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Exploring the accessible conformations of N-terminal acetylated α-synuclein

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Abstract

Alpha synuclein (αsyn) fibrils are found in the Lewy Bodies of patients with Parkinson's disease (PD). The aggregation of the αsyn monomer to soluble oligomers and insoluble fibril aggregates is believed to be one of the causes of PD. Recently, the view of the native state of αsyn as a monomeric ensemble was challenged by a report suggesting that αsyn exists in its native state as a helical tetramer. This review reports on our current understanding of αsyn within the context of these recent developments and describes the work performed by a number of groups to address the monomer/tetramer debate. A number of in depth studies have subsequently shown that both non-acetylated and acetylated αsyn purified under mild conditions are primarily monomer. A description of the accessible states of acetylated αsyn monomer and the ability of αsyn to self-associate is explored.

Keywords

α-synuclein; Parkinson's disease; Intrinsically Disordered Proteins; Acetylation; Monomer; Ensemble; Oligomer; Fibril; Aggregation; Fibril-resistance

1. Introduction

Parkinson's disease (PD) research has sought to answer questions of alpha synuclein (αsyn) function and the mechanism of aggregation surrounding disease pathology. Both remain to be fully articulated today, but several observations have been established and a range of neurodegenerative diseases termed the "synucleinopathies" have been identified[1, 2]. PD in particular is the synucleinopathy characterized by the loss of dopaminergic neurons and is largely considered to be an age-related disease, accompanied in part by age-related deposition of αsyn[3]. αsyn, a major protein component of Lewy Bodies[4, 5] in patients with Parkinson's, is a small primarily neuronal protein that is known to make a structural transition to amyloid fibrils[6-8]. αsyn is expressed abundantly in the nervous system and localizes near presynaptic nerve terminals[9-13]. It is also expressed at high levels in erythrocytes and platelets[14]. αsyn's function is unknown, but there is strong evidence that

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it exhibits lipid binding in vesicles and synaptic membranes[15] and may somehow exert its pathology through this behavior[16]. There is evidence that αsyn functions in assembly of the SNARE complex involved in vesicle transport[17], that it may more generally be involved in synaptic vesicle trafficking and regulation and/or may play a key role in neuronal cell survival[18-22].

The deposition of αsyn has largely been thought to originate from an intrinsically disordered monomer ensemble that under fibril promoting conditions forms amyloid [7, 23, 24], but recently this view of αsyn's native state was challenged[25]. Selkoe and colleagues pushed the biophysical community's long-held view of αsyn as an intrinsically disordered monomer by suggesting that the protein exists in its native state as a fibril resistant helical tetramer. They purified the sample from human erythrocytes, opting to exclude a potentially "harsh" and commonly used boiling step from the purification. Based on this work several questions presented themselves. Do bacterial systems that are commonly used to obtain sample for biophysical characterization not possess the necessary machinery for tetramer assembly? Could the commonly used boiling step during purification denature some key native structure that promoted a helical tetramer of αsyn? Aside from these assembly and purification issues, there was also one molecular difference between the purified samples of Selkoe and colleagues and previous studies, indicative of modification to the monomer by an acetyl group (Ac-αsyn).

This review reports on our current understanding of αsyn within the context of these recent developments and describes the work performed by a number of groups to address the monomer/tetramer debate[25-33]. We summarize major shifts within recently published works addressing these issues in Table 1. Numerous studies indicate that αsyn, both acetylated and non-acetylated, exists as intrinsically disordered monomer conformational ensemble under mild purification conditions. We highlight that the ensemble of monomers is known to develop into a wide range of accessible conformations upon changes of environmental conditions, that it can populate many soluble oligomeric states of varying morphologies and toxicities, and settle into various insoluble fibril or amorphous aggregate morphologies[23], that have largely been studied in the context of PD-related pathology (Figure 1). We discuss the suggestion of a soluble fibril resistant helical tetramer that presumably represents a non-pathological aggregate of αsyn which may have to dissociate before fibril formation can proceed through the monomer (Figure 1). The potential that established methods might disrupt native-stabilizing interactions of a fibril-resistant helical tetramer of αsyn have heightened awareness to cell machinery, to αsyn purification methods, and to the difficulties in choosing appropriate methods of characterization. The extent to which N-terminal acetylation impacts upon the conformation and aggregation behavior of αsyn is discussed separately and it is shown that the acetyl group does not promote the formation of the helical tetramer under mild purification conditions.

2. Overview of non-acetylated αsyn ensemble: monomers and dimers

2.1. Biophysical characterization of the non-acetylated monomer ensemble

The native state of non-acetylated αsyn has been thought to originate from an ensemble of intrinsically disordered monomeric forms, with recognition that the monomers therein are

capable of adopting a wide range of accessible conformations depending on solution and environmental conditions [34-38]. Uversky first spoke of αsyn as the “protein chameleon” [23] due to its ability to respond to its environment and binding partners by varying its foldedness and aggregation state. αsyn is often described as a 140 residue intrinsically disordered protein (IDP) characterized by three distinct regions of the protein: an N-terminal lipid binding repeat region that houses the mutations A30P, E46K, and A53T linked to early onset disease, a hydrophobic non-amyloid component (NAC) region implicated in fibril formation, and an acidic more proline-rich C-terminus suggested to have chaperone activity and possess some key role in modulating structure in the N-terminus[6, 39, 40]. As summarized in Table 1, to study PD related aggregation, αsyn has typically been obtained from overexpression in bacteria, yielding a non-acetylated IDP, as bacteria typically do not modify their proteins by acetylation (Figure 2A)[41-43]. Additionally, while boiling as part of the purification protocol would typically be considered to be harsh for a globular protein, IDP’s are in general characterized by thermostability[34]. Because of this heat stability, αsyn has often been boiled to achieve purity. In addition, IDP’s like αsyn are generally characterized by a highly charged sequence, a lack of stable secondary structure, and a larger than expected Stokes radius compared to spherical and folded proteins of the corresponding molecular weight[34, 44, 45].

