MOLECULAR TRIGGERS OF DISEASE-RELATED SYNuclein AGGREGATION
IN THE PARKINSON’S ENVIRONMENT

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The works in this thesis explores the challenges involved in recapitulating the environment in which the synuclein family of proteins resides, functions and are active participants in Parkinson’s disease (PD) pathophysiology. The link between α-synuclein (αS or asyn) aggregation and PD neurodegeneration has been established by its presence in fibrillar form in intracellular inclusions known as Lewy Bodies (LBs) and associated oligomerization, whereas β-synuclein (βS or βsyn) is thought to be a non-fibrillogenic partner to αS, beneficial to disease. The enclosed work covers two main topics surrounding the environmental sensitivities of these intrinsically disordered proteins’ (IDPs) role in generating disease-associated aggregation: 1) the impact of N-terminal acetylation on αS fibrillation, and 2) the pH responsive source of βS’ inhibited nature. First we generate an N-terminally acetylated αS in response to an investigation of the native oligomeric state of αS in the physiological environment. We find that N-terminal acetylation has minimal impact on the disordered monomeric ensemble of αS and that no evidence of a preference for a tetrameric or other kind of oligomer is found. We find also that acetylated αS forms fibrils of in a similar timeframe and morphology as the non-acetylated form
of the protein. While the impact on our view of αS as an intrinsically disordered monomer ensemble is relatively non-consequential, we go on to show that with at least one binding partner, Cu$^{2+}$, N-terminal acetylation can have a significant impact \textit{in vivo}. Cu$^{2+}$ had been shown \textit{in vitro} to have a unique accelerating effect on αS fibrillation at low, physiological stoichiometries, and given the redox active nature of the metal, and the role of metal dyshomeostasis in neurodegeneration, it had been garnering interest as a significant player in disease-associated αS aggregation. However, we go on to show that N-terminal acetylation occurs at the most significant of three Cu$^{2+}$ binding sites, blocks binding of the metal and abolishes any fibrillation accelerating effect. Therefore, this work forces reconsideration of how the N-terminally acetylated αS interacts with partners \textit{in vivo}, and the ultimate role of Cu$^{2+}$ as an exacerbator of αS pathophysiological aggregation, which may still occur through two other, although much weaker, binding sites at its histidine and C-terminus. In the second part of this thesis, we discover that the model for the physiological environment typically used to draw the conclusion that βS is non-fibrillogenic in contrast to αS, may not be adequate to use to infer things about its intracellular behavior. We discover that mildly acidic pH 5.8, achievable in several intracellular sub-environments, turns on a fibrillation switch and allows βS to form fibrils. We use this pH-responsiveness and a set of α/βS domain-swapped chimeras to study the roots of βS’ less fibril prone nature. We discover through the chimeras that the NAC domain is the most significant determinant of aggregation behavior, and through elimination of pH sensitive sites, and Rosetta modeling of βS on an αS fibril structure, that acidic side chains can modulate the on/off fibrillation switch. This is further demonstrated in pH insensitive Glu→Ala mutants and the pH-responsiveness is suggested to be rooted in glutamate side chains. This work implies that a more complex role for βS in disease and its partnership with αS may be possible than currently understood, and previously undetected. These two projects emphasize that when considering IDP misfolding in neurodegeneration \textit{in vitro}, it is difficult to remove the surface-exposed IDP from its environment in which its behavior is so intricately entwined, but also provides some insight
into factors we must consider in our models, including: co/post-translation modifications, binding partners, and solution environment pH.
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An acknowledgements section of mine wouldn’t be mine if I also didn’t reach farther back in (my) history. An influential English teacher of mine in high-school used to (and still does) say “Science is a euphemism for magic.” At first, you, if you’re a scientist reading this in particular, might find that phrase silly, but carrying it with me has allowed me to continually be amazed by incrementally small advancements and insights through frustrations while conducting research. And what I have loved is that the literary concept of a “willing suspension of disbelief” has allowed me to lean on that phrase, to grow from at first disbelief to the understandings we have as scientists. I am grateful to have been part of such a cumulative body of scientific progress that eventually branched off into my field of disease-associated protein misfolding. I’m grateful to be part of the greater scientific field that will always grow existing and new branches with all
of the collective understanding of many participating minds at its base. Every act toward scientific discovery and understanding is necessarily, I find, an incredibly humbling act. I’m grateful this scientific perspective has touched my life, and hopefully the contributions from my work in Dr. Baum’s group will be small contributions that aides futures of scientists, particularly those seeking out our best health fighting against the disbelief of the devastating effects of neurodegeneration.

Thank you to everyone who has aided my part in the endeavors inside and related to this thesis.
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Figure 1. An illustration of the proposed causative link between αS aggregation and PD progression. In the box above, a close-up view of an αS monomeric ensemble is depicted in light grey. Two αS monomeric forms are highlighted, with their N- and C-terminal domains highlighted to emphasize that molecular level insights have been gained and are being developed as targets in PD, but that the research is ongoing. This is represented by an arrow that leads to a cartoon of a human brain, slightly outside of the monomeric ensemble, that is filled with a representative TEM image of αS fibrils that occur in disease. The entirety of the headspace is overtaken by these fibrils to represent how debilitating PD is, and how important the linked process of αS fibrillation appears to be in disease development.

Neurodegenerative diseases including Parkinson's, Alzheimer's, Amyotrophic Lateral Sclerosis and Huntington's disease are not only incurable and debilitating, but widespread in the aging population. Approximately 1% of people over the age of 60 are affected by Parkinson's disease (PD) and suffers effects from the progressive loss of the dopaminergic neurons in the substantia nigra of the brain. Patients with PD often develop motor symptoms such as bradykenesias, tremors and postural instability, and in later stages of disease, cognitive impairments and dementia. Once in motion, the disease is progressive and patients will largely continue to suffer from these symptoms for the rest of their lives as current disease therapies are
insufficient. Dopamine replacement therapy is among the best available to counter the loss of
dopaminergic neurons, but is also significantly unfavorable. Levadopa, the metabolic precursor to
dopamine, can be administered to deal with motor symptoms of the disease, but with significant
disadvantages. Not only can levadopa adversely affect cognition, but it is also ineffective as a
long-term therapy with patients eventually developing the same, and possibly more erratic motor
symptoms, than a patient who has not received the same therapy. Therefore, while some
medications including dopamine agonists and levadopa, and others to treat psychiatric and
cognitive impairments, can be administered temporarily relieve some symptoms of PD-associated
neurodegeneration, no significant long-term relief or cure to PD currently exists. (1-3)

The basis of PD is complicated because it is multi-factorial in origin, where both genetic and
environmental factors correlate to the development of disease. Huge developments in
understanding the molecular origins of PD came about when it was first recognized that in Lewy
Bodies (LBs), proteinaceous intracytoplasmic inclusions, α-synuclein (αS) is a dominant portion
of the proteinaceous material,(4, 5) and when several familial mutants of αS were associated with
an early onset form of the disease.(6) With its link to disease origins, the understanding of PD as
a “synucleinopathy” has become an important point of focus in identifying drug targets to halt
progression of or cure PD.(7)

The link between neurodegeneration and protein misfolding in general is substantial, where
the progression of disease often correlates with the deposition of normally soluble proteins.
(Figure 1) αS is a 140 amino acid primarily cytosolic protein of unknown function that
accumulates in an aggregated form in LBs in PD, and is described in more detail in introductory
sections elsewhere in this thesis (Chapter 2 in particular). In these inclusions, αS accumulates as
an amyloid deposit, meaning it forms insoluble fibrils of parallel cross-β sheet structure along an
axis often up to several microns in length. (8, 9) While the many processes and their interplay that
surround neurodegeneration include ageing, oxidative stress, conformational changes in proteins,
defective proteolysis and clearance and glial inflammation to name a few, the aggregation of
monomeric αS to misfolded fibrillogenic forms is considered to be a significant causative link or adjacent process, (10-14) as is protein misfolding the case in other neurodegenerative diseases. (15) In Alzheimer's disease, for example, the aggregation of amyloid-β (Aβ) peptide to fibrils has been linked to disease, related by its insolubility and neurotoxicity. While the initial view in targeting protein misfolding in Alzheimer's centered around the idea that fibrils are themselves toxic, years of research have modified an initial “amyloid hypothesis” of Alzheimer's of one which describes the products of fibrillation as neurotoxic agents, to the process of aggregation as being the neurotoxic event. Fibrillogenesis is not often a two state process, and nowadays, the focus on Aβ as causative target of Alzheimer's disease has grown into the amyloid cascade hypothesis, or oligomer hypothesis, to include on and off pathway aggregates, which may include soluble but aggregated oligomeric forms of the monomeric protein, as the actual toxic and neurodegenerative agents.(16) This intermediate including fibrillation process targeted-thinking has carried over into the study of molecular origins of αS and PD, initially urging researchers to ask, can we halt the aggregation process of αS and subsequently control the fate of PD?

Although the amyloid/oligomer-type hypothesis is a way to narrow the field of potential drug targets around αS in PD, αS remains a significantly complicated target. αS belongs to the class of intrinsically disordered proteins (IDPs). Against typical structure-function models of folded proteins, the structure of an IDP does not closely average around typical secondary and tertiary structures characteristic of folded proteins, but rather the non-aggregated αS is described by an ensemble of largely monomeric (and dimeric) interconverting unfolded forms. (17, 18) As the αS monomer ensemble associates within itself, the decision to embrace the aggregated and/or insoluble form comes with a reduction in degrees structural and dynamic freedom of system, where semi-folded or folded oligomeric forms develop. Fibrils of the typical amyloid structure form, of folded monomers at ~4.5 Angstrom interstrand spacing along a fibril axis of up to several microns in length, form, (9) as do oligomers of a heterogeneous nature. The aggregation process itself, therefore, further narrows the drug target, and has lead to exploration of
immunotherapies which directly target unwanted aggregated forms, and the development of molecules like disaggregases which can pry apart amyloid into monomeric or lower-order aggregated forms. While some successes have come from this research, there are several disadvantages to only approaching these aggregated forms as disease targets. The first is obviously that it is currently unknown whether oligomers or fibrils themselves are the neurotoxic agents, and the study of transient, still heterogeneous and lowly populated oligomeric forms remains elusive and challenging. Additionally, even though “fibrils” in general share structural features, several polymorphs of synuclein fibrils have been suggested in the literature, there is no controlled way to develop different polymorphs in vitro, and it’s not known if they all exhibit neurotoxic effect in vivo. Perhaps the most significant disadvantage of approaching these aggregates as targets, however, comes from the fact that their appearance and accumulation may only be associated with later stages of disease, when it is expected to be even harder to get many dysregulated cellular processes back under control and normalized. (19-21) Are there early events in the monomeric ensemble associations that tip it toward an aggregation prone system, before significant dysregulation and dyshomeostasis in cellular processes and damage to cellular material occurs in the intracellular environment?

PD is, for example, closely associated with oxidative stress. Whether oxidative stress itself is a causative agent of disease is currently unknown, but we can certainly understand that it may play many roles in αS aggregation and neurotoxicity. Small modifications to oxidation susceptible side chains of the monomer can dramatically alter (often accelerating) the aggregation process, (22-24) and once formed, it is currently relatively unexplored how immunotherapies or disaggregases, for example might function upon oxidatively modified forms of the protein. αS, it should be noted, is particularly susceptible to modification along the length of its chain. IDPs generally engage in weak intracellular cloud-like interactions in the cell. (25) There ability to bind many targets reversibly and relatively weakly serves to make them ideal to function as hubs of intracellular regulation and communication. Additionally, they function in sensitive ways in
what may be a regulatory role, because they are unfolded and the majority of the chain is exposed to the environment. (18, 26) This allows both binding to multiple targets and a sensitivity of the unfolded monomer to modification in the cellular environment in detecting partners, but also open to modification in the pathophysiological environment. Potentially these modifications may affect not only the solvent-exposed monomers, but also easily affect the surface exposed parts of αS in the aggregated states it samples. Differently post-translationally modified chains of αS may also function differently in the cell, binding partners differently, and so perhaps the most ideal target, despite an inherently enormous structural complexity, may be rooted in the primarily cytosolic monomeric form, before a loss of the soluble function of the monomer, or gain of toxic function of the aggregated forms occurs in substantial amounts.

Although αS is in its origin an IDP monomeric ensemble, the ensemble is not as unfolded as would be expected for a completely solvent-exposed, non-interactive chain. Some “tertiary” contact dynamics have been identified in αS, where contacts are sampled more than expected between regions of the N- and C-terminal domains of the protein. (27-29) While there are many instances where the collapse in bulk hydrodynamic correlates with aggregation propensity, the lowly populated N- to C-terminal reactions sometimes thought to be responsible for this contraction of the ensemble, have in bulk also been labeled as protective. (30) Therefore, in this work, we explore how local modifications at several trigger points along the αS polypeptide chain can contribute to the aggregation propensity of the ensemble and ultimately direct disease progression. For example, the C-terminal portion of αS remains solvent exposed in the fibril, (9) and many significant inhibitory interactions have been attributed to it, and its relationship with the other domains of the protein, including the N-terminal portion. Therefore, when a N-terminal modification of N-terminal acetylation was highlighted in the αS literature and was suggested to alter the native oligomeric state of the protein, (31) We investigated how and if this small modification impacts the aggregation processes the protein engages in. (32) We then further asked, how secondary processes in the cell, like the binding to redox active metals across the
length of the solvent exposed monomeric polypeptide, and its subsequent behavior in the face of metal dyshomeostasis accompanying oxidative stress, could impact disease progression through the αS aggregation process. In doing so, we not only demonstrate that single-site modifications are important considerations when identifying drug targets, but that an imbalance of the ensemble can occur from several points of the protein. (33) To further develop a molecular level view of an aggregation prone or inhibited ensemble, we explore the non-fibrillating and co-localizing homolog of αS, β-synuclein (βS). We develop a set of chimeric proteins, which swap α/βS domains, and study them in the context of an environmentally sensitive pH-dependent fibrillation switch. We are able to localize the loss of inhibition to processes at specific acidic side chains, and also show modulation that comes from the entirety of the nature of the monomeric ensemble. (34) These insights have the capacity to develop into both important spatial and temporal considerations when identifying drug targets and developing effective therapeutic strategies in PD.
2. CHAPTER 2: A REVIEW OF THE AGGREGATION STATES OF αSYN IN LIGHT OF N-TERMINAL ACETYLYATION

2.1. CITATION AND LICENSE


- The details of reuse of the above listed paper in Chapter 2 of this dissertation is detailed in chapter 7.1.

2.2. PUBLICATION INFORMATION

2.2.1. TITLE

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

2.2.2. AUTHOR INFORMATION

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2.2.3. ABSTRACT AND KEYWORDS

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

2.2.4. 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

2.2.5. ACKNOWLEDGEMENTS

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2.3. 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 (35, 36). 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 (37). αsyn, a major protein component of Lewy Bodies (5, 38) in patients with Parkinson's, is a small primarily neuronal protein that is known to make a structural transition to amyloid fibrils (39-41). αsyn is expressed abundantly in the nervous system and localizes near presynaptic nerve terminals (42-46). It is also expressed at high levels in erythrocytes and platelets (47). αsyn's function is unknown, but there is strong evidence that it exhibits lipid binding in vesicles and synaptic membranes (48) and may somehow exert its pathology through this behavior (49). There is evidence that αsyn functions in assembly of the SNARE complex involved in vesicle transport (50), that it may more generally be involved in synaptic vesicle trafficking and regulation and/or may play a key role in neuronal cell survival (51-54).

The deposition of αsyn has largely been thought to originate from an intrinsically disordered monomer ensemble that under fibril promoting conditions forms amyloid (40, 55, 56), but recently this view of αsyn's native state was challenged (31). 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 (31, 32, 57-63). We summarize major shifts within recently published
works addressing these issues in (*The* most recent report by Selkoe and colleagues suggests “metastability” of the tetramer.

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(55), that have largely been studied in the context of PD-related pathology (Figure 2). 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 2). 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.
Figure 2 A schematic of αsyn accessible conformations. 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 (solid arrow). The relationship between the unfolded monomeric ensemble and the proposed tetramer is a subject of investigation (dashed arrow).

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<tr>
<td>Source</td>
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<tr>
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<td>–</td>
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<td>5, 7*</td>
<td>6, 8</td>
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*The most recent report by Selkoe and colleagues suggests “metastability” of the tetramer.

Table 1. Historical description of shifts in αsyn purification approaches and conformational properties.
2.4. OVERVIEW OF NON-ACETYLATED ASYN ENSEMBLE: MONOMERS AND DIMERS

2.4.1. 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 (64-68). Uversky first spoke of αsyn as the “protein chameleon” (55) 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 (39, 69, 70). 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 3A) (64, 71, 72). 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 (64). 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 (64, 73, 74).
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(64, 66, 76). However, the protein is not fully extended for a protein of its size, implying a slight compaction of the monomeric ensemble(66, 77). Evidence for contact between the C-terminus and both the NAC and N-terminal regions of the protein from nuclear magnetic resonance (NMR)(27, 68, 78-84), electron paramagnetic resonance(85) and molecular dynamic studies(28, 81) indicates a possible source of this compaction, as well as some transient secondary structure(84, 86-88). 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(27,
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. Rather, this is a dynamic interaction, and observation of slight compaction is the result of observations on a highly averaged bulk ensemble.

