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Review

Dynamic structural flexibility of α-synuclein

Danielle E. Mor [a,1], Scott E. Ugras [b,1], Malcolm J. Daniels [c,1], Harry Ischiropoulos [a,b,c,d,e,f,*]

[a] Biomedical graduate studies in Neuroscience, Raymond and Ruth Perelman School of Medicine at the University of Pennsylvania, PA 19104, United States
[b] Biomedical graduate studies in Biochemistry and Molecular Biophysics, Raymond and Ruth Perelman School of Medicine at the University of Pennsylvania, PA 19104, United States
[c] Biomedical graduate studies in Pharmacology, Raymond and Ruth Perelman School of Medicine at the University of Pennsylvania, PA 19104, United States
[d] Children's Hospital of Philadelphia Research Institute, PA 19104, United States
[e] Department of Pediatrics, Raymond, and Ruth Perelman School of Medicine at the University of Pennsylvania, PA 19104, United States
[f] Department of Systems Pharmacology and Translational Therapeutics, Raymond, and Ruth Perelman School of Medicine at the University of Pennsylvania, PA 19104, United States

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ABSTRACT

α-Synuclein is a conserved, abundantly expressed protein that is partially localized in pre-synaptic terminals in the central nervous system. The precise biological function(s) and structure of α-synuclein are under investigation. Recently, the native conformation and the presence of naturally occurring multimeric assemblies have come under debate. These are important deliberations because α-synuclein assembles into highly organized amyloid-like fibrils and non-amyloid amorphous aggregates that constitute the neuronal inclusions in Parkinson's disease and related disorders. Therefore understanding the nature of the native and pathological conformations is pivotal from the standpoint of therapeutic interventions that could maintain α-synuclein in its physiological state. In this review, we will discuss the existing evidence that define the physiological states of α-synuclein and highlight how the inherent structural flexibility of this protein may be important in health and disease.

Contents

1. Introduction

α-Synuclein is a soluble protein that is highly conserved in vertebrates and abundantly expressed in nervous tissue (Jakes et al., 1994). It was first discovered in 1988 in association with purified synaptic vesicles from the *Torpedo* electric ray (Maroteaux et al., 1988). Soon afterward α-synuclein was found to be widely distributed across the mammalian brain and localized to presynaptic nerve terminals, suggesting functions related to neurotransmission (Iwai et al., 1995). Independent of these reports, α-synuclein was identified as the precursor

* Corresponding author at: Children's Hospital of Philadelphia Research Institute and Departments of Pediatrics and Systems Pharmacology and Translational Therapeutics, the Raymond and Ruth Perelman School of Medicine at the University of Pennsylvania, PA 19104, United States.
E-mail address: ischirop@mail.med.upenn.edu (H. Ischiropoulos).
[1] Authors contributed equally.
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to a hydrophobic peptide found in Alzheimer's disease senile plaques, termed the non-Aβ component of Alzheimer's disease amyloid (NAC) (Uéda et al., 1993). The α-synuclein gene was also dynamically regulated during song learning in zebra finch, supporting a role in synaptic plasticity (George et al., 1995).

The discovery of a mutation in the α-synuclein gene that was associated with autosomal dominant inheritance of Parkinson's disease (PD) provided the impetus for a major shift in α-synuclein research (Polymeropoulos et al., 1997). PD is a neurodegenerative disorder primarily characterized by the loss of dopamine-producing neurons in the substantia nigra pars compacta resulting in motor impairment. Since the original publication of the A53T mutation, several mutations, as well as multiplications of the α-synuclein gene have been linked to PD (Chartier-Harlin et al., 2004; Krüger et al., 1998; Lesage et al., 2013; Pasanen et al., 2014; Proukakis et al., 2013; Singleton et al., 2003; Zarranz et al., 2004; Ferese et al., 2015) Furthermore, several antibodies against α-synuclein robustly detect the well-known pathoanatomical features of PD, Lewy bodies and Lewy neurites, in postmortem brain tissue from patients with sporadic PD as well as other related neurodegenerative disorders (Baba et al., 1998; Spillantini et al., 1997; Takeda et al., 1998). The finding that wildtype α-synuclein was detected in Lewy bodies and Lewy neurites prompted the publication of numerous studies that investigated the biochemistry and biology of α-synuclein. Despite the rather impressive body of work several fundamental questions remain: What is the physiological function of α-synuclein? What is the structure of native α-synuclein? What factors contribute to the induction of aggregation-competent conformational states of α-synuclein? In this review, we will briefly review the evidence for the different biological functions and discuss ongoing efforts to precisely define physiological structures of α-synuclein.