The αsyn monomer is both unfolded and extended, as it was first reported to have a larger Stokes radius than expected for globular protein of similar molecular weight and a primarily random coil circular dichroism (CD) spectrum[34, 36, 46]. However, the protein is not fully extended for a protein of its size, implying a slight compaction of the monomeric ensemble[36, 47]. Evidence for contact between the C-terminus and both the NAC and N-terminal regions of the protein from nuclear magnetic resonance (NMR) [38, 48-55], electron paramagnetic resonance[56] and molecular dynamic studies[52, 57] indicates a possible source of this compaction, as well as some transient secondary structure[55, 58-60]. The compaction may be at least be partially driven by hydrophobic patches located in the C-terminal (residues 115-119, and 125-129) associating with and shielding both the hydrophobic N-terminal and NAC regions[50, 61]. It should be emphasized that evidence for contact between the N- and C-termini does not imply a static closed picture of αsyn as an IDP[59, 62]. Rather, this is a dynamic interaction, and observation of slight compaction is the result of observations on a highly averaged bulk ensemble[48].

Under conditions promoting pathological aggregation of αsyn, conformational shifts in the ensemble are observed. There is evidence that these interactions may keep the N-terminus from pathological misfolding[39, 63], as their release is associated with increased fibril formation [38, 50, 52, 64, 65]. For example, when the solution pH is lowered, there is a structural rearrangement of the monomer ensemble with enhanced contacts NAC-C terminus and C-C contacts resulting from charge neutralization and compaction of the C-terminal region[38, 54, 58, 66]. Environmental or experimental shifts that reduce the net charge or increase hydrophobicity of the protein [67-69], or interaction with small molecules or metal ions[70-72] can change subtly, but significantly, both the long-range and short range contacts and conformations sampled in the monomeric ensemble.

Therefore, small changes to the αsyn monomer can potentiate big effects on aggregation behavior, yet only small differences to the monomer ensemble. For example, the familial mutations (A30P, E46K,A53T) of αsyn are structurally comparable, as they are similarly unfolded and have similar radii of gyration, but they have distinct kinetics of fibril formation[55, 73-79]. NMR spectroscopy has revealed that mutations affect chemical shifts surrounding the mutation site and that we can correlate these shifts to region-specific shifts in the population of transient secondary structure. These relatively small shifts in transient secondary structure populations can explain bulk differences in fibril formation rates[59, 80, 81].

The N-terminal region is also known to adopt helical structure upon binding lipids, representing a more dramatic conformational shift of the monomer ensemble[82-84]. NMR groups have demonstrated that αsyn displays chemical shifts characteristic of a mostly unfolded peptide, but that the first 100 residues transiently populate helical structure. When bound to lipids or micelles, however, chemical shifts of these residues indicate a structured helical environment.[85, 86]. Bax characterized the structure of the micelle bound form of the protein, and the fact that αsyn adopts helical structure at its N-termini through its repeat region upon binding lipids membranes and micelles has become well-known[82]. Because αsyn localizes near synaptic nerve termini[9], its lipid-induced helical structure[35, 87-89] may be crucial in understanding the protein's function at the membrane, yet it is still unknown how this N-terminally helical monomer conformation is related to the trigger of fibril formation.

2.2. Dimers: equilibrium species and pathological intermediates

Dimers can exist in a small population in pseudo-equilibrium with the monomer ensemble. The Baum lab demonstrated that indeed antiparallel transient inter-chain contacts between the C-terminal hydrophobic patches and the N-terminal region (residues: 3-15 and 35-50) could be detected by using NMR paramagnetic relaxation enhancement experiments[54]. Electrospray ionization mass spectrometry (ESI-MS) and electrospray ionization-ion mobility spectrometry-mass spectrometry (ESI-IMS-MS) obtained under similar conditions has shown that the predominant oligomeric form we observe in αsyn is the dimer. This soft ionization technique has revealed the "conformational heterogeneity" of αsyn, where the monomers and dimers themselves exist in both extended and compact conformations[30, 90, 91], suggesting that the ensemble view of αsyn also extends into its higher oligomeric states.

It is unknown whether this pseudo-equilibrium anti-parallel dimer is on pathway to the fibril formation. It is reasonable to assume that inter-chain N-N species, which adopts the same parallel orientation as monomer units as in the core of the fibril, lies further along the pathway to fibril formation than anti-parallel oriented monomers[92]. This would imply reorientation of monomer units as an obligatory step before formation of the fibril. However, at least one report demonstrates that toxic prefibrillar amyloid aggregates adopt an antiparallel orientation[93], and in this sense we cannot draw any analogy to this pseudo-equilibrium dimer population that exists with the fibril accessible monomer ensemble, and how the monomers therein may interact with it.

