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ORIGINAL PAPER

Mechanistic aspects of Parkinson's disease: α-synuclein and the biomembrane

Klaus Beyer

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Abstract A key feature in Parkinson's disease is the deposition of Lewy bodies. The major protein component of these intracellular deposits is the 140-amino acid protein α-synuclein that is widely distributed throughout the brain. α-synuclein was identified in presynaptic terminals and in synaptosomal preparations. The protein is remarkable for its structural variability. It is almost unstructured as a monomer in aqueous solution. Self-aggregation leads to a variety of β-structures, while membrane association may result in the formation of an amphipathic helical structure. The present article strives to give an overview of what is currently known on the interaction of α-synuclein with lipid membranes, including synthetic lipid bilayers, membraneous cell fractions, synaptic vesicles and intact cells. Manifestations of a functional relevance of the α-synuclein–lipid interaction will be discussed and the potential pathogenicity of oligomeric α-synuclein aggregates will be briefly reviewed.

Keywords Parkinson's disease · Synuclein · Membrane interaction · Protein aggregation · Protein misfolding · Vesicles

Introduction

The deposition of misfolded and insoluble protein aggregates is a common trait in neurodegenerative diseases. A particularly well-studied example for such aberrant protein folding is the accruement of Lewy bodies and Lewy neurites which are characteristic features of the sporadic and, to some extent, of the hereditary variants of Parkinson's disease (PD) and of other neurological disorders such as Dementia with Lewy Bodies (DLB) [1]. The major component of these intracellular deposits is the 140-amino acid protein α-synuclein (αS). This highly conserved protein is abundant in the central nervous system where it constitutes about 0.5–1.0% of the entire cytosolic protein content [2, 3]. It is a member of a protein family that comprises the homologous α-, β- and γ-synucleins. Elevated levels of αS mRNA have been detected in brains of early onset familial PD patients [4] and in brains of patients suffering from the sporadic form of the disease [5]. This underscores the essential role of αS in the pathogenesis of PD and other synucleopathies.

The primary structure of αS is characterized by seven imperfect 11-mer repeats containing a KTKEGV consensus motif, by a hydrophobic region in the centre of the amino acid sequence and by an acidic C-terminus (Fig. 1). The 11-mer repeat sequence of αS bears resemblance to sequence motifs found in apolipoprotein A-I that are believed to constitute amphipathic helical lipid-binding domains [6]. In an early study, using recombinant αS, folding into an amphipathic α-helix was indeed observed when the protein was brought into contact with a negatively charged lipid–water interface [7]. This finding initiated numerous subsequent research activities which will be addressed in detail in the present review article. The hydrophobic domain, comprising residues 61–95, was originally identified as ''non-amyloid-beta component'' (NAC) of Alzheimer's disease plaques [8]. It seems plausible to associate this sequence domain with the aggregation propensity of αS. The homologous β-synuclein (βS), which is distinct from αS by the absence of the central hydrophobic sequence, is

K. Beyer (✉)
Laboratory of Alzheimer's and Parkinson's Disease Research, Department of Biochemistry, Ludwig Maximilian University, 80336 Munich, Germany
e-mail: kbeyer@med.uni-muenchen.de

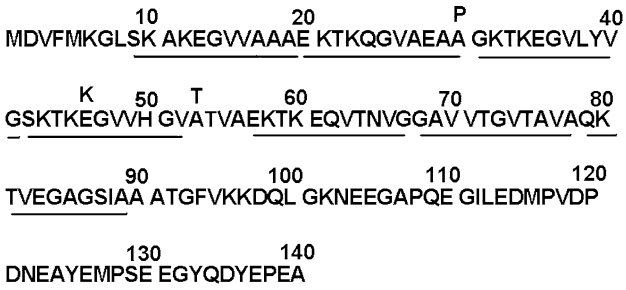


Fig. 1 Amino acid sequence of α-synuclein. Seven imperfect 11-mer repeats are underlined. Point mutations are indicated on top of the affected sequence positions

much less prone to self-aggregation. Rather it has been argued that interaction of βS with αS inhibits the formation of aggregates [9, 10].

Alpha-synuclein was first discovered in the presynaptic nerve terminals of the electric organ of the ray *torpedo californica* where it was found to be associated with the cholinergic synaptic vesicles [11]. Later the protein was identified within presynaptic terminals of the brain and the colocalization with synaptosomal membranes or with synaptic vesicles was confirmed [2, 12–15]. These observations and subsequent studies led to the conjecture that the synucleins may be involved in the maintenance of the synaptic vesicle reserve pool of the brain [16–19]. It must be admitted, however, that a satisfactory solution to the problem of synuclein function, e.g. from studies using transgenic organisms [20–24], has not been achieved so far.

Most cases of PD are of the late onset idiopathic type. There are rare inherited autosomal dominant cases that are associated with point mutations in the αS gene. An A53T mutation was first identified in a large kindred of Italian and Greek origin [25]. Later, two other point mutations, A30P [26] and E56K [27], were found in German and Spanish families, respectively. Both, the A53T and the A30P mutations lead to accelerated formation of protein oligomers, while only the A53T variant protein readily forms large amyloid fibrils [28]. The E46K mutant binds to negatively charged vesicles with a higher protein/lipid ratio than the wild type protein [29], and seems to be even more effective than the other mutations in promoting the formation of high molecular weight aggregates in a catecholaminergic cell line [30].

