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Review

α-Synuclein misfolding and Parkinson's disease

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ABSTRACT

Substantial evidence links α-synuclein, a small highly conserved presynaptic protein with unknown function, to both familial and sporadic Parkinson's disease (PD). α-Synuclein has been identified as the major component of Lewy bodies and Lewy neurites, the characteristic proteinaceous deposits that are the hallmarks of PD. α-Synuclein is a typical intrinsically disordered protein, but can adopt a number of different conformational states depending on conditions and cofactors. These include the helical membrane-bound form, a partially-folded state that is a key intermediate in aggregation and fibrillation, various oligomeric species, and fibrillar and amorphous aggregates. The molecular basis of PD appears to be tightly coupled to the aggregation of α-synuclein and the factors that affect its conformation. This review examines the different aggregation states of α-synuclein, the molecular mechanism of its aggregation, and the influence of environmental and genetic factors on this process.

1. Introduction

α-Synuclein is a 140-amino acid protein, which is encoded by a single gene consisting of seven exons located in chromosome 4 [1]. This protein was first described by Maroteaux et al. in 1988 as a neuron-specific protein localized in the pres*yn*aptic nerve terminals and *nucle*us, and hence was referred to as *synuclein* [2]. Although this protein is at the focus of systematic research in several laboratories, its exact function is still unknown. α-Synuclein attracted significant interest in 1997 after a mutation in its gene was found to be associated with the familial cases of early-onset Parkinson's disease [3], and its aggregates were found to be the major components of Lewy bodies, the hallmarks of PD [4]. Since then, several observations have firmly established α-synuclein's involvement in the pathogenesis of PD. Among the strongest pieces of evidence are the following:

- Autosomal dominant early-onset PD is induced as a result of three different missense mutations in the α-synuclein gene, corresponding to A30P, E46K, and A53T substitutions in α-synuclein [3, 5,6], or as a result of the overexpression of the wild type α-synuclein protein due to gene triplication [7–9];
- Antibodies to α-synuclein systematically detect this protein in Lewy bodies (LBs) and Lewy neuritis (LNs), the hallmark lesions of PD. Here, a substantial portion of total protein in these inclusions is composed of α-synuclein [4, 10];
- The production of wild type α-synuclein in transgenic mice [11] or expression of WT, A30P, or A53T α-synuclein in transgenic flies [12] leads to motor deficits and neuronal inclusions reminiscent of PD;
- Transgenic nematodes *Caenorhabditis elegans* that overexpress wild-type and mutant forms of human α-synuclein (A30P and A53T) caused an accumulation of α-synuclein in dopaminergic (DA) neurons [13]. These worms failed to modulate the locomotor rate in response to an availability of food, a function normally attributed to dopaminergic neurons [13]. Furthermore, worms expressing human α-synuclein under control of the promoter for dopamine transporter (DAT) displayed age- and dose-dependent dopaminergic neurodegeneration [14,15];
- Overexpression of human α-synuclein in the baker's yeast *Saccharomyces cerevisiae* recapitulated many important features seen in PD [16]. Such transgenic yeast represents an important cellular tool that is now commonly used to confirm established and decipher new clues explaining the devastating pathological role of α-synuclein in PD [16];
- α-Synuclein-positive deposits were shown to accumulate in several animal models where Parkinsonism was induced by exposure to different neurotoxicants [18].

Furthermore, in recent *in vitro* study, where two populations of human dopaminergic neuronal cells were cocultured: one overexpressing α-synuclein (donor cells) and the other without α-synuclein overexpression (acceptor cells), α-synuclein pathology was shown to be

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propagated by direct neuron-to-neuron transmission of α-synuclein aggregates. Here, α-synuclein was transmitted *via* endocytosis to neighboring neurons. This eventually led to the formation of Lewy-like inclusions in the acceptor cells, with increasing numbers of these Lewy-like inclusions directly correlated with increasing expression levels of α-synuclein in donor cells [17].

These and other important observations correlating α-synuclein and PD pathogenesis have been reviewed in detail elsewhere [19–23].

These *in vivo* results are supported by numerous *in vitro* studies, which established that the recombinant α-synuclein easily assembles into amyloid fibrils and oligomers under a variety of conditions. Aggregation of α-synuclein is modulated by point mutations associated with familial PD, various environmental factors, posttranslational modifications, and interaction with cellular membranes and different proteins, including chaperones and β- and γ-synucleins. Structural differences between various aggregated forms of this protein may be correlated with their effect *in vivo*.

2. Structural properties and conformational behavior of α-synuclein

2.1. Structural properties

α-Synuclein is an abundant brain protein of 140 residues, lacking both cysteine and tryptophan residues. This protein is present in high concentration at presynaptic terminals and is found in both soluble and membrane-associated fractions of the brain. α-Synuclein was estimated to account for as much as 1% of the total protein in soluble cytosolic brain fractions [24]. Several possible functions for α-synuclein have been suggested, including synaptic vesicle release and trafficking, fatty acid binding and physiological regulation of certain enzymes, transporters, and neurotransmitter vesicles, as well as roles in neuronal survival [21]. The involvement of α-synuclein in the control of the neuronal apoptotic response and in the protection of neurons from various apoptotic stimuli was demonstrated [25]. Knockout of all synucleins (there are three members of the synuclein family in the vertebrata: α-, β-, and γ-synucleins) in mice leads to age-dependent neuronal dysfunction indicating that synucleins are important contributors to long-term operation of the nervous system [26]. α-Synuclein was shown to physically interact with at least 30 proteins, underlying its important role in cell signaling [21, 27,28].

At the level of amino acid sequence, the structure of α-synuclein can be divided into three regions: residues 1–60, which contain four 11-amino acid imperfect repeats (coding for amphipathic α-helices) with a conserved motif (KTKEGV); residues 61–95, which contain the hydrophobic and highly amyloidogenic NAC region and three additional KTKEGV repeats; and the highly enriched in acidic residues and prolines C-terminal region, residues 96–140. The first two regions comprise a membrane-binding domain, whereas the C-terminal tail is thought to contain protein–protein and protein–small molecule interaction sites.

In 1996, Weinreb et al. [29] showed that in solution, α-synuclein has a much larger Stokes radius (34 Å) and sediments more slowly ($s_{20,w} = 1.7$ S) than globular proteins of similar molecular mass, suggesting that it is either elongated or unfolded. Subsequent studies supported the idea that α-synuclein is a natively unfolded protein. For example, Fig. 1 presents some of the data obtained in the laboratory of Prof. Anthony L. Fink [30]. At neutral pH, far-UV CD spectrum of α-synuclein was characterized by a minimum in the vicinity of 196 nm and the absence of bands in the region of 210–230 nm (Fig. 1A), while FTIR spectrum was dominated by a broad peak at 1650 cm^{-1} (Fig. 1B). These spectra suggested that the majority of the molecule was unfolded [30].

The hydrodynamic properties of α-synuclein analyzed by size exclusion chromatography and small-angle X-ray scattering (SAXS) were in agreement with the results of CD and FTIR studies. However, the Stokes radius measured for α-synuclein by size-exclusion chromatography was notably lower than that calculated for a completely unfolded polypeptide chain of appropriate molecular mass [30,31]. R_g values calculated for α-synuclein at neutral pH from SAXS data using the Guinier approximation (40 ± 1 Å, Fig. 1C) were also significantly smaller than those estimated for a random coil polypeptide of the same length (52 Å) [30]. The analysis of SAXS data in the form of Kratky plots provided information on the packing density and the overall conformation of a polymer molecule [32–39]. The scattering curve for a globular conformation is proportional to Q^{-4} at large values of Q, while the scattering intensity from the expanded chain molecule is proportional to Q^{-2} in the moderate Q region and to Q^{-1} in the high Q region. Thus, the Kratky plot for a globular conformation shows a clear peak, whereas the plot for a chain-like conformation has a plateau and then rises monotonically [37]. Fig. 1D shows that the profile of the Kratky plot of α-synuclein at neutral pH was typical for a random coil conformation. Therefore, at neutral pH α-synuclein was shown to be essentially disordered, but more compact than a random coil [30].

2.2. Conformational behavior of α-synuclein

The natively unfolded nature of α-synuclein is determined by its relatively low hydrophobicity and high net charge. It is expected that alterations in the protein environment leading to an increase in its hydrophobicity and/or decrease in net charge can induce partial folding [30]. In fact, these two structural parameters can be modulated *via* changes in the environment. For example, the excess negative charge of α-synuclein at neutral pH (pI = 4.7) is expected to be neutralized by acidification of the protein solution, and the overall hydrophobicity of a protein is expected to increase with increasing temperature.

In agreement with this hypothesis, α-synuclein became more ordered at pH 3.0 or at high temperature [30]: it gained some ordered secondary structure (Fig. 1A and B), became a bit more compact (Fig. 1C), and developed a rudimentary nucleus of a tightly packed core (Fig. 1D) [30]. Furthermore, Fig. 1E shows that a protonation of α-synuclein resulted in the transformation of this natively unfolded protein into a partially folded conformation with a significant amount of ordered secondary structure, some compactness, a rudimentary nucleus of a tightly packed core, and a high affinity for ANS [30]. Comparable folding/compaction was observed for the protein at high temperatures, and an increase in temperature was sufficient to induce the reversible formation of some ordered secondary structure in α-synuclein (Fig. 1F) [30].

2.3. Functional misfolding of α-synuclein

The partial compactness of α-synuclein represents an illustration of a functional misfolding concept, according to which intrinsically disordered proteins (IDPs) contain the preformed binding elements which might be involved in a set of non-native intramolecular interactions. In this way, a polypeptide chain of an IDP misfolds to sequester the preformed elements inside the non-interactive or less-interactive cage, therefore preventing these elements from the unnecessary and unwanted interactions with non-native binding partners [40]. Data below provide support for this conclusion.

Recently, Eisenberg and coworkers have been able to crystallize several peptides derived from the first 72 residues of α-synuclein fused to maltose binding protein for increased solubility [41]. Their data showed that residues 1–13 and 20–34 of α-synuclein formed α-helices under the crystallization conditions, whereas the rest of the sequence remained substantially unfolded [41]. In NMR studies, the C^{α} secondary chemical shifts analysis of the unbound α-synuclein revealed that this protein is largely unfolded and devoid of tertiary structure [42]. However, NMR [43–47], EPR [48], molecular dynamics [44], and electron transfer studies [49] demonstrated a relative compactness of α-synuclein compared to

what would be expected for a fully unfolded peptide chain, as well as the presence of transient long-range contacts within the protein structure. These data indicated that the compactness of α-synuclein structure was due primarily to clustering of hydrophobic residues. Similar to other natively unfolded proteins, α-synuclein can be described as a highly dynamic ensemble of preferred conformations [50,51]. Structural constraints for this conformational ensemble have been obtained by NMR and EPR spectroscopy. For example, an overall preference for helical structure was found in the N-terminal 100 residues, and a specific region, from residues 6 to 37, was proposed to have nascent or transient α-helical structure [42].

Based on the measurements of ^{15}N relaxation rates in the unbound form of α-synuclein, it has been concluded that regions around residues 20 and 120 possessed decreased mobility [52]. The existence of long-range contacts between the C-terminal tail and the central region of the protein was established using the paramagnetic relaxation enhancement (PRE) [53]. The presence of long-range contacts suggested that the native state of α-synuclein was composed of a more compact ensemble of species than would be expected for a random coil state [53]. When the putative structural ensemble of the unbound α-synuclein molecules was generated by MD simulations using the CHARMM force-field and 20 protein replicas, it became evident that the protein ensemble samples had non-random conformations involving, in particular, contacts between residues ~120–140 of the C-terminus and residues ~30–100 in the central region of the protein sequence [53]. Analogous PRE experiments, in which the nitroxide radical MTSL was attached to the introduced cysteines at positions 18, 90, and 140, suggested that the numerous long-range interactions were present, leading to the formation of a hydrophobic cluster that comprised the C-terminal part of the highly hydrophobic NAC region (residues 85–95) and the C terminus (residues 110–130), probably mediated by M116, V118, Y125, and M127. Within the C-terminal domain, residues 120–130 were shown to contact residues 105–115, and the region in the vicinity of residue 120 also interacted with the N terminus in the vicinity of residue 20 [46]. The existence of these long-range interactions was suggested to play a role in inhibition of the spontaneous α-synuclein oligomerization and aggregation [46, 53]. The autoinhibitory conformations fluctuate in the range of nanoseconds to micro-seconds, corresponding to the time scale of secondary structure formation during folding [46]. This nascent highly dynamic tertiary structure was shown to be released by α-synuclein interaction with polyamine or by temperature increase; *i.e.*, under conditions that induce α-synuclein aggregation *in vitro* [46].

The C-terminus of α-synuclein (residues 120–140) is very acidic and negatively charged (−8 net charge: 8 negative charges, no positive charges), whereas the central region (residues 30–100) is slightly positively charged (+3 net charge: 9 positive and 6 negative charges). The electrostatic attraction between these two regions might, at least in part, be responsible for the long-range interactions in α-synuclein. This hypothesis is supported by accelerated fibrillation of α-synuclein induced by polyamine that binds and neutralizes the negative charges of C-terminus [47, 54]. Furthermore, a hydrophobic cluster was found at the C-terminus of α-synuclein. This cluster was shown to be stabilized by the long-range interactions as well [46,47]. These long-range hydrophobic and electrostatic interactions in native α-synuclein were affected by the methionine oxidation [55].

Fig. 2 illustrates the structural diversity of α-synuclein and represents 3D-structure of this protein bound to vesicles [48, 56]; 3D-structures of C- and N-terminal fragments of this proteins bound to a single domain camelid antibody [57] or to a coiled-coil domain of synphilin-1 [58]; and some representatives of the ensemble of structures found for this protein in the unbound form [46]. Fig. 2 clearly shows that α-synuclein and its N- and C-terminal fragments adopt very different structures in various complexes and in the unbound form. Here, partial compaction of the unbound α-synuclein driven by the sequestering of the protein's N- and C-termini *via* their interactions with each other and with the central NAC region represents an illustration of the functional misfolding mentioned above [40].