As the monomeric ensemble is shifted towards more fibril prone conditions, previously described conformational changes (Section 2.1), as well as changes in population of oligomeric species occurs. Incubation at high temperature is one external factor inducing this shift[94]. Under these conditions, soluble oligomers of αsyn spontaneously associate and a dimer is the predominant oligomeric species of αsyn to appear alongside formation of the fibril, along with smaller populations of higher-order oligomers. Biophysical characterization of this intermediate which includes some dimer shows it has more hydrophobic patches exposed than the native monomer[36]. This on-pathway dimer that appears at the time of fibril formation may be conformationally dissimilar from the pseudo-equilibrium dimer population previously described, which may not necessarily be correlated with fibril formation. There is some evidence that formation of at least one species of dimer is the rate limiting step of fibril formation[95] and cysteine mutants have shown that particular dimer linkages accelerate fibril formation *in vivo* and *in vitro*[95]. This implies accessibility of many distinct conformations for the dimer, in the same way as the monomer, and presumably varying degrees of membrane affinity and cytotoxity Additionally, dimers are not the sole on-pathway oligomeric species that appear during events of PD pathology. Observations of higher order oligomers, that occur alongside fibril formation and in response to other events appear to include a large slew of different species, which we address in the upcoming section.

3. Heterogeneity of the αsyn oligomeric structures and their pathogenicity

3.1. The role of oligomers in *in vivo* pathology

The motivation to understand whether there is a helical tetramer of Ac-αsyn lies not only in desire to accurately portray the protein *in vivo*, but also to understand how oligomers in particular function in disease-related pathology of PD. There is ample evidence that soluble oligomers are the real pathogenic species of neurodegenerative disease, whereas fibrils serve as reservoirs of misfolded, irreversibly modified deposited protein better-off removed from solution[96-103]. Because amyloid deposits were first detected in brains of sick individuals, it was assumed that they were the neurotoxic species, but because amyloid is such a common structural motif, the ability to form amyloid is now considered a general property of a polypeptide in solution[104]. Over and over, conversion of IDPs into amyloid aggregates has not been observed to be a simple two-state transition. Oligomer formation has been established as an important mechanistic step in fibril formation, for example as in Alzheimer's disease[102, 105]. As briefly described in αsyn, soluble oligomeric intermediates commonly appear as insoluble fibrils form[106-108] and the situation may be quite similar to that established for AD.

What determines if a protein will form soluble oligomeric species, or if an amyloid fibril will form? It seems that a polypeptide will sample many parallel or antiparallel conformations before a final structural state is preferred[109]. This arises from a competition between hydrophobic forces and side chain interactions, versus the propensity of the polypeptide chain to form β-sheet like hydrogen bonds[110]. The prefibrillar oligomer is thought to be the cytotoxic species, as toxic inter-chain associations are sampled that a monomer alone could not support. The fluorescent probe 8-anilinonaphthalene-1-sulfonic

acid binds to exposed hydrophobic patches. Its binding demonstrates that the most toxic species are associated with greater overall surface hydrophobicity[94, 111-113]. In fact, overall greater hydrophobicity is associated with increased chance for exposed hydrophobic portions of the sequence to exhibit toxicity through interaction with the membrane. This may as well be the case for αsyn [112, 114].

Oligomeric PD pathology may be rooted in membrane association, where oligomers of αsyn can perturb membrane integrity and cause cell death by altering transport across the membrane [115-118]. At least one report demonstrates that *in vivo* membrane associated αsyn oligomers correlate with toxicity rather than inclusion formation[119] but also that the degree of oligomer toxicity is related to an array of structurally diverse morphologies that can form. Interestingly, of the familial mutants implicated in PD, A30P and A53T have different kinetics of fibril formation relative to the wild type monomer, but both share the property of an accelerated oligomerization[120, 121]. These mutants may exert their pathology through the formation of pore-like oligomers that form alongside fibril formation[122].

3.2. Oligomers associated with the fibril formation pathway are highly heterogeneous

During the time course of fibril formation early prefibrillar oligomers and late soluble oligomers, which are not a part of the fibril have been observed in αsyn[107, 123] Their isolation and structural characterization has been of great interest, and some shared features of fibril accelerating or inhibiting species have been characterized. It was postulated that an on-pathway amyloidogenic transition occurs through partially folded oligomeric species originating in the dimer[36]. Soluble aggregates first appear that maintain the helical character of the monomer, but lose some disorder in favor of β-rich structure as they age. β-rich intermediates build as fibril formation proceeds and begin to get consumed at the end of the lag phase[107, 111]. This conversion into more β-rich species may describe the formation of initial aggregates and their conversion into amyloid-like aggregates., The conformational conversion between oligomeric types observed by Dobson and colleagues is also accompanied by direct observations of conversion into a more toxic form and highlights that certain oligomer types can be either toxic or non-toxic. [106]. AFM has been used to observe β-rich spherical and annular oligomer morphologies prior to fibril formation of αsyn. The initial aggregates appear to be spherical aggregates. They have been shown to convert to more spherical compact species, and then into annular species upon further incubation[124]. Annular species of αsyn are known to induce membrane leakage[103, 125], but spheroidal species can bind brain-derived membranes quite tightly, as well[124]. Spherical morphologies seem to disappear once the fibril has formed, whereas annular species may sometimes coexist with the fibril[124]. Oligomer induced toxicity is relevant to the entire fibril formation process.

Soluble oligomers may appear after the fibril has formed, or their formation may instead be preferred. Late stage distinct oligomeric species appear once fibrils have formed and they are also β-rich [108, 111, 123, 126]. Some suggest they occur from dissociation of the fibril or that they represent end-products of a fibril resistant-soluble oligomerization pathway and

may not be converted into fibril. At the end of fibril formation 10-20% of protein exists as such a non-fibrillar oligomer[127].