The single amino acid variants may aggravate an intrinsic nucleation propensity of the protein [31–33]. As an alternative, the amino acid replacements may impair membrane binding and coil-helix transition of αS which may then redirect the unstructured protein into the aggregation pathway. Overproduction of the protein may also account for aggregation which is a nucleation-dependent process that requires a minimum concentration of the monomer. Multiplication of the αS gene is indeed associated with different phenotypes of PD which may be a consequence of a persistently enhanced αS monomer level [34–36].

The most common autosomal recessive forms of parkinsonism are linked to mutations in the *parkin* gene. The structure of Parkin with its C-terminal RING domains points at an E3 ubiquitin protein ligase function of the protein [37–39]. A number of parkin substrates have been identified. The literature on the existence of authentic Parkin substrates and on the role of Parkin in the proteasomal degradation of αS is still controversial; however, see [40] for a recent review. Another link to the proteasomal system is the finding that mutations in the ubiquitin C-terminal hydrolase UCH-L1 causes PD. DJ-1 is another protein that is associated with recessive parkinsonism [41]. Although DJ-1 has some structural homology with cysteine proteases or with chaperones, the true function of the protein is not known. Association with the outer mitochondrial membrane gave rise to the assumption that DJ-1 protects against oxidative stress and other consequences of mitochondrial damage [41–43]. Other proteins associated with heritable forms of PD are the mitochondrial kinase PINK1 [44] and LRRK2/dardarin, a very large protein with multiple enzymatic sequence domains [45]. Although the linkage of these proteins to PD has been established, more research will be required to elucidate their physiological role.

Finally, oxidative stress and impairment of mitochondrial function may account for an early onset of the disease. Defects in the mitochondrial respiratory complex I, as a result of the accidental contact with bipyridinium compounds such as the drug metabolite MPP^+ [46] or the herbicide paraquate [47], were shown to be the risk factors for PD associated with aggregation of αS [48]. To summarize, it may be concluded that alterations of the conformational preference of the protein (e.g. by critical point mutations or by oxidative damage), an increased intracellular αS abundance or a defective degradation pathway will end up in αS oligomerization as a common disease causing consequence.

Here I will not go further into a discussion of the biomedical and clinical aspects of the disease, since excellent topical review articles are available that cover most of these issues [24, 40, 49–57]. I will also refrain from discussing the less well-studied role of the αS homologs, β-synuclein and γ-synuclein, and their interaction with αS [9, 10, 58]. Rather, the present review article intends to give an overview of what is known so far on the interaction of αS with lipids and bilayer membranes. This interaction evinces a remarkable dichotomy, e.g. lipid interaction may promote or inhibit αS fibrillization; the binding of αS may be deleterious for the membrane integrity or it may be

beneficial for the maintenance of the bilayer integrity, depending on the membrane composition, on the aggregational state of the protein and on the details of the experimental conditions.

Interaction with model membranes and lipid-induced protein folding

A remarkable property of αS is its structural malleability (Fig. 2). The protein is essentially unstructured in dilute aqueous solution, i.e. it belongs to a class of proteins referred to as natively unfolded [59]. Upon contact with a lipid–water interface the N-terminus acquires a helical structure while the C-terminus remains unstructured [60–62]. In Lewy bodies, however, the protein is arranged in fibrils with a so-called cross-β-structure [63, 64].

The fact that αS is heat stable and natively unfolded and the absence of cysteinyl residues from the amino acid sequence facilitated isolation, purification and handling of the recombinant protein [65, 66]. This enabled a number of biophysical studies designed to elucidate the physiological role of αS and the pathways into misfolded and aggregated states which may eventually end up with cell damage and disease. It turned out that, after a lag time, the recombinant wt and A53T mutant proteins form a fibrillar material in vitro that resembles the brain-derived proteinaceous aggregates [67]; this material has been thoroughly characterized by Thioflavin fluorescence and circular dichroism spectroscopy [58].

Binding to model membranes and simultaneous helix formation was first demonstrated for synelfin, which is a protein from canary brain with striking homology to human αS [7, 68]. The authors of these studies came up with three fundamental conclusions, i.e. membrane binding of αS requires lipids with a negative net charge; the protein undergoes a structural transition upon binding from random coil into an α-helical conformation; and αS binds preferentially to vesicles with a small radius of curvature as obtained by sonication of a lipid suspension. The 11-residue periodicity in the N-terminal region of the primary structure suggested that αS winds up into an amphipathic or ''sided'' helix upon vesicle binding which is a structural feature akin to the exchangeable apolipoproteins [6, 7, 66, 69].