2. The physiological function(s) of α-synuclein

The initial studies indicated that α-synuclein is not required for neuronal development or synapse formation, but instead may modulate synaptic activity. In rodents, α-synuclein is detected close to the time of birth and continues to increase until one month of age, when it reaches a steady-state level that is maintained throughout adulthood (Shibayama-Imazu et al., 1993). Similarly, in cultured rat neurons the development of synapses precedes α-synuclein expression and translocation to axonal terminals (Murphy et al., 2000; Withers et al., 1997). The hypothesis that α-synuclein regulates synaptic activity was directly tested in mice lacking α-synuclein. α-Synuclein null mice develop normal brain architecture and synaptic contacts, and do not exhibit gross behavioral phenotypes (Abeliovich et al., 2000). However, subtle abnormalities in activity-dependent neurotransmitter release have been observed. Upon repeated stimulation, dopaminergic synapses from α-synuclein null mice sustain highly elevated dopamine release (Abeliovich et al., 2000; Yavich et al., 2004). Functional redundancy among α-synuclein and the other synuclein family members, β- and γ-synuclein, may account for the mild phenotypes observed in the single knockout. In α/β-synuclein double knockout mice, synaptic plasticity appears unaltered relative to α-synuclein single knockouts, although dopamine levels in the striatum are reduced (Chandra et al., 2004). The importance of synucleins is particularly highlighted by α/β/γ-synuclein triple knockouts, which have decreased life span and late-onset synaptic dysfunction compared with wildtype mice (Burré et al., 2010; Greten-Harrison et al., 2010). Triple knockouts in another study had motor deficits and decreased striatal dopamine, along with abnormal dopamine neurotransmission (Anwar et al., 2011). Collectively, these reports emphasize the important role of the synucleins in long-term synaptic maintenance and plasticity.

2.1. Synaptic vesicle trafficking

Examination of the role of α-synuclein in the synaptic vesicle cycle has yielded conflicting results. Depletion of α-synuclein from rodent hippocampal neurons both in vivo and in vitro induces a significant loss of undocked synaptic vesicles, suggesting that α-synuclein acts to replenish or maintain the resting and/or reserve vesicle pools (Cabin et al., 2002; Murphy et al., 2000). In contrast, another study found that increasing α-synuclein in rodent hippocampal neurons reduces the recycling pool of vesicles (Nemani et al., 2010). The effect of α-synuclein on vesicles docked at the plasma membrane prior to exocytosis is similarly unclear. Knockout or knockdown of α-synuclein in rodent hippocampal neurons results in either a decrease or no change in the number of docked vesicles (Cabin et al., 2002; Murphy et al., 2000). Conversely α-synuclein expression in PC12 cells causes an accumulation of vesicles at the plasma membrane and impairment of exocytosis (Larsen et al., 2006). However, in mice modestly overexpressing α-synuclein (levels are not associated with neurotoxicity), hippocampal synapses display a redistribution of vesicles away from the active zone. The density of vesicles in synaptic boutons is also reduced, consistent with α-synuclein-mediated inhibition of vesicle clustering. This is supported by α-synuclein-induced defects in vesicle re-clustering following endocytosis in rat hippocampal neurons (Nemani et al., 2010). Still, opposing results have been obtained from yeast, in which α-synuclein expression results in massive accumulations of vesicles that co-localize with Rab GTPases (Gitler et al., 2008; Soper et al., 2008). Likewise, α-synuclein has been shown to restrict vesicle diffusion away from synapses in mouse hippocampal neurons (Wang et al., 2014). Several lines of evidence, therefore, support the participation of α-synuclein in synaptic vesicle trafficking, though the specific steps for which it may be most important, i.e. vesicle docking, recycling and/or re-clustering, remain unclear.

2.2. Chaperone-like activity and neurotransmitter release

α-Synuclein and the other synuclein family members may act as molecular chaperones, facilitating neurotransmitter release. Cysteine-string protein α (CSPα) is a chaperone that is essential for synaptic health; its deletion in mice leads to a decrease in SNARE protein complexes, nerve terminal degeneration, motor impairment and death. When expressed in CSPα-deficient mice, α-synuclein is able to rescue this degenerative phenotype and restore levels of SNARE complexes in synaptic terminals. Moreover, mice lacking both α-synuclein and CSPα exhibit an exacerbated phenotypic decline (Chandra et al., 2005). These findings suggest that α-synuclein is able to complement the activity of CSPα in promoting synapse integrity. Direct evidence for the interaction of α-synuclein with SNARE complexes was documented by co-immunoprecipitation of α-synuclein with SNARE proteins and specific binding to the vesicle-associated SNARE protein synaptobrevin-2. In mammalian cells and purified in vitro systems, α-synuclein dose-dependently facilitates SNARE complex assembly (Burré et al., 2010). Additional support for chaperone-like activity includes sequence homology between α-synuclein and 14-3-3 protein chaperones as well as the association of α-synuclein with 14-3-3 and its binding partners in rat brain (Ostrerova et al., 1999). α-, β-, and γ-synucleins are also able to prevent the aggregation of denatured proteins in vitro (Souza et al., 2000a), further supporting a conserved chaperone-like function of synucleins and the existence of several protein–protein interactions that facilitate synaptic function.