There are many pathways which have been identified toward soluble aggregates. Organic solvents have been used to model membranes, and it has been shown that a helical rich monomer will eventually associate into a helical rich oligomer that also appears stable [128]. Covalently cross-linked non-fibrillar oligomers are also well known to form under oxidative or nitrative stresses. Nitration, for example, inhibits fibril formation through the formation of inhibitory higher-order oligomers than the dimer [129]. This mix of species only further describes the range of the secondary structures, morphologies and pathologies that oligomers of αsyn are capable of populating[92, 94, 130-132]. Increased oxidative stresses and increased metal levels have been correlated with PD, so this class of stable non-fibrillar oligomers that form under stresses are potentially important players in the mechanism of aggregation as well[133].

Various pathways available to soluble oligomer, not surprisingly results in a very heterogeneous population of possible oligomers. Oligomer morphology has been shown to be highly dependent on solution conditions, including the presence of lipids[124, 134-137]. Also, incubation with different types of metals generates partially folded structures[138-140] that go on to form a variety of oligomeric structures. Whereas incubation with Cu^{2+}/Fe^{3+}/ Ni^{2+} produce spherical oligomers of 0.8-4 nm particles, incubation with Co^{2+}/Ca^{2+} produces yields pore-like annular rings 70-90 nm in diameter[141].

3.3. Stabilization of non-fibrillar oligomers that appear to be non-pathogenic

In previous paragraphs our focus was on oligomers more closely linked to pathology, but as mentioned, some oligomers can be stabilized in non-fibrillar forms not capable of adopting cross-β structure on their own and may not necessarily be linked to cytotoxicity (Figure 1). The αsyn monomer that has been modified by methionine oxidation of the αsyn monomer to the sulfoxides is one example of these non-toxic non-fibrillar species. This modification at methionine residues promotes the stabilization of an oligomer that appears slightly more unfolded than monomeric αsyn. While probably not covalently cross-linked, these oligomers exhibit stability and do not go on to form fibrils[142-144]. Furthermore, these oligomers do not exhibit toxicity toward dopaminergic neurons, suggesting that particular conformational features are indeed necessary to exert pathology as an oligomer (Figure 1) [144]. Interaction with small molecules like the flavonoid baicalein can also prevent formation of the fibril by stabilizing soluble oligomeric end products and these oligomers also do not disrupt membranes[145]. Structurally these species are spherical, have a well developed secondary structure, are relatively globular with a packing density intermediate between globular protein and pre-molten globule and very high thermodynamic stability. In contrast oligomers stabilized by modification of the monomer with 4-hydroxy-nonenal are non-fibrillar, but are also toxic[145]. Could a helical tetramer be similarly stabilized, such that the stabilization in the oligomer conformation is more favorable than in amyloid, and could it also share conformational features of non-pathology with aforementioned species? Descriptions of size, morphology or overall conformation may not be sufficient to describe the cytotoxicity of an oligomer of asyn. For example, in amyloid-β two oligomers of similar

size but dissimilar toxicity have been identified, where the more toxic species adopts a conformation in which hydrophobic regions remain more exposed[146].

4. αsyn is N-terminally acetylated *in vivo*

Before attention was drawn to the possible role of the acetyl group by the recent report[25] of tetramer formation of αsyn, αsyn was studied from a variety of sources, some of which were mammalian and were likely to be N-terminally acetylated. Although the acetyl group had not previously warranted an explicit examination, drawing comparisons between in-cell work *and in vitro* work could be challenging. Therefore, the Bartels *et al.* report clearly suggested that co- or post-translational modifications (PTM's), namely acetylation, may have significant influence on αsyn structure and aggregation properties so specific investigation of the acetyl group naturally followed in the reports we describe in this review(Table 1). PTM's to αsyn are known to regulate/modify αsyn's propensity to aggregate[132, 142, 147]. It has been known for some time that αsyn in human tissues is acetylated, but the role of N-terminal acetylation is unclear, as it is seen in both healthy and individuals sick with synucleinopathies. Two mass spectrometry (MS) studies of αsyn from human tissues, both report that the base mass of the protein before any other modifications is the acetylated form -- consistent with that reported by Selkoe and colleagues from red blood cells (RBCs)[25, 148, 149]. The report indicated that acetylation of αsyn was not limited to neuronal tissue; however, the site of acetylation was not identified.

4.1. Bacteria lack the machinery for N-terminal acetylation

Mammals modify the proteins they produce with many more PTM's than yeast and bacteria, as these may play a role in more complex signaling pathways[150, 151]. N-terminal acetylation is a well-known modification in eukaryotic cells. Up to 80% of proteins are modified by N-terminal acetylation in mammals, whereas bacteria rarely acetylate their N-termini and if they do, by distinctly different mechanisms [43]. The aforementioned MS studies[148, 149] indicated that acetylation occurs at the N-terminus, where an acetyl group has removed the α-amino charge of the initiating amino acid by covalent modification at that site.