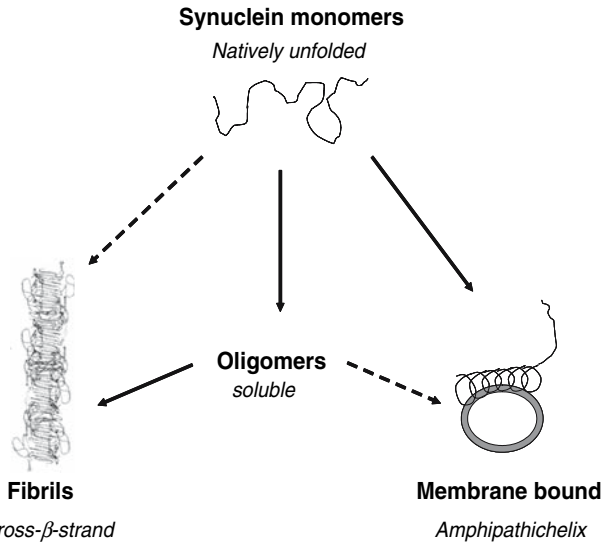


Fig. 2 Simplified representation of the structural variability of α-synuclein. Transitions supported by experimental evidence are designated by solid arrows

Deletion mutants of αS were constructed in order to map the essential sequence determinants for lipid binding and for the concomitant random coil–helix transition of the protein [69]. It turned out that the complete N-terminus, encoding the 11-mer repeats (approximately residues 1–102), is needed for vesicles consisting of a mixture of phosphatidylcholine and phosphatidylserine, whereas the affinity for vesicles containing phosphatidic acid was almost unaffected by the deletions, as inferred from the lipid-induced helicity of the protein. A slight reduction of the lipid affinity was noted by these authors for the A30P mutation whereas the A53T mutant had almost no effect on the vesicle binding of the protein [69–72]. The A30P but not the A53T substitution interferes with the formation of a proper sided helix at the N-terminus as shown recently by NMR spectroscopy [73].

The structure-promoting association with acidic vesicles [7] prompted a more detailed investigation of the lipid specificity of the αS–membrane interaction. Somewhat controversial results were obtained regarding the specificity of the interaction with charged lipid headgroups, e.g. a high affinity for phosphatidic acid was reported [7, 69, 74, 75] which was not found in other studies [66, 76]. Using a thin layer chromatography overlay technique it was shown that αS binding to acidic membranes is augmented in the presence of phosphatidylethanolamine (PE), a lipid class that constitutes 15–24% of the total lipid content of synaptic vesicles [66]. The authors argued that the enhanced binding of αS in the presence of PE relieves curvature stress induced by this phospholipid with its intrinsic propensity for negative surface curvature. It was also demonstrated by atomic force microscopy (AFM) that planar lipid multibilayers on a mica substrate were gradually disrupted in the presence of both wt and A53T αS [66]. The other disease-associated mutant known at that time, A30P, was characterized as ''defective'' with regard to membrane interaction as it showed weaker binding, according to CD spectroscopy and small angle X-ray diffraction [72].

More recently, Rhoades and colleagues studied the headgroup selectivity of the αS–lipid interaction using fluorescence correlation spectroscopy (FCS) [75]. A

fluorescent label was introduced after an appropriate site-specific amino acid replacement (S9C), which allowed evaluation of the autocorrelation functions in terms of free vs. lipid-bound protein fractions. This technique is particularly suited for an investigation of small protein and lipid concentrations. The authors observed preferential αS binding to vesicles containing 1-palmitoyl-2-oleyl-phosphatidic acid (POPA) as compared to vesicles composed of 1-palmitoyl-2-oleyl-phosphatidylserine (POPS), in agreement with earlier reports [7, 69]. When POPS vesicles were titrated with αS the molar ratio of bound protein to total lipid reached a maximum of ~1:83; further protein addition led to a decreasing ratio, in contrast to the fixed stoichiometry that can be expected for a simple saturation behaviour. The authors argue that this effect may be a consequence of bilayer destabilization with increasing protein concentration. The FCS- technique also revealed weaker binding in the presence of zwitterionic lipids, indicating that negative surface charge is not the only factor that determines αS binding [75].

Another recent study, using spin label electron spin resonance spectroscopy, investigated the lipid selectivity of wt, mutant and C-terminally truncated αS (residues 1–92). Various spin-labelled lipids were studied in a host matrix of fluid phase dimyristoyl phosphatidylglycerol (DMPG). The increment in hyperfine splitting obtained upon protein addition yielded relative association constants, indicating that the truncated αS has a higher selective affinity for most lipid species than the wt or mutant proteins. This was attributed to the removal of negative charges from the C-terminus which may further account for the affinity of the truncated version for zwitterionic lipids [77]. The abolishment of electrostatic repulsion may also explain why removal of the negatively charged C-terminus of αS strongly increases the aggregation propensity of the protein [78–81]. Partial proteolysis, e.g. by the 20S proteasome, and the fact that the truncated variant promotes the aggregation of the full length protein may then initiate a vicious cycle of nucleation and aggregation [80, 81]. The observation that the truncated protein in Lewy bodies from patients amounts to approximately 15% is therefore remarkable with regard to the initiation of αS aggregation in vivo [82, 83].

Solid-state NMR techniques were recently employed to investigate how full length αS as well as N- and C-terminal αS-peptides affect the headgroup conformation and lipid phase structure in multilamellar large vesicles (MLV). Small variations of the headgroup conformation and, to some extent, disruption of the bilayer were detected by ^{2}H- and ^{31}P-NMR. These observations were not surprising, however, regarding the unusually high protein/lipid molar ratios employed in this study. More interestingly, the authors were able to demonstrate that both peptides were capable of inducing lateral lipid demixing in the MLV membranes which led them to conclude that both, N- and C-terminal αS domains, contribute to membrane interaction [84].