2.3. Putative role in neurotransmitter synthesis and reuptake

Published evidence indicates that α-synuclein-mediated protein-protein interactions may modulate dopamine synthesis and recycling. α-Synuclein may inhibit the activity of tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis. α-Synuclein and TH co-immunoprecipitate from rat striatal tissue and MN9D dopaminergic cells and α-synuclein was shown to inhibit TH activity in MN9D and PC12 cells, potentially through PP2A phosphatase-mediated reduction of serine 40 phosphorylation of TH (Peng et al., 2005; Perez et al.,

2002). α-Synuclein may also interact with and inhibit the activity of aromatic amino acid decarboxylase, which catalyzes the conversion of L-DOPA to dopamine (Tehranian et al., 2006). Thus, α-synuclein may serve as a negative regulator of dopamine synthesis, though further validation of these findings is necessary. Several reports have also implicated α-synuclein in the regulation of the dopamine transporter (DAT), though the evidence is conflicting with regards to the functional consequences. Direct binding of α-synuclein to DAT has been demonstrated in multiple studies. However, α-synuclein does not appear to alter DAT function, but rather in various cellular contexts can promote or inhibit DAT trafficking to the plasma membrane (Oaks and Sidhu, 2011). Elucidating the relationship between α-synuclein and DAT requires further investigation.

3. α-Synuclein structural flexibility

3.1. Primary sequence

The primary sequence of α-synuclein consists of 140 amino acids with a predicted molecular mass of 14,460.16 Da and an isoelectric point of 4.67 (Fig. 1). The sequence of α-synuclein is composed of three functionally defined domains. The N-terminal region (amino acids 1–60) is characterized by the presence of unique and highly conserved sequence of imperfect tandem repeats with a central consensus motif of K(A)–T(A,V)–K(V)–E(Q,T)–G(Q)–V(A). These motifs spanning residues 10–86 are projected to form two amphipathic α-helices and are characteristic of several proteins such as apolipoproteins that bind reversibly to membranes (George et al., 1995; Maroteaux et al., 1988). Indeed the structure of membrane bound α-synuclein contains two α-helices (amino acids 3–37 and 45–92) in a roughly antiparallel arrangement with a short linking region (Ulmer et al., 2005). These helices are stabilized by interaction with a variety of phospholipid bilayers, though α-synuclein interacts preferentially with membranes of high curvature and an abundance of acidic phospholipids, properties consistent with those of synaptic vesicles (Davidson et al., 1998; Zhu et al., 2003). Upon interaction with membranes of low curvature α-synuclein adopts a distinct secondary structure characterized by a single extended helix that includes both previously described helical domains and the linker region (amino acids 38–44) (Ferreon et al., 2009; Georgieva et al., 2010; Trexler and Rhoades, 2009). All known mutations associated with familial PD (A30P, E46K, H50Q, G51D, A53E, and A53T) are found in the N-terminal domain (Krüger et al., 2008; Lesage et al., 2013; Pasanen et al., 2014; Polymeropoulos et al., 1997; Proukakis et al., 2013; Zarranz et al., 2004). These mutations, with the exception of G51D, A53E, and A30P, increase the propensity of α-synuclein to form insoluble aggregates and produce morphologically distinct aggregate species (Ghosh et al., 2014; Giasson et al., 1999; Greenbaum et al., 2005; Lesage et al., 2013; Mahul-Mellier et al., 2015; Narhi et al., 1999). Though the precise mechanism by which these mutations promote aggregation has not been conclusively shown, evidence implicate an accelerated formation of oligomers (Conway et al., 2000) likely due to the destabilization of the native N-terminal conformation (Bertoncini et al., 2005a; Burré et al., 2015; Coskuner and Wise-Scira, 2013; Dettmer et al., 2015).

Amino acids 61–95 compose the hydrophobic NAC domain (Uéda et al., 1993). This region contains a sequence of amino acids (71–82) necessary and sufficient for α-synuclein self-assembly into amyloid fibrils (Giasson et al., 2001). Recently the crystal structures of residues 68–78 (termed NACore), and residues 47–56 (PreNAC) were resolved by the use of micro-electron diffraction, revealing that strands in this region stack in-register into β-sheets that are typical of amyloid assemblies (Rodriguez et al., 2015).

The C-terminal domain (96–140) is rich in negatively charged amino acids (contains 10 glutamate and 5 aspartate residues) and was originally proposed to be essential for maintaining the solubility of the protein. The presence of 5 proline residues, which are known to induce turns and disrupt secondary protein structure, suggested that this region is devoid of secondary structure (George et al., 1995; Ulmer et al., 2005). However, the C-terminus was shown to form transient, long-range interactions with the N-terminus resulting in the formation of multiple compact monomeric structures (Bertoncini et al., 2005a; Dedmon et al., 2005). These compacted structures of α-synuclein are temperature sensitive and are resistant to aggregation. The data also indicated that at elevated temperatures the C-terminus assumes an extended conformation that liberates N-terminal associations and enables aggregation (Bertoncini et al., 2005b; Dedmon et al., 2005). Moreover, C-terminally truncated forms of α-synuclein aggregate faster than full length protein (Hoyer et al., 2004; Li et al., 2005). Truncated α-synuclein has been detected in the brains of both control (non-disease) and PD patients. Cleavage of full-length protein at residues D115, D119, N122, D125 and Y133 was documented in α-synuclein extracted from LBs (Anderson et al., 2006).