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, as their release is associated with increased fibril formation. 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. Environmental or experimental shifts that reduce the net charge or increase hydrophobicity of the protein, or interaction with small molecules or metal ions 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. 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.

The N-terminal region is also known to adopt helical structure upon binding lipids, representing a more dramatic conformational shift of the monomer ensemble. 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(77, 110). Bax et al. 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(108). Because αsyn localizes near synaptic nerve termini(42), its lipid-induced helical structure(65, 108, 111, 112) 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.

2.4.2 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(83). 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(32, 113, 114), 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(115). 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(116), 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(29). 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(66). 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(117) and cysteine mutants have shown that particular dimer linkages accelerate fibril formation in vivo and in vitro(118). 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 cytotoxicity. 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.

2.5. / 3 HETEROGENEITY OF THE ASYN OLIGOMERIC STRUCTURES AND THEIR PATHOGENICITY

2.5.1. / 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(119-126). 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(127). Over and over, conversion of IDPs into amyloid aggregates has not been observed to be a simple two-state transition(128). Oligomer formation has been established as an important mechanistic step in fibril formation, for example as in Alzheimer's disease(125, 126, 129, 130). As briefly described in αsyn, soluble oligomeric intermediates commonly appear as insoluble fibrils form(129, 131, 132) 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(133). 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(134). 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(29, 123, 135-139). 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(137, 140).

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(141-144). At least one report demonstrates that in vivo membrane associated αsyn oligomers correlate with toxicity rather than inclusion formation(140) 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 (100, 120). These mutants may exert their pathology through the formation of pore-like oligomers that form alongside fibril formation (145).

2.5.2. OLIGOMERS ASSOCIATED WITH THE FIBRILLATION 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 (129, 146). 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 (66). 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 (129, 135). 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 (131). 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 (147). Annular species of αsyn are known to induce membrane leakage (126, 148), but spheroidal species can bind brain-derived membranes quite tightly, as well (147). Spherical morphologies seem to disappear once the fibril has formed, whereas annular species may sometimes coexist with the fibril (147). 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(132, 135, 146). 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(69).

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(149). 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(150, 151). This mix of species only further describes the range of the secondary structures, morphologies and pathologies that oligomers of αsyn are capable of populating(29, 115, 117, 152, 153). 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(152, 154-157).

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(147, 158-161). Also, incubation with different types of metals generates partially folded structures(96, 162, 163) that go onto form a variety of oligomeric structures. Whereas incubation with Cu²⁺/Fe³⁺/Ni²⁺ produce spherical oligomers of 0.8–4 nm particles, incubation with Co²⁺/Ca²⁺ produces yields pore-like annular rings 70–90 nm in diameter(164).

2.5.3/3.3 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 2). 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 onto form fibrils(165-167). 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 2)(166). 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(168). Structurally these species are spherical, have a well developed secondary structure, and 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(168). 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 αsyn. 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(169).

2.6. 4 ASYN IS N-TERMINALLY ACETYLATED IN VIVO

Before attention was drawn to the possible role of the acetyl group by the recent report (31) 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 report by Selkoe and colleagues 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 (153, 165, 170-173). 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)(31, 175, 176). The report indicated that acetylation of αsyn was not limited to neuronal tissue; however, the site of acetylation was not identified.

2.6.1.4.1 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 (177). 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 (72). The aforementioned MS studies ohrfelt (175, 176) 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 (174, 178). 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(177, 179, 180). Therefore, depending on the type of cell to synthesize αsyn, the protein may or may not be acetylated (Figure 3A and B). 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(177).

2.6.2./4.2 POSSIBLE ROLE OF N-TERMINAL ACETYLATION IN VIVO

Recognizing that αsyn acetylation does indeed occur, one study prior to Selkoe and colleagues’ investigated the role of NatB activity in a yeast model by disrupting NatB activity(109). 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(181). 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(182). N-terminal acetylation may also regulate degradation pathway or be responsible for structural effects at the N-terminus(183). 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(184) 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(185). 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(186).

2.7. /5 ASYN 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 (31) 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, so that *Escherichia coli* derived boiled αsyn, too, will migrate near the position of the tetramer at 58 kDa on a native gel (31).

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 (64). Therefore Bartels et al. 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/asyn monomer). At the same time, a higher lipid binding capacity for this “native” asyn 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, Thioflavin T (ThT) fluorescence did not indicate that RBC ac-asyn 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. (57) 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.

2.8. SUBSEQUENT STUDIES INDICATE THAT ASYN EXISTS AS A PRIMARILY UNFOLDED MONOMER

A rapid period of overlapping work began to determine the oligomeric state of asyn from various cell sources, under non-denaturing conditions and to investigate the role of the acetyl group modification. Lashuel and colleagues examined asyn 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).

In response to report by Bartels et al., Lashuel and colleagues determine that αsyn exists as an unfolded monomer within neuronal sources. Fauvet et al. 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 and colleagues 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. 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 demonstrated a sensitivity of CN-PAGE to small differences in the protein composition and went onto 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 \( \alpha \)syn co-migrate with recombinant \( \alpha \)syn. 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(63, 188). 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(63). 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(31) did differently, but Fauvet et al (63) 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 \( \alpha \)syn protocol and cannot replicate the dynamic tetramer observations of Wang et al.

Concurrently, Rhoades and colleagues sought to determine if the nature of the purification method (61)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. Trexler et al. is the first to use a bacterial co-expression system to generate Ac-\( \alpha \)syn (Figure 3C). In this co-expression system developed by Mulvihill et al.(75), the yeast analog to NatB is cloned into a bacterial plasmid, allowing overexpression of \( \alpha \)syn 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 \( \alpha \)syn close to 100% of the time in E.
coli. Trexler et al. 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 and colleagues also encounter 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.

2.9. CONTINUED DISCUSSION ON THE OLIGOMERIZATION STATE OF ASYN

In response to the studies that indicate that cellular αsyn is an unfolded monomer(58, 63), Selkoe and coworkers(62), 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 4 °C as opposed to 37 °C. 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.
2.10. N-TERMINAL ACETYLATION OF MONOMERIC ASYN INDUCES 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. (32), 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 (59) 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 (58) show by in-cell NMR that non-acetylated αsyn is a disordered monomer in the macromolecular environment of the cell. Lashuel's group (59) 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-αsyn is unfolded monomer (Table 1).

Table 1.

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 (59, 60). However, the N-terminal acetyl group affects the transient secondary structure as observed by NMR (32, 59, 60). Residue-specific NMR chemical shift analysis shows that there is an increase in the transient helical propensity at the initiating N-terminus (32, 59, 60) 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 (189, 190).
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(59) and Bax(60) found no significant differences in fibril formation rate, Kang et al.(32) 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.

2.11. CONCLUSIONS

Recent studies that suggested that αsyn 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\(^{(166)}\). 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 \(\alpha\text{syn}\) to adopt many conformations in a variety of oligomeric states, working from the monomer ensemble of Ac-\(\alpha\text{syn}\) 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 \(\alpha\text{syn}\).
3. **CHAPTER 3: A DETAILED LOOK AT THE EFFECT OF N-TERMINAL ACETYLYATION ON THE αSYN MONOMERIC ENSEMBLE**

3.1. **CITATION AND LICENSE**

- The work published in the above listed paper comprises the entirety of Chapter 3 of this dissertation, and its reuse in this dissertation is detailed in Chapter 7.2.

3.2. **PUBLICATION INFORMATION**

3.2.1. **TITLE**

N-terminal acetylation of α-synuclein induces increased transient helical propensity and decreased aggregation rates in the intrinsically disordered monomer

3.2.2. **AUTHOR INFORMATION AND CONTRIBUTIONS AND FUNDING**

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Lijuan Kang and Gina M. Moriarty contributed equally to this work.
3.2.3. **ABBREVIATIONS**

αsyn, alpha synuclein; Ac-αsyn, acetylated αsyn; BOG, β-octyl glucoside; EM, fluorescence electron microscopy; ESI-IMS-MS, ion mobility spectrometry combined with ESI-MS; ESI-MS, noncovalent electrospray ionization mass spectrometry; IDP, intrinsically disordered protein; NatB, N-acetyltransferase B; SEC, analytical size-exclusion chromatography; ThT, Thioflavin T

3.2.4. **ACKNOWLEDGEMENTS**

The authors thank Elizabeth Rhoades for helpful discussions and for the suggestion of the NatB co-expression system to make acetylated α-synuclein; Vikas Nanda for access to CD; and Mr. Valentin Starovoytov for assistance with TEM images.

3.3. **INTRODUCTION**

Alpha synuclein (αsyn) is a small primarily neuronal protein that is known to make a structural transition to amyloid fibrils in several neurodegenerative diseases. It is a major component of Lewy Bodies in patients with Parkinson's, a disease resulting from a loss of dopaminergic neurons.\(^{(5, 191)}\) While a large body of evidence over many years has supported the characterization of αsyn as an intrinsically disordered monomer, a recent study by Bartels et al. in which αsyn was isolated from red blood cells, as well as neuronal and non-neuronal cell lines, reported that in its physiological form, αsyn exists as a helical tetramer that is resistant to amyloid formation, and has a mass corresponding to the sole modification of the monomer by an acetyl group.\(^{(31)}\) Shortly thereafter, a GST recombinant αsyn protein purified from the micellar reagent β-octyl glucoside (BOG), similarly showed the existence of a dynamic αsyn tetramer.\(^{(57)}\) In response to these papers, Faivert et al. and an assemblage of groups went on to demonstrate that αsyn isolated from rodent and human nervous system tissues and erythrocytes presents as an intrinsically disordered monomer. In this work, Fauvet et al. was the first to address the role of the acetyl group, referred to in the Bartel et al. paper, and showed the acetylated and
nonacetylated proteins migrate similarly on nondenaturing gels. A follow-up report by Trexler et al. has indicated that recombinant acetylated αsyn (Ac-αsyn) is monomeric under physiological conditions, but that it may display a greater preference for helical structure and higher-order oligomerization states when purified in the presence of BOG.

It has been demonstrated that soluble and insoluble fractions of brain tissues from patients suffering from Parkinson's and from dementia with Lewy bodies universally contain N-terminal Ac-αsyn. While an uncommon modification to prokaryotic proteins, the N-termini of eukaryotic proteins are often processed at the initiating amino acid with the addition of an acetyl group by N-acetyltransferase complexes. The role of N-terminal acetylation, however, is poorly understood, but has been suggested to affect the thermal stability of proteins. Because N-terminal Ac-αsyn is now believed to be the physiologically relevant species in the brain, it is critically important to characterize the conformational properties and fibrillation kinetics of this protein to understand how acetylation impacts on the mechanism of fibril formation and disease.

Here we present the first direct experimental evidence that N-terminal acetylation affects the secondary structure propensities and kinetics of fibril assembly of Ac-αsyn, relative to the nonacetylated protein. Using NMR, noncovalent electrospray ionization mass spectrometry (ESI-MS), ion mobility spectrometry combined with ESI-MS (ESI-IMS-MS), Thioflavin T (ThT) fluorescence, and electron microscopy (EM), we demonstrate that the 100% N-terminal acetylated recombinant αsyn protein purified under mild physiological conditions presents primarily as a disordered monomer. Our results highlight that N-terminal acetylation impacts on secondary structure propensity in important functional regions including the N-terminal and His-50 metal binding regions and the regions of the three familial mutations A30P, E46K, and A53T. The removal of the positive charge at the N-terminus arising from acetylation thus has short and long-range conformational effects that impact on the distribution of states sampled by the intrinsically disordered αsyn monomer and on the rate of fibril assembly.
3.4. RESULTS AND DISCUSSION

To determine the oligomeric status and the conformational properties of Ac-αsyn and to compare this with αsyn, Ac-αsyn was generated from an E. coli coexpression system containing the yeast N-terminal acetyltransferase B (NatB) and purified using mild physiological purification conditions (herein described as “mild” purification) that avoid steps involving the application of heat or salting out (herein described as “harsh” conditions) (see Supporting Information for detailed protocols of “mild” and “harsh” purification). Taking into account recent suggestions that harsh purification steps such as boiling can destroy tetramer formation or that purification under nonphysiological conditions can promote higher-order oligomerization states of αsyn, a mild physiological purification protocol that applies only homogenization and liquid chromatography was adopted for Ac-αsyn in this work. ESI-MS confirms that purified Ac-αsyn coexpressed with this eukaryotic modification system exists as 100% acetylated protein (observed mass 14502.5 Da, expected mass 14502.1 Da) [Figure 4 (A,B)].

Investigation of the existence of higher-order oligomeric states in the Ac-αsyn was performed using ESI-MS and ESI-IMS-MS (Figure 4), along with solution methods including analytical size-exclusion chromatography (SEC) and migration on a native gel (Figure 7). The latter two methods show that both proteins elute at the same volume as a single peak using SEC and migrate at the same position on the native gel. These data show that the acetylated protein exhibits indistinguishable hydrodynamic dimensions from the nonacetylated protein purified under mild conditions. Furthermore, comparison by native gel, SEC, 1H-15N HSQC and ESI-MS (Figure 8) of the nonacetylated αsyn purified by harsh or mild purification protocols indicate that they are essentially indistinguishable, consistent with other comparisons made by Fauvet et al.(63)

ESI-IMS-MS experiments add further information about the oligomerization status of Ac-αsyn, as the population distributions can be obtained quantitatively from these experiments by the ability of ESI-IMS-MS to separate peaks of identical m/z and to quantify their population and conformational properties.(196) Comparison of the ESI-IMS-MS spectra of Ac-αsyn and αsyn
indicate that acetylation does not perturb the oligomerization status of αsyn, as both αsyn and Ac-αsyn appear predominantly monomeric (~90–95%), with the presence of a small population of dimeric species (~5–10%), where the former appears in both more compact and extended forms at physiological pH (Figure 4(A,B)). These data are consistent with previous analyses using ESI-MS,(113, 114, 197) and interchain NMR PRE experiments that have shown weak dimer N- to C-terminal interchain interactions in αsyn under physiological conditions.(83) Because of the suggestion in the recent literature that Ac-αsyn purified under micellar BOG conditions can shift the monomer populations toward oligomeric species,(61) the possibility of higher-order transient oligomeric species purified under physiological conditions was further probed using a higher cone voltage (170 V) in ESI-IMS-MS, which favors detection of large noncovalent aggregates. These experiments revealed no difference between the oligomeric distribution of acetylated and nonacetylated samples and no evidence for the population of higher-order species, consistent with results obtained by Trexler et al. when purification was performed under physiological conditions.(61) While the biophysical techniques inform about the hydrodynamic radii, ESI-IMS-MS provides definite evidence that both proteins are predominantly monomeric in aqueous solution at pH 7.4.
Residue-specific analysis of Ac-αsyn by NMR was next pursued to enable the secondary structure propensities of Ac-αsyn and αsyn monomers to be compared (Figure 5). Backbone assignments by triple resonance experiments confirmed that Ac-αsyn is acetylated on the N-terminal residue Met-1 (Figure 9). An overlay of the 1H-15N spectrum of Ac-αsyn and αsyn at pH 7.4, shows that the two proteins share a high degree of similarity, except at the first nine N-terminal residues (Figure 5A). Both Ac-αsyn and αsyn display narrow chemical shift dispersion, characteristic of a predominantly unfolded protein, consistent with analyses using far UV CD (Figure 10). Acetylation of the N-terminus results in the appearance of the Met-1 and Asp-2 resonances in the Ac-αsyn 1H-15N HSQC spectrum possibly because of changes in hydrogen exchange rates arising from the modification at Met-1. Additionally, acetylation results in upfield shifting of residues observable in both spectra in the region of the first nine N-terminal residues.
residues, [inset Figure 5A] further supporting the site of acetylation as Met-1 and demonstrating the extent to which N-terminal acetylation alters the conformational properties of the polypeptide chain.

The secondary structure propensity of Ac-αsyn was next examined by analyzing NMR chemical shift perturbations and was compared with that of αsyn (Figure 5). A number of methods have been developed to determine the secondary structural propensities of unfolded proteins.(198-200) Here we use secondary structure propensity scores (SSP)(199) which represent ensemble-averaged values over a distribution of states to obtain the secondary structure propensities of both Ac-αsyn and αsyn. Paralleling the chemical shift deviations observed in $^1$H-$^{15}$N HSQC spectra [Figure 5 (A)], increased SSP values up to 0.3 are observed for the first 12 residues in the N-terminal region of Ac-αsyn. These values are significantly larger than those observed previously for αsyn and mutants of this protein(84, 87, 88, 107) and represent a significant stabilization of transient helix and hence a redistribution of the structural ensemble sampled by the monomeric protein within the N-terminal region [Figure 5(B)]. Longer-range perturbations, although small, are observed, in other regions of the N-terminus in the regions between residues 28–31, 43–46, 50–66 and are marked by a decrease in β-sheet propensity in Ac-αsyn [Figure 5(B)]. By contrast, the NAC and C-terminal regions [Figure 5(B)] remain relatively unperturbed by acetylation. The change of secondary structure propensities arising from acetylation may relate to important structural and functional properties of the protein. Specifically, changes are observed at Met-1, Asp-2, and His-50, the high affinity copper binding regions,(49, 201-203) and at the three familial mutants A30P, E46K, and A53T associated with Parkinson's disease that affect the rate of fibril formation.(100, 104) Together, the results reveal that N-terminal acetylation of αsyn does not by itself cause the intrinsically disordered protein (IDP) to self-assemble into tetrameric or other higher oligomeric forms, however, marked short range and subtle long-range effects of N-terminal acetylation are observed on the disordered monomer.
Figure 5. NMR characterization of Ac-αSyn. A. $^1$H-$^{15}$N HSQC spectra of Ac-αsyn (magenta) versus αsyn (blue) at 15°C. The changes in the first nine residues of Ac-αsyn are indicated in the spectrum. The Δδ (ppm) for the inset is calculated by the expression $((\Delta H)^2 + (0.159*\Delta^{15}N)^2)$ (inset). B. Top: Schematic of αsyn divided into N-terminal, NAC and C-terminal regions. Middle: SSP analysis of Ac-αsyn (magenta) and αsyn (blue) using $^{13}$Cα and $^{13}$Cβ chemical shifts as input and a five residue sliding window with Zhang et al. random coil references. (205) $^{13}$C assignments for Ac-αsyn were obtained from a 350 μM doubly labeled protein in PBS buffer at 15°C and $^{13}$C assignments for αsyn were used from a previous report. (84) Bottom: Differences of SSP (ΔSSP = SSP(Ac-αsyn) – SSP(αsyn)) between Ac-αsyn and αsyn with boxes shown at positions of familial mutations. $^1$H-$^{15}$N HSQC comparison of αsyn purified under mild and harsh conditions indicate that they are very similar (Figure 8).