A specificity for a lipid headgroup also emerged when it was shown that αS tends to oligomerize at the surface of small vesicles (SUV) containing phosphatidylinositol (PI) or phosphatidylinositol-4,5-bisphosphate (PIP2) [85], in contrast to the helix formation observed earlier in the presence of other negatively charged lipids [7]. Most notably, the presence of polyunsaturated fatty acids (PUFA) in the PI species strongly promoted oligomerization of the protein. This finding was further substantiated by incubation of αS with SUV composed of 1-palmitoyl-2-oleyl-phosphatdylcholine (POPC) and either PC or PA containing arachidonic acid chains. Oligomer formation was induced even in the presence of micellar arachidonic acid alone. The oligomer formation in the presence of PUFA was not due to dityrosin cross linking [86, 87] under oxidative conditions as the removal of all four tyrosines from the sequence had no effect on the oligomerizaton propensity [85].

Despite the wealth of information available now there are still somewhat controversial data regarding the conditions and consequences of αS–lipid interaction. Zhu et al. observed enhanced αS fibrillization (as detected by Thioflavin fluorescence) in the presence of PC/PG–SUV at lipid/protein molar ratios ≤ 20 but complete inhibition of protein fibrillization when the ratio was ≥95. Fibril formation was retarded but not inhibited in the presence of LUV, in agreement with the notion that tight lipid interaction of αS needs interfacial membrane curvature [74]. A systematic investigation of the interaction with various non-ionic and anionic detergent micelles and with phospholipid vesicles came to similar conclusions, i.e. the presence of an anionic interface promotes the αS fibrillization as long as the protein is at high relative abundance with respect to the interface [88], whereas an excess of interfacial area results in inhibition of protein aggregation [89]. Thus, for an appraisal of the aggregation propensity of αS in vivo it seems desirable to have a better knowledge on local intracellular concentrations of the protein.

While a preference for curved membranes was already noted in the earliest communications, binding of αS to multilamellar or to large unilamellar vesicles (LUV) or even multilamellar liposomes (MLV) has been later described by several authors [72, 75]. A common characteristic of these reports was the presence of phosphatidylserine (PS) in the vesicle membranes, indicating that the phosphoserine headgroup strongly promotes the membrane interaction of αS. Narayanan and Scarlata, using a number of fluorescence techniques, reported on αS binding to charged (100% PS) and neutral LUV. There was very little helix formation, even in the presence of 100% PS, which may be a consequence of the transient character of the

protein–lipid interaction under the real time equilibrium conditions of these experiments [89].

The striking preference for vesicles with a small radius of curvature suggests that there is an intrinsic propensity in the αS structure for interaction with a topologically stressed bilayer–water interface. Particular stress will certainly accumulate in a small unilamellar bilayer vesicle (SUV) below the chain-melting transition temperature (T_m) of the lipids. The resulting lipid ordering is obviously inconsistent with local bilayer curvature in the vesicle membrane. A high protein-vesicle affinity and simultaneous helix folding was indeed observed in the presence of SUV composed of synthetic phospholipids with saturated chains and zwitterionic headgroups such as dipalmitoyllecithin (DPPC) or dimyristoyllecithin (DMPC) below the temperature T_m [18]. Isothermal titration calorimetry revealed an unexpectedly large exothermic heat upon αS binding below but not above the chain-melting temperature of these phospholipids. This could only be reconciled with a disorder–order transition or rather freezing of lipids residing in defect structures that inevitably arise as a consequence of the presence of planar gel-state domains in the vesicle membrane; see Fig. 3 for a schematic representation.

The αS-induced membrane ordering was subsequently verified by electron spin resonance spectroscopy and by fluorescence techniques [90]. Besides the phospholipid vesicles in a stressed gel state there was also a remarkable affinity for vesicles composed of sphingomyelins and for sphingomyelin–cholesterol mixtures, suggesting that sphingomyelin and cholesterol containing bilayer domains, similar to the putative raft domains in biological membranes, promote the interfacial binding and concomitant helix folding of αS. Interestingly, sphingomyelin which is believed to be a constituent of lipid rafts was found in Levy bodies [91, 92]. Moreover, recent work suggests that lipid rafts provide a platform for αS–membrane interaction in vivo (see below).

Taken together, these observations are in accord with the belief that the actual physiological function of αS consists of stabilizing the synaptic vesicle reserve pool [16].

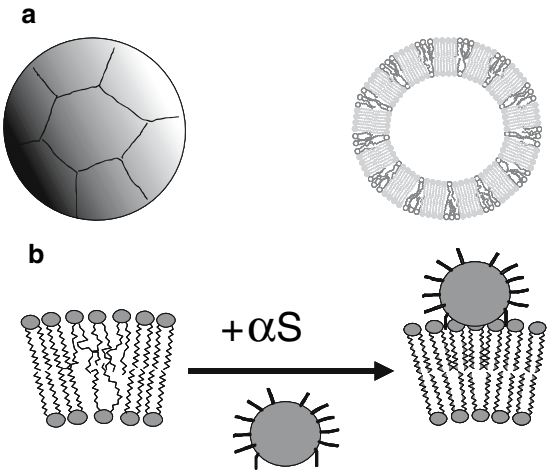


Fig. 3 (**a**) Schematic representation of defect lines in the bilayer membrane of a small unilamellar vesicle below the chain-melting temperature of the lipids. (**b**) Hypothetical ordering of the lipid acyl chains in the defect zones upon binding of the amphipathic α-synuclein helix