N-terminal acetylation is carried out mostly co-translationally by a group of enzymes known as N-acetyltransferases (Nat) in eukaryotes[152, 153]. Mammalian cells have these complexes, and yeast an analogous enzyme complex, but bacteria do not. Nat's catalyze the transfer of an N-acetyl group from acetyl-coenzyme A to the N-termini of proteins with sequence specificity. Different Nat's (types A-F in eukaryotes) work upon different initiating amino acid substrates, dependent upon the identity of the first two to three amino acids of the protein polypeptide[154]. Therefore, depending on the type of cell to synthesize αsyn, the protein may or may not be acetylated (Figure 2A, 2B). Specifically, N-acetyltransferase B (NatB) has αsyn as a substrate, producing acetylated αsyn (Ac-αsyn). NatB targets proteins beginning with Met-Asn-, Met-Glu- or as in the case of αsyn, Met-Asp-. Substrates of NatB are acetylated nearly 100% of the time, as the acidic amino acids in the second position are thought to stimulate the transfer of the acetyl group[151].

4.2. Possible roles of N-terminal acetylation *in vivo*

Recognizing that αsyn acetylation does indeed occur, one study prior to Bartels *et al*'s investigated the role of NatB activity in a yeast model by disrupting NatB activity[155]. They found NatB activity to be essential for proper membrane targeting of αsyn. Without NatB activity, non-acetylated αsyn is produced, and a much more diffuse cytoplasmic localization of αsyn compared to those with whole NatB activity (producing Ac-αsyn) was observed. While this *in vivo* effect was observed in this one instance for Ac-αsyn, the role of N-terminal acetylation is not generally well understood[156]. One study suggests that N-terminal acetylation represents an early sorting step, where acetylated proteins are targeted toward the endoplasmic reticulum, unless they remain non-acetylated and are kept localized to the cytosol instead[157]. N-terminal acetylation may also regulate degradation pathways[158] or be responsible for structural effects at the N-terminus[159]. Levels of acetylation may be related to regulation of other post translational modifications. NatB, specifically, has been shown to induce elevated phosphorylation levels in yeast[160] consistent with the aforementioned yeast study of αsyn, where decreased levels of phosphorylation were observed when the protein remained non-acetylated and localized in the cytosol[155]. Acetylation may not be necessary at all for proteins, but some examples do point to a necessity. For example, tropomyosin requires N-terminal acetylation so that it may bind to actin[161].

5. αsyn is proposed to be a helical tetramer in its native state

Selkoe and colleagues strived to isolate αsyn under physiological conditions and have challenged the existence of the αsyn monomeric ensemble[25] by proposing a fibril resistant helical tetramer form of the protein (Table 1). In contrast to the typical protocol in which αsyn has been derived from bacterial systems, overexpressed and denatured, they purified αsyn from gently-treated RBCs known to have a high endogenous expression level of human Ac-αsyn. From both RBC lysate and endogenously expressed αsyn from neuronal and non-neuronal cells lines, Selkoe and colleagues showed on Clear Native PAGE (CN-PAGE), that αsyn migrates near the tetramer position against folded, globular protein standards. The unusual migration of an IDP against globular standards was not unfamiliar. Native gels are unreliable objective determinants of molecular weight, as protein migration through the acrylamide matrix depends strongly on protein charge and shape. IDP's typically display a Stokes radius of a much higher molecular weight species, and this has previously been attributed to enhanced interactions with the matrix[36], so that *E. coli* derived boiled αsyn, too, will migrate near the position of the tetramer at 58 kDa on a native gel[25].

Selkoe and colleagues' report also stated that they obtained a CD spectrum that indicated largely helical structure that was sensitive to irreversible heat denaturation. Isolation from human cell line 3D5 (which are M17D cells stably expressing αsyn) yielded similar results to RBC derived αsyn, and they showed that αsyn derived from *E. coli* was random coil even after non-denaturing purification, consistent with previous reports[41]. Therefore Selkoe and colleagues implied that expression in human cell lines and a non-denaturing purification are necessary to "preserve" this native tetramer structure. If this is indeed the native form of

αsyn, non-denaturing methods of purification and mammalian machinery may be necessary to observe it. When denatured, RBC αsyn became random coil, and migrated more similarly to *E. coli* derived αsyn on a native gel, rather than the mildly purified helical sample.

Helical structure of αsyn can be induced by its interaction with membranes. Therefore, it might logically follow that the milder purification did not fully remove helix inducing lipids. However, treatment with Lipidex and subsequent phosphate analysis indicated the sample was relatively pure in that regard (0.25 mol phosphate/αsyn monomer). At the same time, a higher lipid binding capacity for this "native" αsyn was demonstrated with surface plasmon resonance. They also employed some unbiased methods of MW determination including sedimentation equilibrium-analytical ultracentrifugation (SE-AUC) and scanning transmission electron microscopy both indicating a tetramer. Bartels *et al.* also observed one other unprecedented trait of the sample – that under standard fibril assay conditions, ThioflavinT (ThT) fluorescence did not indicate that RBC ac-αsyn formed any fibrils *in vitro* – clearly also in contrast to previously observed results and the *in vivo* condition (Table 1).

Not too long after, Wang *et al*.[135] similarly reported a dynamic tetramer form of the protein. The protein was obtained by recombinant expression methods and was modified by a 10 residue N-terminal tag left over from a glutathione S-transferase (GST) construct, making it difficult to compare directly with the tetramer obtained from RBCs. The purification method was similarly "non-denaturing" but included the non-physiological detergent beta-octyl glucopyranoside (BOG) typically used to purify membrane bound protein. Perhaps N-terminal acetylation was somehow mimicked by the cleaved GST-tag and would prove to be important in the context of a non-denaturing purification. Researchers now would interpret data in light of a greater possibility of the tetramer, and more carefully consider their assumption that their expression and purification methods did not preclude an accurate representation of the protein *in vivo*.