While N-terminal acetylation has been shown to increase helicity in peptides, (190) the data presented here represent the first investigation of acetylation at the N-terminus of a full-length IDP. The increased helicity in Ac-αsyn can be rationalized by stabilization of the helix macrodipole, (206) where removal of the α-amino positive charge upon acetylation is favorable to the overall dipole moment of the helix that this IDP transiently samples. The acetyl group is also known to form a highly favorable helix N-cap, in which the acetyl-carbonyl group interacts favorably with unsatisfied hydrogen bond donors in the N-terminal turn of the helix. (189, 207)

The fibrillation properties of Ac-αsyn and αsyn were also examined using ThT fluorescence to provide macroscopic information about the role of N-terminal acetylation in modifying the efficiency of fibril nucleation and elongation. The normalized ThT fluorescence of Ac-αsyn and
αsyn at pH 7.4 exhibit typical sigmoidal curves for fibril assembly (Figure 11). The lag times of the samples were highly variable, thereby ruling out analysis of the role of N-terminal acetylation in fibril nucleation. The elongation rates are slower in the acetylated versus nonacetylated αsyn proteins for all experimental trails. For both the lag times and elongation rates we note that the aggregation kinetics are sensitive to the purification methods [Figure 6(A)]. The change in fibrillation rate may result from stabilization of the N-terminal region of the protein by acetylation, or changes in secondary structure propensities at residues 50–66, which have been shown previously to have significant effects upon the kinetics of fibril formation,(17, 84, 87) or both. Physicochemical changes resulting from acetylation may affect electrostatic and hydrophobic interactions, resulting in alterations in transient long-range contacts between the highly charged C-terminal region and the helix stabilized N-terminal region. This redistribution of states may have effects upon fibril formation.

This work presents the first NMR structural characterization of N-terminal Ac-αsyn and illustrates the effect of N-terminal acetylation on the fibrillation rates of the protein. We demonstrate conclusively using ESI-IMS-MS that the equilibrium states that are sampled in the Ac-αsyn are primarily monomer and a small population of dimer. NMR data support the view that both Ac-αsyn and αsyn exist at pH 7.4 as intrinsically disordered monomers, and further shows that N-terminal acetylation results in significant stabilization of transient helical propensity in the first 9 residues of the protein along with longer range changes in secondary structure between residues 50–66 and around the three familial mutants A30P, E46K, and A53T. These regions represent important functional regions associated with metal ion binding and with familial mutants that affect aggregation rates. We show that N-terminal acetylation not only changes the distribution of the intrinsically disordered monomeric conformers within the structural ensemble of αsyn, but that this cotranslational modification disfavors fibril formation, presumably caused by the conformational redistribution of the monomeric protein.
3.5. MATERIALS AND METHODS

3.5.1. PROTEIN EXPRESSION AND PURIFICATION

Expression of acetylated protein was achieved by coexpressing the αsyn plasmid and the NatB plasmid in E. coli. using a procedure similar to that described by Trexler et al.(61) The NatB plasmid was kindly gifted by Dr. Daniel Mulvihill. Acetylated αsyn was purified only under “mild” physiological conditions and nonacetylated αsyn protein was purified under both “mild” physiological and “harsh” conditions for comparison (see Supporting Information for detailed descriptions of Ac-αsyn expression and “harsh” and “mild” purification protocols).

3.5.2. BIOPHYSICAL CHARACTERIZATION

NMR assignments, ThT fluorescence and TEM experiments were performed as described previously.(84) Native gel, SEC and CD procedures are described along with Supporting Information Figures S1 and S2, respectively. ESI-IMS-MS experiments were performed using similar procedures to Smith et al.(196)αsyn proteins (35 μM) were dissolved or buffer exchanged
into 165 mM ammonium acetate, pH 7.4 and the sampling cone voltage was varied from native conditions (30 V) to conditions that allow for the detection of large noncovalent species (170 V).

3.6. ACKNOWLEDGEMENTS

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3.7. SUPPLEMENTARY INFORMATION

3.7.1. PROTEIN EXPRESSION OF ACETYLATED ASYN

The acetylated αsyn (Ac-αsyn) was produced by co-expressing the αsyn plasmid and NatB plasmid in E. coli. The NatB plasmid was a kind gift from Dr. Daniel Mulvihill. The αsyn and NatB plasmids were first co-transformed into E. coli. E. coli cells were cultured in LB medium (Sigma) with 25 μg/L chloramphenicol (Sigma) and 50 μg/L ampicillin (Fisher) and grown in Erlenmeyer flasks at 37 °C with vigorous shaking. Expression was induced by addition of 1 mM isopropyl-β-D-1-galactopyranoside (Fisher) once the cell culture had reached an OD₆₀₀ of 0.4-0.6 and incubation was then allowed to proceed at 20 °C overnight. Cells were harvested the next morning. Uniform isotope labeling of acetylated αsyn was performed in standard minimal M9 medium containing ¹⁵NH₄Cl (CIL, Inc.) as the sole nitrogen source and ¹³C-glucose (CIL, Inc.) was substituted for ¹²C-glucose (Sigma) when ¹³C/¹⁵N doubly labeled samples were expressed.

3.7.2. PROTEIN PURIFICATION

Ac-αsyn was purified only under “mild” conditions and αsyn protein was purified under both “mild physiological” and “harsh” conditions. Comparison of αsyn purified under harsh or mild purification protocols by ¹H-¹⁵N HSQC, ESI-MS, CD, native gel and SEC indicate that they are essentially indistinguishable.

(i) Mild protocol: Pelleted cells were retrieved from storage at -80°C and suspended in 25 mM Tris HCl buffer, pH 7.7. The cell pellet was then homogenized three times at 10,000-15,000
psi. The cell lysates were centrifuged for 30 minutes at 13,000 rpm. The supernatants were then passed through a 0.22 µm filter before being loaded onto a HiTrap Q 5 mL column on an AKTA FPLC system (GE Healthcare LifeSciences). The column was equilibrated with 25 mM Tris HCl, pH 7.7 and αsyn was eluted by applying increasing concentrations of up to 500 mM NaCl. αsyn usually eluted at ~250 mM NaCl. αsyn enriched fractions were concentrated to 2-3 mL using an Amicon 10k MWCO centrifugal filter (Millipore) before being loaded onto a Superdex 75 HiLoad 26/600 prep grade size exclusion column (GE Healthcare LifeSciences). The column was equilibrated with 25 mM Tris HCl and 250 mM NaCl or phosphate buffered saline (PBS) before sample injection. Pure protein fractions were detected by SDS-PAGE. Fractions containing αsyn were then collected and flash-frozen in liquid nitrogen before storage at -80 °C.

(ii) Harsh protocol: Purification of αsyn was as previously described(84). Briefly, pelleted cells were suspended in PBS and homogenized three times at 10,000-15,000 psi. The cell lysates were centrifuged and streptomycin sulfate (Fisher) (10 mg/ml) was added to the supernatant and the mixture was stirred at 4 °C for 30 minutes, followed by centrifugation. Ammonium sulfate (Sigma) (0.361 g/ml) was added to the supernatant and the mixture was stirred at 4 °C for 30 minutes, followed by centrifugation. The pellet was re-suspended in PBS and boiled for 20 minutes. After the mixture cooled down, the mixture was centrifuged. The supernatant was dialyzed against 25 mM Tris HCl buffer overnight. The supernatants were then passed through a 0.22 µm filter before being loaded onto a Hitrap Q column on an AKTA FPLC system (GE Healthcare LifeSciences). The column was equilibrated with 25 mM Tris HCl, pH 7.7 and αsyn was eluted by applying increasing concentrations of up to 500 mM NaCl. αsyn usually eluted at ~250 mM NaCl. The αsyn enriched fractions were dialyzed four times against ammonium bicarbonate (Sigma), flash-frozen in liquid nitrogen, lyophilized and stored at -20 °C.

3.7.3. SUPPLEMENTARY FIGURES
Figure 7_SI.1. Ac-αsyn and αsyn purification. Ac-αsyn and αsyn both purified under mild conditions elute at the same position in analytical SEC and migrate similarly by native gel electrophoresis. Additionally they migrate as previously observed for unfolded monomers2; 3. A. Analytical SEC profiles of purified Ac-αsyn (lower panel) and αsyn (upper panel). All αsyn samples were thawed from storage at -80°C or from lyophilized powder stored at -20°C. The samples were prepared by dissolving them in PBS if needed, and passing them through an Amicon 100k MWCO filter to remove large aggregates. Samples were concentrated to 100 µM if needed with an Amicon 10k MWCO centrifugal filter (Millipore). 100 µL was loaded onto the column. Samples were eluted at 0.5 mL/min on a Superdex 200 10/300 (GE Healthcare Lifesciences) column in PBS, pH 7.4. B. Native gel electrophoresis Lane 1: BSA (66 kDa); Lane 2: Ac-αsyn (14.502 kDa); Lane 3: αsyn (14.460 kDa) ; Lane 4: αsyn G132C (14.506 kDa and 29 kDa). BSA marks the migration position of a 66 kDa globular protein. G132C αsyn, which also migrates as monomer and covalently linked dimer on SDS-PAGE marks the migration of a unfolded monomer of 14.5 kDa and dimer on the native gel. Native gel electrophoresis was performed on 13% acrylamide home-made gels which were prepared according to standard protocol in the absence of sodium dodecyl sulfate. Ac-αsyn and αsyn samples purified from mild conditions, BSA (Sigma), and lyophilized G132C αsyn purified from harsh conditions were loaded onto gels with Native Sample buffer (Bio-Rad). Gels were run at 140V for 3 hours and stained with SimplyBlue SafeStain (Life Technologies Corporation).
Figure 8_SI.2. Comparison of harsh and mild purification protocols. αSyn purified using mild (blue) and harsh conditions (green)(84) are biochemically and structurally indistinguishable. A. Analytical SEC (conditions described in S1); B. Native gel electrophoresis (conditions described in Figure 7_SI). Lane 1: BSA, Lane 2: mildly purified αsyn; Lane 3: harshly purified αsyn; Lane 4: harshly purified G132C αsyn. C. ¹H-¹⁵N HSQC NMR spectra obtained by Lashuel also showed that αsyn purified under harsh and mild conditions exists as a disordered monomer that gives rise to similar ¹H-¹⁵N HSQC spectra(208).
**Figure 9_SI.3. Acetyl group $^{13}$CA/B NMR signature.** The CBCA(CO)NH (left) and HNCACB (right) spectrum of Ac-αsyn. The experiments were acquired on a Varian 800 MHz spectrometer (350 μM) at 15 °C. Met-1 is labeled as M1. The rectangular box designates the $^{13}$C methyl carbon of the acetyl group that is shown by a star on the schematic representation of Ac-αsyn at the top of the graph.
Figure 10_SI.4. Circular Dichroism of αsyn and Ac-αsyn. Far UV CD spectra of the 10 μM Ac-αsyn (magenta) and αsyn (blue) using the mild purification protocol in 10 μM sodium phosphate buffer, pH 7.4 at 37°C indicates the random coil nature of both proteins by the characteristic minima at 195 nm. Samples were thawed from storage in PBS buffer at -80°C and passed through Amicon 100k MWCO centrifugal filter (Millipore) filter. Samples were then buffer exchanged five times into 10 μM sodium phosphate buffer, pH 7.4 to remove chloride ions using an Amicon 10k MWCO centrifugal filter. Spectra were obtained on an AVIV420SF circular dichroism spectrophotometer. Measurements were made at 37 °C from 190-260 nm at a step size of 0.5 nm. The averaging time was 4 seconds, the settling time was 1 second, and three scans were averaged. The contribution from buffer was subtracted and data were normalized to concentrations obtained from absorbance at 280 nm (ε_{280}=5120 M^{-1} cm^{-1}) prior to dilution for measurement to approximately 10 μM.
Figure 11_SI.5. ThT fibrillation of αsyn and Ac-αsyn. The normalized ThT fluorescence data of Ac-αsyn (magenta) and αsyn (blue). Protein was thawed and exchanged to PBS buffer pH 7.4, NaCl 137 mM (dissolved from Sigma PBS tablet) using Amicon 10k MWCO centrifugal filter (Millipore). The solution was filtered through Amicon 100k MWCO centrifugal filter (Millipore) to remove the big aggregates and the final protein concentration was around 150 µM ($\varepsilon_{280}=5120$ M$^{-1}$ cm$^{-1}$) with 20 µM ThioflavinT for fluorescence measurements. 100 µL of the mixture was then pipetted into a well of the 96-well clear-bottom black-wall plate (Costar) and sealed with clear sealing film (Axygen) to prevent the evaporation during incubation. Measurements were recorded at 37°C with linear shaking at 600 rpm. ThT fluorescence was recorded at 30-min intervals using a POLARstar Omega reader (BMG, Inc.). Both Ac-αsyn and αsyn form fibrils within 230 hours. The data were fitted using the Boltzmann function under the sigmoidal category in Origin software. The data were normalized using initial and final values from the Boltzmann fit.
4. CHAPTER 4: CU$^{2+}$ BINDING INDUCED FIBRILLATION IN THE PRESENCE OF N-TERMINAL ACETYLAITION

4.1. MOTIVATION FOR THE WORK

At the time this work was published, there was an extensive ongoing effort in the literature to characterize the details of Cu$^{2+}$ binding to αS because it appeared to be a potentially relevant intracellular partner that accelerated its fibrillation at relatively low concentrations in vitro. Cu$^{2+}$ is also a redox active metal, and given the significant role oxidative stress plays in PD, and in accelerating and exacerbating deleterious effects of αS aggregation, Cu$^{2+}$ could likely play an important role in related processes.

Then, when the report was published that garnered the urgent attention of the community of researchers on αS in PD, suggesting that αS is natively a helical tetrameric form, this had the potential to alter the way everyone crafted the hypotheses that drove their research. Although significant evidence was again gathered to support the view of αS an as intrinsically disordered monomeric ensemble in vivo, the endeavor did highlight a key co-translational modification to the protein. αS is ubiquitously N-terminally acetylated in the body. While we have shown that N-terminal acetylation exerts a relatively mild effect on the αS monomeric ensemble, and development of the fibril, there was still the potential, that this modification could play a big role in the cellular environment.

Because N-terminal acetylation occurs at a key Cu$^{2+}$ binding site, we felt a study of Cu$^{2+}$ binding to the N-terminally acetylated form would big a significant contribution to the ongoing efforts to understand Cu$^{2+}$ and metal-binding, which can frequently occur to the intrinsically disordered monomeric form of αS impacts the protein in disease.

4.2. PUBLISHED WORK:

4.2.1. CITATION AND LICENSE

• The entirety of Chapter 4.2 is republished from the above listed published work, and its reuse in this dissertation is detailed in Chapter 7.3

4.2.2. PUBLICATION INFORMATION

4.2.2.1. Title

A Revised Picture of the Cu(II)–α-Synuclein Complex: The Role of N-Terminal Acetylation

4.2.2.2. Author Information and Funding

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4.2.2.3. Abstract

α-Synuclein (αS) is an amyloidogenic intrinsically disordered protein implicated in Parkinson’s disease, for which copper-mediated pathways of neurodegeneration have been suggested. We have employed nuclear magnetic resonance, circular dichroism, electrospray ionization mass spectrometry, and thioflavin T fluorescence to characterize interactions of Cu²⁺ with the physiological acetylated form (Ac-αS). Significantly, N-terminal acetylation abolishes Cu²⁺ binding at the high-affinity M1-D2 site present in the nonacetylated protein and maintains Cu²⁺ interactions around H50/D121. Fibrillation enhancement observed at an equimolar Cu²⁺
stoichiometry with the nonacetylated model does not occur with Ac-αS. These findings open new avenues of investigation into Cu\(^{2+}\)-mediated neurodegenerative pathology suggested in vivo.