Structure of membrane-bound αS

The structural transition upon membrane binding of αS that had been first characterized by CD-spectroscopy [7, 69], has been now thoroughly investigated by a number of NMR laboratories. The structuring of the protein was studied in the presence of detergent micelles, which proved necessary for high-resolution NMR work [62, 73, 93–98]. A cornerstone paper by Eliezer et al. demonstrated that αS assumes a bipartite structure upon binding to a sodium dodecyl sulphate micelle [93]. Using standard triple resonance techniques the signals observable in the proton-nitrogen correlation spectrum (HSQC) could be assigned to the N-terminal 100 αS amino acids, indicating that these residues are protected from rapid exchange with the surrounding solvent. Thus, the N-terminus folds into an amphipathic helix that associates with the micelle–water interface whereas the acidic C-terminus of the protein remains unstructured.

More insight into the micelle-induced αS structure became available from the carbon ($^{13}C\alpha$) secondary shifts and from sequential NH-NH Nuclear Overhauser Enhancements (NOE), which showed that there are two helical regions in the N-terminal sequence, interrupted by a single helix break around residue 42 [60, 95]. A comparison with other proteins featuring the peculiar 11-mer repeat motives gave rise to the conclusion that the αS helical pitch is characterized by 3 turns over 11 residues (3/11 helix) rather than by 5 turns over 18 residues as in the standard version of an α-helix [95, 99].

More recent NMR work revealed a distinct secondary structure of micelle-associated αS, i.e. the two helices comprising residues 3–37 and 45–92, are oriented antiparallel and connected by an ordered antiparallel linker region, while the C-terminal protein tail remains highly mobile [62]. The most conspicuous property of this arrangement was a long-range curvature within the two helical regions. The curvature radius was larger than that of a spherical micelle which led the authors to speculate that this intrinsic geometry is adapted to the radius of synaptic vesicles. These conclusions were also based on residual dipolar couplings obtained after partial immobilization of the protein in a negatively charged polymer matrix and on proton relaxation enhancements by Mn^{2+} bound in a covalently

attached cysteaminyl–EDTA complex [62]. Similar techniques were employed for a comparison of micelle binding of the A30P and A53T mutant αS proteins. It turned out that the former replacement perturbs the helical structure and the molecular dynamics of the protein whereas the A53T mutant is essentially indistinguishable from the wt protein. Cross-relaxation experiments also showed the partial insertion of the helices into the micellar interface [73]. Similarly, paramagnetic relaxation enhancement of the αS backbone NH resonances in the presence of nitroxide labelled fatty acids indicated that the helical residues 61–95 are partially embedded in the SDS micelle [98].

It must be noted that SDS (and other) micelles have finite lifetimes characterized by two relaxation times in microsecond and in millisecond time ranges [100]. Thus, the interaction with SDS micelles may be not well suited to reproduce the binding of αS at the curved lipid–water interface of intracellular organelle membranes. The dipolar interaction which interferes with high resolution NMR spectroscopy of an immobilized protein can be useful, however, in combination with solid state NMR or spin label ESR techniques, for the determination of interresidue distances. The method of site-directed spin labelling was employed for an assessment of the chain arrangement in the αS amyloid cross beta structure [101] and of the structure of the protein bound at small unilamellar vesicles composed of phosphatidylcholine and phosphatidylserine [102]. Spin labelling at 47 single sites, combined with an examination of the accessibility for oxygen or for a paramagnetic nickel complex, enabled a detailed analysis of the αS topology with respect to the vesicle interface. This analysis confirmed the 3/11 helical arrangements and also suggested a bent shape of the helical part of the protein, in agreement with the NMR work [62, 95].

As an aside it should be mentioned that NMR spectroscopy, using paramagnetic proton relaxation enhancement after site-specific spin labelling, indicated that the ''natively unfolded'' αS molecule is not entirely without conformational preference. Although it is customary to assume that the monomeric protein has no secondary structure in an aqueous environment, this sensitive technique revealed a class of conformations that is stabilized by the interaction between C-terminal residues and the central hydrophobic portion of the protein (the so-called NAC domain). It was assumed that this transient interaction retards the aggregation of the protein and thereby stabilizes the monomeric state [96, 103].

Lipid binding in vivo and in cultured cells

Another line of experiments dealt with αS binding to intracellular membranes for which preliminary evidence had been already given in early reports on the distribution of αS among subcellular fractions [68, 104–106]. Accordingly, a study of αS distribution in rat brain homogenate showed that 15% of the protein was membrane bound and that this fraction aggregated readily after prolonged incubation, in contrast with αS separating with the cytosol [107]. Inhibition of mitochondrial respiration by Rotenone in αS overexpressing Cos7 cells also resulted in aggregation in the membrane fraction, indicating that oxidative stress is particularly effective in promoting the aggregation of the membrane-bound αS fraction. The primary aggregates of approximately 90 kDa molecular weight gradually disappeared after 48 h in favour of larger structures. These results seem to be in conflict with other reports showing that membrane interaction inhibits rather than accelerate aggregation and fibril formation of αS [89, 108].