All the data summarized above clearly show that α-synuclein belongs to the family of intrinsically disordered proteins (IDPs), and more specifically to the subfamily of the most disordered members of this protein family, known as natively unfolded proteins, which are characterized by a unique combination of low overall hydrophobicity, low sequence complexity and high net charge [49]. Generally speaking, IDPs exist as dynamic and highly flexible structural ensembles, either at the secondary or at the tertiary structure level. In contrast to ordered proteins whose 3-D structure is relatively stable and Ramachandran angles vary only slightly around their equilibrium positions with occasional cooperative conformational switches, intrinsically disordered proteins or regions exist as dynamic ensembles in which the atom positions and backbone Ramachandran angles vary significantly over time with no specific equilibrium values, and typically undergo non-cooperative conformational changes. IDPs include both extended (random coil-like) regions with perhaps some secondary structure, and collapsed (partially folded or molten globule-like) domains with poorly packed side chains [59].

3. Pathways and molecular determinants of α-synuclein misfolding and aggregation

3.1. Structure and polymorphism of α-synuclein fibrils

Two structural classes of amyloid fibrils have been proposed: those derived from folded proteins, and those derived from intrinsically disordered proteins. Amyloid fibrils are formed from folded proteins by either the refolding mechanism or by a gain-of-interaction model [60]. In the refolding model, proteins such as insulin convert from native structures to fibrils by initially unfolding, and then refolding into a secondary structure that is rich in β-sheets. Fibrils formed in this way are stable due to backbone hydrogen bonding, rather than side chain-side chain interactions. Alternatively, native proteins such as transthyretin (TTR), yeast prion Sup35, superoxide dismutase (SOD), and β_2-microglobulin undergo a limited conformational change to expose a short segment of a previously inaccessible region. This region can then interact with the surfaces of other molecules to form fibrils without causing a major perturbation in the protein's native structure. Fibrils are formed through 1) direct stacking of exposed regions (TTR and SOD) [61,62], 2) swapping of two β-sheet containing domains from two monomers to form a cross-β spine (Sup35 and β2-microglobulin) [63,64], or 3) strand-swapping between two adjacent monomers [65]. All of these native-like proteins are rich in β-structure, and as a result form fibrils with minimal alterations to their native structures.

α-Synuclein belongs to a class of intrinsically disordered amyloid proteins that form fibrils by converting either all or part of the previously unstructured polypeptide into well-defined, β-sheet rich secondary structures. Other examples of these amyloids include islet amyloid polypeptide (IAPP), tau, and Aβ. The atomic structure of cross-β spines in α-synuclein fibrils was first determined by X-ray crystallography and by X-ray diffraction of synthetic human synuclein filaments and filaments extracted from DLB and MSA brains [66]. These studies were later confirmed by X-ray diffraction of many short amyloid peptides, including a small segment from α-synuclein [64, 67]. Together, these studies revealed that α-synuclein fibrils are composed of several protofilaments containing a cross-β structure in which β-strands are arranged in parallel, and the β-sheets are in-register with highly ordered amino acid side chain patterns exposed on the surface of the β-sheets. Furthermore, the side-chains protruding from the two β-sheets of the cross-β spine interdigitate in a self-complementary manner to give rise to highly ordered structures known as steric zippers. These steric zippers run up and down the fibril axis and exclude

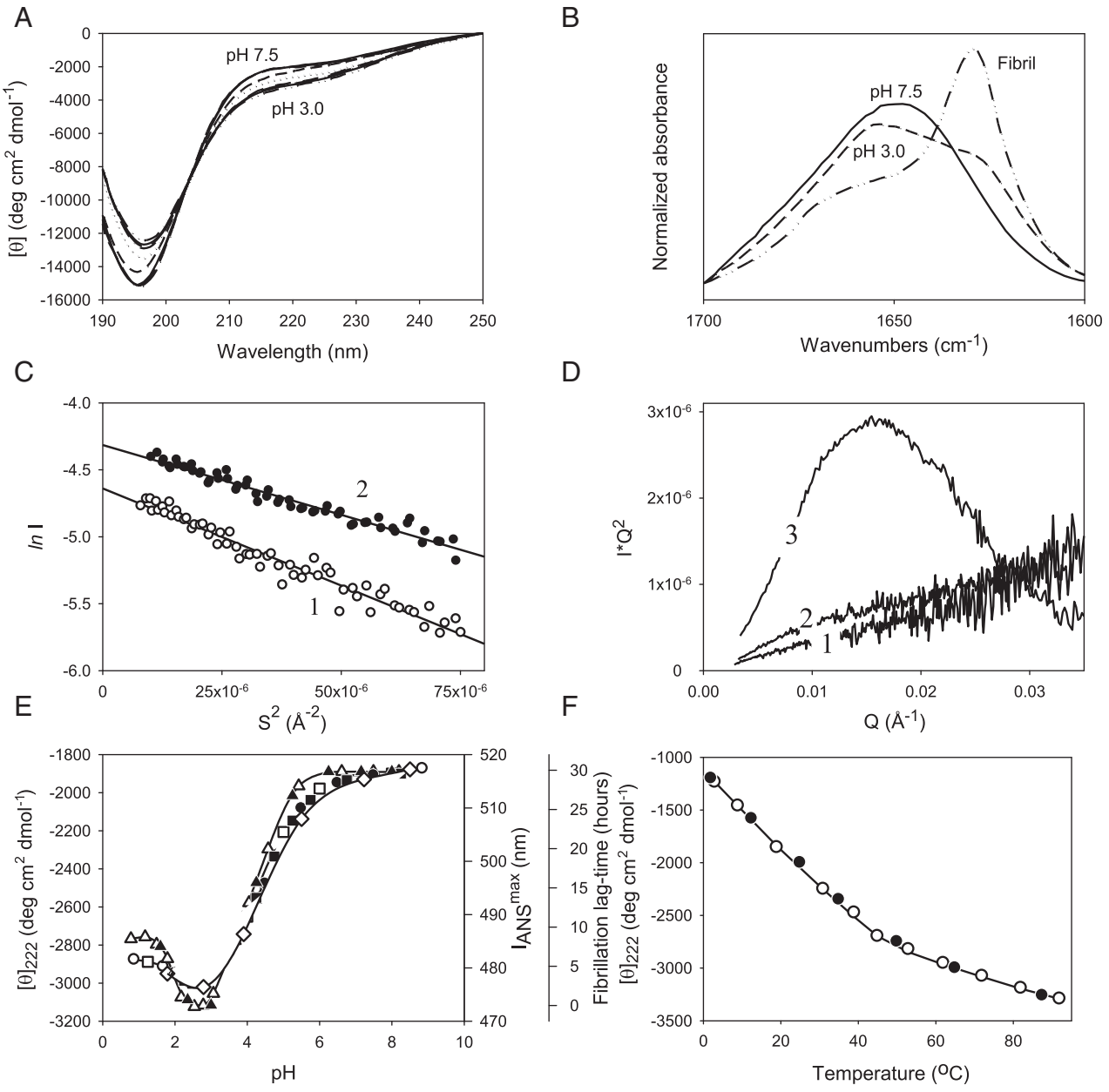

Fig. 1. Structural properties and conformational behavior of human α-synuclein. A. Far-UV CD spectra measured under different conditions. B. FTIR spectra measured for natively unfolded, partially folded and fibrillar forms of α-synuclein. Guinier (C) and Kratky plot (D) representation of the results of SAXS analysis of human α-synuclein at different experimental conditions: 1 — pH 7.5; E. pH-Induced partial folding of α-synuclein. F. Temperature-induced folding of α-synuclein. Modified from [30].

water from the interface between the β-sheets. The steric zipper interface between β-sheets is not unique to fibrils formed by α-synuclein, but is a highly conserved common motif that is fundamental to parallel β-sheet-rich amyloid fibrils formed by many amyloid proteins, such as tau, the PrP, insulin, IAPP, lysozyme, and β_2-microglobulin [67].

Recent studies using high resolution cryo-electron microscopy, atomic force microscopy, and solid state and quenched hydrogen/deuterium exchange NMR spectroscopy have revealed that α-synuclein fibrils exhibit a distinct structural polymorphism. These morphological differences are likely due to variations in the folding of the β-sheets, differences in the molecular packing between sheet interfaces, or interactions of side chains with the environment. In fact, subtle changes in buffer conditions such as the pH, temperature, ion concentration, and external variables such as agitation or toxins can drastically influence the folding and aggregation processes of α-synuclein. Cryo-electron microscopy and solid state NMR revealed that the morphology of both recombinant α-synuclein (30–110) fibrils and filaments extracted from PD patient brains can be classified as either straight or twisted ribbons [68,69]. At the molecular level, both types of fibrils share a common five-layered parallel, in-register β-sheets core that consists of a five-layered β-sandwich [68, 70]. However, these two types of fibrils differ significantly in the arrangements of protofilaments. Recent evidence from solid state and quenched hydrogen/deuterium exchange NMR further proposes that the straight fibrils have protofilaments (β4 and β5) aligned unidirectionally with each other to form a fibril, whereas in the twisted fibril type, two protofilaments (residues 20–30) twist around each other, giving rise to a sub-protofilament that can twist again with another sub-protofilament to form a fibril. These results show how the differences in the fibril architecture at the molecular level are translated into differences in their morphology.

The observation that various amyloid proteins assemble into highly ordered β-sheet-rich fibrillar aggregates independent of their primary amino acid sequence suggested that amyloid proteins misfold

following a common conserved pathway, in which soluble monomers form intermediate, transient oligomeric structures and then assemble into more structurally ordered fibrillar aggregates. In accordance with this theory, purified α-synuclein assembles into β-sheet-rich fibrillar aggregates under specific conditions *in vitro* [71,72]. The formation of α-synuclein fibrils occurs in a nucleation-dependent manner, where the rate-limiting step is the spontaneous formation of small metastable oligomeric intermediates, also known as fibril nuclei [73–75]. Such intermediates result from partial folding and aggregation of unstructured α-synuclein, and exist in rapid equilibrium with its monomeric form. Once fibril nuclei have formed, fibrils are then grown by a "dock and lock" mechanism, in which monomers initially bind to, or "dock onto", the exposed regions of a fibril in a reversible manner. The docking step is then followed by an irreversible re-organization of the fibril surface, which generates the most optimal surface area for further fibril growth [76,77]. A similar nucleation-dependent principle applies to α-synuclein oligomerization, such that the formation of oligomers is also a highly ordered process that involves an intrinsic rate-limiting lag phase.

α-Synuclein aggregation can occur through multiple pathways that can give rise to structurally distinct oligomeric and fibrillar species. As described in the preceding section, the process of amyloidogenic protein misfolding is highly influenced by many environmental and intrinsic factors, such as mutations, the pH of the environment, the presence of chaperones, the aggregation propensity of the protein in question, and so forth. Nevertheless, a wide range of amyloidogenic proteins have been shown to assemble into common oligomeric and fibrillar conformations. This fact suggests that amyloid misfolding is largely mediated by peptide-backbone interactions, and not by interactions of the side groups [78].

3.2. Structures of α-synuclein oligomers

Oligomers of α-synuclein, similar to those of other amyloidogenic proteins, are highly structurally diverse. Some of them are β-sheet rich, while others are primarily disordered. Recent studies identified several distinct populations of α-synuclein oligomers and obtained their structural information. For example, Giehm and coworkers identified wreath-like oligomers with a diameter of approximately 18 nm [79]. These oligomers were able to disrupt the membranes and easily assembled into fibrils. Hong et al. identified the oligomers that formed in parallel with fibril formation [80]. Their morphology depended on salt concentration in solution. These oligomers did not incorporate into fibrils but disrupted the lipid membranes. Apetri et al. found that oligomers formed at the early stages of α-synuclein aggregation are primarily α-helical [81]. Glabe and coworkers developed conformation-specific antibodies to distinguish between different structural classes of amyloid oligomers [82,83]. They identified at least three structural classes of amyloid oligomers (fibrillar, prefibrillar and annular) and found that α-synuclein formed oligomers from all three classes, depending on the experimental conditions [83–85]. The oligomeric structures formed by α-synuclein in the presence of metal ions, small molecules, chaperones, and chemical modifications will be discussed in detail below.

These examples show the conformational diversity of α-synuclein oligomers. This structural diversity is translated to variability in cytotoxicity and biological activity.

3.3. Propagation of α-synuclein aggregates in animal models of PD and in vitro

The seeding and propagating properties of α-synuclein aggregates have been elegantly demonstrated by recent cell culture studies. In human embryonic kidney (QBI-293) cells that are overexpressing α-synuclein, exogenous α-synuclein fibrils promoted and induced endogenous α-synuclein to form LB-like intracellular inclusions. These intracellular aggregates exhibited typical pathological characteristics earlier described for LBs and LNs from PD brains, such as hyperphosphorylation, ubiquitination, insolubility in detergent, recognition by conformation-specific antibodies, and staining by thioflavin S dye [86]. Intracellular propagation of α-synuclein fibrils was dependent on the presence of a fibril-forming core, further confirming *in vitro* experiments that demonstrated nucleation-dependent fibrillation of α-synuclein [71, 87]. Seeding of endogenous α-synuclein is not specific to the fibrils, but was also previously described for α-synuclein oligomers, in which three distinct types of oligomers promoted intracellular oligomer seeding of endogenous α-synuclein in cultured human neuroblastoma (SH-SY5Y) cells [88]. Lastly, the propagation and spreading of misfolded synuclein is reported in cases where human PD patients that received embryonic nigral transplants. In these cases, the embryonic dopaminergic neurons grafted into PD patients developed α-synuclein inclusions and have reduced the dopamine transporter over a period of 14 years [89]. Taken together, these *in vitro* and *in vivo* studies synergistically suggest that misfolded α-synuclein aggregates propagate and spread in a disease through an amyloid-specific, nucleation-dependent seeding mechanism that is similar to the self-propagating mechanism of infectious prion proteins [90,91].