The C-terminus appears to be important for the interaction of α-synuclein with other proteins and for the interaction with small molecules (Burré et al., 2012; Burré et al., 2010; Conway et al., 2001; Mazzulli et al., 2006; Souza et al., 2000b; Woods et al., 2007). Additionally, it contains the major sites of metal binding and post-translational modifications. Binding of iron, copper, and other metals has been shown to influence α-synuclein function and aggregation (Uversky et al., 2001a). Addition of Fe(III), but not Fe(II) to preformed oligomers of α-synuclein accelerates aggregation, raising the question of metal binding at different points during the aggregation process (Kostka et al., 2008). Cu(II) is unique among metals at accelerating aggregation of α-synuclein at physiologically relevant concentrations. The sole histidine residue H50 in α-synuclein was found to be critical for Cu(II) binding (Rasia et al., 2005) whereas other divalent metal ions, including Mn(II), Co(II), Ni(II) and Fe(II), preferentially bind to the C-terminus of α-synuclein at residues D121, N122, and E123 (Binolfi et al., 2006).

3.2. Post-translational modifications

α-Synuclein undergoes a number of post-translational modifications, including N-terminal acetylation, serine and tyrosine phosphorylation, lysine ubiquitination and tyrosine nitration (Oueslati et al., 2010; Barrett and Greenamyre, 2015). α-Synuclein purified under mild

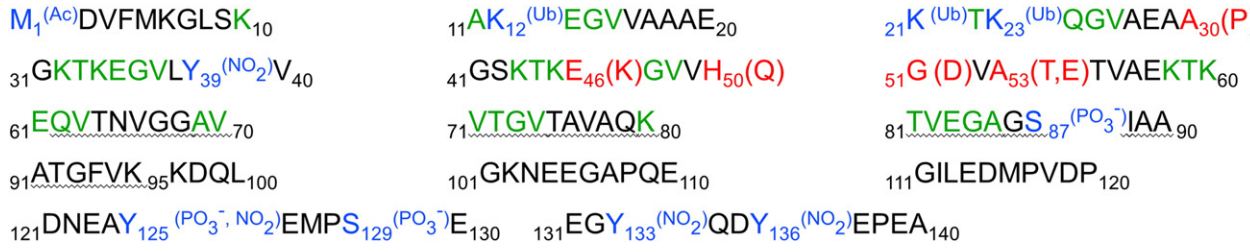

Fig. 1. Primary sequence of human α-synuclein. Green color indicates the imperfect tandem repeats. Known mutations are indicated in red. The hydrophobic NAC domain is underlined. The major sites of posttranslational modifications identified in vivo are highlighted in blue (Ac, acetylation; Ub, ubiquitination; NO_2, nitration; and PO_3^- phosphorylation).

conditions is acetylated in the N-terminus. The N-terminal acetylation may account for the formation of an oligomeric form of the protein with partial α-helical structure (Trexler and Rhoades, 2012). However, semisynthetic production of N-terminally acetylated α-synuclein demonstrated that modified and unmodified versions of the protein share similar secondary structure, aggregation propensities, and membrane binding (Fauvet et al., 2012). NMR studies indicated that the first 12 residues undergo a chemical shift due to N-terminal acetylation. This modification also appears to stabilize the helicity of the N-terminus within the context of the full-length protein, and increases the affinity of α-synuclein for lipids (Dikiy and Eliezer, 2014).

Mass spectrometry-based methodologies revealed that α-synuclein extracted from human Lewy bodies was phosphorylated at S129 (Fujiwara et al., 2002). An antibody raised against phosphorylated S129 was then used to show that α-synuclein was phosphorylated at this site only in subjects with disease and that S129 phosphorylated α-synuclein was present only in the Triton-X- and Sarkosyl-insoluble, urea soluble fraction. These data indicated that some form(s) of aggregated α-synuclein and not the soluble protein is targeted for phosphorylation at S129. Indeed in vitro data showed that purified fibrils of α-synuclein are substrates for casein kinase 1 or 2 (Waxman and Giasson, 2008). Other data indicated that polo-like kinase (PLK) 2-mediated phosphorylation of S129 increased autophagy-mediated degradation of α-synuclein, suggesting that phosphorylation may be a neuroprotective mechanism to accelerate clearance of aggregated protein (Oueslati et al., 2013). In addition to the monomeric α-synuclein, S129 phosphorylated bands with apparent molecular weight of 22 kDa and 29 kDa were observed in the detergent insoluble extract (Hasegawa et al., 2002). These bands were also immunoreactive with anti-ubiquitin antibodies suggesting that S129 phosphorylated α-synuclein is also targeted for mono- and di-ubiquitination. It has long been established that the core of Lewy bodies stains positive for both α-synuclein and ubiquitin whereas the surrounding halo is immunoreactive for α-synuclein (Hasegawa et al., 2002). Of the 15 lysine residues in α-synuclein, the major sites of LB-derived α-synuclein undergoing ubiquitination were residues K12, K21, and K23 (Anderson et al., 2006; Hasegawa et al., 2002; Sampathu et al., 2003).

A number of spectroscopic methodologies (CD and NMR) were employed to explore the effect of S129 phosphorylation on the structure of α-synuclein. CD data revealed that phosphorylation of S129 did not affect secondary structure, such that both non-phosphorylated and phosphorylated S129 exhibited random coil structure (Paleologou et al., 2008). NMR data revealed a number of chemical shifts that occur due to phosphorylation. While the residues surrounding S129 exhibited the greatest perturbation, residues 1–90 also exhibited detectable chemical shifts (Paleologou et al., 2008) This likely reflects the previously documented long-range interactions of the C- and N-termini. The potential effects of phosphorylation of S129 on the structure of the protein were not faithfully reproduced by mutation of S129 to either E or D, two common phosphomimics used to study the structural consequences of phosphorylation. For example, phosphorylation at S129 increased the hydrodynamic radius of the protein, whereas S129 E/D mutants did not (Paleologou et al., 2008).