6. Subsequent studies indicate that αsyn exists as a primarily unfolded monomer

A rapid period of overlapping work began to determine the oligomeric state of αsyn from various cell sources, under non-denaturing conditions and to investigate the role of the acetyl group modification. Lashuel and colleagues examined αsyn from mouse, rat and human brains and addressed the issue of the source, the purification and the characterization methods of the protein and their impact on the oligomerization state (Table 1)[29].

In response to report by Bartels *et al*., Lashuel and colleagues determine that αsyn exists as an unfolded monomer within neuronal sources. Lashuel *et al*.[29] examined bacterial lysates under denaturing and non-denaturing conditions (with and without a boiling purification step respectively) against a range of non-globular standards, including: 1) *E. coli* derived unfolded monomeric αsyn 2) disulfide linked A140C αsyn including some dimer and 3) Ac-αsyn. Regardless of purification, samples from bacterial lysates elute and migrate at identical positions on a size exclusion chromatography (SEC) column or CN-PAGE. This indicated that the various samples are either all unfolded monomers, all more compact

tetramers, or that coincidentally these structures migrate at identical positions. Coupled now with a far UV spectrum of a primarily random coil protein, rather than a helical spectrum observed by CD, however, Lashuel's bacterial αsyn appears to be unfolded regardless of whether it has been boiled and it resembles unfolded monomeric αsyn. A random coil spectrum is not necessarily synonymous with a monomeric protein. Static light scattering (SLS) was used as a more unbiased molecular weight determinant alongside elution from SEC[162]. While data from size exclusion chromatography (SEC), indicated a Stokes radius close to a globular standard at 64 kDa, SLS indicates a protein of 14 kDa. Therefore bacteria, consistent with Selkoe and colleagues' observations, do not assemble into a helical tetramer, even without boiling.

Lashuel and coworkers[29] demonstrated a sensitivity of CN-PAGE to small differences in the protein composition and went on to use CN-PAGE to explore the role of mammalian machinery and denaturation by boiling. Whether endogenous or overexpressed, whether boiled or not, whether isolated from bacteria or present in mouse, rat samples or HEK293,HeLa,SH-SY5Y,CHO,and COS-7 mammalian cell lines -- identical CN-page migration and sometimes SEC-SLS, repeatedly indicated the unfolded monomer. Across research groups, acrylamide percentages, purification protocols and the source, the samples of αsyn co-migrate with recombinant αsyn. To test whether factors present in cell could promote tetramerization, they examined fresh or aged samples, since aged samples are expected to be more oligomer-rich, along with a control of exogenously added recombinant protein. *In vivo* oligomer-specific enzyme-linked immunosorbent assay (ELISA) could not detect any other oligomers in the samples, confirming that purification has not disturbed this observation[29, 163]. In addition, the report explored the possibility that the tetramer population could be dynamic and unstable, so that if the protein for some fraction of the time populates a tetrameric state, it would have a different cross-linking profile than a protein that populates primarily the monomer state. They observed that no significant amount of oligomers beyond the dimer are observed, indicating that DSS could not effectively capture a tetramer either. This report additionally repeated the RBC purification procedure[29]. Unable to replicate the tetramer, it was still concluded that disordered monomer is isolated from RBC's. It is not clear what Selkoe and colleagues[25] did differently, but Fauvet *et al*. [29] does note that samples of sufficient quantity and purity could not be obtained using this purification, even with another hydrophobic interaction chromatography (HIC) step, suggesting some complicating interactions in either sample. Fauvet *et al.* also attempted the GST-constructed αsyn protocol and cannot replicate the dynamic tetramer observations of Wang and colleagues.

Concurrently, Rhoades and colleagues sought to determine if the nature of the purification method[32] or N-terminal acetylation had enough biophysical consequence to promote the fibril resistant tetramer. They examined samples purified with and without BOG and the N-terminal acetyl-group. Rhoades is the first to use a bacterial co-expression system to generate Ac-αsyn (Figure 2C). In this co-expression system developed by Mulvihill et a[164], the yeast analog to NatB is cloned into a bacterial plasmid, allowing overexpression of αsyn into more unsophisticated expression systems. The yeast NatB is shown to function in bacteria to produce N-terminally acetylated proteins, and it seems to acetylate αsyn close

to 100% of the time in *E.coli*. Rhoades finds that N-terminal acetylation and non-physiological purification including BOG were necessary for observation of helical oligomeric αsyn. Non-acetylated or BOG free αsyn was disordered and presumably monomeric, but the CD spectrum of Ac-αsyn purified in the presence of BOG was helical. Rhoades also encounters the complication that disordered monomer and helical tetramer have similar hydrodynamic radii, but coupled with SE-AUC, which is "independent" of molecular shape, Ac-αsyn(BOG) was shown to have a sedimentation curve that exchanged with an oligomer. That the sample was specifically tetrameric is not clear. While the report by Trexler et. al, does not exclude the possibility that N-terminally acetylated αsyn has a higher affinity for membranes and/or BOG itself, the work was further provocative towards the role of acetylation in helicity and oligomerization.