3.4. α-Synuclein aggregation and cell death

There are two important questions regarding α-synuclein aggregation in PD: which species present during the aggregation of α-synuclein could be responsible for the neuronal death, and can the neurotoxicity even be ascribed to a single aggregate type? Toxicity may be exerted by specific populations of α-synuclein aggregates directly and/or mediated *via* various routes through proteins involved in different cellular processes [92–94].

The mechanisms proposed to describe the neurotoxicity of α-synuclein and its aggregates can be grouped into three major classes — mechanical disruption of cellular compartments/processes, toxic gain of function, and toxic loss of function. One of the most commonly accepted examples of the former is permeation of cellular membranes by amyloid aggregates. α-Synuclein oligomers can bind to lipid membranes and disrupt membrane bilayers [94–96]. Certain oligomeric forms of α-synuclein were shown to penetrate membranes, forming pore-like channels [79, 97]. Membrane permeation by amyloid oligomers without pore formation has also been proposed [98]. It is believed that this is one of the main mechanisms of toxicity for protein aggregates.

Alternatively, impairment of α-synuclein degradation *via* proteasome inhibition by the aggregated species and copper-dependent generation of ROS have been proposed as possible mechanisms for neurotoxicity of α-synuclein aggregates [93, 99]. It is possible, and in fact quite likely, that multiple toxic aggregated species of α-synuclein that utilize different mechanisms of toxicity are present *in vivo*. In addition, several studies stress that α-synuclein-related neurotoxicity might arise from a loss of function (summarized in [99]). All the factors mentioned above are not necessarily mutually exclusive, but instead may be synergistic.

The capability of the PD-related A30P mutation to dramatically accelerate the initial oligomerization of α-synuclein and to significantly retard the formation of mature fibrils [100–103] is only one piece of evidence which suggests that oligomeric intermediates of α-synuclein, rather than mature fibrils, may in fact be the disease-associated species of the protein [104], and that oligomers, not fibrils, are cytotoxic [105,106]. Several additional facts in support of the idea of oligomer toxicity are listed below [107,108]:

- In cell models, toxicity is usually seen without heavily aggregated α-synuclein, leading to the suggestion that some soluble species mediate toxicity [109];
- Detectable aggregation of α-synuclein and deposition of this protein into insoluble fractions occur later than cell death *in vitro* [110];

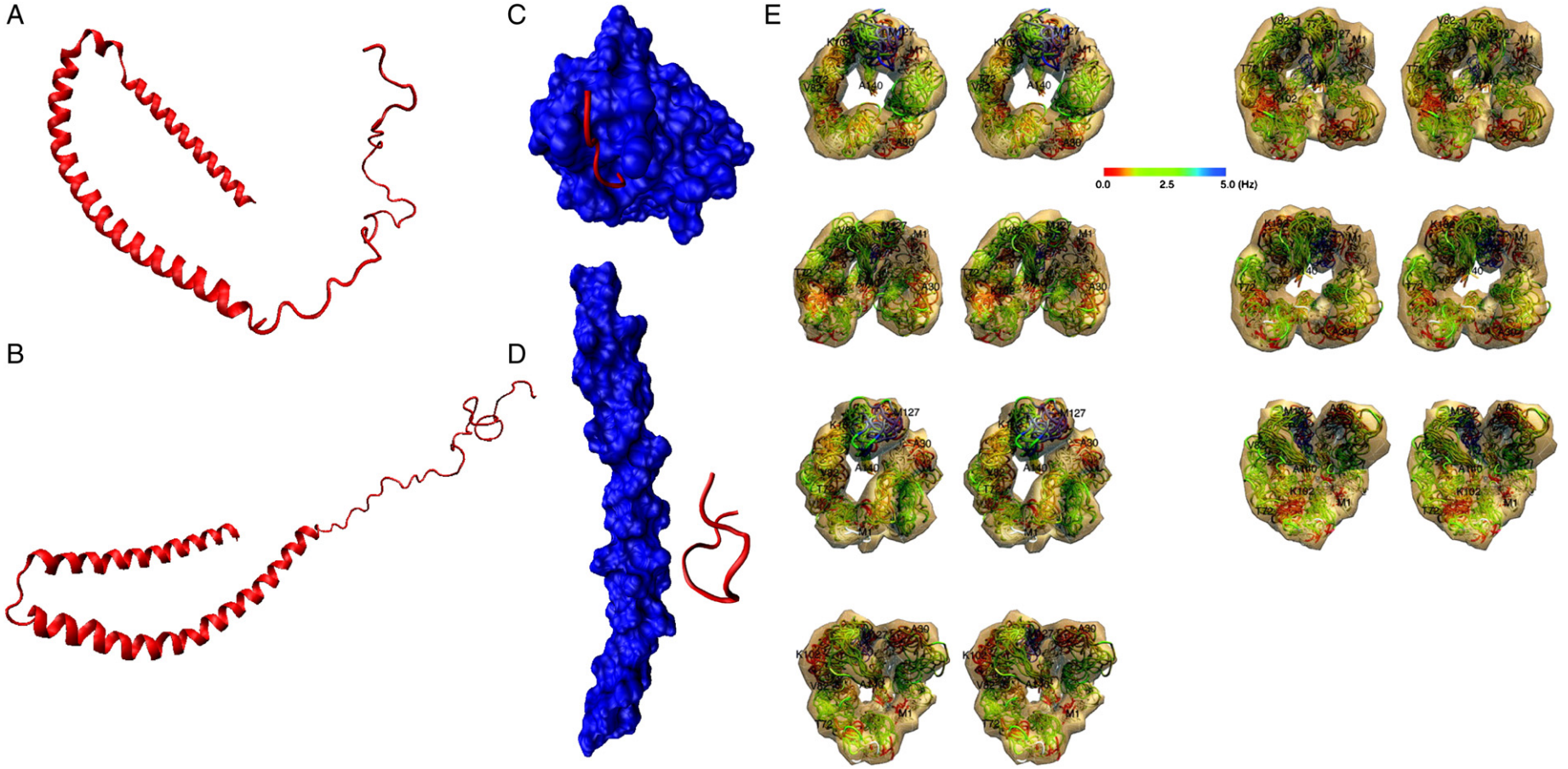

Fig. 2. Structural characterization of α-synuclein and its fragments in bound and unbound states. A and B. 3D-structures of α-synuclein bound to vesicles (PDB IDs: 1XQ8 and 2KKW) [48, 56]. C. 3-D structure of the C-terminal fragment bound to a single domain of camelid antibody (PDB ID: 2X6M) [57]. D. 3-D structure of the N-terminal fragment of α-synuclein bound to the coiled-coil domain of synphilin-1 (based on PDB ID: 2KES) [58]. E. Representative conformations of the unbound α-synuclein calculated from PRE data. Shown are the stereo-pairs of seven most populated clusters containing 80, 75, 46, 39, 25, 24, and 20 structures representing 50% of all calculated conformations. Each of these seven clusters represents the 10 lowest-energy structures within an atomic density map calculated from all conformations contained in each cluster. RDCs were mapped onto the structures with the use of a continuous color scale, together with the representatives of the ensemble of native structures found for this protein in the unbound form. In each cluster, positions of some of the key residues (M1, A30, T72, V82, K102, M127, and A140) are indicated. Plot E is modified from [46]. This figure is adapted from [40].

- Transgenic mice expressing A53T and WT exhibited neurodegeneration outside the *substantia nigra* without fibrillar inclusions [111];
- Lentiviral-based expression of human α-synuclein in rat *substantia nigra* resulted in selective dopaminergic toxicity with nonfibrillar inclusions [112];
- The α-synuclein-containing inclusions in some animal models do not contain fibrils, and the fibril-containing inclusions found in the fly PD model can occur in the absence of neurodegeneration [113,114];
- Loss of dopaminergic neurons was the highest in transgenic mice expressing oligomer-forming E35K and E57K α-synuclein mutants and the lowest in those expressing a fibril-forming A53T mutant [115].

This data allows us to make a conclusion that, similar to other neurodegenerative diseases, there is a lack of correlation between α-synuclein fibril accumulation and neurotoxicity, making it likely that oligomers are the neurotoxic species.

4. Roles of posttranslational modifications

The effect of post-translational modifications (PTMs) on protein secondary structure and susceptibility to conformational changes can be dramatic [116]. Both the spectrum and the range of the PTM-induced changes are very broad, as they are produced by such diverse processes as proteolysis, phosphorylation, lipidation, S-nitrosylation, nitration, oxidation, glycosylation, methylation, adenosine diphosphate (ADP)-ribosylation, acylation (acetylation, isoprenylation, myristoylation), ubiquitination, sumoylation, sulfation, farnesylation, and many others [117]. Protein aggregation is a highly cooperative process, and even a small subpopulation of modified α-synuclein could have a substantial impact on kinetics and product distribution. Here we will survey the most common types of spontaneous modifications in α-synuclein and their known effects on its conversion into disease-related forms.

The peculiarities of α-synuclein PTMs and their roles in modulation functions and aggregation of this protein have been covered in an excellent review [118]. It has been pointed out that out of >300 known PTMs [116,117], only a few have been described for α-synuclein [118]. This includes phosphorylation, nitration, dityrosine crosslinking, methionine oxidation, glycosylation, ubiquitination, sumoylation, AGE adduct formation, crosslinking by transglutaminase, truncation, and N-terminal acetylation. The known sites of PTMs in α-synuclein are shown in Fig. 3 and discussed below.

4.1. Phosphorylation

In human α-synuclein *in vivo*, serine 129 was established as a major phosphorylation site, with a second phosphorylation site located at serine 87 (Fig. 3A, red circles). Both of these sites are highly conserved. Casein kinases CK1 and CK2 [119], and G-protein coupled protein kinases are believed to be responsible for phosphorylation at these sites. It is interesting to note that the degree of α-synuclein phosphorylation (mostly at Ser-129) is significantly elevated in α-synuclein deposits in DLB, MSA, and PD brains [120–122]. It has been estimated that 90% of α-synuclein in LBs is phosphorylated at Ser-129 [120].

The effect of phosphorylation at Ser-129 on aggregation of α-synuclein has been studied by expressing the S129A α-synuclein mutant incapable of phosphorylation or the S129D mutant as a mimic of phosphoserine [123–125]. The authors found that α-synuclein phosphorylation at this position enhanced the formation of aggregates, whereas treatment with the casein kinase 2 inhibitor or S129A mutation had the opposite effect [124]. Reduction of Ser-129 phosphorylation by promoting the phosphatase activity in transgenic mice leads to decrease in α-synuclein aggregation and improved motor performance [126]. This data shows that Ser-129 phosphorylation promotes aggregation of α-synuclein. Phosphorylation at Ser-87, on the other hand, expands the structure of α-synuclein, increases its conformational flexibility, and blocks its aggregation *in vitro*[127]. Phosphorylation of Tyr125 was also found in the human brain and was shown to attenuate the conversion of α-synuclein to toxic oligomers [128,129]. The effect of phosphorylation of the structure and aggregation of α-synuclein has been found to be highly dependent on the position of the modification.

4.2. Oxidative modifications

4.2.1. Tyrosine oxidation

As α-synuclein does not have cysteines and tryptophanes, the primary targets for oxidative modifications are its methionine and tyrosine residues. The α-synuclein primary sequence contains four tyrosine residues: Tyr-39, Tyr-125, Tyr-133, and Tyr-136 (Fig. 3, blue circles). These tyrosine residues are conserved in all α-synuclein orthologs and in β-synuclein paralogs, suggesting that these residues might play important functional roles [21]. Common chemical modifications of tyrosine residues are nitration and oxidative dimerization (Fig. 4A and B). Tyrosine residue can be converted to 3-nitrotyrosine *via* spontaneous or peroxidase-catalyzed reaction with peroxynitrite [130]. This modification decreases the pKa value of the tyrosine hydroxyl by approximately 3 units to 7.2. It has been shown that all four tyrosines in α-synuclein can be subjects to nitration [131–135] both *in vitro* and in LBs from the brains of PD patients. However, in a cellular model of PD, only a significant increase in nitration of Tyr-39 was detected while nitration levels of other tyrosine residues were unchanged [136]. The difference could be due to a higher accessibility of Tyr-39 to a nitrating agent. Nitration of either Tyr-39 or C-terminal tyrosines *in vitro* leads to decreased binding of α-synuclein to lipid membranes. Nitration of C-terminal tyrosines leads to further unfolding of α-synuclein [135, 137]. Nitrated α-synuclein was unable to form fibrils by itself (probably due to oligomer formation) but its presence accelerated fibril formation from unmodified protein [135]. Nitrated α-synuclein was highly toxic to dopaminergic neurons and caused motor dysfunction in rats, presumably due to the same oligomer formation [138]. Treatment of α-synuclein with oxidizing or nitrating agents can also result in oxidative crosslinking of tyrosines [139] and other residues [132]. Tyrosine crosslinking has been shown to promote oligomerization of the protein and inhibit its transition to fibrils [132, 139,140].