Subsequent studies found additional sites of phosphorylation. Elevated levels of phosphorylated α-synuclein at residue S87 were detected in human brains with Alzheimer's disease, Lewy Body disorders, and multiple system atrophy (Paleologou et al., 2010). S87 phosphorylation alters the biophysical properties of α-synuclein, including inhibition of fibril formation and reduction in membrane binding (Paleologou et al., 2010). Additionally, phosphorylated α-synuclein at residue Y125 was detected in Drosophila expressing human wildtype α-synuclein as well as in human brains, though levels were decreased in disease compared with aged-matched healthy controls (Chen et al., 2009).

The proximity of the α-synuclein phosphorylation sites to the metal binding sites raised the question of how phosphorylation may affect metal ion interactions. This was investigated by the use of C-terminal peptides containing residues 119–132 that were either unmodified, phosphorylated at Y125 or at S129 (Liu and Franz, 2005). By exploiting the luminescence properties of Tb^{3+}, it was found that phosphorylated Y125 showed enhanced Tb^{3+} binding relative to wildtype or phosphorylated S129. Additionally, phosphorylated Y125 preferentially bound to trivalent rather than divalent metal ions. To investigate this further, longer C-terminal fragments comprised of residues 107–140 that were either unmodified of monophosphorylated at Y125 or S129 were tested for their affinity to various metal ions. By using a fluorescence quenching assay, the dissociation constants of the metal ion complexes and the α-synuclein peptides were determined. These data indicate that either phosphorylation at Y125 or S129 increases the binding affinity for Cu (II) and Fe(II), but not Fe(III). Furthermore, phosphorylated Y125 has a greater affinity for Pb(II) than wildtype, but phosphorylated S129 has an even greater affinity than phosphorylated Y125. Additionally, tandem MS indicated that phosphorylation causes the metal ion binding sites to shift towards the C-terminal end of α-synuclein (Lu et al., 2011).

α-Synuclein within Lewy bodies is nitrated on all four tyrosine residues (Giasson et al., 2000). Chemical nitration of α-synuclein results in the formation of both tyrosine nitrated monomers and nitrated dimers (Souza et al., 2000b). Immunoelectron microscopy confirmed that nitrated monomers and dimers are incorporated into amyloid fibrils. Purified nitrated α-synuclein monomer by itself was unable to form fibrils, whereas the nitrated dimer accelerated aggregation of unmodified α-synuclein (Hodara et al., 2004). Additionally, nitration at residue Y39 in the N-terminus decreased binding to synthetic vesicles and prevented the protein from adopting α-helical conformation (Hodara et al., 2004). These observations were recently confirmed and elegantly expanded by the generation of site-specifically nitrated α-synuclein using protein semisynthetic chemistries (Burai et al., 2015). Using the synthetic nitrated α-synuclein the data showed that nitration did not interfere with phosphorylation of S129 by PLK3 and reaffirmed that intermolecular interactions between the N- and C-terminal regions of α-synuclein are critical in directing nitration-induced oligomerization of α-synuclein (Burai et al., 2015).

3.3. Native conformation(s) of α-synuclein

Fig. 2 depicts the rapid growth in the number of publications identified in PubMed using the term synuclein and highlights key studies that explored the native structure and conformation of the protein. Early biochemical studies of α-synuclein isolated from bacterial expression systems or α-synuclein expressed in rodent tissues indicated that it is monomeric with limited secondary structure. Electrophoretic separation of α-synuclein purified without heating on 6, 10, or 14% acrylamide gels estimated an apparent molecular weight of 20 ± 3 kDa. However, the values of sedimentation coefficient ($S_{20,w} = 1.7S$), stokes radius (34 Å), analysis on native gels and derivation of the frictional coefficient ($f/f_o = 2.09$) indicated an apparent molecular weight in the range 57–58 kDa (Weinreb et al., 1996). To reconcile this apparently anomalous behavior it was proposed that monomeric α-synuclein achieves minimal structure in simple solutions and this rather extended unstructured conformation resembles a globular protein with a larger apparent molecular weight. This assumption was further corroborated by examination of purified monomeric α-synuclein by CD, FTIR and small angle X-ray scattering, which failed to identify significant secondary structural features. Furthermore, minimal shifts in the spectroscopic features of α-synuclein were observed when the protein was placed in solutions that would increase hydrophobicity and neutralize negative charges indicating that the protein is natively unstructured, joining a growing group of proteins sharing similar biochemical and biophysical characteristics (Uversky et al., 2001b). NMR and CD data, however, indicated that α-synuclein assumes increasingly folded secondary structure when exposed to conditions that promote aggregation (low pH and high temperature) or upon interaction with phospholipids. Collectively these data indicated that

