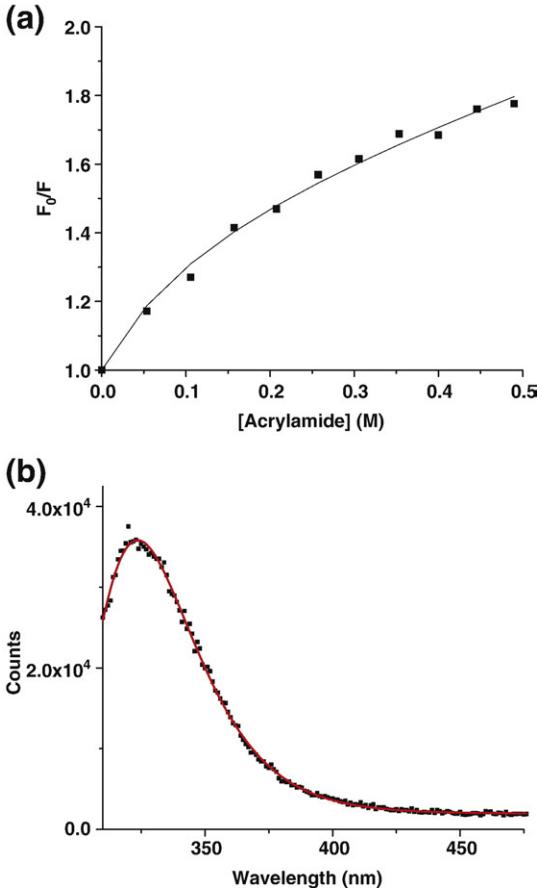

Fig. 5. Trp fluorescence from fibrillar αS. (a) Stern–Volmer plot of fibrillar 69W αS. Residual monomeric αS contributes to the fluorescence signal; therefore, a two-component fit was used to describe the experimental data. (b) Trp emission spectrum of 69W αS fibrils, with an emission maximum at ~324 nm.

considerably more protected compared with the oligomers.

Discussion

Elucidating the conformation of intermediates in the aggregation of αS remains an important question that is still unresolved. Although oligomeric αS is known to contain secondary structural elements,[18,19] more detailed information on how individual monomers are arranged in the aggregate is not available. Trp fluorescence is an extremely useful tool to learn about protein structure and has successfully been used to monitor αS conformational changes.[20,26–28] The data presented here allow a unique view on how individual monomers might be arranged in an oligomeric aggregate.

It is important to realize that there is no strict definition of the αS oligomer. In terms of size, low-molecular-mass dimers and trimers as well as ~400-kDa high-molecular-mass spherical aggregates are referred to as oligomeric.[21] In addition, oligomers can have different functional properties, such as toxicity and seeding ability.[11] We have used a preparation method similar to that used by Volles *et al.*[18] that resulted in stable oligomers that are known to interact with lipid vesicles.[14] The αS Trp mutants used in this study formed amyloid fibrils, and oligomers prepared from these mutants had properties similar to those prepared from WT αS. We therefore conclude that the inserted Trp residues did not significantly affect αS conformational properties.

Monomeric αS has the characteristics of an intrinsically disordered protein.[29,30] In solution, the protein does not have defined secondary and tertiary structures. Although the Stokes radius is larger compared with a globular protein of similar weight, the protein is most likely not fully extended but rather partially collapsed.[30,31] Long-range interactions between the N- and C-termini of the protein have been reported.[32] Our results support this view of the αS monomer. The Trp emission maxima of around 348–349 nm for mutants 4W, 39W, 69W, 90W and 124W and around 355 nm for 140W point to a polar environment. Generally, the emission wavelengths are slightly blue shifted compared with free NATA in water (λ_{max}=355 nm). Since the Trp emission wavelength can also be influenced by neighboring residues,[24] acrylamide quenching studies give additional information. Trp fluorescence was efficiently quenched with typical Stern–Volmer constants of around 10–20. Thus, for monomeric αS, all residues are considerably solvent exposed, which is consistent with other reports.[33]

The aggregation of αS is driven by a hydrophobic stretch of amino acids in the middle of the protein sequence.[34] Although the exact fibril structure of αS is not yet resolved, it is generally considered that approximately residue 35 to residue 100 form the amyloid core of the fibril.[33,35] This core is densely packed and protected from proteolysis[36] and hydrogen–deuterium exchange.[33] How the structure of the oligomeric species is related to the fibrillar structure is currently not known. Our data show that especially the residues in the N-terminus and in the hydrophobic core (mutants 4W, 39W, 69W and 90W) sense a considerably more apolar and protected environment compared with the monomer. The peak wavelength of the Trp emission for these residues is blue shifted to around 336–338 nm. In addition, quenching by acrylamide is reduced to Stern–Volmer constants of typically around 4. However, these values differ from those for completely buried Trp residues in proteins.[37,38] We have observed significantly larger blue shifts and smaller Stern–Volmer constants from fibrils from Trp mutant 69W αS. Thus, compared with αS fibrils, oligomers are more likely to be less densely packed. The C-terminus appears to be the part of the protein that is most exposed to the solvent for oligomeric αS. The emission wavelength showed a smaller blue shift and the Stern–Volmer constants were higher compared with the other mutants. The fact that the C-terminus remains relatively exposed is consistent with it being highly soluble and containing many negatively charged residues.