There is also evidence of αS interaction with intracellular lipid stores. Expression of the protein in HeLa cells and supplementation of the cells with fatty acids resulted in a striking intracellular redistribution of the protein, i.e. αS accrued on the phospholipid monolayer surrounding the intracellular lipid droplets, which partially protected the triglycerides from hydrolytic cleavage. Cross-linking experiments led to the suggestion that dimers or trimers of the protein are associated with the droplet surface [109].

Based on the analysis of αS immunoreactivity in extracts from a mesencephalic neuronal cell line, one group of authors argued that free fatty acids play a particular role in the formation and maintenance of soluble protein oligomers [110]. The authors reported that PUFA but not monounsaturated or saturated fatty acids promote the formation of fatty acid-binding αS oligomers [111] which is reminiscent of earlier work by Perrin et al. mentioned above [85]. Likewise, elevated PUFA levels were detected in PD and DLB brains as well as in extracts from A53T synuclein-transfected cells, which led to the speculation that both the PUFA level in the cell and the aggregational state of αS are coordinated by the αS–PUFA interaction [112]. The fatty acid affinity of αS remains somewhat obscure, however, e.g. the suggestion that αS may have properties of a fatty acid binding protein [110] was not borne out when the interactions of unsaturated fatty acids with αS and with a genuine intracellular fatty acid-binding protein were compared by nuclear magnetic resonance spectroscopy [113].

Considerable interest was focussed in recent years on the role of lipid phase separation giving rise to microdomains in cellular and subcellular membranes. It is customary to assume that undisturbed microdomains can be isolated by low-temperature detergent treatment due to the resistance of these structures against detergent solubilization [114]. Using this technique it was demonstrated that recombinant wild type αS associates with detergent-

resistant membranes in permeabilized HeLa cells and in the presence of synaptosomal membranes from transgenic mice expressing human αS, whereas the disease-related A30P mutation abolishes these interactions [71]. It should be noted that the detergent-based raft isolation has been criticized as it may artificially induce lipid demixing and lateral domain formation in a complex biological membrane [115]. Additional in vitro experiments using small unilamellar vesicles, the composition of which mimicked that of lipid rafts, however, supported the results obtained with fractionated cell membranes or subcellular membranes, i.e. αS binds to mixed vesicles composed of equimolar amounts of cholesterol, brain phosphatidylserine and other brain phospholipids. Surprisingly, decreasing the concentration of cholesterol resulted in increasing αS binding while the binding was optimal in the presence of a mixture of PS species with 18:1 and 22:4 chains [116]. The special preference for the simultaneous presence of phospholipids containing oleic acid and of phospholipids with polyunsaturated chains such as the 22:4 species has been taken as an indication for phase separation in the membrane. Although an experimental proof for this notion is lacking, this idea is appealing as it conforms with reports suggesting that αS binds preferably to domain boundaries in the lipid–water interface [18, 90].

Transient interactions with intracellular membranes were observed when bifunctional crosslinking reagents were applied to cells expressing αS and mutant αS variants. Association with the membrane fraction and a crosslinking product of slightly elevated molecular weight were observed only in the presence of cytosol, suggesting that an unknown soluble factor mediates the membrane interaction [117]. The authors could not exclude, however, that their observations were due to crosslinking with phospholipids in the cytosolic cell fraction. Another recent study also demonstrates the involvement of a cytosolic factor in the regulation of the αS partitioning between cytosol and membraneous compartments [118]. These authors, unlike the former workers, show that an unknown brain-specific cytosolic protein promotes the release (rather than binding) of membrane-bound αS from synaptosomal as well as from synthetic membranes (see next paragraph). Clearly, more research will be required to address the critical issue of regulation of the membrane bound αS pool.

Is there a relation between membrane binding and αS function?

Although the physiological role of αS is unknown the protein is likely to have a protective functions in the context of vesicular transport and neurotransmission [16, 119]. Examination of hippocampal tissue obtained from αS-deficient transgenic mice by electron microscopy and electrophysiology revealed rapid depletion of the synaptic vesicles, suggesting that the protein is associated with the maintenance of the vesicle reserve pool [17]. The modulation of vesicular trafficking could also be demonstrated when αS was expressed in a yeast model system [70].

The dopaminergic system is particularly vulnerable to oxidative stress which is most likely linked to mitochondrial dysfunction. Damage to lipids, proteins and nucleic acids has been consistently detected post mortem in the substantia nigra of sporadic PD patients [120, 121]. Both, plasma and organelle membranes in the brain are rich in polyunsaturated lipids that are highly susceptible to peroxidation by reactive oxygen species (ROS). It is interesting in this regard that the four methionine residues of αS are easily oxidized to form the corresponding sulfoxides [122]. Based on this finding it could indeed be demonstrated that interfacial binding of the monomeric (but not of the fibrillar) protein protects small unilamellar vesicles consisting of unsaturated phospholipids from lipid oxidation in the presence of hydrogen peroxide [123]. The putative function as an antioxidant, however, may be contrasted with the simultaneous observation that oxidized αS tends to form oligomers that are suspected of being in fact the toxic species [122]. The intracellular concentration, which potentially increases by upregulation of the protein, may be the crucial factor that determines whether αS has a protective or a deleterious effect associated with oxidative stress. As an example, protein aggregation, oxidative stress and altered mitochondria were observed as a result of αS overexpression in a hypothalamic cell line. This result also gave rise to the suggestion that the protein may be involved in the regulation of mitochondrial activity [124].