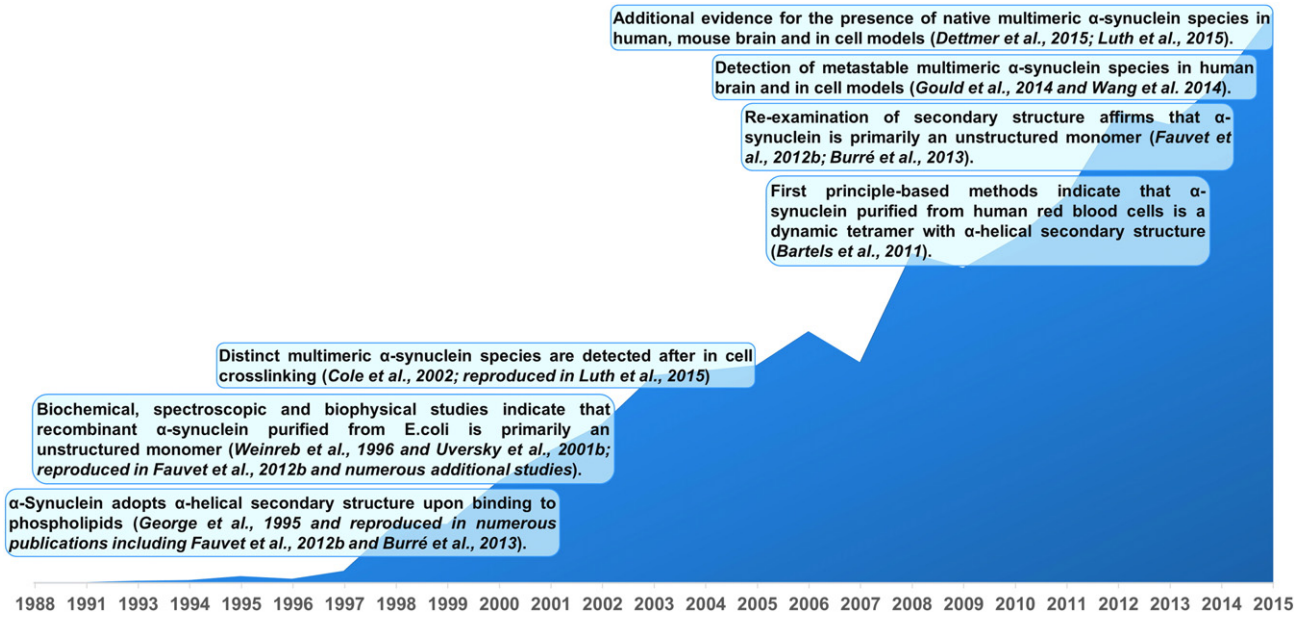

Fig. 2. The graph depicts the number of publications retrieved from PubMed using the search term "alpha synuclein" from a single publication in 1998 to 862 in 2015. Significant milestones that examined the native structure and conformations of α-synuclein are displayed.

native α-synuclein is primarily an unstructured monomer, which can assume different compact conformations that resist aggregation, adopts α-helical conformation upon binding to lipids and undergoes conformational changes prior to oligomerization and formation of amyloid fibrils (Uversky et al., 2001b). However, the methodologies employed to quantify the molecular weight of α-synuclein in these elegant studies were not based on first principles and therefore a lingering uncertainty remains regarding the native size of the protein. Moreover, crosslinking experiments in both intact cells expressing α-synuclein and lipid-free lysates revealed the stabilization of high molecular weight α-synuclein multimers (consistent with dimers, trimers, and larger multimers). These multimers were not reduced by dilution of lysates before crosslinking, nor by reducing the concentration of crosslinker from 1 mM to 8 μM, suggesting that they represented endogenous protein complexes (Cole et al., 2002).

Examination of the α-synuclein native state was reignited in 2011 with the publication of results indicating that α-synuclein exists natively as a tetramer, rather than a monomer. Methodologies that are based on first principles were employed to examine the molecular weight and size of α-synuclein extracted under non-denaturing conditions from human red blood cells. Analytical ultracentrifugation produced a sedimentation equilibrium value of 4.78 S, indicating a molecular weight of 57.8 kDa. Analysis of particle geometry by scanning transmission electron microscopy revealed the presence of roughly spherical molecules with a diameter of approximately 3.0–3.5 nm. Automated sampling of 1000 α-synuclein particles showed a distribution of molecular weights between 10 and 175 kDa with a peak distribution at 55 kDa. These findings constitute the most direct measurements of the native molecular weight of α-synuclein. The tetrameric species were shown to have α-helical conformation and were resistant to aggregation (Bartels et al., 2011).

Complimentary observations were made using recombinant GST-tagged α-synuclein purified from bacterial expression systems under non-denaturing conditions. Single-particle electron microscopy of purified α-synuclein revealed complexes of sizes and internal geometries consistent with trimers and dimers, which were corroborated by measurements of the hydrodynamic radii and elution on native state PAGE. As observed previously, these species were more resistant to aggregation than denatured monomer. CD also showed that several α-synuclein mutations associated with early onset PD (A30P, E46K, A53T) exist in less ordered conformations than wildtype α-synuclein. These mutants were also more prone to aggregation (Wang et al., 2011). However, using the same α-synuclein construct that contains a 10-residue N-terminal extension, which forms multimers when isolated from *Escherichia coli*, NMR studies indicated that only a small fraction of α-synuclein assembles into α-helical trimers and tetramers and the majority remains as a disordered monomer (Gurry et al., 2013). These data indicated that several potential conformers of α-synuclein may exist in equilibrium. The observation that α-helical trimers and tetramers constitute only a small fraction of the total α-synuclein may explain other studies in which in-cell NMR was used to probe for the structure of α-synuclein and reported primarily the presence of unstructured monomer. NMR data of α-synuclein in intact cells failed to detect stable or highly populated α-synuclein multimers and confirmed the intrinsically disordered nature of the protein in *Escherichia coli* regardless of its purification method (Binolfi et al., 2012). Collectively these studies generated an apparent controversy and stimulated several additional studies that explored the native size and structure of α-synuclein.