4.2.3. BODY OF TEXT

The link between oxidative stress and protein aggregation in neurodegenerative disorders, including the assembly of α-synuclein (αS) amyloid fibrils in Parkinson’s disease (PD), is ever-expanding. Potentially aberrant redox-active metal chemistry in the highly respiratory neural environment may induce formation of harmful oxidants. Endogenous biometals may therefore reinforce oxidative neurodegeneration by dysregulation of metal transport and metabolism. An emerging role for copper in PD is suggested on the basis of its abnormal distribution within the brain. In PD patients, overall depressions of copper levels in the brain and increased levels of copper in the cerebrospinal fluid have been observed. Moreover, spatial imaging detects a complex localization of copper within the PD midbrain (209).

α-Synuclein is a 140-residue intrinsically disordered protein (IDP) for which an abundance of evidence reveals that the native form undergoes N-terminal acetylation in vivo. (31, 175, 176, 210) Despite the initial recognition of this co-translational modification by Anderson et al., a general awareness of N-terminal acetylation among in vitro αS researchers arose when Bartels et al. rediscovered this mammalian modification within the context of a possible helical tetrameric form of the protein.(31) Following this investigation, several in vitro studies have focused on the role of acetylation in αS (59-61, 211, 212). However, the majority of in vitro studies on the Cu\(^{2+}\)–αS complex predate an interest in the acetylated protein form, and the commonly accepted picture of the Cu\(^{2+}\)–αS complex is rooted in a nonacetylated αS model (i.e., nonAc-αS). Multiple lines of evidence reveal that Cu\(^{2+}\) binds nonAc-αS at three specific sites (Figure 12A) (201-203, 213-215), namely the, (1) high-affinity N-terminal M1-D2 site 1, (2) lower-affinity H50 site 2 also located at the N-terminus, and (3) non-stoichiometric low-affinity C-terminal site 3 anchored around D121. Although an extensive literature debate (216)(please see references therein) addresses the relative involvement of sites 1 and 2, one widely accepted model of the highest-
affinity complex of Cu$^{2+}$ with nonAc-αS is centered at site 1, where Cu$^{2+}$ is readily anchored by the freely available amino-terminal nitrogen. (216)

In this report, we characterize the impact of N-terminal acetylation within full-length Ac-αS on the conformation and fibrillation of the Cu$^{2+}$-αS complex using nuclear magnetic resonance (NMR), electrospray ionization mass spectrometry (ESI-MS), visible circular dichroism (CD) spectroscopy, and thioflavin T (ThT) fluorescence amyloid assays. We demonstrate that Ac-αS lacks the highest-affinity amino-terminal Cu$^{2+}$ binding site, maintains low-affinity binding with the H50 and D121 sites, and undergoes altered amyloid formation modulated by the presence of Cu$^{2+}$. These findings suggest that Ac-αS is the appropriate experimental model for in vitro Cu$^{2+}$ binding studies and copper-mediated αS pathology in PD.

NMR has assumed a key role in sequence specific mapping of Cu$^{2+}$ binding, and we employ this approach to examine interactions of Cu$^{2+}$ with full-length Ac-αS. Figure 12A depicts the main putative anchoring residues (M1D2, H50, and D121) at each of the three Cu$^{2+}$ binding sites as described in previous studies employing nonAc-αS.(201-203, 213, 216) Normalized $^1$H–$^{15}$N heteronuclear single-quantum coherence (HSQC) spectroscopy intensities in the presence of 0.5 equiv of Cu$^{2+}$ pinpoint the regions where paramagnetic Cu$^{2+}$ localizes in nonAc-αS and Ac-αS (Figure 12B). Significant differences in signal attenuation are observed at site 1 in the presence of acetylation, while smaller intensity differences are observed at weaker affinity sites 2 and 3. A Cu$^{2+}$ titration shows that the differences seen with 0.5 equiv of Cu$^{2+}$ remain similar up to an equimolar stoichiometry (Figure 14), demonstrating that N-terminal acetylation abolishes the relevance of high-affinity site 1 Cu$^{2+}$ binding in Ac-αS and preserves the binding at sites 2 and 3.

The NMR data indicate somewhat enhanced line broadening within the proximity of D121 at site 3 in Ac-αS relative to non-Ac-αS (Figure 14B and Figure 16C of the Supporting Information) despite the fact that their C-termini do not differ in sequence or modification. To evaluate whether the Cu$^{2+}$ binding profile is altered at the C-terminus due to N-terminal acetylation, we have employed Mn$^{2+}$, which binds exclusively to the C-terminus, as an alternate
paramagnetic probe.(213) The Mn\(^{2+}\) attenuation profiles are nearly superimposable (Figure S3A of the Supporting Information) for nonAc-\(\alpha\)S and Ac-\(\alpha\)S, suggesting that N-terminal acetylation alone does not account for the differences in intensity of the Cu\(^{2+}\) -bound Ac-\(\alpha\)S complex. Cu\(^{2+}\) titration data (Figure 16B,C of the Supporting Information) indicate that enhanced line broadening in region 3a versus region 3b is observed across the Cu\(^{2+}\) concentration range, suggesting the Cu\(^{2+}\) ion distributes itself differently at site 3 when site 1 is acetylated. Whereas Mn\(^{2+}\) binds only sites 3a and 3b, Cu\(^{2+}\) binds across the length of the protein with both N- and C-terminal contributions.

Therefore, attenuation differences observed at site 3a may be partially derived from Cu\(^{2+}\) -mediated N-terminal interactions. Future mutagenesis studies at Cu\(^{2+}\) binding sites will elucidate the extent of independence or cooperativity between N- and C-terminal sites. Consistent with our NMR data, previous studies of short N-terminally acetylated \(\alpha\)S peptide models similarly reflect an inability to bind Cu\(^{2+}\).(217, 218) The limitation of these peptide model studies is that Cu\(^{2+}\) interactions are isolated to single binding sites, making it difficult to account for the complete range of interactions between multiple Cu\(^{2+}\) binding sites within the full-length protein. In this respect, our NMR results demonstrate that Cu\(^{2+}\) binding in the full-length N-terminal acetylated protein can still occur at sites 2 and 3 in the absence of the participation of site 1. In addition, Cu\(^{2+}\) binding may also function as a probe of preexisting long-range contacts in \(\alpha\)S, or metal-mediated interactions between Cu\(^{2+}\) binding sites distributed throughout the length of the protein. Visible CD difference spectra of Ac-\(\alpha\)S and nonAc-\(\alpha\)S in the presence of Cu\(^{2+}\) (Figure 12C) indicate, as the NMR data suggest, the inability of Ac-\(\alpha\)S to complete a coordination sphere with site 1 atoms. Spectral profiles of the nonAc-\(\alpha\)S–Cu\(^{2+}\) complex reported previously are characterized by the codevelopment of negative ellipticity arising from a deprotonated backbone nitrogen charge transfer transition at ~300 nm and weak positive ellipticity (d → d) at ~600 nm(216) up to an equimolar ratio, reflective of a 1:1 stoichiometry. While Figure 12C presents expected ellipticity changes for the nonAc-\(\alpha\)S–Cu\(^{2+}\) complex, the profile of Ac-\(\alpha\)S is virtually
indistinguishable from the baseline even at a 3:1 copper:protein stoichiometry (Figure 15). Ac-αS does not form a proposed (5,6)-membered ring site (219) with Cu²⁺, as acetylation blocks the anchoring α-amino nitrogen at M1. This may arise from the combined effect of weaker initial anchoring and the inability of a more helical geometry induced by N-terminal acetylation (32, 59, 60) to provide successive stabilizing ligands from the D2 nitrogen and side chain oxygen. A D2A mutation in a nonAc-αS N-terminal peptide also disrupts stable M1-D2 coordination (216) thereby supporting the view that high-affinity anchoring of Cu²⁺ to the M1 site requires this suite of ligands.

ESI-MS is used to evaluate the relative populations of Cu²⁺-bound complexes of nonAc-αS and Ac-αS in a titration from 0 to 10 equiv of Cu²⁺ (Figure 12D,E). In nonAc-αS, the first equivalent of Cu²⁺ shifts most of the unbound species to a population containing one bound Cu²⁺ atom, whereas in Ac-αS, a significant proportion of the unbound species persists. This reflects the absence of site 1 binding in Ac-αS, which is consistent with the NMR and CD data (Figure 1B,C). A higher number of Cu²⁺ equivalents shifts the population further toward species that contain multiple bound Cu²⁺ atoms. This observation suggests that sites 2 and 3 contribute to a lesser extent, corroborating the general consensus of nonAc-αS–Cu²⁺ binding published to date. In Ac-αS, the addition of ≥2 equiv of Cu²⁺ shifts the populations toward bound complexes containing one, two, or more Cu²⁺ atoms, in a fashion reflecting a possible lower-affinity binding to sites 2 and 3 alone (Figure 12E).
Figure 12. Characterization of Cu(II) binding to αS and Ac-αS. A) Schematic highlighting the putative main anchoring residues at each Cu$^{2+}$ site, color-coded for their presence in nonAc-αS (magenta) and Ac-αS (dark blue). (B) Ratio of paramagnetic Cu$^{2+}$ 1H–15N HSQC attenuation in the presence of 0.5 equiv (200 μM αS and 100 μM Cu$^{2+}$). (C) Visible CD spectra of 200 μM nonAc-αS and Ac-αS in the presence of 1.0 equiv (200 μM) of Cu$^{2+}$ (D) ESI-MS profiles of the +8 peak in the presence of 0, 1, 2, and 10 equiv of Cu$^{2+}$ for nonAc-αS and Ac-αS. (E) Schematic illustrating a possible avenue of Cu$^{2+}$ binding reflective of the bound metal ratios observed via ESI-MS. In nonAc-αS, an initial event of 0–1 equiv of Cu$^{2+}$ represents binding at high-affinity site 1, while sites 2 and 3 are occupied at a higher number of equivalents with weaker affinity as observed in Ac-αS.

Much of the attention that Cu$^{2+}$ has received stems from its unique role in accelerating nonAc-αS amyloid formation in vitro.\(^{(213, 220)}\) In view of the lower Cu$^{2+}$ affinity implied by the absence of site 1 binding in Ac-αS, we have conducted ThT fluorescence amyloid assays (Figure 13. ThT fibrillation kinetics in the presence of and absence of Cu(II) in αS and Ac-αS.) to assess whether Cu$^{2+}$ binding at sites 2 and 3 is capable of accelerating amyloid formation. ThT assays conducted with nonAc-αS reveal that Cu$^{2+}$ binding at the lowest stoichiometries accelerates fibrillation in terms of growth rate and lag phase as noted in previous studies.\(^{(220)}\)
Conversely, ThT assays of Ac-αS reveal that 1 equiv of Cu²⁺ imparts a relatively minor effect on aggregation kinetics, while 2 equiv may more dramatically impact the aggregation kinetics (Figure S4 and the discussion of the Supporting Information). These trends are consistent with the Cu²⁺-bound population distributions of Ac-αS observed in the ESI-MS experiments, at low and higher stoichiometries, suggesting that weak Cu²⁺ binding at sites 2 and 3 may have a complex effect on aggregation kinetics. Interestingly, the biological relevance of Cu²⁺–Ac-αS interactions might not derive solely from the acceleration of fibrillation. In fact, previous studies have demonstrated that Cu²⁺-induced oligomers can represent the toxic species. The mechanism by which the aggregation kinetics are altered by interactions of Cu²⁺ with sites 2 and 3 requires further investigation into the conformational steps leading to formation of soluble oligomers and ordered fibrils.

In summary, the impact of N-terminal acetylation on Cu²⁺ binding reported herein effectively reshapes the consideration of Cu²⁺-mediated pathology in vivo. In conjunction with recent evidence gleaned from biophysical studies, our findings suggest that the acetylated protein is essential for characterizing Cu²⁺–αS interactions in vitro. While the complexity surrounding coordination of Cu²⁺ to the nonacetylated protein remains, N-terminal acetylation elegantly resolves the issue of the relative contributions of the M1 and H50 binding sites and localizes Cu²⁺-induced pathology to H50 and the C-terminus within Ac-αS. The results of this report are particularly intriguing in view of the role that copper assumes in localization of the protein coupled with the impact of acetylation on αS membrane affinity and reshaping. Our revised model may assist in elucidating the mechanisms by which Cu²⁺ induces formation of β-like conformers or mediates the balance between protective and pathological intra- and/or interchain interactions.
4.2.4. **SUPPLEMENTARY INFORMATION**

4.2.4.1. **Methods**

4.2.4.1.1. **Protein expression**

Protein expression of non-acetylated α-synuclein (nonAc-αS) and acetylated α-synuclein (Ac-αS) was performed as described in Kang et al.1 (32) 15N labeled samples were prepared for NMR and non-labeled samples were prepared for circular dichroism (CD) spectroscopy, electrospray ionization-mass spectrometry (ESI-MS) and the Thioflavin T (ThT) fluorescence assay. Ac-αS was produced by co-expressing the αS plasmid and N-acetyltransferase B (NatB) plasmid in E. coli. The NatB plasmid was a kind gift from Dr. Daniel Mulvihill.(75) Cells were harvested and frozen at -80°C until purification. Frozen pellets were thawed and re-suspended in phosphate buffered saline (PBS), 137 mM NaCl, pH 7.4 and homogenized three times at 10-15,000 psi. The lysates were centrifuged at 14,000 rpm for 30 minutes. The supernatants were stirred with 0.010 g/mL streptomycin sulfate at 4°C for 20 minutes and centrifuged again. The supernatant was stirred for 1 hour with 0.361 g/mL (NH4)2SO4 at 4°C and centrifuged once more. The pellets were re-suspended in PBS, 137 mM NaCl, pH 7.4 and boiled in a hot water bath for 15 minutes followed by centrifugation. The supernatants were dialyzed overnight at 4°C against Buffer A (25 mM Tris, pH 8.0). Samples were applied to a HiTrap Q column on an AKTA FPLC system (GE Healthcare Life Sciences) at 4°C. The column was equilibrated with 8 column volumes of Buffer...
A. A stepwise gradient at 0, 15, 30, 50 and 100 % 25 mM Tris 500 mM NaCl pH 8.0 (4 °C) was applied and the proteins eluted at 50 %. The nonAc-αS and Ac-αS containing fractions were pooled and dialyzed four times into 20 mM NH₄HCO₃ prior to lyophilization and storage at -20°C.

4.2.4.1.2. **NMR**

¹H-¹⁵N HSQC spectra were acquired on a Bruker 600 MHz spectrometer. Lyophilized samples were dissolved in 90% 20 mM MES, 20 mM MOPS, 100 mM NaCl pH 7.1 and 10% D2O. Samples were passed through an Amicon 100K MWCO centrifugal filter (Millipore) to remove undissolved aggregates. A 10K filter was employed as needed to concentrate samples for the Cu²⁺ titration to 300 µM and to 200 µM for the Mn²⁺ titration. Aliquots of metal were added to a 500-600 µL sample volume from an aqueous 0.015 M stock solution of CuSO₄·5H₂O (Aldrich) and MnCl₂·4H₂O (Sigma) prepared from weighing the solid without further preparation. ¹H-¹⁵N- HSQC spectra were collected from the hsqcetpf3gpsi pulse sequence at 15°C. Spectra were processed with nmrPipe3 and centered peak intensities were obtained as data heights from Sparky, which were used to calculate the normalized signal attenuation. Assignments for nonAc-αS and Ac-αS were transferred from those published previously in PBS, 137 mM NaCl, pH 7.4. The intensities of spectrally overlapped resonances that may yield unreliable data heights are designated in Figures 1B and S3A by an empty white circle at the indicated residue. For ease of language in discussion of the experimental data, we use the term “equivalents” to imply a 1:1 stoichiometry (i.e. 200 µM protein to 200 µM total Cu²⁺ meaning one “equivalent” for both nonAc-αS and Ac-αS).

4.2.4.1.3. **Visible Circular Dichroism**

Visible circular dichroism (CD) spectra were recorded on an Aviv Model 420 Circular Dichroism spectrophotometer at 15.0°C. Solutions of nonAc-αS and Ac-αS at 200 µM were prepared as detailed in the NMR Methods section in 20 mM MES, 20 mM MOPS, and 100 mM NaCl pH 6.5 employing similar experimental conditions as described in a previous report.
Wavelength scans were acquired in a 1 cm quartz cuvette over the range of 750.0 to 250.0 nm at 2 nm intervals employing an averaging time of 4 seconds and a bandwidth of 1 nm. Four scans were acquired and signal averaged as depicted in Figure 12C. Data of Ac-αS reported in Figure 15 was recorded at 300 nm by signal averaging for 10 minutes employing a time constant of 6 seconds and bandwidth of 1 nm. For nonAc-αS, the protein standard was titrated with Cu$^{2+}$ by adding µL aliquots of a 0.015 M CuSO$_4$ stock (described in the above section) to a sample volume of 1.2 mL. Data are expressed as the differential ellipticity relative to protein in the absence of Cu$^{2+}$ at the indicated metal:protein ratios.

4.2.4.1.4. **Electrospray Ionization mass spectrometry**

Electrospray ionization mass spectrometry (ESI-MS) experiments were performed on a Finnigan LTQ spectrometer (Thermo Electron Corporation) equipped with an electrospray ionization (ESI) source. ESI-MS data was acquired in positive ion mode with a spray voltage of 5 kV, a capillary temperature of 200°C, and a 32V capillary voltage. Samples were injected at 13 µL/min, sampled for 5 minutes, and measured across the 600-4000 m/z range. 50 µM samples of nonAc-αS and Ac-αS were prepared as described in the NMR Methods section in 50 mM NH$_4$CH$_3$COO buffer, pH 7.5 and a 100X concentrated CuSO$_4$ stock solution was used for the desired equivalent amount.