4.2.2. Methionine oxidation

Methionine residues are also susceptible to oxidation to sulfoxide and ultimately sulfone (Fig. 4C). All four methionines in α-synuclein (Met-1, Met-5, Met-116, and Met-127) located outside the repeat-containing region (see Fig. 3, yellow circles) are highly susceptible to oxidation to methionine sulfoxide *in vitro*[141–144]. Oxidized methionines often disrupt protein structure, since methionine sulfoxide is significantly more polar and rigid than methionine [145]. Methionine-oxidized α-synuclein was found to be more highly unfolded than the non-oxidized protein [141, 143,144], less prone to form oligomers and fibrils, and even able to inhibit the fibrillation of non-modified α-synuclein [143]. The inhibition α-synuclein fibrillation by methionine oxidation was shown to be proportional to the number of oxidized methionines. It has been proposed that methionine oxidation disrupts end-to-end association of α-synuclein required for fibril formation and thus directs its aggregation toward less structured, non-toxic oligomers [55, 146]. Methionine sulfoxides have been shown to bind metal ions with some multivalent ions able to act as a bridge between two or more of them. Such inter- or intramolecular coordination of multiple methionine sulfoxides could significantly alter the protein structure. Indeed, fibrillation of oxidized α-synuclein was promoted by Ti^{3+}, Zn^{2+}, Al^{3+}, and Pb^{2+} ions but not by Hg^{2+}, Cu^{2+}, and Ca^{2+} [144].

4.2.3. Modification by oxidative dopamine adducts

Since PD pathology is associated with dopaminergic neurons, the interaction between α-synuclein and dopamine has been extensively investigated [147]. Dopamine is known to bind to α-synuclein non-covalently, inhibiting its fibrillation and stabilizing the oligomers [148]. However, dopamine is highly susceptible to oxidation, and its oxidation products form adducts with α-synuclein [146, 149]. These adducts drive aggregation of α-synuclein into primarily unstructured, SDS-resistant oligomers [146, 149,150].

Overall, oxidative modification can significantly alter the aggregation pathway of α-synuclein, usually toward oligomer formation. The structure and toxicity of these oligomers depend on the nature of modification and other experimental variables.

4.3. Lysine modification

4.3.1. Ubiquitination

Ubiquitin is a small protein that can be enzymatically attached to lysine residues of various cellular proteins. Ubiquitination is used to target proteins for proteolytic degradation. Although α-synuclein contains 15 lysine residues, only Lys-6, Lys-10, and Lys-12 were shown to be ubiquitinated *in vivo* (see Fig. 3, green boxes) [151]. Since ubiquitination is not required for the degradation of the α-synuclein monomer, it appears that α-synuclein ubiquitination occurs after its aggregation [152]. Not surprisingly, ubiquitinated α-synuclein is present in both LBs during PD [153] and in cytoplasmic inclusions during MSA [154]. The effect of monoubiquitination of α-synuclein on its aggregation depends on the site of modification. For example, α-synuclein monoubiquitinated at Lys-6 aggregated much slower than the unmodified protein [155]. However, monoubiquitination of α-synuclein by SIAH ligase at multiple lysine residues promoted the formation of cytotoxic aggregates both *in vitro* and *in vivo*[156]. Overexpression of Parkin (a ubiquitin ligase) or ubiquitin in *Drosophila* had a protective effect against α-synuclein-mediated neurodegeneration, presumably by targeting α-synuclein aggregates for proteolytic degradation [157–159]. It appears that ubiquitination of α-synuclein at Lys 6 interferes with its aggregation, but its modification at other residues may be promoting aggregation.

4.3.2. SUMOylation

Small ubiquitin-like modifiers (SUMOs) are small proteins that display significant structural similarities to ubiquitin and can also form protein adducts in a similar fashion. Only one yet unidentified lysine residue at the protein N-terminus was shown to be modified by SUMO1 (Fig. 3, brown box) [160]. SUMOylation of α-synuclein was shown to promote its aggregation and decrease its toxicity in COS-7 cells [161].

4.3.3. Modification by advanced glycation end-products

Reducing sugars and sugar-derived aldehydes can react with the amino groups of the proteins to form Schiff base adducts. Rearrangement of these adducts can lead to the heterogeneous set of adducts known as advanced glycation end-products (AGEs) [162]. It has been shown that AGEs and α-synuclein were similarly distributed in LBs of PD and LBD patients and that α-synuclein was crosslinked by AGEs [163]. This crosslinking was shown to promote α-synuclein aggregation and ROS generation in SH-SY5Y cells [164]. Formation of α-synuclein-AGE adducts with either D-ribose or methylglyoxal shifted its aggregation pathway toward oligomer formation [165,166]. These oligomers were cytotoxic and had a molten globule-like secondary structure [165,166].

4.3.4. Modification by lipid-derived aldehydes

Highly reactive aldehydes (for example, 4-hydroxy-2-nonenal and malondialdehyde) are also produced by lipid peroxidation [167]. Similar to sugar-derived aldehydes, these lipid-derived aldehydes react with α-synuclein and promote the formation of stable β-sheet rich cytotoxic oligomers [168–170]. Interestingly, oligomers formed after

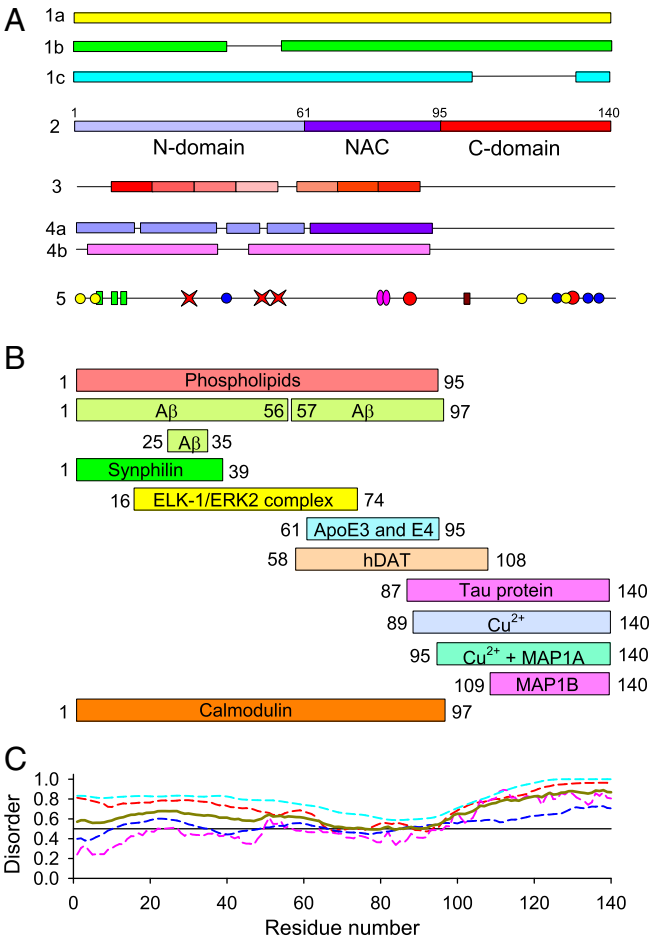

Fig. 3. Schematic representation of α-synuclein structure with the emphasis on: A, peculiarities of primary structure; B, the putative interaction domains; C, intrinsic disorder predictions. Plot A shows three AS isoforms of α-synuclein (1a, 1b, and 1c); three formal structural domain (2); seven imperfect repeats (3); predicted (4a) and experimentally determined α-helices (4b); sites of posttranslational modifications (methionines, yellow circles; tyrosines, blue circles; phosphorylation sites (red circles); ubiquitination, green boxes; sumoylation, brown box; tTG crosslinking sites, red ovals) and PD-related mutations (three red stars) (5). Plot B represents interaction domains responsible for binding of several ligands and proteins. The numbers on the bars correspond to the residues in α-synuclein sequence. Modified from [21]. Plot C represents results of the intrinsic disorder prediction using IUPred (pink dashed line); RONN (blue dashed line); PONDR VSL2 (red dashed line) and PONDR VL3 (cyan dashed line). The results averaged over these for predictions are shown as solid dark yellow line.

reactions with 4-hydroxy-2-nonenal and 4-oxo-2-nonenal had different structures and morphologies [169].

Overall, lysine modification of α-synuclein tends to promote the formation of oligomers at the expense of fibrils. This is likely due to the ability of more flexible structures of oligomers to accommodate these modifications. Similar behavior has been observed for other amyloidogenic peptides and proteins [171–173].

4.3.5. Transglutaminase crosslinking

Both inter- and intramolecular crosslinking between lysine and glutamine residues is catalyzed by a tissue transglutaminase [174]. In PD nigral dopamine neurons, α-synuclein was shown to be heavily crosslinked in this fashion, and the extent of crosslinking correlated with the disease progression [175,176]. The exact localization of modification sites in the protein is not known as yet [175]. For the full-length α-synuclein Gln79, Gln99 and Gln109 were found to serve as crosslink acceptors and Lys60 was identified as one of the crosslink donors (see Fig. 3, red ovals) [177,178]. Intramolecular crosslinking of α-synuclein with low concentrations of transglutaminase resulted in altered conformational and immunological properties of the protein [177]. In these

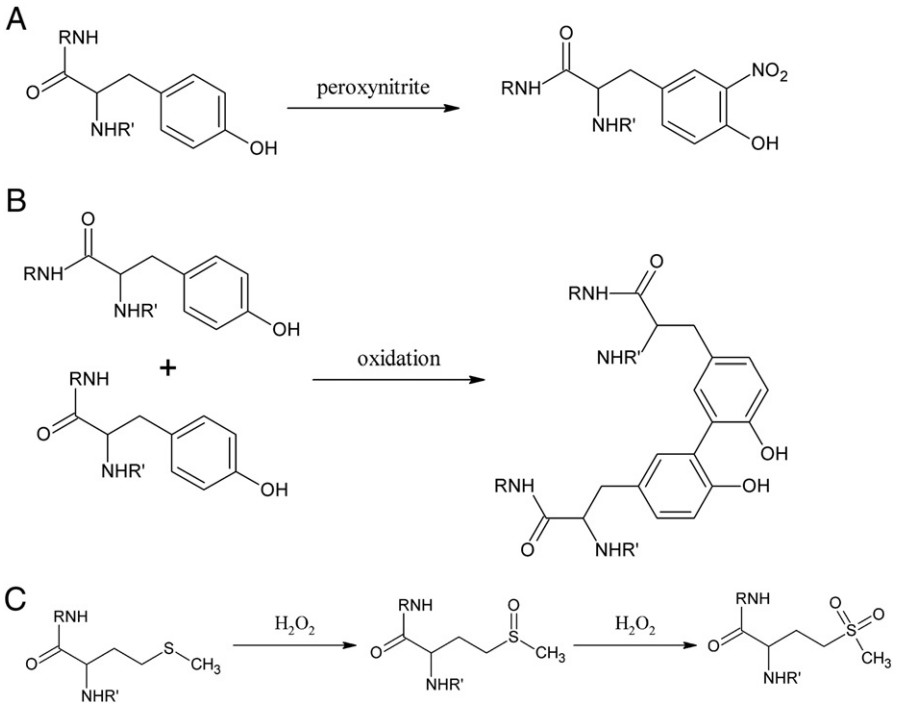

Fig. 4. Methionine and tyrosine oxidation scheme. A — Tyr nitration, B — Tyr dimerization, and C — Met oxidation.

conditions, Gln79 and Gln109 residues were crosslinked to lysine residues at the N-terminal region of the protein. Crosslinks inhibited α-synuclein fibril formation and promoted oligomer formation [177, 179]. These crosslinked oligomers were primarily unstructured and unable to disrupt lipid bilayers [179]. α-Synuclein crosslinking in the presence of lipid bilayers primarily produced a Lys58-Gln99 crosslink [178]. α-Synuclein crosslinked in this fashion accelerated fibril formation from the wild-type protein as detected by ThT fluorescence [178]. Overall, the effects of α-synuclein crosslinking on its aggregation depend on the position of the crosslink, with most crosslinks promoting the formation of unstructured, relatively non-toxic oligomers.

4.4. Truncation

N-terminal and C-terminal truncations are common modifications of α-synuclein, and α-synuclein fragments are often associated with LBs [180,181]. C-terminal truncations are the most common modifications, with the cleavage site usually located between residues 115 and 135 [182]. The resulting α-synuclein fragments form fibrils more readily compared to the full-length protein, and were proposed to initiate its aggregation *in vivo* and *in vitro* [66, 180,181, 183]. Detailed studies have shown that removal of the C-terminal domain that serves as an intramolecular chaperone destabilizes the monomeric state of α-synuclein and accelerates its aggregation [47, 184]. The increased aggregation propensity of α-synuclein fragments correlates with their higher toxicity *in vivo*. Co-expression of α-synuclein fragments truncated at residues 110 and 120 with wild-type protein in the cell cultures resulted in increased cell death [180, 185]. Experiments in transgenic animals expressing α-synuclein fragments also usually showed neuronal loss and lower dopamine levels, especially if wild-type α-synuclein and its fragments were co-expressed [186–188].

4.5. N-terminal acetylation

Analysis of synuclein forms present in LBs from DLB patients by two-dimensional immunoblot analysis and mass spectroscopy revealed that N-terminal acetylation was a common posttranslational modification of this protein [182]. N-terminal acetylation of cytosolic proteins is catalyzed by the N-terminal acetyltransferase B complex (NatB). This is a rather common modification, especially for the proteins in which the initiating Met residue is retained [189]. The exact roles of this modification for modulation of α-synuclein structure, function, aggregation, and cytotoxicity are not known as of yet. However, it is believed that N-terminal acetylation is required for the proper interactions of α-synuclein with the membrane [190]. In transgenic yeast, α-synuclein showed no cytotoxicity when expressed in a strain lacking NatB activity [190].

4.6. PTMs and intrinsically disordered nature of α-synuclein

Fig. 3C represents the results of disorder prediction for human α-synuclein by several disorder predictors: PONDR® VL3 [78, 191], PONDR® VSL2 [192], RONN [193], and IUPred [194]. It can be seen that α-synuclein is predicted to be almost completely disordered by all these predictors (as disorder probability scores ≥ 0.5 correspond to a prediction of disorder), emphasizing that its sequence is typical of the intrinsically disordered proteins. Therefore, Fig. 3 clearly shows that all functionally important sites of α-synuclein, as well as all its PTM sites, are located inside the disordered sequence. This emphasizes the importance of the intrinsically disordered nature of this protein for its function.