A re-examination of the native state of α-synuclein reasserted that the behavior of α-synuclein from various sources was consistent with a disordered monomer. This behavior was observed with protein extracted and isolated under both denaturing and non-denaturing conditions. CD spectra previously attributed to tetrameric assemblies were not reproduced using isolated monomer, but were replicated with the addition of small unilamellar vesicles. Natively isolated α-synuclein before or after boiling that disrupts secondary structure migrated as high molecular weight α-synuclein bands in native PAGE, which was attributed to the rather expanded size of the unstructured monomer in solution. These findings reaffirmed that the majority of native α-synuclein is a monomer with minimal secondary structure (Fauvet et al., 2012). Further support was provided by similar explorations in the mouse brain, which indicated that the predominant native form of α-synuclein is an unstructured monomer. α-Synuclein exhibited random coil structure in solution, readily aggregated over time, and adopted α-helical structure only upon membrane binding (Burré et al., 2013).

α-Synuclein multimers were detected in postmortem non-diseased human brain using mild protein extraction methods, but no further

purification. These α-synuclein multimers had Stokes radii ranging from 33.2–37.5 Å, sedimentation coefficients ranging from 1.4S to 3.8S and apparent molecular weights ranging from 53–70 kDa in native gradient gels. The multimers were detected by anti-α-synuclein antibodies that recognize different epitopes and the multimer identity was confirmed by mass spectrometry. Consistent with previous observations, melting point thermostability analysis showed progressive loss of the α-synuclein multimers and heating of the brain extracts above 55 °C collapsed the higher molecular weight α-synuclein conformers into the 53 kDa species, which corresponds to the unstructured monomer. These data indicated the presence of α-synuclein conformers, defined as conformationally diverse α-synuclein multimers, in the human brain. Therefore it appears that both monomer and metastable multimers coexist and that interactions with lipids, other proteins, or small molecules may transiently stabilize these species (Gould et al., 2014). This was further supported by controlled bimolecular fluorescence complementation methodologies in different cell types that found α-synuclein metastable conformers assembled in synapses. It was suggested that the function of these multimeric α-synuclein conformers is to restrict recycling of synaptic vesicles and thus reduce neurotransmitter release (Wang et al., 2014).

Additional support for native multimeric species comes from recent studies in which serial purification of α-synuclein from non-pathological human cortical tissue was performed. Removal of lysate components other than protein followed by sequential removal of proteins though size exclusion, anion chromatography, and thiopropyl sepharose 6b separation, resulted in the isolation of >90% pure α-synuclein. Each step of serial purification resulted in a progressive loss of α-synuclein immunoreactive high molecular weight bands observed after disuccinimidyl glutarate crosslinking and SDS-PAGE separation. Analysis of α-synuclein secondary structure by CD found that the sequentially purified protein had greater α-helical content than the recombinant α-synuclein. However, a high degree of variability in secondary structure was observed between purified samples raising questions about the stability of these helical conformations (Luth et al., 2015). Furthermore, crosslinking experiments conducted in brain tissue from mice expressing wildtype or A53T human α-synuclein in the absence of mouse α-synuclein showed that the A53T mutation reduced the presence of soluble multimeric α-synuclein (Dettmer et al., 2015).

4. Concluding remarks and perspectives

Collectively the studies on the native structure indicate a remarkable conformational plasticity and structural flexibility of α-synuclein. The ability of the protein to adopt N-terminal α-helical conformation through its association with lipids has been well documented. The association with lipids has been shown to prevent fibril formation (Martinez et al., 2007; Zhu and Fink, 2003) and may also stabilize physiological multimeric species that together with the monomer regulate SNARE complex assembly and recycling of synaptic vesicles (Burré et al., 2014; Wang et al., 2014). However, other groups have demonstrated a role for phospholipid membranes in promoting pathological α-synuclein aggregation, potentially by acting as a scaffold for amyloid nucleation. This event may preferentially occur at low lipid to protein ratios, when monomeric α-synuclein is free in solution and can participate in nucleation (Galvagnion et al., 2015; Ysselstein et al., 2015).