4.2.4.1.5. **ThT fluorescence**

Thioflavin T (ThT) fluorescence was monitored for 150 µM nonAc-μS and Ac-μS in 20 mM MES, 20 mM MOPS, and 100 mM NaCl adjusted to pH 7.4 as described previously(84) with the addition of 3 mm PTFE beads (Taylor Scientific) to each well in a COSTAR-96 well plate. CuSO$_4$ stocks were prepared as described in the NMR Methods section at 100X excess and 1µL added to each well at the desired concentration. Figure 13 presents the average of three or four time-dependent ThT fluorescence assays for nonAc-μS and Ac-μS in the absence and presence of CuSO$_4$. The raw data for 0, 1, and 2 Cu$^{2+}$ equivalents with nonAc-αS and Ac-αS appears in Figure 17.
4.2.4.2. Figures

Figure 14_SI.1. Heat map representation of Cu(II) binding to Ac-αS and αS. A) The putative main anchoring residues at each of the three sites are indicated in boxes colored for their presence as Cu$^{2+}$-binding sites in nonAc-αS (magenta) and Ac-αS (blue). B) and C) Heat-map representations of paramagnetic signal attenuation for Cu$^{2+}$ titrations within nonAc-αS (B) and Ac-αS (C) in 20 mM MOPS, 20 mM MES, 100 mM NaCl, pH 7.1. 1H-15N HSQC signal attenuation is normalized relative to the intensity in the absence of Cu$^{2+}$ (I0) and is represented during the course of the titration up to a 1:1 stoichiometry by a color gradient. Full signal attenuation (I/I0 = 0) is represented in black while no attenuation (I/I0 = 1) appears as white.
Figure 15_SI.2.Visible circular dichroism Cu$^{2+}$ titration of nonAc-αS and Ac-αS at 300 nm. Circular dichroism differential ellipticity at 300 nm for 200 μM nonAc-αS (magenta) and Ac-αS (blue) titrated with Cu$^{2+}$ at 15 °C in a buffer system comprised of 20 mM MES, 20 mM MOPS, 100 mM NaCl, pH 6.5. The ellipticity difference (Δmdeg) is reported relative to protein in the absence of Cu$^{2+}$. Results for nonAc-αS are consistent with previously published studies in which the ellipticity monitored at 300 nm reflects a 1:1 metal:protein stoichiometry in the absence of acetylation. In contrast, the ellipticity of Ac-αS is relatively constant and does not develop the same copper coordination sphere up to a 3:1 metal:protein stoichiometry suggesting the absence of high-affinity ligands at the amino terminus.
Figure 16_SI.3. 1H-15N HSQC of nonAc-μS and Ac-μS in the presence of Mn\(^{2+}\) and discussion of C-terminal attenuation by Cu\(^{2+}\). A) \(^{1}\)H-15N HSQC signal height in the presence of 0.4 Mn\(^{2+}\) equivalents for nonAc-αS (magenta) and Ac-αS (dark blue). Sites of Cu\(^{2+}\) binding are indicated at site 1, site 2 and sites 3a and 3b with darker grey shading from left to right. Overlapped peaks in the HSQC are indicated by white circles. B) The C-terminal sequence of αS encompassing site 3 in its entirety (i.e., residues 109-140) is aligned to highlight C-terminal repeats in the sequence and the preferred metal binding regions in each repeat. Preferred metal binding regions site 3a (118-124) and site 3b (134-140) are indicated by solid green lines, and dashed green lines indicate a weaker preference for the sub-region of site 3 which includes the residues between sites 3a and 3b. C) Correlation plots of the attenuation at each Cu\(^{2+}\) equivalent for Ac-αS (dark blue) and nonAc-αS (magenta) highlight the differences observed in Cu\(^{2+}\) - induced C-terminal attenuation between nonAc-αS and Ac-αS. Measurements were collected every 0.1 equivalent over the course of a titration up to 1 equivalent Cu\(^{2+}\) as described in the Methods: NMR section, and the attenuation data at each point was averaged over the indicated residues in the sub-region. The correlation of average attenuation in the indicated sub-region over the course of titration is presented as follows: for the first C-terminal repeat metal binding region (residues 118-124) vs. the entire C-terminus (96-140) (left), for the second C-terminal repeat region (residues 134-140) vs. the entire C-terminus (middle), and the two repeat regions (right).
Discussion of Figure S3: Mn\(^{2+}\) binding is confined to the C-terminal region in \(\alpha S\),(213)\) and the superimposable intensity profiles of Mn\(^{2+}\)-induced attenuation between nonAc-\(\alpha S\) and Ac-\(\alpha S\) in Figure 16A suggest that the N-terminal modification does not alter the C-terminal conformation to a degree which interferes with the ability of the metal to associate with this region. In contrast, Cu\(^{2+}\) binding profiles of nonAc-\(\alpha S\) and Ac-\(\alpha S\) differ at both the N- and C-termini (refer to Figure 12B). The Cu\(^{2+}\) binding profiles exhibit small yet significant differences at the C-terminal site 3a (Figure 12B) in addition to major differences observed within the N-terminus in the presence of acetylation.

We highlight the subtle differences in C-terminal binding to Cu\(^{2+}\) between nonAc-\(\alpha S\) and Ac-\(\alpha S\) in the correlation plots of Figure 16C. The left panel illustrates that the first repeat is preferentially broadened as noted in the observed deviation from linearity relative to the average broadening over the entire C-terminus for both nonAc-\(\alpha S\) and Ac-\(\alpha S\). In contrast, one observes less deviation from linearity in the middle panel, indicating a lower Cu\(^{2+}\) preference for site 3b relative to the entire C-terminus. Furthermore, there is a greater overall preference for site 3a within Ac-\(\alpha S\) as observed in a slice of the titration (Figure 12B). The right panel of Figure 16C compares broadening between the two sub-regions 3a and 3b and highlights the enhanced Cu\(^{2+}\) specificity of Ac-\(\alpha S\) at site 3a relative to nonAc-\(\alpha S\) over the course of the titration.

Previously, Eliezer and colleagues demonstrated that truncation of the C-terminus does not affect Cu\(^{2+}\) binding at the N-terminal domain.(202) In the full length acetylated model, however, modification at the N-terminus impacts on binding at the C-terminal region. When acetylation blocks the main Cu\(^{2+}\) binding site 1 in the full length protein, a subtle effect to the C-terminus is detected. In the same report, the authors examine the impact of diethylpyrocarbonate (DEPC) modification upon Cu\(^{2+}\) induced attenuation. Rasia et al.(201) previously employed DEPC to chemically block the histidine binding site. Unbeknownst to these authors at the time, DEPC treatment modifies the amino-terminus (site 1) and blocks Cu\(^{2+}\) binding. Eliezer and colleagues
examined this site 1-blocked species via NMR and observed a similar enhancement of attenuation at site 3a. These investigators rationalized the enhanced attenuation in terms of a decreased competition between Cu\(^{2+}\) binding sites when the amino-terminal site 1 is removed in the DEPC modified species.\(^{(202)}\) In light of the information from the Mn\(^{2+}\) binding profiles presented herein, we offer an alternative interpretation of the Cu\(^{2+}\) profiles, namely that Cu\(^{2+}\) can serve as a probe of interactions between the N- and C-domains already known to exist in \(\alpha\)S and/or that Cu\(^{2+}\) mediates cross-talk between the N- and the C-termini, potentially catalyzing amyloidogenic structures.
Figure 17_SI.4. Raw Thioflavin T fluorescence profiles of nonac-as and Ac-as in the presence of 0, 1 and 2 Cu²⁺ equivalents. The normalized Thioflavin T intensity profiles for nonac-as (magenta panel outline, A-C) and Ac-as (dark blue panel outline, D-F) fibrillation assay in the presence of 0 (black), 1 (red) and 2 (blue) Cu²⁺ equivalents. The data for each sample type was acquired in triplicate or quadruplicate and the raw curves are presented in grey dotted lines in each panel. The average profiles are indicated by the solid colored line overlay in each panel. An average of the 0 and 1 Cu²⁺ equivalent data is presented in Figure 2 and discussed in the main text. Consistent with removal of the high-affinity site 1, a marked effect at a 1:1 stoichiometry is observed by the fibrillation enhancement of nonac-as in the presence of 1 Cu²⁺ equivalent, whereas a negligible impact is observed in the case of Ac-as, consistent with the removal of site 1. Herein, we include the data for 2 Cu²⁺ equivalents to demonstrate that a more marked effect is observed in Ac-as when a larger population of the protein is potentially bound to Cu²⁺, illustrating the dissimilarity in behavior relative to nonac-as at the same Cu²⁺ ratios. At present, we do not understand the “overshoot” observed for nonac-as in the presence of 2 Cu²⁺ equivalents (Panel C). In light of the experimental challenges posed by in vitro metal-protein studies and the ligand (Cu²⁺) solubility in a neutral buffered solution, we are particularly cautious in interpreting the observed Ac-as aggregation kinetics at such high Cu²⁺ ratios. Given in vivo evidence for Cu²⁺-mediated pathology, the Ac-as-Cu²⁺ interaction may presumably impact a variety of oligomerization and fibrillation pathways via sites 2 and 3, a working hypothesis that is currently under investigation.

4.3. FUTURE WORK AND COMMENTARY
This work should dramatically shift how all future Cu\(^{2+}\)-αS aggregation-related studies are shaped. If αS should encounter Cu\(^{2+}\), authors must pay attention to the model in which they are working and understand if the protein should be expected to be N-terminally acetylated. If any aggregation acceleration is observed, for example, authors must be careful to acknowledge if binding occurs only at site 2 and 3. Additionally, future investigation of the non-acetylated form likely do not have any physiological relevance, nor does Cu\(^{2+}\) binding at sites with weaker affinity likely pose a significant risk \textit{in vitro} as Cu\(^{2+}\) is unlikely to bind Ac-αS in any significant population.

Since the time of publication, another non-physiological aspect of Cu\(^{2+}\) binding has inspired a whole new set of investigations. In the intracellular environment Cu\(^{2+}\) is unstable, as the intracellular environment is reducing. Therefore, in the cell, αS is likely to encounter monovalent Cu\(^{+}\), and several reports since have investigated the binding of the reduced form of the metal to αS. Because in contrast to Cu\(^{2+}\) which is paramagnetic, Cu\(^{+}\) is diamagnetic and structural information is more easily accessed via NMR. Cu\(^{+}\) binding sites have been identified at both the N- and C-terminal domains, and binding of the metal alters transient secondary structures. Interestingly the methionines which mediate the binding, are also sites which are easily modified in oxidative environments. Therefore, Cu\(^{+}\) binding of the redox active metal to these sites may be involved in some sort of homeostasis, especially because Cu\(^{2+}\) is the stable form of the metal in the extracellular environment, and methionine oxidation may somehow be coupled to αS bridging the barrier of metal oxidation state. Further investigations of reduced copper will provide a more physiological view of how the metal could be involved in PD.
CHAPTER 5: UNDOING OF THE FIBRIL INHIBITED NATURE OF βS

5.1. MOTIVATION FOR THE WORK

β-synuclein (βS) co-localizes with αS. Although the proteins share similar cellular space, and ~60% sequence identity, they behave in contrasting ways at the point of disease. It has been generally accepted that in the physiological environment, αS is a disease-related amyloidogenic protein in PD, and βS is in contrast a fibril-inhibited synuclein that plays a role in ameliorating disease. Therefore, researchers looking to solve the puzzle of neurodegeneration and halt the progression of PD have asked – where do the roots of βS’ inhibition lay? In its sequence, in its structure, or in its dynamics? Perhaps, post-translational modifications, its relations with cellular partners, or the dynamic nature of its intracellular processing is most closely related to its inhibition? These questions ultimately are driven by the curiosity of the research community asking -- can we engineer a way for αS behavior to be more βS-like, less engaged with the process of toxic aggregation in PD, or to enhance beneficial aspects of their partnership, and use what we learn to provide a better therapy for the many who suffer and will be suffering from Parkinson’s disease?

While βS appears to evade capture into insoluble Lewy Bodies, and presents as non-amyloidogenic in in vitro experiments, how sure are we that βS is nothing but benign? Some sequence swaps have been identified which exert an effect deterring βS from its inhibitory nature, some in vitro (non-physiological) environmental conditions have been identified that at least suggest the ability of βS to adopt an αS-like fibrillar structure, and so too have there been recent instances where βS forms aggregates which perhaps contribute to the pathophysiological environment in model systems. These instances, and a latent ability of βS to be fibril-competent, have forced us to reconsider the exclusive classification as a benign, non-aggregating partner to αS in PD. While the protein appears less aggregation prone overall, is its potential engagement in an aggregation pathway part of what can contribute to the pathophysiological environment? In the
following paper, we present both intrinsic and extrinsic factors that work to engage an on/off switch of βS fibrillation, which should be accessible to βS in the physiological environment, and which may imply a more complex and under-detected role of βS in disease.

5.2. PUBLISHED WORK

5.2.1. CITATION AND LICENSE


- The above listed work makes up Chapter 5.2 of this thesis, and its reuse is again detailed in Chapter 7.4

5.2.2. PUBLICATION INFORMATION

5.2.2.1. Title

A pH dependent switch promotes β-synuclein fibril formation via glutamate residues.

5.2.2.2. Author Information and Funding

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Abstract and Keywords

Alpha-synuclein (αS) is the primary protein associated with Parkinson's disease, and undergoes aggregation from its intrinsically disordered monomeric form to a cross-β fibrillar form. The closely related homolog beta-synuclein (βS) is essentially fibril resistant under cytoplasmic physiological conditions. Toxic gain of function by βS has been linked to dysfunction, but the aggregation behavior of βS under altered pH is not well understood. In this work, we compare fibril formation of αS and βS at pH 7.3 and mildly acidic pH 5.8, and demonstrate that pH serves as an on/off switch for βS fibrillation. Using αS/βS domain-swapped chimera constructs and single residue substitutions in βS, we localize the switch to acidic residues in the N-terminal and NAC (non-amyloid-βcomponent) domains of βS. Computational models of βS fibril structures indicate that key glutamate residues (E31, E61) in these domains may be sites of pH-sensitive interactions, and variants E31A and E61A show dramatically altered pH-sensitivity for fibril formation supporting the importance of these charged sidechains in fibril formation of βS. Our results demonstrate that relatively small changes in pH, which occur frequently in the cytoplasm and in secretory pathways, may induce the formation of βS fibrils and suggest a complex role for βS in synuclein cellular homeostasis and Parkinson's disease.
KEYWORDS: Parkinson disease; beta-synuclein; computer modeling; fibril; neurodegeneration; nuclear magnetic resonance (NMR); protein aggregation; synuclein

5.2.2.4. Abbreviations

The abbreviations used are: αS, α-synuclein; βS, β-synuclein; LB, body; PD, Parkinson’s disease; IDP, intrinsically disordered protein; NAC, non-amyloid-β component; PRE, paramagnetic spin relaxation experiment; AFM, atomic force microscopy; MTSL, S-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl)methyl methanesulfonothioate; ThT, thioflavin T; r.m.s.d., root mean square deviation; RDC, residual dipolar coupling; HSQC, hetero- nuclear single quantum coherence; ssNMR, solid state NMR.

5.2.3. Introduction

α-Synuclein (αS), the primary protein component of intra-cytoplasmic inclusions known as Lewy bodies (LB) in Parkinson’s disease (PD), is a 140-residue, predominantly monomeric, intrinsically disordered protein (IDP)(4, 32, 63, 88, 212, 225). It is abundant in the cytosol but present in many organelles(226, 227). The monomers of αS can aggregate to soluble oligomers as well as insoluble oligomers and fibrils, which are considered to be associated with the pathogenesis of PD(39). A closely related family member, β-synuclein (βS) that co-localizes with αS, has ~60% sequence identity with αS but has not been detected in LBs of PD patients(5, 52). Instead, it has been proposed that βS may delay αS fibril formation and ameliorate αS toxicity in vivo by inhibiting αS aggregation(95, 228, 229). Furthermore, despite the high sequence similarity, βS itself does not form fibrils in vitro at cytoplasmic pH without facilitating agents(230). However, recent reports highlight a role for a possible toxic gain-of-function of βS in two different model systems(231, 232), and βS is a component of vesicle-like lesions in the hippocampus, whose formation accompanies dementia in PD(233). In addition, two βS mutations, V70M and P123H, were found in sporadic and familial dementia with LBs and have been
suggested to be involved in lysosomal pathology(234-236). There is an emerging role for βS in the pathophysiology of PD, but the molecular determinants of this role remain poorly studied.