5. Modulating α-synuclein aggregation by various environmental factors and interactions with other proteins and small molecules

5.1. Effect of macromolecular crowding

The environment inside a living cell is extremely crowded. The concentration of macromolecules, including proteins, nucleic acids, carbohydrates, and small solutes within a living cell can be as high as 400 mg/ml [195], with the intracellular solutes taking up about a half of the total cellular volume [195–198]. The extracellular environment is crowded as well, with protein concentrations in plasma as high as 80 mg/ml [198]. Obviously, the volume occupied by solutes is unavailable to other molecules, a phenomenon known as “excluded volume effects” [195, 199]. In crowded environments the structures

of both folded and natively unfolded proteins become more compact [200,201]. Aggregation of intrinsically disordered proteins is significantly accelerated by crowding because aggregates and aggregation intermediates are more compact than unfolded monomers [200, 202–204]. For example, the fibrillation of prion protein and tau (244–441) fragment was also accelerated by the presence of crowding agents [205]. However, the effects of crowding on the structure and aggregation of folded proteins are more complex. For example, crowding had only small effects on high-affinity protein–protein complexes [206], while formation of a low-affinity complex between GFP analogs CyPet and YPet [207] was disrupted, and these proteins aggregated instead. When aggregation of a model β-hairpin peptide was studied in the presence of crowding agents, the lag time for aggregation was increased but the aggregation rate was not affected [208].

In case of α-synuclein, high concentrations of various types of crowding agents (polyethylene glycols, polysaccharides or other proteins) were shown to accelerate its fibril formation *in vitro*[209,210]. The effect increased with the increasing length of polymer, as well as with the increasing polymer concentration. However, the lag time and the fibril elongation rate were affected differently by different crowding agents with the stronger effect observed on the nucleation rate of fibril formation [211–213]. The formation of α-synuclein protofibrils was also accelerated by the presence of the crowding agents [210]. The effects of other aggregation-promoting additives (metal ions, pesticides) were cumulative with the effects of the crowding agents [211, 213,214]. It seems clear that in the crowded cellular environment, α-synuclein is even more susceptible to aggregation than in dilute solution.

5.2. The effect of anions

Anions were shown to induce partial folding of α-synuclein at neutral pH, forcing the formation of an amyloidogenic partially folded intermediate and accelerating fibril formation in this protein. The magnitude of the fibrillation accelerating effect followed the position of the anion in the Hofmeister series, indicating that the major role of anions in α-synuclein fibrillation is their modulation of protein–water interaction, although electrostatic effects have to be taken into account as well [215]. Therefore, the enhanced fibrillation of α-synuclein in the presence of anions is the result of the loss of the uncompensated charge, which is a factor promoting the soluble unfolded conformation, and an increase in the preferential hydration, which promotes partial folding and aggregation by strengthening hydrophobic interactions. Both nucleation and fibril growth are affected by a combination of these two effects. The addition of small quantities of salts eliminates the strong electrostatic repulsion between the residues in the negatively charged C-terminal domain of α-synuclein, giving rise to its partial folding to the amyloidogenic conformation. High concentrations of salts act similar to crowding agents to bring about the dehydration of α-synuclein, further promoting its aggregation [215].

5.3. Role of environmental toxins: pesticides

Several lines of evidence point to environmental exposure as a potential contributing factor in the pathogenesis of PD [216–222]. Both epidemiological and clinical observations reveal pesticides and herbicides, especially paraquat and rotenone, as important environmental PD risk factors [223]. Since rotenone inhibits mitochondrial electron transfer and paraquat catalyzes ROS formation, both of them induce oxidative stress. Administration of paraquat or rotenone to mice or rats led to the overexpression of α-synuclein, presumably to combat the oxidative stress caused by the pesticides [18, 224–226]. Higher levels of α-synuclein led to accumulation of α-synuclein-containing aggregates within the neurons of *substantia nigra*. In addition, these pesticides were shown to promote aggregation of α-synuclein *in vitro* in a dose-dependent manner [224, 227,228]. It has been proposed that their effect is due to the specific stabilization of the amyloidogenic partially folded conformation of α-synuclein, although they may also promote oxidative modification of the protein.

5.4. Role of environmental factors: metals and α-synuclein aggregation

The possible role of heavy metals in the etiology of PD has been proposed early on based on the results of epidemiological studies [229–235] and from the postmortem analysis of the brain tissues of PD patients [236–238]. For example, the analysis of the PD mortality rates in Michigan revealed that counties with an industry in the paper, chemical, iron, or copper related-industrial categories had significantly higher PD death rates than counties without these industries [234]. An epidemiological study conducted in Quebec established that an increased risk for PD is associated with long-term occupational exposure to manganese, iron, and aluminum [232,233]. A population-based case–control study in Detroit suggested that chronic occupational exposure to manganese or copper, or to dual combinations of lead, iron, and copper, is associated with increased incidences of PD [229, 235]. In addition to epidemiological data, postmortem analysis of brain tissues from patients with PD shows a considerable increase in total iron, zinc, and aluminum content of the Parkinsonian *substantia nigra* compared to control tissues [236–239]. Another study revealed that the central nervous system tissues of fish exposed to elevated metal ion concentrations had increased levels of α-synuclein aggregates [240]. Overall, increased levels of heavy metals in the environment appeared to correlate with increased incidence of PD.

While metal ions can cause brain damage directly [241–245], the effect of metals on PD is at least partially due to their effect of the aggregation of α-synuclein. Several possible mechanisms for metal-stimulated aggregation of α-synuclein can be envisaged. The simplest would involve direct interactions between α-synuclein and metal ions leading to structural changes in α-synuclein. *In vitro* experiments indeed showed that α-synuclein aggregation is facilitated by the presence of Cu^{2+}[246], and other metal ions [247]. Redox-active metal ions may also be able to influence misfolding of α-synuclein *via* its oxidation. Since we discussed the effect of oxidation on α-synuclein earlier, here we will consider the direct effects of metal ions on α-synuclein conformation and aggregation.

Fig. 5 shows that at physiological conditions in the presence of millimolar concentrations of various metal cations, α-synuclein adopted a partially folded conformation. This conformation is characterized by an increased amount of ordered secondary structure (Fig. 5A), changed environment of tyrosine residues (Fig. 5B), increased protection of these residues from quenching (Fig. 5C), and by the appearance of solvent-accessible hydrophobic surfaces as detected by the fluorescent probe ANS (Fig. 5D) [247]. The ability of a given cation to induce structural changes in α-synuclein was proportional to the cation's charge density [247]. It was proposed [247] that metal ions stabilize the partially folded conformation of α-synuclein by decreasing the electrostatic repulsion between the negative charges in this protein. To some extent this situation is similar to the anion-induced folding of acid unfolded globular proteins, when the electrostatic repulsion is reduced by binding of counter-ions and protein conformation becomes more compact [30, 36, 248–251]. Cations that were the most efficient in promoting partial folding of α-synuclein also converted the highest proportion of this protein to amyloid fibrils, presumably due to a high aggregation propensity of this partially folded conformation. A more detailed analysis of effect of individual metal ions on α-synuclein aggregation is presented below.

5.4.1. Aluminum

Exposure to aluminum was one of the conditions linked to the PD pathology *via* the epidemiological studies and the postmortem analysis of the brain tissues of PD patients [232,233, 236–239]. Incubation

of α-synuclein with Al^{3+} leads to the changes in the far-UV CD, UV absorbance, intrinsic fluorescence and ANS fluorescence spectra consistent with the formation of the partially folded intermediates described above [247, 252]. At low protein concentrations (<35 μM) these changes occurred simultaneously, were rapid, reversible, and independent of protein concentration, indicating an intramolecular conformational change [30]. Al^{3+}-induced partial folding of α-synuclein was also confirmed using the selective non-covalent adduct protein probing mass spectrometry (SNAPP-MS), which utilized interactions between 18-crown-6 ether and lysine residues to probe protein structure in the presence and absence of metal ions [253]. It has been shown that these interactions were altered dramatically in the presence of 3 μM Al^{3+}, suggesting that Al^{3+} binding caused a significant change in the conformational dynamics of the monomeric form of α-synuclein [253].

$AlCl_3$ was also shown to promote α-synuclein aggregation. At high α-synuclein concentrations, the addition of Al^{3+} induced the formation of oligomers detected by light scattering [247]. These oligomers possessed a significant amount of ordered secondary structure and readily converted to fibrils. The addition of 2.5 mM $AlCl_3$ shortened the lag-time for α-synuclein fibril formation ~3-fold, and increased the apparent rate of fibril formation ~1.5-fold [247]. α-Synuclein fibrils formed in the presence of Al^{3+} had an altered morphology which consisted of twisted ribbons with a periodicity of about 100 nm [254]. In the presence of 20% ethanol, Al^{3+} promoted α-synuclein oligomer formation [255].

5.4.2. Calcium

A recent study revealed that α-synuclein regulates the pathways of Ca^{2+} entry inside the cells [256]. In another study, a link between Ca^{2+} homeostasis, α-synuclein, and cytosolic dopamine was established, suggesting that interplay between these three molecules can be responsible for the selective death of the dopaminergic neurons in the *substantia nigra*[257]. Using a microdialysis technique it was shown that α-synuclein binds Ca^{2+} with an IC_{50} in micromolar range. The Ca^{2+}-binding site was assigned to a C-terminal domain of this protein [258]. These findings show the importance of α-synuclein–Ca^{2+} interactions *in vivo*.

Like other metal ions, Ca^{2+} increased the aggregation rate of α-synuclein. The addition of Ca^{2+} directed the aggregation of α-synuclein to a mixture of annular oligomers (70–90 nm in diameter, 4 nm in height) and spherical oligomers (10–20 nm in diameter) [259,260]. Each annular particle induced by Ca^{2+} appeared to be composed of a ring of several spherical particles. No annular oligomers were found when α-synuclein lacking the C-terminal 15 amino acids was co-incubated with Ca^{2+}, indicating that the C-terminal Ca^{2+}-binding domain was involved in the formation of annular oligomers. Interestingly, soluble 30–50 nm-sized annular α-synuclein oligomers were isolated by a mild detergent treatment from glial cytoplasmic inclusions purified from MSA brain tissue [261], and it has been proposed that the formation of such aggregated species inside the neurons can be influenced by the increased intracytoplasmic Ca^{2+} concentration [262]. This is an indication that Ca^{2+}-dependent aggregation of α-synuclein may occur *in vivo* as well.

In addition, Ca^{2+} was shown to modulate the interaction of α-synuclein with the cell membranes. While in the absence of Ca^{2+} α-synuclein interacts with lipid membranes *via* the N-terminal domain, the addition of Ca^{2+} promotes additional interaction between the membrane and the C-terminal domain that may lead to aggregation of this protein [263].

5.4.3. Copper

In addition to binding to C-terminal region of α-synuclein with low affinity like other metal ions, Cu^{+} and Cu^{2+} ions also bind with nanomolar affinity at the N-terminus of the protein [264–267]. The high copper affinity of α-synuclein suggests that most of this protein is copper-bound *in vivo*. Copper binding may be integral for a physiological function of α-synuclein since it is believed to be involved in copper and iron metabolism and possess a copper-dependent ferrireductase enzymatic activity [268].

Significant effort has been devoted to the investigation of Cu^{2+}-binding by α-synuclein. Initially, the primary Cu^{2+}-binding site was shown to involve His50 as the anchoring residue and other nitrogen/oxygen donor atoms in a square planar or distorted tetragonal geometry [264]. The acidic C-terminus of the protein was shown to coordinate a second Cu^{2+} equivalent with a 300-fold lower affinity [264]. Several recent studies [267, 269–273] utilized EPR spectroscopy and site-directed mutagenesis to characterize the Cu^{2+}-binding sites of α-synuclein in more detail. The conclusion was that the highest affinity Cu^{2+} binding site is located at the first few N-terminal residues of α-synuclein, and the Cu^{2+} binding constant at this site is around 100 nM. Some studies came to the conclusion that His50 is also involved in Cu^{2+} binding at this site; however, other studies disputed that. When NMR spectroscopy was used to identify lower-affinity Cu^{2+}-binding sites, as many as 16 different sites capable of binding Cu^{2+} were found [274], with most of the lower-affinity sites located in the acidic C-terminal region.

Using AFM-based single-molecule mechanical unfolding it has been shown that the presence of Cu^{2+} significantly enhances the relative abundance of the β-like structure in monomeric α-synuclein [275]. It has been proposed that upon Cu^{2+} binding, α-synuclein adopts a more structured conformation that could promote its aggregation [247, 267, 274, 276]. Cu^{2+}-synuclein complex is also capable of redox cycling, resulting in the generation of oxygen radicals that lead to oxidative stress and chemical modification of α-synuclein [277,278]. Removal of Cu^{2+} ions from α-synuclein by another Cu^{2+}-binding protein abolished ROS generation [278].

Cu^{2+} was shown to be an effective accelerator of α-synuclein aggregation even at physiologically relevant concentrations without altering the morphology of the resultant fibrillar structures [247, 264]. Studies using α-synuclein fragments allowed Brown and coworkers to propose that Cu^{2+} binding may result in dimerization or oligomerization of α-synuclein [276]. The appearance of α-synuclein oligomers in the presence of Cu^{2+} has been confirmed by ESI-MS [279]. It was also shown that neurotoxicity of α-synuclein oligomers was increased by the presence of Cu^{2+}[280]. Cu^{2+}-loaded cytotoxic oligomers of α-synuclein were isolated and shown to possess a unique stellate morphology by EM analysis [280].

Overall, α-synuclein was shown to bind copper ions with high affinity and may play a role in copper metabolism *in vivo*. Copper binding accelerates the aggregation of α-synuclein and influences its pathological effects.