7. Continued discussion on the oligomerization state of αsyn

In response to the studies that indicate that cellular αsyn is an unfolded monomer[27, 29], Selkoe and coworkers[33],with an even further heightened awareness to experimental conditions, most recently reported that endogenous αsyn is predominantly tetramer. Using *in* vivo cross-linking as their primary tool, they identify several factors which might matter in terms of isolating the tetramer. During overexpression, particularly in protein derived from IPTG induction, more monomer is found. More monomer is also isolated when cross-linking is done at 4C° as opposed to 37C°. The tetramer is "preserved" in a concentration dependent manner at the time of lysis, where a higher concentration at the time of lysis favors the tetramer. This suggests that macromolecular crowding in cell may favor folding and stabilization of the native non-pathological tetramer. For this reason and for the fact that the Ac-αsyn level is endogenously high in erythrocytes, Selkoe and colleagues' considers RBC's an ideal system to obtain Ac-αsyn. These results may reflect of the experiments themselves, or may reflect the preference of the monomer to associate with itself, even in the presence of other binding partners, but that it is also stabilized in the monomeric form.

8. N-terminal acetylation of monomeric αsyn induces helix formation and affects lipid binding

Because Ac-αsyn is now recognized to be the physiologically relevant species in the brain, its biophysical characterization has been pursued. Questions that have been raised include the monomer or oligomeric preference of the Ac-αsyn species, its conformation, interactions with membranes and ability to form fibrils. Kang *et al*.[30], show that recombinant 100% acetylated Ac-αsyn purified under mild physiological conditions exists primarily as a monomeric protein. ESI-IMS-MS experiments indicate a small population (5-10%) of dimer that is consistent with previous observations of dimer species in solution. Lashuel and colleagues[28] use similar techniques as in their first report and again do not observe any higher-order oligomers, now in the acetylated protein. This suggests that acetylation by itself is not sufficient to favor a helical tetramer. Selenko and colleagues [27] show by in-cell NMR that non-acetylated αsyn is a disordered monomer in the macromolecular environment of the cell. Lashuel's group[28] additionally compares Ac-αsyn and αsyn with in-cell NMR and draws similar conclusions. Although the possibility of exchange with higher-order

oligomers cannot be ruled out, the predominant cellular form indicated by these experiments of Ac-asyn is unfolded monomer (Table 1).

The conformational properties of Ac-αsyn have also thus far been investigated and it is suggested that there is minimal change in the hydrodynamic radius and intra-chain long-range interactions, if any[28, 31]. However, the N-terminal acetyl group affects the transient secondary structure as observed by NMR[28, 30, 31]. Residue-specific NMR chemical shift analysis shows that there is an increase in the transient helical propensity at the initiating N-terminus[28, 30, 31] that may arise as the acetyl group masks the alpha-amino positive charge and interacts favorably with the helix dipole moment. Additionally, the acetyl group itself is a good N-cap, favoring hydrogen bonding for an N-terminal alpha helix at the initiating residues[165, 166] .

At least one report suggests the membrane binding properties of Ac-αsyn are strongly altered by acetylation and indicates a two- fold higher lipid affinity. It is suggested that the increase in N-terminal transient helix may be critical to initiating membrane binding. Preformed transient helix at the N-terminus may therefore play an important role in the recognition of binding partners, may be important for membrane recognition, or may imply that lipid mediated association of the hydrophobic surfaces of helices may relevant to routes of self-association of the acetylated monomer.

Fibril formation of Ac-αsyn was investigated by measuring the fibrillation kinetics using ThT fluorescence. While groups of Lashuel[28] and Bax[31] found no significant differences in fibril formation rate, Kang *et al*.[30] found that N-terminal acetylation slows the rates of fibril formation by approximately a factor of two. Clearly the acetyl group alone cannot inhibit fibril formation, but it does impart a small inhibitory effect, which may arise from a redistribution of the monomeric protein ensemble.

9. Conclusions

Recent studies that suggested that asyn exists as a soluble, tetrameric, fibril-resistant form of αsyn were provocative, and a monomer/tetramer debate followed. The discussions about the accessible states of αsyn have raised many important questions related to cellular machinery, αsyn purification methods and the extent to which acetylation impacts on a monomer-oligomer equilibrium. A number of in depth studies have subsequently revealed that both non-acetylated and acetylated αsyn purified under mild or harsh conditions is primarily a monomer.

Despite the controversy surrounding the notion of a helical non-pathological tetramer, the concept of a soluble, non-pathological αsyn oligomer was perhaps not new. Biophysical studies have shown that αsyn can be induced to self-associate into a heterogeneous variety of soluble oligomers, some of which may be beneficial, or non-pathological. For example, methionine oxidation, arising from conditions of oxidative stress, stabilizes a fibril resistant oligomer of αsyn that is non-toxic to dopaminergic cells[144]. This may be consistent with the regulatory role methionine oxidation is suggested to have, sometimes being beneficial.

In order to understand the interplay between aggregation prone and aggregation resistant kinetic pathways from the unfolded monomer, the initial interchain associations between monomers within the starting ensemble and associations present in already-isolated stable soluble oligomers may need to be considered further. Defining the properties that drive these different species may lend to our understanding of how to enhance fibril resistant, fibril prone and/or non-toxic pathways *in vivo*. Because of the great ability of αsyn to adopt many conformations in a variety of oligomeric states, working from the monomer ensemble of Ac-αsyn we may (again) isolate stabilizing interactions of a helical oligomeric species that does not tend toward fibril and we may begin to better elucidate shared features of non-pathology and fibril resistance amidst the entirety of the currently known heterogeneous oligomer population of αsyn.