Understanding the complex relationship of synucleins with neurodegeneration requires consideration of their structures and functions in diverse subcellular environments, beyond the cytoplasm. Although the function of αS is still unclear, membranes and synaptic vesicles are sites of function for αS(237, 238), which may have a role in dopamine transmission, trafficking, and regulation of the vesicular pool(238), and βS may play similar roles(239). During their functional life cycle, synucleins likely experience a wide range of pH microenvironments, including environments more acidic than the neutral pH maintained in the neuronal cytosol(240, 241). Intravesicular pH drops along the endocytic pathway to ~6 and in late endosomes and lysosomes to as low as 4.5 (240, 242). In addition to its role in vesicular fusion and trafficking, αS is subject to lysosomal degradation(13), and it may enter the slightly alkaline mitochondrial environment(243). Furthermore, pH dysregulation accompanying oxidative stress can result in cytosolic acidification(244), and oxidative stress is a significant contributor to PD pathology(245). Given the ubiquitous presence of synucleins in different cellular compartments, it is crucial to investigate the environmental pH dependence of βS and αS aggregation to understand synuclein homeostasis and its dysregulation in PD.

In their monomeric forms, αS and βS synucleins are model IDPs that exist as unfolded protein ensembles, only transiently adopting small degrees of local secondary structure (88, 95, 105, 199, 246). IDP side chains will be exposed to the cellular environment to a significantly greater extent than buried side chains of a folded protein, thereby making them more susceptible to environmental changes but uniquely able to respond rapidly to these changes and perform regulatory roles(18, 247). Subcellular pH differences between organelles may have more profound effects on IDPs, increasing local hydrophobicity as side-chain charge decreases. Altered charge/hydrophobicity balance will therefore change the conformational preferences in a particular region of an IDP and modulate its aggregation propensity(248-251). The distribution of
charge and hydrophobicity differs significantly between αS and βS. A comparison of their sequences shows that synuclein proteins, including αS and βS, have a three-domain organization and contain the following (Figure 18): 1) an N-terminal amphipathic domain of alternating charge and hydrophobicity, 2) a central hydrophobic core domain known as the non-amyloid component (NAC), and 3) an acidic C-terminal domain, which is comparatively more proline-rich and highly charged. αS and βS differ most in their hydrophobic NAC domains where the βS NAC is 11 residues shorter. The N-terminal domains share the highest sequence identity, differing only in six point mutations. The C-terminal domain of βS is more acidic and more proline-rich than αS, and it constitutes a larger percentage of the polypeptide length compared with αS. This distribution of charge/hydrophobicity implies that the pH-dependent behavior of these proteins may be mediated by charge changes in the N-terminal, NAC, and C-terminal domains upon alteration of the environmental pH. Although the NAC region is known to be necessary for aggregation of αS (252), the susceptibility of the N, NAC, and C domains of αS and βS to promote aggregation, and the interdependence of these domains to regulate aggregation in a pH-dependent manner, is not well-understood.

**Figure 18** Sequence comparison of αS and βS. Aligned sequences of βS and αS in a three-domain organization: N (amphipathic) terminus, NAC (hydrophobic), and C terminus (acidic) domains. Charged residues are highlighted, and ones with potentially altered pKa values in our experimental pH range are colored as follows: free N terminus, dark blue; histidine, light blue; glutamic acid, dark red; aspartic acid, orange. Identical residues in the aligned position are indicated by a vertical dash between the two sequences. Sequence gaps are indicated by a horizontal dash and a pale yellow color.
Altered microenvironments may also alter the stability, structure, and formation rates of the aggregated state of synucleins, which, in turn, may determine their toxicity(253). αS in a pathogenic fibrillar form exists in parallel, in-register β-sheets(8). A novel Greek-key topology for the αS fibril has been determined by solid-state NMR experiments to a high level of molecular detail(9). Alternative topologies, derived from modeling based on experimentally determined structural restraints, have also been proposed(254), and other experimental results suggest an inherent polymorphism and environmental sensitivity of the αS aggregate morphology(255-257). Environmental changes, such as those in pH, may exert their effects by stabilization of one or more of these fibrillar topologies similar to the effect of familial mutations switching the preference between two different fibril topologies, as recently proposed by Nussinov and co-workers(254). In several amyloidogenic systems, exposure to mildly acidic pH results in fibril formation and/or alters fibril stability and structure(255, 258-262). As the fibrillation reaction is under kinetic control, a small change in the protonation states of key charged residues, leading to the formation of aggregation-competent species, can dramatically alter the reaction rate(260). As Vendruscolo and co-workers(249) have demonstrated for αS, mildly acidic pH (pH 5.2) can contribute to fibrillation acceleration relative to neutral pH by amplification of secondary fragmentation processes. Structurally, altered protonation of side chains has been shown to alter the registry of the fibril structure(255), and may relieve inter- and intra-chain repulsion between like charges, as well as provide additional stability to the fibril in the formation of cooperative side chain-mediated hydrogen bond networks ("polar zippers" (263)).

Here, we investigate the role of mildly acidic pH on the propensity for synuclein fibril formation. We demonstrate the presence of an environmentally sensitive pH mechanism in βS that serves as an on/off switch for fibrillation at mildly acidic physiological pH, thereby modifying the view that βS is non-fibrillogenic. By constructing a set of αS/βS domain-swapped chimeras, we show that although the NAC domain is indeed the strongest determinant of
fibrillation propensity, the βS N-terminal domain and its interactions with the βS NAC domain can significantly modulate its fibril formation. Structural studies with NMR and AFM show that there are no pH-dependent conformational changes in the intrinsically disordered monomer but rather that the pH switch may derive from the ability of βS to form fibrils due to a new charge distribution. Computational modeling of βS fibrils indicates that a Greek-key motif observed in αS fibrils can be maintained in models of βS fibrillar structures. Fibril models also reveal two glutamate side chains that may underlie the observed pH-dependent fibrillation of βS, and single substitutions to alanine at these positions lead to significantly altered aggregation behavior. These findings provide insight into the complex interplay of charge and hydrophobicity for fibril formation in different cellular microenvironments, and they suggest that βS may play a multifaceted role in disease and homeostasis in PD.

5.2.4. RESULTS

5.2.4.1. βS Forms Fibrils at Mildly Acidic pH

To investigate the impact of pH on αS and βS fibrillation, we performed thioflavin T (ThT)-based fibrillation assays with purified proteins that were incubated at two different pH values. Fibrillation kinetics of αS and βS measured at near-physiological pH 7.3 and mildly acidic pH 5.8 show that αS fibrillation is accelerated at pH 5.8 relative to neutral pH, whereas βS exhibits a striking pH-dependent on/off fibrillation switch (Figure 19, A and B); at pH 7.3 βS does not form fibrils, although at pH 5.8 βS forms fibrils with an apparent half-time of greater than 60 h (Figure 19B). Aggregation of βS has similar (slow) lag and (rapid) elongation phases characteristic of amyloid-like fibril formation by αS and many other fibrillogenic proteins, suggesting that βS aggregates are fibrillar in topology. Indeed, upon visualization of βS aggregates with AFM (Figure 19, C–F), fibrillar structures similar to αS aggregates at both pH values were observed. Both fibrils have similar overall topologies and heights of ~5–8 nm.
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Figure 19 pH-dependent ThT fibrillation of αS and βS and AFM imaging. 1 mg/ml αS mg/ml αS (A) and βS (B) were incubated at 37 °C in a plate reader, and fibril formation was monitored with a ThT-binding assay in 20 mM MES, 20 mM MOPS, 100 mM NaCl, pH 5.8 (blue), and pH 7.3 (red). Normalized average fluorescence values were used for plotting the curve, and the standard deviations calculated from at least three replicates are indicated by shading in a lighter color. The samples that did not show any fluorescence are indicated by a horizontal line for comparison. AFM imaging was carried out at the end of the fibrillation process when samples that formed fibrils reached the plateau phase. AFM images for αS at low pH 5.8 (C) and high pH 7.3 (D) showed fibrils. βS forms fibrils at pH 5.8 (E) and spherical oligomers (F) at pH 7.3. The oligomers do not exhibit fluorescence in the ThT-binding assay (B; red curve).

We next investigated the molecular determinants of the observed pH-dependent fibril formation by βS by considering elements of the protein that may change their charge when the pH is changed from 7.3 to 5.8 (supplemental Figure 27). A histidine side chain (theoretical pKa~6.4), His-65, which is present in the βS NAC region (but not in αS NAC), is a likely candidate. Additionally, a previous study showed that several Asp and Glu residues, distributed throughout the N-terminal, NAC, and C-terminal domains, had altered pKa values in the αS monomeric state(264), suggesting that the titration of one or more of the homologous residues in βS could also impart the observed pH sensitivity for aggregation. The N terminus (with a theoretical pKa of 7.7 ± 0.5 (265)) may have a small charge difference in this pH range, in the monomeric state. Therefore, we investigated the following three potential candidates for the pH-
dependent switch: 1) the His-65 residue in the NAC region; 2) the free-N terminus; and 3) the acidic residues in the N-terminal, NAC, and C-terminal domains.

To uncover which of the three sites identified above contribute to the pH-dependent βS fibrillation, two of the sites were made insensitive to pH changes (between pH 5.8 and 7.3); the H65N mutation converts the His at position 65 in the βS NAC region to the polar charge-invariant Asn, which is also the identity of the residue at position 65 in αS (Figure 20A). We found that this variant exhibited similar on/off pH-dependent fibrillation kinetics compared with the wild-type βS protein, suggesting that the His-65 is not responsible for the pH-dependent switch. Next a pH-insensitive N terminus was designed by enzymatic N-terminal acetylation of the H65N mutant (4), thereby creating a variant that is pH-insensitive both at the N terminus and in the NAC region of βS. Despite simultaneous elimination of possible pH sensitivity at the N terminus and His-65, pH-dependent fibrillation is still observed (Figure 20B). Therefore, these results suggest that the third putative pH-sensitive element, the series of acidic residues in the N-terminal, NAC, and C-terminal domains, is likely to be the primary determinant of the observed pH sensitivity of βS fibril formation.

Figure 20 pH-dependent fibril formation of H65N-βS and acetylated H65N-βS. pH-dependent fibril formation for H65N-βS (A) and a variant in which the free N terminus is acetylated in the H65N variant (B) are shown. ThT fibrillation assays for both variants show pH dependence, indicating that neither site is responsible for the observed pH-dependent switch.
5.2.4.2. *Domain-swapped synuclein chimeras reveal the interplay of synuclein domains in βS pH-regulated Fibril Formation*

βS has eight Glu/Asp residues in the N-terminal domain, 19 in the C-terminal domain, and 1 in the NAC domain (Figure 18) compared with 7, 15, and 2, respectively, in αS. An intractable number of combinations can be generated by swapping these residues between αS and βS. Therefore, to further delineate the contribution of the individual synuclein domains to the observed pH-dependent fibrillation of βS, and to investigate the domain–domain interactions underlying fibrillation of αS and βS, we utilized segmental mutagenesis. A set of eight chimeric proteins that contain segmental domain swaps between the N-terminal amphipathic domains (residues 1:60), the NAC hydrophobic domains (residues 61–95 in αS; residues 61–86 in βS), and the C-terminal acidic domains (residues 96–140 in αS; residues 87–134 in βS) were designed and isolated. The eight chimeric proteins are designated as αS WT (AAA), βS WT (BBB), BBA, ABB, ABA, BAB, BAA, and AAB with the notation A and B indicating the protein from which the domain is taken (αS and βS, respectively), and had lengths that range from 130 to 143 amino acids.

Examination of the fibrillation kinetics of this set of chimeras at the two pH values shows a clear grouping defined by the NAC domains, indicating that the NAC region is a key determinant of fibrillation behavior. Strikingly, the XAX (where X represents an A or B domain in the indicated position) chimeras that contain the αS NAC are always fibrillogenic at both pH values, whereas those containing the βS NAC, XBX are non-fibrillogenic at high pH, but ThT-fluorescent and therefore cross-β structure-positive, with the exception of ABB, at low pH (Figure 21, A–D). Additionally, XXB chimeras typically have a longer lag time than XXA chimeras suggesting that the βS C-terminal domain may be involved in inhibitory processes (supplemental Figure 28).
Figure 21 pH-dependent αS/βS domain-swapped chimera fibrillation. ThT-binding fluorescence assay for chimeras that contain the αS-NAC domain (XAX) at low (A) and high (B) pH and chimeras that contain the βS NAC domain (XBX) at low (C) and high (D) pH are shown. Normalized averages of fluorescence values are used to plot the curves, and the standard deviation calculated from at least three replicates is shown by shading in a lighter color. The chimeras that did not show any fluorescence are indicated by a horizontal line. Fluorescence traces for AAA and BBB constructs are identical those in Figure 19 but are reproduced here for comparison.

The observed fibrillation trends of chimeras enable a delineation of the contribution of each domain and domain–domain interactions to fibrillation. For example, the contribution of the N-terminal domain to low-pH fibrillation can be investigated by comparing fibrillation of ABB and BBB chimeric proteins. ABB chimeric protein fibrillation is significantly diminished compared with WT βS (BBB) despite having an identical NAC domain (Figure 21D). AFM-derived images show that βS (BBB) forms robust, αS-like, fibrils at mildly acidic pH, whereas the ABB protein forms spherical globules (Figure 22, A and B). Replacing the C-terminal domain of the ABB construct with the corresponding αS domain, resulting in the ABA chimera, restores the ability for β-sheet structures (as detected by ThT binding) at pH 5.8. However, the resulting aggregates do not resemble amyloid fibrils (Figure 22D). In contrast, the chimeric protein BBA forms BBB-
like fibrils at pH 5.8, suggesting that the pH-dependent interaction between βS NAC and βS N-terminal domains is a significant contributor to inducing fibrillation at mildly acidic pH (Figure 22C). In the XAX set, the αS NAC region, on the other hand, is necessary and sufficient for fibrillation at both pH values, and the flanking N- and C-terminal domains modulate its fibrillation to a less striking degree.

![AFM images of low pH 5.8 ABX and BBX fibrils](image)

**Figure 22**. AFM images of low pH 5.8 ABX and BBX fibrils. Non-contact mode AFM images for low pH samples of chimeric constructs with a βS NAC domain in a 2.5 X 2. Mm spot size are shown. A, WT βS; B, ABB; C, BBA; and D, ABA chimeric proteins. Scale bar, 500 nm.

Taken together, the observed fibrillation behavior of H65N βS mutant, N-terminally acetylated βS (Figure 20) and XBX chimeric variants (Figure 21) indicate that 1) the βS NAC region plays a predominant role in fibril formation at pH 5.8, 2) pH-dependent changes in the NAC and N-terminal domains critically modulate the aggregation propensity of βS, and 3) the
presence of the βS C terminus consistently slows the aggregation kinetics suggesting that this region plays an inhibitory role in fibril formation.

5.2.4.3. αS and βS monomer conformation ensembles are largely unperturbed by pH

To determine whether the observed pH-dependent aggregation of βS is a result of changes in the conformational ensemble of monomeric βS, pH-dependent structural rearrangements of the monomer were explored by measuring residual dipolar couplings (RDC) and intrachain paramagnetic spin relaxation experiments (PREs) at the two pH values (Figure 23). 1H-15N HSQC comparison of βS at pH 7.3 and 5.8 shows small chemical shift perturbations primarily at the sites of ionizable side chains, suggesting that the protonation states of these residues are being affected in this experimental pH range (Figure 23A). Strikingly, no significant pH-dependent rearrangement is observed via the RDCs or PREs for βS or αS (Figure 23, B–E). In the RDC experiment, which is a measure of order in the monomer, the profile is the same at the two pH values; but notably, the C-terminal domain of βS between residues ∼90 and ∼115 is more ordered than that of αS, consistent with previous results (Figure 23, B and C)(27, 266, 267). In the PRE experiment, a spin label is placed in the NAC domain adjacent to the C-terminal domain to probe transient interactions with the neighboring N- and/or C-terminal domains. αS-90-MTSL and βS-79-MTSL pH-dependent PRE measurements indicate that the monomer conformations are populated differently in αS and βS with the NAC interacting more strongly with the C terminus in αS and the NAC interacting more strongly with the N terminus in βS consistent with previous results (Figure 23, D and E)(268). The stronger interaction between the βS NAC and N-terminal domain compared with αS may contribute to its greater sensitivity to pH changes as observed by the lack of Tht-fluorescent aggregation of the ABB chimera. Nonetheless, these long-range interactions in the monomer appear to be unperturbed by pH change. Although our results do not rule out the existence of an additional rarely populated conformational state at low
pH values that is not detectable by these NMR experiments due to its transient nature, we conclude that αS and βS monomer conformations do not exhibit any significant pH-dependent conformational change. The differences in the fibrillation behavior of βS at low and high pH values, therefore, may arise from a difference in formation rates and/or stabilities of higher-order (oligomeric and/or fibril) species.
Figure 23 RDCs and intrachain PREs. A) βS chemical shift perturbation across the pH range 7.3-5.8 calculated using the normalized CSP expression \( ((^1H)^2 + (0.159*^{15}N)^2)^{1/2} \). Three putative pH-dependent events are indicated as follows: at the free N terminus (dark blue), at His-65 (light blue), and groups of aspartic and glutamic acids (-COOH). Although these acidic residues are concentrated at the C terminus, they are also present in the N-terminal domain indicated as dark red (glutamic) and orange (aspartic acid) circles. pH-dependent amide RDCs are shown for αS in B and βS in C with low pH 5.8 (blue) and high pH 7.3 (red). \(^1H-{^{15}N}\) HSQC-IPAP spectra were collected in the absence or presence of C8E5 aligning media in 20 mM NaPi, 100 mM NaCl at both pH values. pH-dependent intra-chain PRE values are shown for A90C-MTSL-labeled αS in D and A79C-MTSL-labeled βS in E measured in 20 mM NaPi, 100 mM NaCl buffer and after the addition of 10 mM sodium ascorbate. High pH 7.3 PRE values are shown in red and low pH 5.8 values in blue. Open circles indicate points that are overlapped. Black lines are calculated as the expected profile for a random coil chain as in Sung and Eliezer (88).
5.2.4.4. Modeling the structure of βS fibrils suggests sites of pH sensitivity

Structural studies with AFM (Figure 19) showed that fibrils formed by βS at low pH adopt overall topologies that are similar to αS fibrils under high-pH conditions. Therefore, we investigated the molecular structure of βS fibrils. Recently, an αS fibril structure was determined using solid-state NMR techniques and was reported to have a Greek-key like architecture(9). Given the similarity between αS fibrils at high and low pH, we used this structure as our starting model to ask if and how βS is capable of adopting and maintaining a similar fibril structure. Using Rosetta software, the aligned βS sequence was threaded onto the backbone structure of fibrils obtained for αS, and the relative stability of βS fibrils was calculated in the context of the Greek-key polymorph using Rosetta Symmetry(269).