5.4.4. Iron

The interconnection between iron homeostasis, α-synuclein aggregation, and PD is very strong. As it was already pointed out, numerous epidemiological studies [229–235] and the postmortem analysis of the brain tissues [236–238] have linked the heavy metal exposure and metal accumulation in the brain with the PD pathogenesis. Postmortem analysis of brain tissues from patients with PD revealed that the *substantia nigra* of the PD brain is characterized by a shift in the Fe^{2+}/Fe^{3+} ratio in favor of Fe^{3+} and a significant increase in the Fe^{3+}-binding protein, ferritin. Glutathione content was also shown to be significantly lower, confirming the change in the redox status of the environment [237]. The aggregation of α-synuclein may be contributing to this phenomenon since α-synuclein has been proposed to act as a ferrireductase [268], and its aggregation is likely to decrease or abolish this activity. The evidence for the ferrireductase activity of α-synuclein is the ability of Cu^{2+}-bound α-synuclein to catalyze reduction of Fe^{3+} to Fe^{2+}*in vitro* and increase in Fe^{2+} levels in the cells overexpressing α-synuclein *in vivo* [268].

As a transition metal closely associated with ROS formation, iron has been suspected to contribute to PD because of its ability to promote

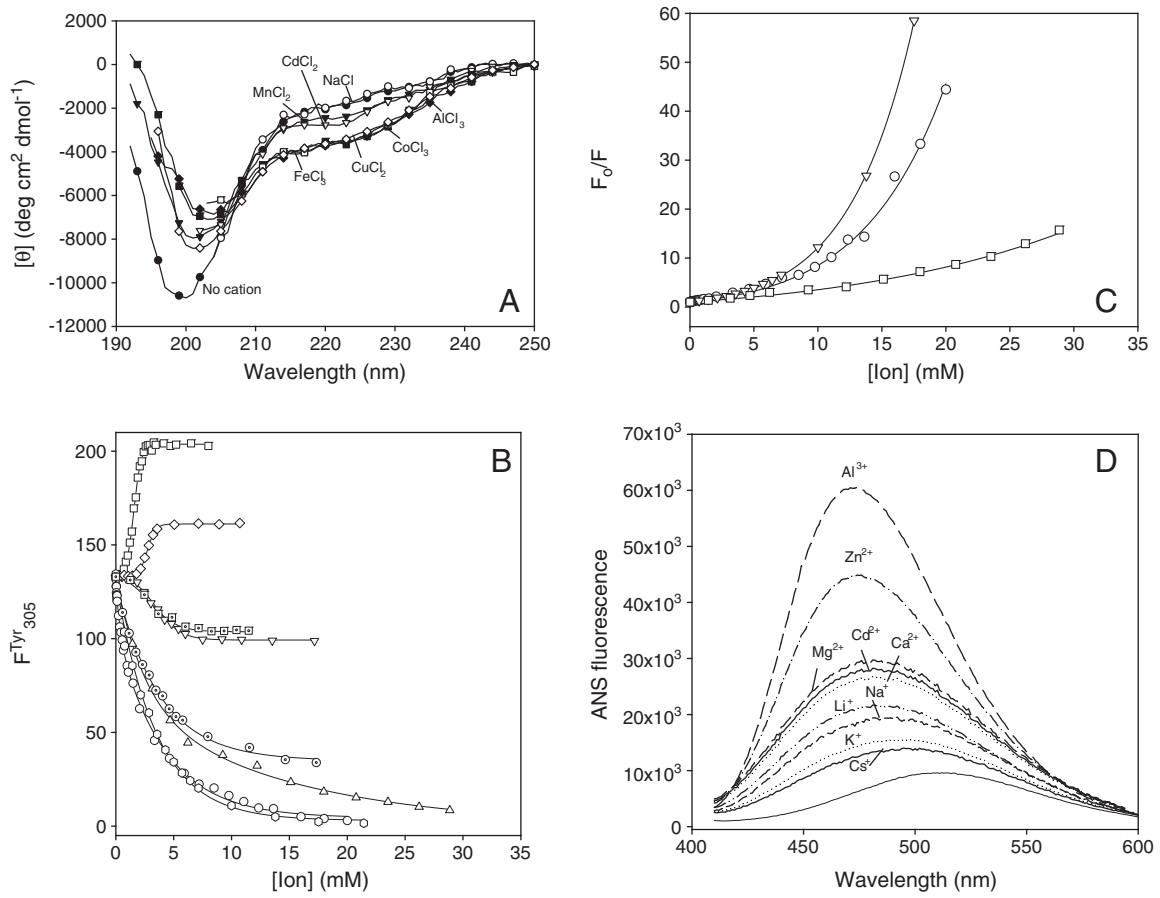

Fig. 5. Metal ion-induced conformational changes in α-synuclein. A. Far-UV circular dichroism spectra of 35 μM α-synuclein measured in the absence or presence of 2 mM of the indicated metals. B. Comparison of the effect of metal ions on the intrinsic α-synuclein fluorescence. Titration curves measured for Al^{3+} (squares), Zn^{2+} (diamonds), Mn^{2+} (inverse triangles), Fe^{3+} (circles), Fe^{2+} (triangles), Cu^{2+} (hexagons), Co^{3+} (dotted circles) and Co^{2+} (dotted squares). C. Stern–Volmer plots for α-synuclein fluorescence quenching by Cu^{2+} (circles), Fe^{3+} (inverse triangles) and Fe^{2+} (squares). D. ANS fluorescence spectra measured for free dye (solid line) and in the presence of 7 μM α-synuclein and 10–50 mM of the chloride salts of mono-, di- and trivalent cations. Modified from Ref. [30].

oxidative damage. Indeed, similar to copper, Fe^{2+}-bound α-synuclein produced ROS *via* redox cycling [281]. However, iron is able to directly influence the aggregation of the α-synuclein as well [282]. Investigation of α-synuclein oligomer formation in the presence of iron and alcohols at the single particle level using single-molecule fluorescence techniques and AFM showed that both alcohols and Fe^{3+} were effective inducers of α-synuclein oligomerization at micromolar concentrations [88, 255]. The morphologies of the resulting oligomers were different, with alcohols inducing small oligomers and ferric ions inducing the formation of larger oligomers. It is worth noting that Fe^{3+} only caused an effect on α-synuclein aggregation when added in the presence of intermediate concentrations of ethanol (~5%), suggesting that the effect of Fe^{3+} depended on the presence of the intermediate I species. Although both oligomers could seed fibril formation, only Fe^{3+}-induced oligomers were SDS-resistant and could form ion-permeable pores in a lipid bilayer that were blocked by the oligomer-specific A11 antibody [83, 255]. Recently, the bioluminescent protein-fragment complementation assay (BPCA) was implemented to directly analyze the formation of toxic α-synuclein oligomers in the cell culture. The assay confirmed that Fe^{3+} promoted α-synuclein oligomer formation in living cells [283].

The binding of Fe^{3+} was also shown to alter the morphology of α-synuclein fibrils. The addition of Fe^{3+} induced the formation of shorter and thicker fibrils from both wild type and mutant α-synuclein [266]. These observations provide strong support for an important role of the ferric iron in the formation of toxic α-synuclein oligomers *in vivo* [255, 283]. Iron contributes to aggregation of α-synuclein by both direct binding to the protein, leading to alteration of the aggregation pathway, and by production of ROS that oxidize α-synuclein.

5.4.5. Lead

Exposure to lead is known to produce aggresome-like inclusion bodies in target cells as a toxic response [284]. This process was shown to be controlled by both metallothionine and α-synuclein. In fact, Pb^{2+} exposure produced a rapid increase in α-synuclein expression in cells stably expressing metallothionine. Expression then decreased over 48 h as Pb^{2+}-induced aggresome-like inclusion bodies containing both metallothionine and α-synuclein were formed [284]. In an *in vitro* study, Pb^{2+} was one of a few metal ions shown to overcome the methionine oxidation-induced inhibition of the α-synuclein fibril formation [144]. It appears that, like other metal ions, lead promotes aggregation of α-synuclein.

5.4.6. Magnesium

There is significant evidence that PD is associated with lower levels of magnesium in brains. Atomic absorption and atomic emission spectroscopy revealed lower concentrations of magnesium in the caudate nucleus in Parkinsonian brains in comparison with the control [285]. This finding was confirmed by ICP-MS analysis of PD and control brains, which revealed that Mg^{2+} concentration was lower in cortex, white matter, basal ganglia, and brain stem of PD brains compared to control brains [286]. Magnesium concentration in cerebrospinal fluid decreased with the duration and severity of PD [287]. Epidemiological studies suggest that a low dietary intake

of magnesium is associated with a higher risk of PD [288,289]. A study of low Mg^{2+} intake over generations in rats showed increased neurodegeneration of *substantia nigra*, a link that has been proposed for increased incidence of PD and ALS in Guam [290]. The reason for correlation between Mg^{2+} levels and PD is unclear, and could be due to either direct effect of Mg^{2+} on protein aggregation or to indirect effects (*e.g.* inhibition of calcium signaling or requirement for magnesium as a nutrient).

The effect of Mg^{2+} on α-synuclein aggregation is rather complex. When α-synuclein was incubated in the presence of high Mg^{2+} concentrations (>10 mM), large aggregates composed of densely packed short fibrils were rapidly formed [291]. However, at low concentrations Mg^{2+} inhibited α-synuclein aggregation induced either spontaneously or by incubation with iron [292]. Mg^{2+} was also able to modulate the interaction between α-synuclein and several herbicides and inhibit the herbicide-induced aggregation of α-synuclein [293]. In this essence, Mg^{2+} was very different from other metals ions, all of which strongly promoted the herbicide-induced α-synuclein aggregation. Furthermore, Mg^{2+} counteracted the effect of other metal ions when they were present together [293]. In line with these observations, it was shown that millimolar concentrations of Mg^{2+} protect dopaminergic neurons in the *substantia nigra* from MPP^{+}-mediated degeneration in transgenic rats [294]. These observations suggest that the interaction of Mg^{2+} with α-synuclein might play a neuroprotective role by inhibiting the aggregation of the latter.

5.4.7. Manganese

Chronic manganese intoxication, manganism, produces an irreversible syndrome which bears a striking resemblance to PD, including fixed gaze, bradykinesia, postural difficulties, rigidity, and tremor [295]. Parkinsonism due to chronic manganese intoxication can be separated from PD by the presence of dystonia and mental status changes [296]. LBs have never the been observed in manganese-induced parkinsonism, and the major effects of manganese toxicity were found in the cells of the striatum and globus pallidus which are not dopaminergic [297].

Although manganism is clearly different from PD, there is a possible link between chronic exposure to Mn^{2+} and PD [298,299]. For example, a recent study by Lindquist and coworkers found that PD-related protein PARK9 is a Mn^{2+} transporter that helps protect the cells from manganese toxicity [300]. Knockdown of this protein increases α-synuclein misfolding and toxicity, suggesting the possible involvement of Mn^{2+}. Mn^{3+} is able to oxidize α-synuclein resulting in the immediate formation of di-tyrosine cross-links [247]. Since di-tyrosines can be formed both intra- and intermolecularly, this modification represents an additional structure- and aggregation-promoting factor.

In fact, co-incubation of α-synuclein with Mn^{2+}*in vitro* induced a partial folding of the protein and its fibrillation [247]. Curiously, when SK-N-MC neuroblastoma cells stably expressing the human dopamine transporter were transfected with human α-synuclein and exposed to 30–300 μM $MnCl_2$, the viability of cells overexpressing α-synuclein after 72 h of exposure to Mn^{2+} was dramatically reduced, suggesting that Mn^{2+} may cooperate with α-synuclein in triggering neuronal cell death such as seen in manganese parkinsonism [301]. A similar effect has been observed in rat mesencephalic cells [302]. This data indicates a possible connection between Mn^{2+} and α-synuclein *in vivo*, although it's not clear whether it's due to direct interaction between them.

5.4.8. Zinc

Zinc has been studied as one of potential environmental factors exposure to which might favor PD, but no correlation between occupational exposure to zinc and PD progression has been found [230, 234–235, 303]. A recent epidemiological study showed a positive correlation between higher levels of zinc and a decreased risk of PD [289]. However, the analysis of the Parkinsonian *substantia nigra* revealed the enhanced level of zinc in comparison with the control tissues [237–239]. Zinc was shown to be an effective promoter of α-synuclein aggregation and specifically its fibrillation *in vitro* [247, 304]. When the fibrillation of the oxidized form of wild type α-synuclein and its Met-minus mutants were studied, the presence of Zn^{2+} was shown to induce a dramatic acceleration of fibril formation for all of the oxidized proteins studied in a mutation-dependent manner [142]. The rates of fibrillation for the oxidized α-synucleins in the presence of Zn^{2+} could be arranged in the following order: M5L/M116L/M127L > M116L/M127L > M5L ≈ M116L ≈ M127L [142]. This suggested that methionine sulfoxides of the oxidized α-synuclein are not directly involved in the coordination of Zn^{2+} as double and triple Met → Leu mutants aggregated faster than WT or single mutants. Interaction with Zn^{2+} decreased the propensity of the Leu-substituted α-synucleins to oligomerize, indicating that the presence of Zn^{2+} caused partitioning in favor of the fibrillation pathway [142]. Zinc was also one of the several metals shown to dramatically accelerate the herbicide-induced fibrillation of α-synuclein [293]. Overall, zinc promotes fibrillation of α-synuclein at the expense of its oligomerization *in vitro*. Its effect *in vivo* is unclear, and may in fact be protective.

5.4.9. Interaction of α-synuclein with metal ions: general considerations

In general, there are at least two major types of interactions of the α-synuclein with metal ions: low-affinity and high-affinity binding. The low-affinity binding (in the millimolar range) is driven by the electrostatic interactions of the metal ions with the negatively charged side chains of α-synuclein and is mostly directed to C-terminal domain of the protein. Many metals can interact with α-synuclein non-specifically, resulting in the effective neutralization of the Coulombic charge-charge repulsion and stimulating the partial folding of α-synuclein. The resulting partially folded conformation of α-synuclein is more aggregation-prone [247]. The C-terminus of α-synuclein also contains a specific, albeit also low-affinity, metal binding site ^{119}DPDNEA^{124}. Metal binding at this sequence may be driven not only by electrostatic interactions but also by the residual structure of the α-synuclein C-terminus [305]. The higher-affinity metal-binding site has been assigned to several N-terminal residues and a slightly lower-affinity site is ascribed to the imidazole ring of the sole histidine residue [306].