A membrane-related property is the inhibition of phospholipase D2 by the synucleins [125, 126]. It was shown that all isoforms (α-, β-, γ-synuclein) were capable of inhibiting the recombinant phospholipase [127]. One can assume that phosphatidic acid, i.e. the product of phospholipase D activity, has a preference for negative curvature in a lipid bilayer which can be attributed to the average molecular shape of the phospholipid. The regulated production of phosphatidic acid may be necessary to facilitate bilayer fission as part of the process of synaptic vesicle budding from the cell membrane [128]. Another membrane-associated function of αS may involve the regulation of the dopamine transporters in the vesicle membrane and in the cytoplasmic membrane of the synapse, i.e. αS-mediated endocytosis of the transporter prevents intracellular dopamine accumulation and oxidative stress [19, 129]. It is long known that cytosolic dopamine causes oxidative stress [130, 131] and initiates apoptosis [132]. Thus, the synucleins may be involved in the regulation of vesicle recycling and in the maintenance of dopamine

homeostasis. A link with inositol-mediated intracellular calcium signalling was recently established when it was shown that αS binds with high affinity to membranes containing phosphatidylinositol-4,5-bisphosphate and that it modulates the activity of phospholipase-Cβ [133]. Regarding the critical role of calcium signalling in central cellular events, such as vesicle trafficking and dopamine uptake, this finding deserves further research to elucidate its meaning within the framework of synaptic neurotransmission.

A recent paper by Wislet-Gendebien and colleagues reported on cytosolic factors that elicit the release of αS binding to intracellular membranes (118]. The disease-associated mutations, A30P and A53T, significantly enhanced the cytosol-mediated dissociation rate of the protein from presynaptic membranes. Interestingly, there was no difference with regard to lipid binding between the A30P and A53T mutant proteins, in contrast to the earlier finding that the A30P mutation impairs association with purified or artificial membranes [15, 72, 116]. Thus, failure of proper regulation of the αS partition by brain-specific cytosolic proteins may be implicated in the pathogenesis of PD. The authors note that such a cofactor may serve as a pharmacological target for the control of αS aggregation, although the putative regulator protein has not been identified so far.

Membrane interaction and the pathogenicity of αS oligomers

A number of molecular mechanisms have been held responsible for the downstream neurotoxicity of αS aggregation, including impairment of the proteasomal system, damage to mitochondria, apoptosis, sensitization to oxidative stress and dopamine-mediated toxicity; see [40] for a comprehensive review article. In the recent literature there is a broad consensus regarding the toxicity of oligomeric species of the protein. Nonfibrillar oligomers that appear en route from the unstructured protein monomers to mature fibrillar aggregates are believed to represent a major cause for the pathophysiology of Parkinson's disease. This implicates that the well-ordered, high-molecular weight cross-beta structures, as detected by electron microscopy and X-ray scattering techniques [64], represent a dead end state rather than the actual culprit of the disease [32, 134–136]. Here I will give a brief overview of the current opinion on soluble αS oligomers and on their potential for membrane damage which may be associated with cell toxicity.

The concept of protofibillar intermediates (or prefibrillar αS oligomers) has been particularly put forward by Peter Lansbury and co-workers [137]. The separation of protofibrillar αS was achieved by column chromatography where the protofibrils eluted in the void volume of the column [138–141] or simply by incubation of αS until fibril formation commences after the typical lag time of the process, followed by centrifugation to remove any fibril material [74]. Aggregation intermediates were typically composed of 20–25 monomers. Atomic force microscopy and electron microscopy revealed an annular species with an outer diameter of 10–12 nm and an inner diameter of 2–2.5 nm, while circular dichroism spectroscopy was indicative of a β-sheet rich structure [142]. A summary of isolation and purification protocols for these oligomers has been recently published [143].

The salient feature of the intermediate aggregates with regard to their toxic potential, however, was tight membrane binding and the permeabilization of unilamellar phospholipid vesicles composed of acidic phospholipids. An influx of Ca^{2+} ions into the vesicles, as detected by Fura-2 fluorescence, was attributed to the formation of pores consisting of αS oligomers in the vesicular bilayer [50, 139, 144]. Lysis or solubilization of the vesicles could be excluded which provided additional support for the assumption of oligomeric protein pores. Small vesicles were more prone to permeabilization than larger ones, which conforms with the binding preference of the αS monomer [7]. The same authors later discovered annular and even tubular structures when the oligomeric aggregates of A30P and A53T αS were studied by electron microscopy and image reconstruction [140]. These structures resembled the cytolytic β-barrel toxins of certain bacteria, suggesting that the annular shape directly accounts for a non-specific pore mechanism of the protofibril toxicity [142]. Spherical protofibrils were also shown to bind much more tightly to brain-derived microsomal and mitochondrial membranes than monomers or fibrils. According to atomic force microscopy they eventually convert to the larger annular structures [141].