In Fig. 3 we propose a model which incorporates and summarizes the existing knowledge regarding α-synuclein biology and structure. The steady state levels of α-synuclein are carefully regulated by protein synthesis and removal by several pathways such as the ubiquitin-proteasome pathways and autophagy (Webb et al., 2003). Controlling the steady state levels of this protein by regulating synthesis and degradation may be the first critical defense in preventing aggregation. Conformational change to α-helical rich structures, and stabilization of metastable multimers is achieved by specific interactions with vesicular phospholipids and proteins. The sequestration of α-synuclein in association with membrane vesicles and with other proteins may be of critical importance for preventing aggregation. Therefore these dynamic equilibria maintain functionality and promote assemblies that are resistant to aggregation. Catastrophic events that may include inappropriate post-translational modifications will disassemble the multimers as well as transform aggregation-incompetent monomers to aggregation-competent species. The first step in the pathway to amyloid fibril formation is the generation of a dimer that is either held together by hydrophobic interactions induced by increased conformational transition to β-sheet structure or upon covalent cross-linking. Following this nucleation event (Wood et al., 1999) the hydrophobic patch of amino acids between residues 71–82 appears to be primarily responsible for allowing additional α-synuclein monomers to assemble to form oligomeric structures. This transition is the committed rate limiting step for aggregation and must

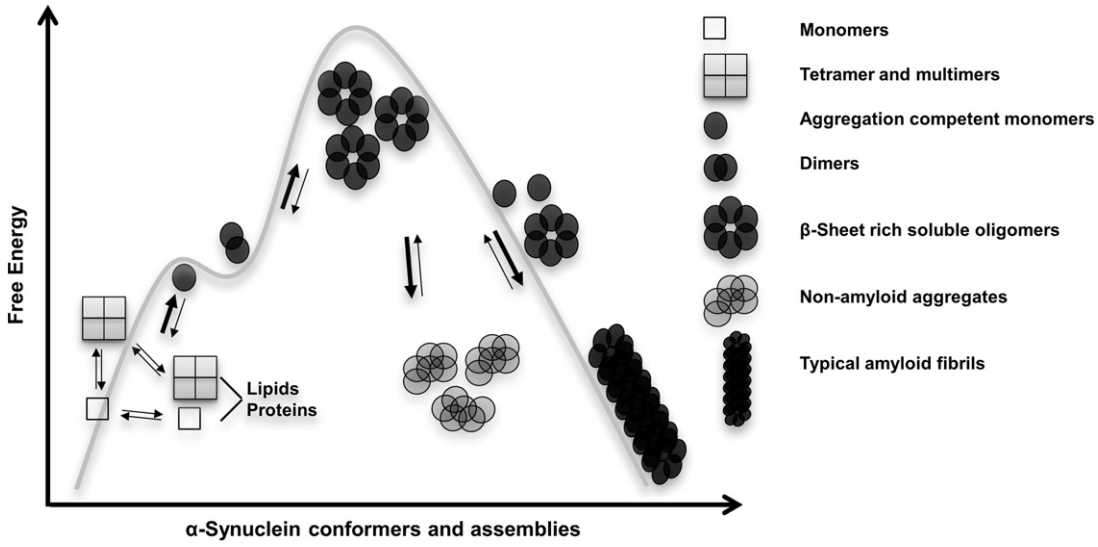

Fig. 3. Free energy landscape of possible α-synuclein conformers and multimeric assemblies. The conversion of native α-synuclein to aggregation-competent monomers may depend on dissociation from stabilizing interactions with lipids and/or proteins as well as dissociation of the metastable tetrameric species. α-Synuclein aggregation-competent monomers can then assemble into dimers and larger oligomeric conformers. The generation of α-synuclein oligomers can rapidly lead to formation of stable amyloid fibrils, or 'off-pathway' amorphous aggregates, both of which have been observed in postmortem brain tissue from patients with PD and related disorders.

overcome a relatively large thermodynamic requirement that permits the conversion from an unstructured coil to organized β-sheet conformation. Oligomers are soluble in aqueous buffers and can appear spherical or ring-like by atomic force and electron microscopy (Conway et al., 2000; Lashuel et al., 2002). Soluble, high molecular weight oligomers have been extracted from human brain tissue and their levels appear to be increased in PD brain (Sharon et al., 2003) as well as mouse models of α-synuclein aggregation (Tsika et al., 2010). As oligomers grow, they reach an undefined critical length and are able to assume additional quaternary structure. At this stage, these structures may continue to grow in linear β-sheets, forming polarized protofibrils and eventually fibrils. Fibrils may further arrange into protein inclusions although it remains unclear if other proteins within these inclusions anchor these fibrils. Alternatively, oligomers may remain soluble by interacting with small molecules (Conway et al., 2001) or by incorporating post-translationally modified α-synuclein molecules. These structures remain "off the amyloid fibril pathway" and may constitute what has been described in human postmortem tissue as "dots" or "dust-like" amorphous aggregates (Braak et al., 2001; Duda et al., 2002). At this juncture, it remains unclear which of these assemblies are toxic to neurons. Recent data indicate that several conformationally distinct assemblies (possibly different strains) of α-synuclein generated in vitro will induce the aggregation of endogenous α-synuclein resulting in neurodegeneration (Guo et al., 2013; Luk et al., 2012; Peelaerts et al., 2015; Sacino et al., 2014). The appreciation of different α-synuclein conformers and assemblies as well as their roles in disease may guide potential therapeutic approaches. For example, therapeutic strategies can be centered on preserving and stabilizing the physiological multimeric conformers as well as preventing monomers from aggregating. Alternatively, sequestration and removal of aggregation-competent monomers and oligomers can be considered.

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