There is a significant correlation between the propensity of a metal ion to induce partial folding of the non-oxidized α-synuclein and its ability to promote fibrillation of this protein [247]. Among the most effective stimulators of protein partial folding and subsequent fibrillation were Cu^{2+} and Fe^{2+}, which were shown to interact with α-synuclein specifically, together with non-specifically interacting Al^{3+}, Co^{3+}, and Mn^{2+} [247]. Since there are several different modes of metal ion interactions with α-synuclein, it is not surprising that metal binding induces a wide range of conformational changes in this natively unfolded protein. Metal-induced structural changes vary from a relatively minor gain of residual ordered structure in the presence of some monovalent metals to a relatively more pronounced transformation into the pre-molten globule-like conformation, promoted by interaction with polyvalent ions [247]. Different partially folded monomeric species can assemble into morphologically and structurally different oligomers and fibrils. Illustrative examples of this variability of the metal-stabilized oligomeric forms that include 0.8–4 nm spherical particles induced by Cu^{2+}, Fe^{3+}, and Ni^{2+}; larger 5–8 nm spherical oligomers induced by Mg^{2+}, Cd^{2+} and Zn^{2+}; and 70–90 nm annular oligomers stabilized by Ca^{2+} [259]. The morphology of the amyloid fibrils and the prevalence of fibrillation *vs.* oligomerization pathways were shown to be strongly dependent on the nature of the metal ion as well. Such metal-dependent structural variability of monomeric and oligomeric species, together with the metal-specific fibril morphology, should be taken into account in the analysis of the molecular mechanisms of neurodegeneration. Detailed structural characterization of α-synuclein–metal complexes will shed more light on the molecular mechanisms of synucleinopathies. A better connection between the extensive *in vitro* data on the

effect of metal ions on α-synuclein structure and aggregation and the cellular processes triggered by the exposure to heavy atoms should be established (Fig. 6).

6. Interactions of α-synuclein with other proteins

Several dozen proteins are known to physically interact with α-synuclein [21, 307–310]. Fig. 3B represents the localization of binding sites for some known α-synuclein interaction partners. It is of great interest to note that all these interactions are potentially affected either by familial point mutations in α-synuclein or by one or several of its PTMs, or by both (cf. Fig. 3A and B). A recent proteomic analysis using a SILAC technique (stable isotope labeling by amino acids in cell culture) identified 587 proteins involved in the formation of complexes with α-synuclein in the dopaminergic MES cells, with 141 proteins displaying significant changes in their relative abundance (increase or decrease) after the MES cell were treated with rotenone [311]. The list of the individually identified proteins involved in the interaction with α-synuclein includes, but is not limited, to PLD2 [312], UCH-L1 [313], parkin [314], synphilin [315–317], 14-3-3 protein [109, 318], prolyl-isomerase, Pin1 [319], α_B-crystallin [320], different PKC isozymes, BAD, ERK [318, 321], Rab5A [322], ELK-1/ERK-2 complex [323], ERK-1/2, p38MAPK, and SAPK/JNK mitogen activated kinases (MAPKs) [324], Aβ, [325–327], MAP1B [328], heterodimeric tubulin [28, 329], tau protein [330,331], TBP-1 [332,333], phospholipase D [334], protein phosphatase 2A [335], DAT [336], mitochondrial complex IV enzyme cytochrome oxidase [337], TH [338], aromatic amino acid decarboxylase [339], DJ-1 [340,341], histones [342], and calmodulin [343,344].

Some of these proteins were shown to stimulate α-synuclein aggregation *in vitro* at substoichiometric concentrations. The list of these aggregation promoters includes tau [345], histones [345], tubulin [329, 346], and agrin [181]. Except for histones, these proteins have all been identified as components of LBs and/or GCIs [181, 307–309, 329, 345]. Since these proteins are aggregation-prone in physiological conditions, it is likely that they accelerate the aggregation of α-synuclein by cross-seeding. Cross-seeding of fibril and oligomer formation by the proteins with different sequences is a well-known process both *in vitro* and *in vivo*. For example, protein A amyloidosis is accelerated by fibrils formed by several proteins with unrelated sequences [181, 307, 309, 329, 345]. Cross-seeding of α-synuclein aggregation by PrP^{Sc} and Aβ fibrils has been demonstrated both *in vitro* and in transgenic mouse models [347,348]. α-Synuclein fibrils were also shown to seed the formation of tau aggregates in the cell culture [347]. Cross-seeding has been demonstrated for oligomers of amyloidogenic proteins as well. Kayed and coworkers have shown that both Aβ and α-synuclein oligomers are able to seed formation of cytotoxic tau oligomers [349]. Thus it is quite likely that cross-seeding is also the underlying mechanism for promotion of α-synuclein aggregation by amyloidogenic proteins.

Non-amyloidogenic proteins influence α-synuclein aggregation by other mechanisms. For example, the addition of a peptidyl-prolyl isomerase (PPIase) FKBP accelerated α-synuclein aggregation *in vitro* and resulted in the formation of aggregates with clear fibrillar morphology, while the addition of a PPIase inhibitor suppressed α-synuclein aggregation [350,351]. Proline isomerization is known to be a slow step in protein folding, and PPIase addition is known to accelerate aggregation of other proteins such as β-2-microglobulin [352,353].

6.1. Chaperons

Chaperons provide the cells with potent means to neutralize and scavenge toxic misfolded proteins. Heat shock proteins (Hsp) are a family of chaperones that are both constitutively expressed and induced by different stresses that suppress protein aggregation, and participate in protein refolding and/or degradation. Torsin A, a protein with homology to yeast Hsp104, co-localizes with α-synuclein in LBs and is found in many peripheral tissues and brain regions [354–356]. Other heat shock proteins have also been shown to co-localize with α-synuclein in LBs [357]. The over-expression of torsin A or other heat shock proteins was shown to suppress α-synuclein aggregation in the cellular models [357–359]. Yeast Hsp104 inhibited fibrillization of α-synuclein and its PD-linked mutants *in vitro*[360]. Moreover, Hsp104 was capable of ATP-driven disassembly of oligomers and fibers composed of α-synuclein and its mutants. Disassembly was enhanced by the mammalian Hsp70 chaperone system. Co-expression of Hsp104 and α-synuclein A30P in transgenic rats reduced the formation of α-synuclein inclusions and prevented neurodegeneration [360]. Inhibition of Hsp90 leading to upregulation of Hsp70 resulted in inhibition of α-synuclein aggregation in the cell culture models and *in vivo* [361].

Using immunohistochemistry and confocal microscopy, it has been demonstrated that αB-crystallin, a small chaperone that binds to unfolded proteins and inhibits aggregation, is a prominent component of GCIs and LBs [362]. In cultured proteasome-inhibited C6 glioma cells, transfection with GFP-tagged α-synuclein resulted in ubiquitin- and αB-crystallin-positive aggregates, resembling GCIs in MSA brains [362]. These studies are supported by the detailed *in vitro* analysis, which revealed that αB-crystallin serves as a potent inhibitor of wild-type, A30P, and A53T α-synuclein fibrillation [320]. Overexpression of the chaperone Hsp70 prevented dopaminergic neuronal loss associated with α-synuclein in *Drosophila* and interference with its activity accelerated α-synuclein toxicity [113]. Overexpression of Hsp70 also prevented the formation of α-synuclein aggregates in transgenic mice and its fibrillation *in vitro*[363,364] Hsp70 inhibited α-synuclein aggregation by binding to the hydrophobic core of the protein and stabilizing its non-toxic disordered oligomers [365]. However, annular oligomers of α-synuclein were resistant to unfolding by chaperons and inhibited the Hsp70/Hsp40 system [366]. In addition, LBs in human postmortem tissue were shown to immunostain for Hsp70 and Hsp40. Similar results have been observed for inclusions in the brain tissues from patients with DLB, LBVAD, and NBIAI [113]. These data show that chaperons play an important role in the neuronal arsenal that mitigates α-synuclein toxicity [113].

6.2. β- and γ-synucleins

Conformational analysis revealed that α-, β-, and γ-synucleins are natively unfolded under physiological conditions *in vitro*, and are able to adopt comparable partially folded conformations at acidic pH or at high temperatures [367]. Although both α- and γ-synucleins were shown to form fibrils, β-synuclein did not fibrillate when incubated under the same conditions [367]. Curiously, the addition of either β- or γ-synuclein in a 1:1 molar ratio to α-synuclein solution substantially increased the duration of the lag-time and dramatically reduced the elongation rate of α-synuclein fibrillation [367]. Fibrillation was completely inhibited at a 4:1 molar excess of β- or γ-synuclein over α-synuclein [367]. β-Synuclein inhibited α-synuclein aggregation in animal models as well [368]. The formation of stable mixed tetramers with α-synuclein has been proposed as a mechanism of inhibition [369]. This suggests that β- and γ-synucleins may act as regulators of α-synuclein fibrillation *in vivo*, potentially acting as chaperones. As a result, one possible factor in the etiology of PD would be a decrease in the levels of β- or γ-synucleins [367].

7. Inhibition of α-synuclein aggregation by small molecules

7.1. Small molecules can alter IDP aggregation pathways

Many small molecules inhibit the aggregation of proteins or alter their aggregation pathway. The mechanisms of actions of these compounds usually involve non-covalent or covalent binding to the

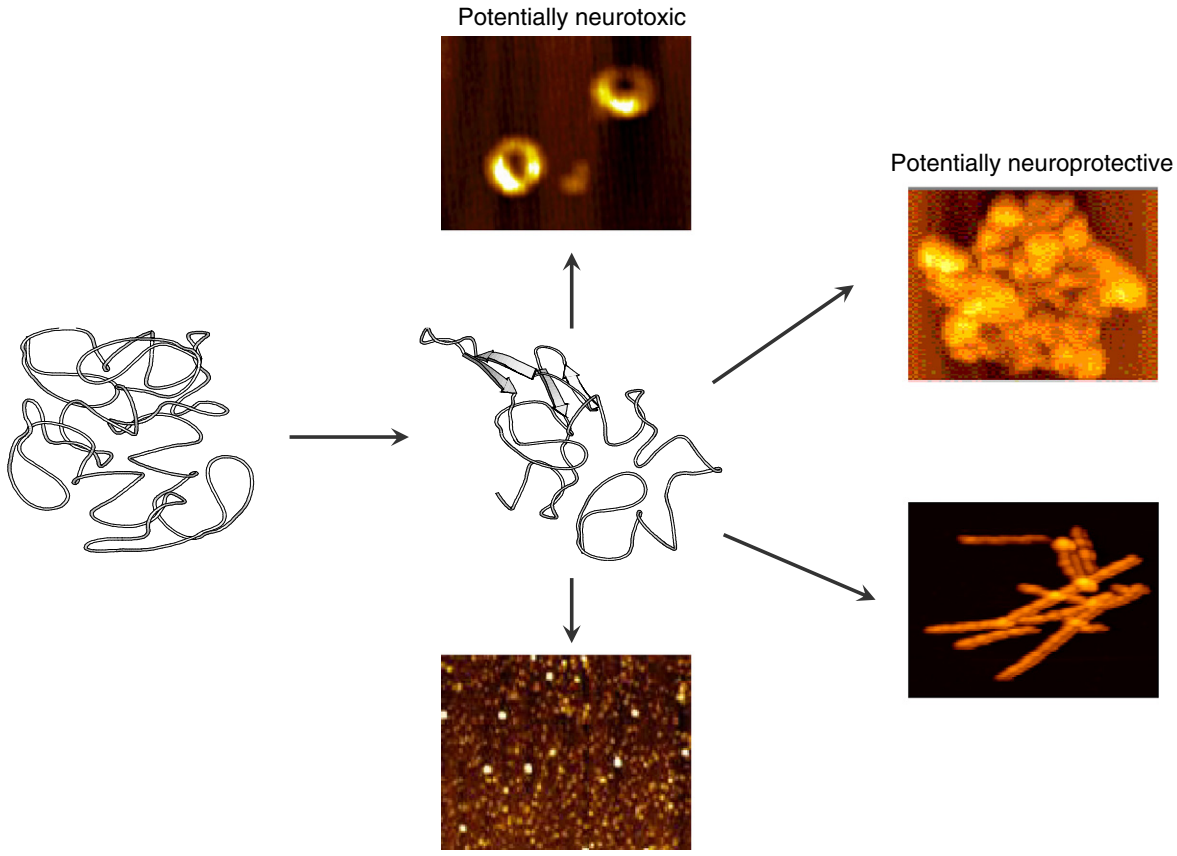

Fig. 6. Different aggregated forms achievable by the partially folded α-synuclein either under the different experimental conditions or during the fibrillation/aggregation process. Partially folded species together with soluble oligomeric forms (doughnuts and spheroids) are potentially neurotoxic, whereas insoluble aggregated forms (amorphous aggregates and fibrils) are potentially neuroprotective.

protein, leading to the change in relative stability of its monomers or aggregates. Usual structure-based drug development approaches are not applicable to IDPs due to their high conformational plasticity. One of the successful approaches has been to use small molecule binding to alter the conformation of IDP aggregates. Since toxicity and biological activity of both oligomers [82, 370,371] and fibrils [372]vary significantly with their structure, small molecules may be able to direct protein aggregation to non-toxic aggregates. There are many examples of this approach in literature. For example, Necula and coworkers [373,374] have shown that many known inhibitors of protein aggregation act by altering the aggregation pathway favoring the formation of either amyloid fibrils or a specific type of amyloid oligomers. This has been also shown for many individual small molecules such as methylene blue [373,374], polyphenols [371, 375–377], and many others.