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Abbreviations

PD	Parkinson’s disease
αsyn	α-synuclein
Ac-αsyn	Acetylated α-synuclein
IDP	intrinsically disordered protein
BOG	beta-octyl glucopyranoside
GST	glutathione S-transferase
CD	circular dichroism
CN-PAGE	clear native PAGE
NAC	non-amyloid component region
PTM	post-translational modifications
RBC	red blood cells
Nat	N-acetyltransferase
NatB	N-acetyltransferase B
SE-AUC	sedimentation equilibrium-analytical ultracentrifugation
ELISA	enzyme-linked immunosorbent assay
ThT	Thioflavin T
SEC	Size exclusion chromatography
SLS	static light scattering
NMR	nuclear magnetic resonance
ESI-MS	electrospray ionization-mass spectrometry

ESI-IMS-MS electrospray ionization-ion mobility spectrometry-mass spectrometry

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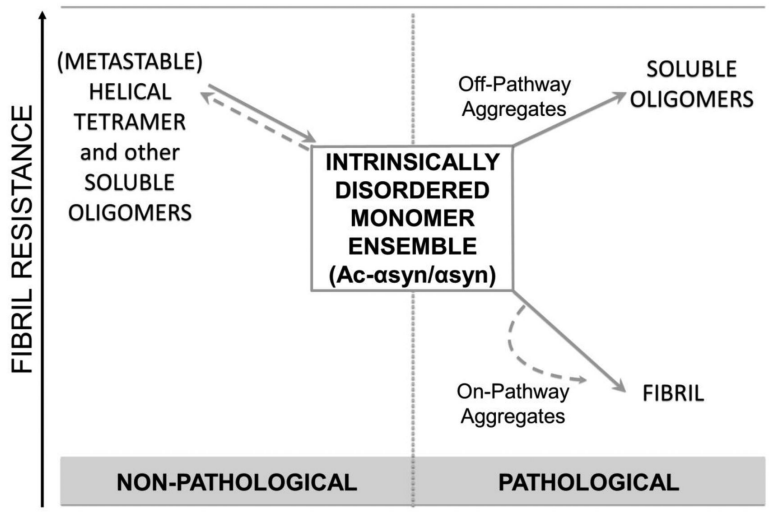

Figure 1. A schematic diagram of the possible accessible states of non-acetylated and acetylated αsyn

The right side represents two possible pathological aggregation pathways from the unfolded monomeric ensemble to 1) insoluble fibrils through on-pathway transient oligomeric intermediates and 2) to off-pathway soluble oligomers. Off-pathway soluble oligomers represent non-fibrillar end products of aggregation. The left side presents 1) the recent proposal that αsyn can exist as a soluble fibril resistant helical tetramer which is acetylated, and 2) other known oligomers that are not toxic such as methionine oxidized oligomers. It is proposed that the non-pathological tetramer needs to dissociate to the monomeric ensemble before pathological aggregation can occur (dark arrow).

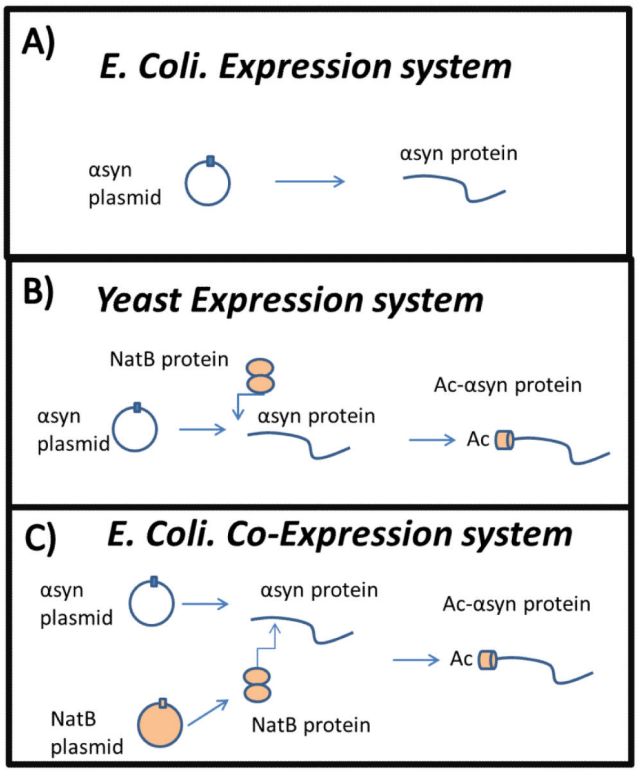

Figure 2. A pictoral representation of the co-expression system designed to generate Ac-αsyn in bacteria

In this figure: ring-like circles represent plasmids, lines represent the αsyn protein, and two ellipses represent the NatB protein. A) Bacteria, lacking NatB, express non-acetylated protein. B) Yeast house yeast-NatB which acts upon αsyn and generates Ac-αsyn. C) To obtain Ac-αsyn within a bacterial expression system, plasmids encoding yeast-NatB can be co-expressed with the plasmid encoding for αsyn so that Ac-αsyn is obtained[164].

Table 1

Historical description of shifts in αsyn purification approaches and conformational properties.

	1996- Dec. 2011	> Dec 2011	> May 2012
Source	Mostly Bacterial	Mammalian	Bacterial / Mammalian
N-terminal acetylation	No	Yes	Yes
Purification protocol	Often denaturing	Non-denaturing	Denaturing and non-denaturing
Average secondary structure	Primarily random coil	Primarily helical	Primarily random coil
Transient initiating N-terminal helix	No	---	Yes
Primary native state	Monomer	Tetramer	Primarily monomer
Fibril Prone	Yes	No	Yes
Referring section within text	2-3	5, 7*	6, 8

*The most recent report by Selkoe and colleagues, suggest “metastability” of the tetramer (section 7).