We first investigated the stability and structure of the αS fibril structure in Rosetta simulations with and without conformational restraints on the ssNMR-derived structure, to generate a baseline for comparison with βS models. We observed that a funnel-like landscape is obtained in both constrained and unconstrained Rosetta simulations with low r.m.s.d. structures corresponding to the lowest energies (Figure 24A). The Greek-key type structure is maintained in the low energy models, indicating the existence of a robust funnel around the ssNMR-derived structure in the Rosetta force field (Figure 24B). Having recapitulated the thermodynamic stability of the αS fibril structure and obtained a baseline for comparison, we next asked whether βS was capable of taking on the same type of Greek-key structure as αS. We first performed a global sequence alignment of αS and βS, and we threaded the corresponding N-terminally aligned residues of βS on the Greek-key motif observed in the αS structure without considering gaps arising due to a shorter length of the βS NAC. This approach required incorporating part of the C-terminal region of βS (residues 87–97) in the threaded model in the αS NAC region. Starting unconstrained simulations from this model resulted in an ensemble that was considerably destabilized and is dominated by structures that deviated significantly from the Greek-key
structure (Figure 24, C and D). In analyzing the models, we observed that the residues needed to form a critical tight turn in the Greek-key structure, which were GAG in αS and were VKR in the threaded starting model; these large side chains are incompatible with the formation of a tight turn in βS. In addition, this threading places the C-terminal residues in a sterically incompatible area of the Greek-key structure. The steric repulsion incurred by packing these side chains likely led to the observed destabilization of the Greek-key structure in this threading.

In light of the nascent N–NAC interactions in the βS monomer ensembles and the unlikely involvement of the highly charged C-terminal domain in amyloid, we next aligned the sequences by incorporating a greater number of the N-terminal portion of the βS sequence in the model. This was possible by threading residues 19–86 of βS instead of residues 30–97 into the Greek key. Interestingly, this alignment of the GAG motif corresponds to replacing the 11 “missing” residues of the βS NAC with the residues from βS N-terminal domain. Performing unconstrained Rosetta Symmetric Relax simulations starting from this alignment, we observed that the stability of the Greek-key-containing structures was restored, and the structures of the observed lowest-energy conformations closely resembled the αS fibril topology (Figure 24, E and F). Although the absolute Rosetta energy values for the βS models are still higher than those obtained for the αS ssNMR structure, a funnel-like shape is observed in both landscapes. Although little structural information is available about the βS fibril to validate or further refine our models, our simulation results indicate that it is possible for βS to adopt a similar amyloid form as αS when the N-terminal domain of βS participates in the core structure of the fibril.
Figure 24. Rosetta score (in Rosetta energy units, REU) versus r.m.s.d. (Å) for the ssNMR-derived αS structure showing a robust funnel. The lowest energy structure is indicated by a red point, and in B the lowest energy structure identified in the simulation is pictured. The N terminus is colored blue; the NAC is colored gray; and the C terminus is red. Trailing ends of the N and C termini are shown in light blue and red, respectively. The GAG-turn compatible motif is highlighted in magenta. Rosetta simulations using a threading in which residues 30–97 from βS are N-terminally aligned with corresponding residues from αS show a lack of stable energy funnel, and higher energy values (C) and a large deviation from the Greek-key ssNMR structure (D). A C-terminally aligned threading for the βS(19–86), which incorporates more of the N-terminal domain in the Greek-key, results in a more stable form compared with the βS(30–97) threading. A robust energy funnel, albeit with increased energy values compared with αS, is restored (E), and the lowest energy structure has a low r.m.s.d. from the Greek-key structure (F).
5.2.4.5. **Single amino acid substitutions of key glutamate side chains alters βS fibrillation behavior**

Computational analyses, the aggregation behavior of chimera, and greater interactions between the N-terminal and NAC domains in the βS monomer observed by NMR indicated that acidic residues in the N-terminal and NAC domains are key determinants of pH-dependent fibril formation. Further examination of low-energy fibril models, and the sequence differences between βS and αS in the N-terminal and NAC domains led us to hypothesize that Glu-31 and Glu-61 side chains contribute to the observed pH-dependent switch. Glu-31 is present only in βS but not αS (residue 31 is instead a glycine). Glu-61, the first residue position of the NAC domain, is the only buried glutamate side chain in both αS and βS fibril models, and it may experience different microenvironments in both the monomer (Figure 23) and the fibril states of αS and βS.

To test the contribution of these identified residues (Glu-31 and Glu-61) to pH-dependent fibril formation, these residues were individually substituted to the pH-insensitive alanine. The E31A βS variant showed a significant increase in the lag time for aggregation at low pH, confirming that Glu-31 contributes to the pH-dependent aggregation behavior (Figure 25A). In contrast, the E61A βS variant lost pH dependence of aggregation and was found to robustly and rapidly aggregate under both low and high pH conditions. AFM characterization showed that aggregates of E61A βS at both pH values adopt a fibril morphology. Thus, strikingly, a single mutation in the βS NAC region (E61A) allows aggregation at high pH with αS-like efficiency (Figure 25B). These results suggest that glutamate switches exert their effects by both promoting and inhibiting fibril formation in a pH-dependent manner, and they highlight how small changes in charge content can dramatically alter synuclein fibrillation behavior.
Figure 25. ThT fluorescence and AFM images of βS mutants E31A and E61A at pH 5.8 and pH 7.3. Aggregation kinetics (as detected by ThT fluorescence) of βS mutants E31A (A) and E61A (B) at low pH (blue), high pH (red), and wild-type βS at low pH (black) are shown. At low pH, E31A shows a significantly higher lag time compared with wild-type βS. AFM images of E31A at low pH (C) and high pH (D) show fibrils and oligomers, respectively, similar to wild-type βS (Figure 19). The E61A substitution abolishes the pH dependence for fibrillation and shows similar lag times at both pH values. Fibril morphologies at low pH (E) and high pH (F) are shown.

The contribution of both identified glutamate residues is consistent with the observed aggregation behavior of chimeric proteins. The chimeric protein ABB, which does not have Glu-31 (αS N-terminal domain has Gly-31), does not form fibrils at mildly acidic pH (Figure 22 and supplemental Figure 30). Whereas Glu-61 from the NAC region is present in both the ABB chimera and wild-type βS (BBB), NMR characterization of the monomer (Figure 23) and computational models of fibrils (Figure 24) indicate that the NAC domain participates in interactions with the N-terminal domain, which differs between these variants. Thus, both identified sites contributing to the pH switch are either absent or have a significantly different microenvironment in the ABB chimera compared with the wild-type βS and BBA chimera, which is consistent with their observed aggregation behavior (Figure 21 and Figure 22).

Although the presence of contiguous βS N-terminal and NAC domains appears to be the key feature for fibrillation at mildly acidic pH, we note that the ABA chimeric protein, which lacks
this feature, does form fibrillar aggregates. The interactions between the N-terminal domain of αS and NAC domain of βS in this chimera are expected to be less fibril-stabilizing compared with BBX chimera, but the lower negative charge of the C-terminal domain in this ABA chimera compared with ABB chimera may relieve inhibition of aggregation to a greater extent and allow formation of ThT-positive oligomers (that are nevertheless distinct from “regular” BBB or AAA fibrils at either pH value; supplemental Figure 30). The balance between fibrillation-promoting interactions in the N-terminal and NAC domains and other inhibitory interactions utilizing all three domains contributes to fibril formation.

Taken together, our results show that a subtle balance of pH-dependent stabilizing as well as inhibitory intra- and inter-chain interactions in all relevant thermodynamic states, monomer, non-fibrillar aggregates, and mature fibrils, underlies the pH-sensitive aggregation behavior of synucleins mediated by key glutamate residues.

5.2.5. DISCUSSION

The key observation in our study is that mildly acidic pH 5.8 renders the otherwise non-fibrillogenic βS fibrillogenic. At high pH (pH 7.3) the βS protein is non-fibrillogenic as previously demonstrated in the literature (30, 95). However, in contrast to αS, which is fibrillogenic at both pH values, βS fibrillation is effectively controlled by an on/off pH-dependent switch. ThT binding-based fibril formation assays of chimeric constructs, AFM-based imaging, point mutagenesis, and atomic resolution modeling of βS fibrils suggest that pH-dependent interactions mediated by key glutamate side chains and involving the N-terminal and NAC domains of βS are key contributing factors of this observed pH-dependent fibrillation of βS. In addition, pH-dependent effects at acidic residues in the C-terminal domain may also play an important role in fibril formation. Given that several subcellular organelles have mildly acidic pH, our data demonstrate that βS aggregation is highly environment-dependent and suggest that
the view of βS as a simple αS aggregation inhibitor, which is not prone to aggregation itself, may need to be reconsidered.

Despite their high sequence similarity, where and how the fibrillation-determining differences between αS and βS reside in their primary sequence and tertiary structures have remained unknown. Their NAC regions are the most disparate in length and sequence, and although deletion of the essential stretch of hydrophobic residues in αS renders it non-fibrillogenic, simply transferring the missing residues from αS to βS has not made the latter fibrillogenic (252, 270, 271). Although swapping other regions of the NAC between the two proteins similarly modulates the aggregation propensity to some extent (270, 272, 273), a complete transfer of fibrillation properties by swapping NAC domains has not been possible. Taken together, previous studies and our results indicate that the formation of βS fibrils is determined not exclusively by its NAC domain but is controlled by interactions between the NAC and other domains, whereas the NAC domain appears to be both necessary and sufficient for αS aggregation.

The set of domain swapped chimeras allowed us to investigate the role of inter-domain interactions in aggregation of βS. The most striking result from comparing the aggregation behavior of low pH ABB (not amyloid-forming) and BBX (fibril-forming) chimeric proteins was that the N-terminal and β-NAC domains together play a key role in the ability to form fibrils at mildly acidic pH. Although the NAC domain is necessary for fibrillation, in βS the N- and C-terminal domains regulate the NAC domain-mediated fibrillation inducing interactions, with interactions between the N-terminal and NAC domains playing a more crucial role in the stabilization of the βS fibril architecture similar to previous observations in αS (210, 274, 275). The C-terminal domain, which is highly enriched in negative charges, and is thus expected to be more pH-sensitive, does not appear to provide the required pH-sensitive stabilizing interactions, but may instead contribute to pH-dependent aggregation via C-terminal inhibitory (N-C and NAC-C) intra/inter-chain interactions as evidenced by the differing lag times (but highly similar fibril topology) between all C-terminally swapped chimeras (supplemental Figure 28 and Figure
(AAX, ABX, BAX, BBX) (30, 68), and by the enhanced aggregation propensity at low pH of the ABA chimera compared with ABB. Janowska et al. (30) also suggest an enhanced interaction of the αN and βC domains relative to the αN and αC domains, which could also be a contributing factor to the lack of formation of fibrils by ABB chimera. Further exploration of the ABX monomer conformational ensemble will provide insight into the subtle balance of pH-dependent stabilizing and inhibitory interactions that determine aggregation behavior.

Analysis of the computational models for βS fibril provides insight into the balance of intra- and inter-chain interactions formed by glutamate residues, which may determine the observed pH dependence of βS fibril formation (Figure 26A). A decrease in pH is expected to lead to greater protonation of glutamate residues involved in interchain fibril-stabilizing interactions, as well as relieve inhibition of aggregation due to charge-mediated interactions. For example, protonated Glu-31 and Glu-61 side chains (at low pH) may themselves form a glutamine-like “polar zipper” (54) interaction (Figure 26B) or stabilize other polar zippers (Figure 26C) to promote fibril formation, whereas their deprotonated forms at high pH may inhibit fibrillation by interchain charge repulsion and more favorable intra-chain interactions as observed in the NMR data (Figure 23). The E31A variant, which has a higher lag time for aggregation compared with wild-type βS (Figure 25A), is predicted to lack the stabilizing polar zipper interaction (Figure 26B). Charge-mediated aggregation-inhibitory interactions at high pH, e.g. interchain repulsion, may be relieved in the E61A variant compared with wild-type βS, and pH-independent (in this pH range) polar zipper interaction ladders formed by surrounding polar residues in the N-terminal and NAC domains, e.g. Gln-50 and Gln-62, may sufficiently stabilize the E61A fibril form at both low and high pH. At high pH, the desolvation penalty associated with burying a negatively charged Glu-61 in the fibril core (Figure 26A) would also be abolished in the E61A variant. Although Rosetta-based homology models of βS fibrils based on Greek-key αS structures appear to be consistent with experimental data, it is possible that other fibril topologies may equally well explain the results. Moreover, the stabilities and morphologies of fibrillation intermediates, and
the impact of mutations on these, including in the context of the various αS/βS chimera, need to be considered for a fuller understanding of fibril formation. Structure determination of the fibrils formed by βS and its variants under different pH conditions may provide further insight into these issues.

**Figure 26. Possible charge-mediated interactions formed by key glutamates in the βS fibril model.** A, location of N-terminal glutamate residues (red) and polar side chains in the N-terminal domain in the βS Greek-key fibril model. B, Glu-31 may form a hydrogen-bonded polar zipper ladder in its protonated form (rotameric preferences modeled using glutamine), thereby promoting fibril formation. C, Glu-61 may form a similar ladder and participate in additional interactions with the Gln-50 ladder at low pH. The Gln-50 ladder is predicted to form in a pH-independent manner in the E61A variant.

Based on the results of point mutagenesis at Glu-61 and Glu-31, chimeric mutagenesis, computational modeling of fibrils, and NMR data on the monomer ensembles, we argue that pH-
dependent aggregation of synucleins is dependent on a subtle balance of various aggregation-promoting (stabilizing) and inhibiting effects. The N-terminal and NAC domain interactions, evident in the NMR data (intrachain) and Rosetta models (intra and interchain), serve to stabilize the fibrils in a pH-dependent manner, whereas charge interactions involving negatively charged side chains throughout the protein chains (including C-terminal domain) may serve to inhibit aggregation. The balance between charge-dependent stabilizing interactions in the fibril state and aggregation–inhibitory interactions in the soluble states contributes to fibril formation.

Our results have several implications for the role of βS in disease. They indicate that although βS may serve in a neuroprotective role at cytoplasmic pH by preventing αS aggregation, this inhibition may be relieved by a switch into its own fibrillation in more acidic organelles, like the lysosome, which enhance protein clearance and enable homeostasis. Several recent reports suggest that βS may contribute to a toxic gain-of-function in different cellular organelles in a different way than αS(231, 232). For instance, defects associated with βS are linked with defects in endoplasmic reticulum-Golgi-associated proteins in yeast, which is a mildly acidic subcellular environment. There is also an emerging role for endolysosomal dysfunction in PD, particularly in relation to recent reports of Gaucher's disease, a lysosomal storage disease(276, 277). Given that membranes and vesicles are likely sites of function for synucleins, and also regulate microenvironments associated with acidification, fibril formation by βS in these microenvironments may lead to dysfunction. Our in vitro work provides molecular level insights into βS conformations at mildly acidic pH values and suggests that the toxic gain-of-function observed in vivo may be related to the formation of βS aggregates and/or a loss-of-function of the soluble cytoplasmic pH form of βS(278). Evaluation of the stability and toxicity of the in vitro generated βS fibrils in cell culture and animal models will be useful in further investigating the role of βS in PD.

5.2.6 METHODS
5.2.6.1. Mutagenesis, expression and purification

For both the αS N65H and βS H65N as well as all cysteine point mutants, site-directed mutagenesis was performed to exchange the appropriate residues using Invitrogen AccuPrime pfDNA polymerase (Thermo Fisher Scientific, Waltham, MA) and the appropriate primers (Integrated DNA Technologies, Coralville, IA). All acetylated and non-acetylated protein were expressed and purified, as described previously (32), and verified for the correct weight and purity with ESI-MS (data not shown).

5.2.6.2. Chimera preparation

To obtain the DNA plasmids required to express chimeras of αS/βS, the Gibson assembly protocol was employed to insert the necessary G-blocks (Integrated DNA Technologies, Coralville, IA) for the AAB, BAB, BBA, and ABA chimeras, and site-directed mutagenesis starting with point mutants K10M of αS and M10K of βS, respectively, was performed for the BAA and ABB chimeras. Expression and purification of unlabeled protein were then performed in the same manner as the wild-type protein and other point mutants used in this study, and the correct molecular weight and purity were confirmed with ESI-MS (data not shown).