7.2. Effects of small molecules on α-synuclein aggregation pathway

Several compounds have been shown to interfere with aggregation of α-synuclein. For example, rifampicin eliminated α-synuclein fibrillation *in vitro*. It was also able to disaggregate preformed α-synuclein fibrils in a concentration-dependent manner, and led to the formation of soluble oligomers composed of partially folded α-synuclein [378]. The flavonoid baicalein is the main component of the traditional Chinese herbal medicine *Scutellaria baicalensis*[379–383]. Micromolar concentrations of baicalein, or its oxidized forms, were shown to inhibit the formation of α-synuclein fibrils and disaggregate preformed fibrils, giving rise to non-toxic soluble oligomers both *in vitro* and *in vivo*[384,385]. Structural analysis of baicalein-stabilized oligomers revealed that their packing density is intermediate between that of pre-molten globules and typical globular proteins (according to the Kratky plot analysis of the SAXS data), and their secondary structure is relatively well-developed (according to the FTIR and CD analysis) [386]. These oligomers were characterized by high thermodynamic stability and were able to inhibit fibrillation of non-baicalein-treated α-synuclein. Similar oligomers or large amorphous aggregates were formed when α-synuclein or its fibrils were incubated with EGCG [387], curcumin [388–390], other polyphenols [391], an anti-PD drug selengiline [392] and many other compounds [393–395]. These compounds were also shown to alleviate the cytotoxicity of α-synuclein aggregates *in vivo* or in cell cultures. In addition, compounds found in cigarette smoke such as nicotine and hydroquinone had the same effect [396]. This data may be relevant to the results of epidemiological studies, showing that smoking and PD incidence are negatively correlated.

In general, we can see that inhibition of α-synuclein aggregation by small molecules usually occurs by redirection of the aggregation pathway of this protein toward large, non-toxic oligomeric aggregates. In addition to this approach, the drugs could be targeted directly to disordered α-synuclein monomer where their binding would promote structure formation and stabilize the monomeric state. While chemical chaperons that function in this fashion (for example, 4-phenyl butyrate) have been shown to alleviate toxicity of α-synuclein aggregates [397,398], it has not been shown that they do so by stabilizing the monomeric state of the protein. However, this method has been successfully applied to transcription factors and we will briefly discuss some examples below.

7.3. Stabilization of IDP monomers by small molecules

The c-Myc transcription factor is an attractive drug target since it is overexpressed in most human cancers and, under normal conditions, regulates large numbers of genes important in key cellular

processes such as growth, differentiation, metabolism, and apoptosis [399]. In order to be active, c-Myc has to heterodimerize with its partner, Max. The unbound forms of both Max and c-Myc are disordered and undergo mutual coupled binding and folding when their leucine zipper domains interact to form a helical coiled-coil [400,401]. One approach to c-Myc inhibition has been to disrupt the formation of this dimeric complex. In a search for effective inhibitors of the c-Myc–Max interactions, high throughput screening led to the discovery of several specific inhibitors [402,403]. These molecules were shown to bind to one of three discrete sites within the Max-binding domain of c-Myc, dissociate c-Myc–max dimers, and stabilize unfolded c-Myc monomers.

EWS-FLI1 acts as a transcription factor modulating a diverse group of target genes [404,405]. The C-terminal disordered region of EWS-FLI1 has been proposed to be responsible for the critical interaction of this TF with other transcriptional regulators [406]. A screen of a library of 3000 compounds revealed a small molecule that was able to bind to EWS-FLI1 and thereby to inhibit interaction of this protein with RNA helicase A [407], suggesting that the lead compound binds at an intrinsically disordered protein–protein interaction site.

These results show that it is indeed possible for small molecules to stabilize IDP in a monomeric form. While this approach has not yet been applied to aggregation-prone IDPs like α-synuclein, it may be successful for them as well.

8. Interaction of α-synuclein with membranes

α-Synuclein contains several class A2 lipid-binding helices, distinguished by clustered basic residues at the polar–apolar interface, positioned ±100° from the center of apolar face; a predominance of lysines relative to arginines among these basic residues; and several glutamate residues at the polar surface [408–410]. These structural features allow α-synuclein to bind to synthetic vesicles containing acidic phospholipids and to cellular membranes [410,411]. In presynaptic termini, monomeric α-synuclein exists in equilibrium between free and membrane- or vesicle-bound states [412]. The equilibrium is tightly regulated, and it has been estimated that approximately 15% of α-synuclein is membrane-bound within the synaptic termini [413].

The binding of α-synuclein to the membranes was shown to be accompanied by a dramatic increase in α-helix content [410,411]. α-Synuclein forms a variety of helical structures upon membrane binding, depending on the composition of the membrane. The binding of α-synuclein to SDS micelles led to the formation of two anti-parallel curved α-helices (Val3-Val37 and Lys45-Thr92) connected by a well-ordered, extended linker, whereas the acidic C-terminal region (Asp98-Ala140) remained unstructured [56]. Sarkosyl micelles promoted a similar structure of the protein, except the linker between two helices was unstructured [48]. However, binding to a lipid bilayer caused α-synuclein to primarily form a single curved α-helix encompassing residues 1–90 [414,415].

Using ^{2}H- and ^{31}P-NMR spectroscopy it has been shown that the association of α-synuclein with negatively charged membranes of multilamellar vesicles (MLVs) has a profound effect upon the integrity of bilayers containing anionic phospholipids, prompting the formation of nonbilayer or small vesicular structures [416]. EPR and fluorescence spectroscopy analysis revealed that the lipid packing in SUVs is also affected by interaction with α-synuclein [417]. In fact, it has been shown that protein binding led to increasing chain melting temperatures and to enhanced cooperativity of the phase transitions, which was attributed to defect healing in the curved vesicle membranes [417]. The binding of α-synuclein to the membranes also increased the membrane curvature and converted the large phospholipid vesicles into tubules and small vesicles [418,419]. Tubule formation was especially favored for membranes with low anionic lipid content [419]. It has also been found that monomeric α-synuclein bound to the lipid membrane can efficiently prevent lipid oxidation. The antioxidant function of α-synuclein was attributed to its facile oxidation *via* the formation of methionine sulfoxide (see above). These findings suggest that the inhibition of lipid oxidation by α-synuclein may be a physiological function of the protein [420].

The interaction of α-synuclein with the membranes alters the kinetics and pathways of its aggregation *in vitro*. The effects of membrane binding varied from inhibition to acceleration of α-synuclein aggregation [421–428]. Membrane-induced formation of both β-sheet rich [425, 429] and α-helical oligomers [430,431] has been observed. A detailed study of α-synuclein aggregation in the presence of phospholipid membranes found that the structures and heterogeneity of the aggregates strongly depended on the experimental conditions [432].

9. PD-associated mutants of α-synuclein

Finding that a small fraction of PD patients have a familial form of parkinsonism with an autosomal-dominant pattern of inheritance raised considerable interest in understanding the role of genetic factors in the etiology of PD [433]. Several families have been identified whose incidence of PD is significantly greater than in age-matched controls [434,435]. Analysis of point mutations present in these families showed three mutations in α-synuclein gene: A53T, A30P, and E46K [3, 5,6]. In families with the A53T mutation, 85% of patients who expressed the mutant gene had clinical features of PD [3]. Furthermore, the triplication of the wild-type gene has been reported in a large family from Iowa [7–9]. Mutations in α-synuclein were shown to produce a fulminant disease that includes Parkinsonism, but is much more widespread and may resemble DLB [107]. Furthermore, the disorder was shown to also be more progressive, tending to have an earlier onset than sporadic PD [107]. These findings strongly indicate that a single mutation in the human α-synuclein gene is sufficient to account for the PD phenotype.

Detailed analysis using a combination of low resolution techniques, such as CD, FTIR, fluorescence, and several hydrodynamic approaches [87, 100–103, 379, 436–439] revealed that the PD-related point mutations A30P, E46K, and A53T do not affect the global structure of human α-synuclein monomer [100,101, 439]. However, NMR spectroscopy revealed that the A30P mutation strongly attenuated the helical propensity found in the N-terminal region of wild type α-synuclein [52]. The A53T mutation was found to exert a more modest influence on local structural propensity, resulting in a slightly enhanced preference for extended conformations in a small region around the site of mutation [52]. The E46K mutation resulted in subtle changes in the conformation of the monomeric protein [440] and enhanced the contacts between N- and C-termini of the protein [441]. These mutations were also proposed to modify long-range transient structure in α-synuclein [47], although this conclusion remains controversial [442]. All three PD-related point mutations were shown to accelerate α-synuclein aggregation *in vitro*[87, 100–103, 437–439, 443]. A30P mutation promoted α-synuclein oligomer formation, while A53T and E46K mutations promoted fibrillation. Overall, it is clear that all three PD-related mutations of α-synuclein alter its secondary structure and promote its aggregation.

10. Conclusions

The molecular basis of PD (and other synucleinopathies) appears to be tightly coupled to the aggregation of α-synuclein. The focus of studies on the molecular mechanisms of PD pathology became strongly α-synuclein-centric due to the two important discoveries made in 1997, the demonstration that a specific mutation in the α-synuclein gene is related to familial cases of early-onset PD [3], and the demonstration that α-synuclein is highly abundant in LBs [4]. Much has been learned about α-synuclein structure, function, and aggregation properties after these discoveries.

Accumulated data show that α-synuclein is an intrinsically disordered protein that can adopt a number of different conformational

and aggregation states depending on the conditions and cofactors. The intrinsically disordered nature of α-synuclein, its chameleon behavior, and conformational plasticity, together with the breadth of functions and the wide spectrum of interactions ascribed to this protein, are believed to be central to its pathogenicity. In a model depicting α-synuclein aggregation, this protein prior to aggregation exists as a mixture of the natively unfolded (non-sticky) form and a set of partially folded conformations (many of which are sticky). Under normal, non-pathological conditions, this equilibrium is essentially shifted toward the non-sticky unfolded conformation. However, the equilibrium is extremely unstable and can easily be shifted toward the amyloidogenic conformation(s) by subtle changes in the protein environment. This partial folding might lead to α-synuclein self-association, which is facilitated by the formation of solvent-exposed hydrophobic clusters on the surface of a partially folded protein.

Among the factors known to shift conformational equilibrium and promote α-synuclein oligomerization and aggregation *in vitro* are: an introduction of PD-related mutations; an increase in concentration; a decrease in pH; an increase in temperature; an addition of amphipathic molecules (e. g. various agrochemicals, such as herbicides or pesticides); an addition of metal ions and other small charged molecules; interaction with charged biopolymers; interaction with other proteins; interaction with membrane; and immersion of a protein into a crowded environment. Different factors may stabilize different partially folded conformations and therefore produce different aggregation states of α-synuclein, such as various soluble oligomers, amorphous aggregates, or amyloid-like fibrils.

There are three major molecular mechanisms proposed to explain the neurotoxicity of α-synuclein and its aggregates — the mechanical distortion of cellular compartments/processes, toxic gain of function, and toxic loss of function. Any of the aggregated species could potentially be neurotoxic. However, the largest cytotoxicity is believed to be expressed by soluble oligomers. The important notion is that although PD and other synucleinopathies are characterized by the accumulation of α-synuclein-containing deposits, these neurodegenerative maladies are multifactorial diseases whose pathogeneses cannot be, and should not be, explained solely by α-synuclein aggregation, and various other factors have to be taken into account. In fact, the pathogenesis of synucleinopathies is very complex in nature, and these maladies are known to be initiated by numerous factors, including genetic predisposition, toxic insults, failure of the chaperone system, proteasomal misfunction, rare early-onset cases of the autosomal origin, oxidative damage, etc. [99].

This multifactorial nature of PD and other synucleinopathies, and a limited understanding of the key molecular events provoking neurodegeneration, are among the major reasons determining the lack of drugs for the successful inhibition and cure of these diseases [444]. Another factor is the lack of the precise knowledge of the nature of the neurotoxic species that accumulate during α-synuclein misfolding and aggregation, and eventually lead to cell death. As a result, the current arsenal of anti-Parkinsonian drugs is not able to halt or retard neuron degeneration, and all drugs developed so far treat disease symptoms.

However, even though synucleinopathies are multifactorial disorders, the detailed analysis of the α-synuclein function, misfunction, and aggregation could provide an important basis for the future development of some effective therapeutic protocols. In fact, since many of the disease-promoting factors are directly or indirectly related to α-synuclein misfolding or abnormal processing, functioning, or aggregation, the identification of small molecules that would be able to inhibit α-synuclein deposition or reverse fibril/oligomer formation may represent a critical first step toward a better understanding of the pathophysiology of proteinaceous inclusion formation in this set of human diseases.

Obviously, great caution should be taken in developing and examining drugs that inhibit α-synuclein fibrillation or promote the disaggregation of preformed fibrils. In fact, since some small α-synuclein oligomers are extremely neurotoxic, halting the fibrillation process at early stages can promote the formation of such small toxic oligomers, and can therefore do more harm than good. Similarly, enhanced neurodegeneration can be promoted by small molecules that are able to disaggregate fibrils to smaller oligomers or soluble aggregates.

There are several potential solutions for these problems. One of them is a search for small molecules stabilizing the intrinsically disordered conformation of α-synuclein or completely blocking its aggregation, or resulting in the complete disaggregation of the preformed aggregates down to the monomeric state. Alternatively, one can search for chemical compounds that can either clear toxic misfolded proteins or protect neurons from their impact. Finally, a very promising approach relies on compounds that promote protein aggregation, accelerate formation of large inclusions, and eliminate the toxic effects of misfolded protein conformations and small oligomers [394,395]. This approach is based on the observation that large proteinaceous inclusions might play a neuroprotective role, and therefore the stimulation of fast formation of large protein aggregates could be used as a therapeutic means for the affected neurons [395].

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