The interaction of oligomeric αS with membranes has been suggested as a possible mechanism of

toxicity.[12] Permeabilization might occur through a pore-like mechanism,[15,18] although the interaction is still not well understood. The membrane binding of monomeric αS has been much better characterized. Monomeric αS specifically binds negatively charged lipids.[39] Approximately N-terminal residue 1 to residue 100 participate in lipid binding and fold into an α-helical conformation.[40] We have previously shown that oligomeric αS also specifically binds to negatively charged membranes.[22] This implies that the lipid binding of oligomeric αS possibly involves the same N-terminal residues. The data presented here support this idea. Upon addition of DOPS LUVs to oligomeric αS, we observed a pronounced blue shift in the Trp emission spectra of the 4W, 69W and 90W mutants. This blue shift could be the result of a direct interaction with the hydrophobic core of the membrane. Alternatively, a conformational change in the αS oligomer could also result in the change in Trp emission. However, both explanations point to changes in the N-terminus upon the interaction of oligomeric αS with lipids. The 39W mutant showed a less pronounced blue shift. The reason for this is unclear; for instance, there is no highly charged residue in this part of the protein that would prevent this region from interacting with the bilayer interior. We note that this position is located directly next to a break in the α-helical lipid binding motif of the monomeric protein.[41] The C-terminal mutants 124W and 140W did not show a blue shift. The high negative charge of the C-terminus might prevent this part of the protein from interacting with the negatively charged membrane. Quenching by acrylamide is generally slightly reduced in the presence of DOPS LUVs, and the C-terminus remains the most solvent exposed part of the protein.

In summary, the data presented here offer site-specific information on the conformational characteristics of αS oligomers. From our data, we conclude that in the oligomeric form of αS, residues 4–90 make up the core of the aggregate, while the C-terminus is the most solvent exposed part of the protein. In addition, we propose that lipid binding of oligomeric αS is mediated by the N-terminus.

Materials and Methods

Purification of αS

The following mutants of αS with single Trp amino acid substitutions were generated by site-directed mutagenesis: phenylalanine to Trp at position 4 (4W αS), tyrosine to Trp at position 39 (39W αS), alanine to Trp at position 69 (69W αS), alanine to Trp at position 90 (90W αS), alanine to Trp at position 124 (124W αS) and alanine to Trp at position 140 (140W αS). Purification of mutant αS was performed similar to that of WT αS.[42]

Preparation of oligomers

Oligomeric αS was prepared by drying stock solutions of αS (250 μM αS in 10 mM Tris–HCl, pH 7.4) in a vacuum evaporator. The protein was redissolved using MilliQ water at a concentration of 1 mM. The solution was then incubated overnight in an Eppendorf Thermomixer at room temperature and 300 rpm. Subsequently, samples were incubated at 37 °C for 2 h without agitation. The resulting solution was filtered through a 0.2-μm centrifuge filter before injection on a Superdex 200 size-exclusion column. Elution was performed in 10 mM Hepes, pH 7.4, and 150 mM NaCl. The oligomeric species eluted in the void volume, and the oligomeric fractions were collected. Oligomer concentration was estimated by measuring the UV absorbance.

Preparation of LUVs

Two milligrams of DOPS from a stock solution in chloroform was deposited in a glass vial to prepare LUVs. The lipids were dried using a gentle stream of nitrogen gas. Residual chloroform was removed by drying the lipid film in a vacuum for at least 4 h. The lipid film was hydrated by the addition of a solution of 10 mM Hepes, pH 7.4, and 150 mM NaCl. LUVs were subsequently obtained by extrusion through a polycarbonate filter membrane with a pore size of 100 nm.

Fluorescence spectroscopy

Fluorescence spectra were recorded on a Jobin Yvon Fluoromax 4 fluorimeter. The excitation wavelength was set at 295 nm to prevent excitation of the tyrosine residues of αS. Fluorescence emission was recorded from 310 to 500 nm. The slit width was set to 5 nm for both the excitation and the emission. Fluorescence spectra were obtained at a protein concentration of around 2 μM. For the quenching experiments, acrylamide from a 40% stock solution (weight/volume) was added in 5-μl aliquots to 500 μl of protein solution. After each addition, a spectrum was recorded. For the measurements in the presence of LUVs, polarizers were inserted into the emission and excitation pathway. DOPS LUVs were added to a final concentration of around 0.6 mM. Possible artifacts due to scattering by the LUVs can be minimized by setting the polarizers in an orthogonal arrangement. Fluorescence spectra were corrected for the instrument response after background subtraction and for inner filter effects due to light absorption by acrylamide. All experiments were performed in duplicate.

Data analysis

The emission peak wavelength λ_{max} and the width of the emission peak Γ were estimated by fitting the emission spectra with a log-normal function,[23]

$$\text{for } \lambda > \lambda_{max} - \frac{\rho\Gamma}{\rho^2 - 1}$$

$$I(\lambda) = I_{max}\exp\left[\frac{\ln 2}{\ln^2\rho}\ln^2\left(1 - \frac{(\lambda - \lambda_{max})(\rho^2 - 1)}{\rho\Gamma}\right)\right] \quad (1)$$

$$\text{and for } \lambda < \lambda_{max} - \frac{\rho\Gamma}{\rho^2 - 1}$$

$$I(\lambda) = 0$$

where ρ describes the asymmetry of the peak and I_{max} is the fluorescence intensity at the peak wavelength.

Fluorescence quenching experiments were analyzed by creating Stern–Volmer plots, which plot the quenching ratio as a function of the concentration of quencher present. The fluorescence emission intensity was determined by integrating the emission spectrum from 310 to 450 nm. The quenching curves were fit with the modified form of the Stern–Volmer equation,

$$\frac{F_0}{F} = (1 + K_{sv}[Q])e^{V[Q]} \quad (2)$$

where F_0 is the fluorescence intensity in the absence of quencher, K_{sv} is the Stern–Volmer constant, [Q] is the molar concentration of quencher and V is the static quenching constant.

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2009.10.021

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