5.2.6.3. HSQC and RDC experiments for αS/βS

αS and βS solutions were prepared to a concentration of 250 µM in the same manner as the wild-type protein and other point mutant used in this study. Briefly, lyophilized protein was dissolved in buffer, passed through a 100K Amicon centrifugal filter, and then concentrated with a 3K Amicon centrifugal filter to 250 mM. These samples were prepared in 20 mM sodium phosphate, 100 mM NaCl, pH 5.8, and 7.3 buffers. 1H-15N HSQC and RDC experiments were prepared and analyzed similarly to previously described protocols (68). We confirm that þS exhibits similar pH-dependent on/off fibrillation behavior in this buffer in Figure 29.

5.2.6.4. MTSL labeling

Site-directed mutagenesis was performed as mentioned before to incorporate cysteine residues at A90 in αS. In βS, a cysteine residues were incorporated at A79. To incorporate the nitrooxide spin
label MTSL (5-(1-oxyl-2,2,5,5-tetramethyl-2,5-di-hydro-1H-pyrrol-3-yl) methyl methanesulfonothioate), the cysteine mutants were first reduced with a 20 times molar ratio of dithiothreitol (DTT) incubated at 4 °C for 4 – 6 h to remove cysteine–cysteine disulfide bonds. A GE Healthcare HiPrepTM 26/10 desalting column was used to remove excess DTT. Immediately after protein eluted from the column, a 20X molar excess of MTSL was added, and the reaction was left to proceed on a shaking platform at 4 °C overnight. Excess MTSL was removed by dialyzing against ultrapure (18 megohms/cm) deionized water before lyophilizing and storing the protein at —20 °C. PRE experiments were performed as described previously from lyophilized MTSL-labeled protein (68), 100 µM protein, and 10 mM sodium ascorbate to reduce the spin label.

5.2.6.5. Thioflavin-T assay
Lyophilized protein samples were prepared to a concentration of 1 mg/ml by filtering first with a 100K filter from dissolved lyophilized protein and concentrating and buffer exchanging with a 3K centrifugal filter. Samples were loaded with 20 µM ThT (Acros Organics, Pittsburgh, PA) into 96-well clear bottom plates (Corning, Corning, NY) at a concentration of 70 µM, sealed with Axygen sealing tape (Corning), and shaken at a rate of 600 rpm at 37 °C for at least 200 h. Between 3 and 6 samples were measured for each sample type. A POLAR Star Omega plate reader (BMG Labtech, Cary, NC) was used to monitor the increase in ThT intensity. Protocol is adapted from the literature (279).

5.2.6.6. AFM protocol
All AFM images were taken on an NX-10 instrument (Park Systems, Suwon, South Korea), using non-contact mode tips (PPP-NCHR, force constant 42 newtons/m; 330 kHz frequency; Nanosensors, Neuchatel, Switzerland). All synuclein samples were obtained after ThioT fluorescence was complete, at a concentration of 70 µM. A 1 × 1-cm² of freshly cleaved mica (obtained from Ted Pella Inc., Redding, CA) had 20 µl of sample deposited on the surface and was allowed to incubate for 5–10 min while covered. Then the surface of the sample was washed
three times with 100 µl of water, and the bottom and edges were dried using filter paper. The surface was allowed to air-dry for 1 h before being imaged. Image processing was conducted using Gwyddion software.

5.2.6.7. Computational modeling

From the ssNMR-derived structure (Protein Data Bank code 2n0a), the fibril core was isolated by removing the edge chains A, B, H, I, and J, and disordered residues 1–29 and 98 –140 from the chains C–G. An idealized symmetric conformation was generated from this modified structure using fibril symmetry (269). A Rosetta Script input .xml file (see supplemental information, Section 4.2.9) was created to mutate the desired residues to match the sequence of the threaded variant. Backbone nitrogen, carbon, and oxygen atoms were constrained to be within 0.2 Å of their initial coordinates in the constrained Relax simulations. The structure was then symmetrically relaxed using the Rosetta Fast relax algorithm in Rosetta 3.6 (269) to generate 1000 output decoys. In unconstrained simulations, the 0.2 Å limit was removed, and symmetric relax was then performed with the Rosetta Fast relax algorithm to produce 4000 decoys.

5.2.7. AUTHOR CONTRIBUTIONS

G. M. M. performed the NMR characterization, contributed to aggregation kinetics, experimental design, data analysis, and manuscript preparation. M. P. O. performed the computational modeling and data analysis, chimera mutagenesis, and protein production and contributed to aggregation kinetics. T. B. A. performed AFM characterization, mutagenesis, and protein production. M. K. J. contributed to experimental design and assisted with chimera mutagenesis. S. D. K. and J. B. contributed to experimental design, data analysis, and preparation of manuscript. All authors reviewed the results and approved the final version of the manuscript.

5.2.8. ACKNOWLEDGEMENTS

We thank Dr. Ana Monica Nunes for helpful discussions.

5.2.9. SUPPLEMENTARY INFORMATION
Figure 27_SI.1. An illustration of pH events in the experimental range for individual amino acids and αS domains in the experimental pH range. Change of charge per pH unit is calculated from the Henderson-Hasselbach equation for the indicated amino acid sidechains with the pKa values measured for those measured in (264), except for the amino terminus which is assumed to be pKa 8. The free-amino terminus is indicated in dark blue, histidine in light blue, glutamic acid in dark red and aspartic acid in orange. The dark red dashed line indicates a –COOH group that titrates with a pKa of 8. Vertical lines indicate the experimental pH range 5.8-7.3.

Figure 28_SI.2. A sequence comparison of the effect of individual αS/βS domains on the t50 of fibrillation. Each panel examines the effect of having A or B in the X position as indicated by the x-axis labels. The four possible combinations of N-terminal swaps are examined in A), NAC swaps in B) and C domain swaps in C). Samples which contain B in the X position are shown in purple. ABB is represented off scale, as it is the only sample non-fibrillogenic at this pH and may be considered to have an indeterminably long t50.
Figure 29_SI.3. ThT fluorescence of βS in alternate buffer at varying pH. βS fibril formation, under the same conditions as described in Methods (600 rpm shaking, 37°C, ~1 mg/mL), also occurs in 20 mM NaPi 100 mL salt as well as in the 20 mM MES 20 mM MOPS 100 mM NaCl buffer used in the main text. Low pH 5.8 is indicated in blue and high pH 7.3 in red. The raw fluorescence values are reported.
Figure 30_SI.4. pH dependent AFM imaging of αS/βS chimeras. All chimeras were imaged from a sample of the 1 mg/mL samples at the plateau phase of fibrillation. In each case a 2.5 X 2.5 μm square was imaged, and the scale bar indicates a length of 500 nm. In A)-D) high pH 7.3 XAX chimeras are shown and in E) -H) low pH XAX chimeras are shown. Similarly in I)-L) and M)-P) are the low pH 5.8 and high pH 7.3 XBX chimeras, where X indicates either an A or B for an αS domain or a βS domain respectively. Panels A, E, I, M, N, O, and P, are the same as Figure 19D,C,E,F& Figure 22C,B, and D, respectively, and are reproduced here to facilitate an overall comparison of all constructs at both pH values.
Example Rosetta Scripts

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5.3. FUTURE WORK AND COMMENTARY

The discovery that βS is fibril competent under conditions similar to those occurring in the cellular environment, and that we have isolated some molecular determinants of this pH dependences give us insight into where to look, what to target, and how to look at the role of synucleins in core issues of PD development and symptoms.

The occurrence of this mildly acidic βS fibril, and key sites and regions in fibrillar development is an important springboard for future work. We have not yet explored the aggregation pathway or fibrillar products in detail, for example by probing whether or not βS fibrillation proceeds through any oligomeric species. If this can occur, we might go on to question
whether the overall more benign nature of βS is related to a halt of the aggregation pathway at the earliest of slightly aggregated point of the pathway. It will then be interesting to know, of possible oligomeric species and fibrillar products, if they exhibit any toxic effects toward the cell, and to understand their stabilities in various pH regulated organelles. Additionally, it may be worth exploring if a more benign nature of βS is not due to a difference in aggregate structurally, but rather if quantitatively βS forms less insoluble species overall. βS’ significantly more charged and less interactive C-terminus may exert a great solublizing effect on the monomer, oligomers or the fibril. It is more likely to interact with solvent in any of these conformations and perhaps supports a colloidal stability of the monomer or aggregate that deters from the initiation through development of a primary nucleation core, or in the amount of insoluble product capable of exerting a possible toxic effect.

Additionally, on a structural level while it appears that βS is capable of forming a Greek key αS fibril we have not yet empirically derived this molecular structure of the fibril. Minimally hydrogen exchange NMR experiments of the fibril will be good to reveal the boundaries of the core as done previously, and explore the idea that we have proposed that the N-terminal region may be recruited to contribute to the stability of the fibril, or in potential contacts of other polymorphs or the oligomeric ensemble. Does the mildly acidic pH solution environment support a development and stabilization of contacts in a given structural rearrangement? It is a key observation to have located an apparent pH switch localized to glutamate 61 in the E61A βS mutant. Using it in further structural studies of molecular details or bulk structure may help to provide key insights.

As described, we have suggested that no significant pH dependent rearrangement of monomer occurs via the view we took with an intrachain PRE label in the late-NAC region of the protein, or the sensitivity of the RDC experiments to detect lowly populated species local or long-range rearrangements. Regardless of the absolute accuracy of these views, it is also worth exploring if a pH dependent difference exists later on in the aggregation pathway. In our group,
we have been able to correlate the earliest interactions of NMR-visible species to different propensities of αS fibril in different pH conditions, and with different partners, namely βS.

The one key aspect which deserves an in detail investigation in future work, is the partnership of αS and βS. At high pH only αS is fibrillogenic, but at mildly acidic pH now both αS and βS are both fibril competent species. To couple this with spatial variation in pH, and the functions of various organelles, it suggests to us that their relationship is different in different organelles.

![Image](image.png)

**Figure 31.** The spatial variation of the αS/αS partnership in the pH regulated organelles. A speculative summary of the key spatial differences in intracellular subenvironments, and how difference in partnership in its relationship to aggregation may be reflected spatially and functionally in different organelles.

A preliminary coincubation experiment of αS and βS in the fibrillation assay that we have performed in previously in this work shows us as expected, that αS fibrillar species form at mildly acidic pH, and when αS and various stoichiometric ratios of βS are present in solution, the ThT fluorescence suggests the presence of two distinct fibrillar species. Additionally, βS no longer exerts any inhibitory (or even accelerating) effect upon αS fibrillation at mildly acidic pH, but
interestingly, βS fibrillation initiation is accelerated by the presence of the αS fibril. We can currently speculate from this point that βS may be more benign in a slightly alkaline environment, being less aggregation prone, and exerting an inhibitory effect on αS aggregation, like the mitochondria. In the lower pH organelles along the endocytic pathway, clearance of both fibrils would be accelerated, which may be useful in the properly functioning environment. If we can use this insight to narrow the source of dysfunction in PD, this will also further elucidate the role of αS aggregation in PD in particular.

**Figure 32** Coincubation fibrillation assay of αS and βS. 1 mg/mL and increasing multiples of 1 mg/mL βS ThT fluorescence fibrillation assays as performed in section 4.2 demonstrate in A) that αS alone appears to form one fibril, and in B) in a coincubated mixture of 1 mg/mL αS to 5 mg/mL βS the fibrillation curve is biphasic. In C and D the $t_{50}$ (hrs) of fibrillation, taken from half maximum of fibrillation curve in αS are shown alone in black and in the coincubation mixture in blue against varying concentration, and the same for βS is D.

It is interesting that αS is designed to be more robustly fibrillogenic, and βS is designed to respond more rapidly and be more sensitive to these environmental conditions. While separately these synucleins may make the difference in PD, dysfunction in their partnership may also be a significant factor in the pathophysiological PD environment. βS may be designed to tune αS, by...
making it less aggregation prone is certain environments, and by facilitating clearance of synucleins in more acidic environments.
Several important insights into pursuing early-stages of αS aggregation as drug targets for PD come from the work described in this thesis. The most significant characteristics of the unfolded monomeric αS ensemble, had thus far appeared to be contained within αS’ minor population of collapsed structures, or structures where recognizable secondary structures are transiently sampled, which are associated with important intra- and inter-chain interactions that occur between distant N and C-terminal domains and adjacent domains. These interactions work to self-regulate the aggregation propensity of the ensemble, but the self-regulation process is likely also extremely sensitive in the IDP.

While previous to our work on N-terminal acetylation, several oxidative and nitrative modifications had been described in the literature that go on to affect the aggregation propensity of the ensemble in significant ways, we highlight through N-terminal acetylation that not all permanent modifications necessarily go on to have an effect directly upon aggregation. N-terminal acetylation nearly indistinguishably alters the WT αS monomeric ensemble, and the acetylated ensemble produces similar fibril within a relatively unchanged timeframe. What N-terminal acetylation ultimately did for the community was to highlight that the physiological model of the αS IDP ensemble we use in vitro to study PD should not be taken for granted. While ultimately in vitro models of aggregation of the cytosolic forms of the protein remain unaffected, other works have shown that small portion of αS that binds to membranes may be more directly impacted by N-terminal acetylation and remains an active area of research. This small modification has, and will go on to contribute to an accurately developed physiological view of αS (aggregation) in PD.

Although N-terminal acetylation itself did not directly impact the cytosolic WT αS ensemble and its aggregation in significant ways, we have shown through our subsequent work involving Cu²⁺ binding to the αS protein, that the acetylated N-terminus would alter the route of metal-
induced disease progression. Before our work was published, a significant effort was underway to describe the effect Cu\textsuperscript{2+} could play in exacerbating disease through its binding in αS. The focus on Cu\textsuperscript{2+} was developing, in particular, because Cu\textsuperscript{2+} appeared to have a fibrillation acceleration effect \textit{in vitro} at stoichiometric ratios, in concentrations which might be close-to-achievable physiologically. Other metals which had accelerating effects only at non-physiological mM concentrations, would be incapable of being reached in the cell. We have shown that the physiological form of the monomer is the N-terminally acetylated form, and that the result of physiological Cu\textsuperscript{2+} binding upon αS is in fact not the fibrillation accelerating effect observed \textit{in vitro} from the previously used non-acetylated model. This work further confirms the importance of developing an accurate physiological model of the αS IDP to study, highlights the important role modifications and binding partners will play \textit{in vivo}, shows that alterations in the fibrillation profile can be rooted at a specific site, and potentially highlights another interesting aspect of fibrillation induction related to the thread between distal domains.

Up to this point, several mechanisms involving the release of C-terminal inhibitory nature in αS were described to accelerate aggregation acceleration, including removal the C-terminus, the binding of metals and polyamines, and \textit{cis-trans} proline isomerization in altering the structural rigidity of the C-terminus. The binding of Cu\textsuperscript{2+} to the N-terminus, even in the non-physiological non-acetylated form, highlights that aggregated acceleration can too be triggered by the N-terminal domain. This may be because an inhibitory thread between interacting N- and C-terminal domains can be disrupted by binding partners of either side of this thread, and highlights that while the NAC peptide for example, is necessary and sufficient for fibrillation, the N and C-terminal domains also contribute to the bulk character of the ensemble or aggregates.

With this in mind, it was of interest of us to explore where the roots of the inhibitory nature of an extremely similar αS synuclein, by its sequence, βS, are contained within the protein. A set of domain-swapped α/β chimeras was developed to pinpoint key regions. In a standard model of the physiological environment often used \textit{in vitro}, sodium phosphate buffer with salt at pH 7.4,
we discovered that the NAC-domains were the biggest determinant of fibrillation, and that the
intrinsic character of the N- or C- terminal domains further modulates the aggregation propensity
of the chimeras. In a related pursuit, we discovered that βS itself, often described as non-
fibrillogenic in the same sort of pH 7.4 buffer conditions, actually becomes fibrillogenic at a
mildly acidic 5.8 which can be accessed in the physiological environment. It turns out, that on top
of the NAC-based behavior of the chimeras, the extrinsic factor of the pH environment proves to
be a significant alteration of the physiological in vitro model we use to study the aggregation
process in vitro. Through a series of deductions, we were able to pinpoint a fibrillation switch
empirically to a pH sensitive residue type, and identify at least one single residue which is
sufficient for the switch. While on its own this work highlights that perhaps we should consider
more deeply the in vivo role of βS in disease, and that certain subcellular compartments and
microenvironments may be more conducive to disease, helping to narrow both the temporal and
spatial ranges of drug targets causing aggregation in vivo, the work also reinforces the ideas that
the physiological model we choose of these IDPs, must be carefully chosen.

An accurate physiological representation of synucleins include, at least, that the protein be
acetylated at its amino terminus, that partners in the in vitro environment should be chosen to be
realistic interaction partners, including in concentration and localization in the in vivo
environment, and that the totality of the physiological environment cannot be represented by one
environmental condition. The sensitivities of an IDP to the models we choose differ significantly
from the majority of folded proteins because of its inherently promiscuous interactive nature and
sensitivity in the environment. Further work exploring molecular details of synuclein aggregation
and associated neurotoxicity will depend strongly on highlighting key physiological events from
the point of view of the best models.
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8. CHAPTER 8: REFERENCES


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