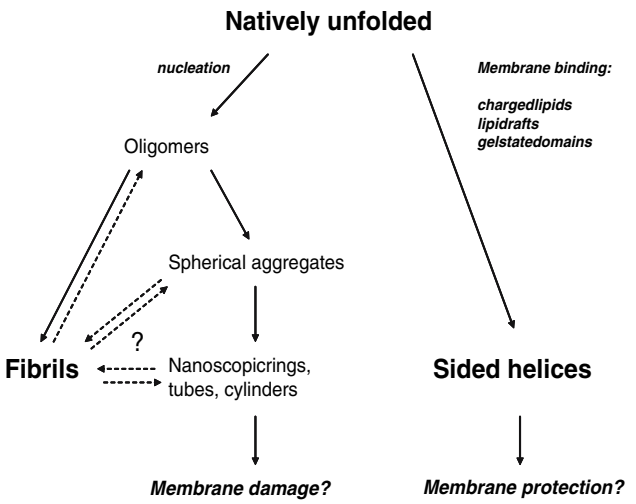
Interestingly, very similar structural features were found for a variant of the Alzheimer amyloid β-peptide [142, 145]. A recent review article comprehensively deals with Aβ aggregation [146]. Altogether, these results point at the pathogenicity of the oligomeric rather than of the mature fibrillar aggregates of αS.

A link between αS toxicity and the oxidation of dopamine was established when it was shown that a number of catechol compounds had an inhibitory effect upon αS fibrillization which results in the accumulation of the cytotoxic nonfibrillar oligomers of the protein. Adduct formation between αS oligomers and the orthoquinone product of dopamine oxidation was demonstrated by radiolabelling and mass spectrometry [147]. A later study identified a binding motif for dopaminochrome, comprising residues 125–129 [148]. If this modification interrupts the fibril maturation it may be speculated that protofibril induced synaptic vesicle permeabilization, followed by

dopamine release and oxidation, launches a fatal feedback process that eventually ends up with loss of neurotransmission and cell death [149]. It may be noted, that these assumption were challenged, i.e. raising the intracellular catechol concentration by overexpression of tyrosine hydroxylase in a cell line expressing simultaneously wt or A53T αS did not result in a conspicuous cytotoxicity [150].

Recently, it became more and more clear that most of the peptides and proteins involved in the different amyloid diseases rather than only αS and the Aβ protein are capable of aggregating into soluble oligomers [151–155], which led to the assumption that there is a common mechanism of amyloid toxicity associated with these proteins (cf. Scheme 1 which is pertinent to αS). Misfolding and aggregation of αS may not solely affect intracellular organelles, but also the outer leaflet of the cytoplasma membrane. A cytotoxic effect was noticed when αS oligomers were added to human neuroblastoma cells [151], i.e. an amyloidogenic protein of intracellular origin attacks the outer cell membrane, indicating that oligomer-mediated bilayer permeabilization may be rather unspecific.

Some authors also strove towards a precise definition of the aggregation intermediates, e.g. ''soluble oligomers'' have been assigned to the nonfibrillar spherical intermediates of 3–10 nm diameter that appear early in the aggregation process while ''protofibrils'' are the more extended later structures with a beaded appearance that are believed to be a result of the coalescence of the oligomers [153]. Techniques were developed for the preparation and purification of oligomers [140, 151] which facilitated an investigation of the membrane-perturbing effects. Thus, incorporation of various amyloidogenic proteins, including αS, into a mica-supported flat membranes allowed for high resolution imaging of annulus shaped oligomers by atomic force microscopy [155]. The pore-like images were highly suggestive, and heterogeneous single channel conductances were indeed observed with these reconstituents. Other authors, however, disagree with the channel interpretation. Rather, again on the basis of oligomer-induced membrane conductance, they concluded from the absence of single channel activity and ion selectivity that the oligomers were only peripherally associated with the membrane which allows ions to cross the perturbed lipid bilayer somehow on their own [154]. Therefore, the molecular mechanism of the detrimental effect of soluble oligomers upon neuronal integrity still remains unclear, although membrane permeabilization has been identified as a possible deadly impairment.



Scheme 1 Summary of current hypotheses: aggregation and folding pathways of α-synuclein

Conclusions and future prospects

There seems to be, now, a broad consensus on several aspects of the amyloid diseases. Most researchers agree that oligomeric aggregation intermediates rather than the characteristic insoluble deposits with a well-defined secondary structure account for the cytotoxicity of misfolded proteins. It is also widely accepted that the disease-causing mechanisms are quite general in different protein misfolding disorders [153]. Moreover, it seems clear that oligomers can exert detrimental effects upon cytoplasmic and intracellular membranes [137, 156]. There is still much confusion, however, as to the bewildering nomenclature for the different aggregation species. There are also numerous problems with reference to the molecular pathology that must be addressed if the very mechanism of the deathly action of these structures is to be understood.

It may be easy to overcome the first problem by strictly replacing ''protofibrils'' with the term ''oligomers'', using an appropriate morphological designation such as ''spherical'' or ''annular'' together with some information on the size of the particles. The more serious problems, however, e.g. considering the putative noxious biomembrane interactions of oligomeric species, clearly require more research. To mention a few of these open questions: Are oligomeric protein structures integrated into a biomembrane in a transbilayer fashion? Is there a selective lipid interaction and what is the role of lipid domains or ''raft'' assemblies? Is there a dynamic equilibrium between mature fibrillar aggregates and oligomers and if so, how does membrane interaction affect that equilibrium?

Extreme efforts have been invested in recent years for a thorough understanding of the role of the synucleins in Parkinson's disease. Therefore, one may expect that more research in this particular field will eventually yield a consistent view on the pathomechanisms of this and other

neurodegenerative diseases which may open new vistas for therapeutic intervention.

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