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Interactions between the Intrinsically Disordered Proteins β -Synuclein and α -Synuclein

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Abstract

Several intrinsically disordered proteins (IDPs) have been implicated in the process of amyloid fibril formation in neurodegenerative disease, and developing approaches to inhibit the aggregation of these IDPs is critical for establishing effective therapies against disease progression. The aggregation pathway of the IDP alpha-synuclein (α S), is implicated in several neurodegenerative diseases known as synucleinopathies and has been extensively characterized. Less attention has been leveraged on beta-synuclein (β S), a homologous IDP that co-localizes with α S and is known to delay α S fibril formation. In this review, we focus on β S and the molecular-level interactions between α S and β S that underlie the delay of fibril formation. We highlight studies that begin to define α S and β S interactions at the monomer, oligomer, and surface levels, and suggest that β S plays a role in regulation of inhibition at many different stages of α S aggregation.

Keywords

alpha synuclein; beta synuclein; intrinsically disordered protein; protein-protein interaction

1. Introduction: Synucleinopathies and Aggregation

The role and actions of intrinsically disordered proteins (IDPs) have become an area of intense interest, with IDPs now being identified to play important roles in many biological processes including cellular signaling,^[1] phase separation,^[2] and transcription and translation.^[3] But beyond their normal physiological functions, IDPs have also been found to contribute to several human neurodegenerative diseases (e.g. Parkinson's disease (PD), Alzheimer's disease, Huntington's disease, spongiform encephalopathies, dementia with Lewy Bodies (DLB)) and non-neuropathic amyloidoses (e.g. type II diabetes, ApoAI amyloidosis, atrial amyloidosis),^[4] which are characterized by the accumulation, misfolding, aggregation and deposition of an IDP into amyloid plaques. While the amyloid diseases involving IDPs are relatively well-known by the general public, it is also true that natively folded proteins also misfold and aggregate to cause human amyloid diseases.^[5] It has been found that folded proteins on-average contain more amyloidogenic sequences than IDPs.^[6]

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Conflict of Interest Statement

The authors have declared no conflict of interest.

Understanding the mechanism of amyloid plaque formation beginning from the native IDP or folded protein, and developing approaches to inhibit aggregation, is critical for establishing effective therapies against disease progression. In the case of PD, DLB, and multiple system atrophy (MSA), the aggregation of the 140-residue IDP alpha-synuclein (α S) has been implicated in the disease etiology.^[7] A second member of the synuclein family, beta-synuclein (β S), is a 134-residue IDP that is co-expressed and co-localizes with α S,^[8] and has been found to inhibit α S fibril formation and reduce the formation of LBs.^[9, 10] The molecular mechanisms of the interaction between β S and α S, and the stages of the aggregation pathway at which these interactions arise, has been the subject of recent investigations. In this review, after a brief introduction of α S and known modulators of its aggregation, we discuss the nature of β S and its interaction with α S along the aggregation pathway to highlight how the IDP β S modulates α S aggregation.

A large body of work exists on trying to understand the conformational preferences of monomeric IDPs, and in establishing conformational ensembles from experimental parameters and computational modelling that accurately reflect the intrinsic disorder.^[11, 12] For α S and β S in particular, an irregular distribution of charged residues allows for different domains of the IDPs to be described as polyampholyte or polyelectrolyte,^[13] which can influence the conformational preferences of the IDP ensemble.^[14] The N-terminal domains of α S and β S exhibit a slight net-positive charge, while the C-terminal domains are negatively charged, with β S more so than α S (Fig. 1). These domains can participate in intra-chain, as well as inter-chain, electrostatic interactions.^[10, 15] The synucleins have been observed to transiently adopt both compact and extended structures, with the N-terminal domain displaying a higher propensity to form α -helices, especially in the presence of membranes, while the C-terminal domain tends to adopt more extended structure, likely due to the higher proportion of proline residues present (Fig. 1).^[16, 17, 18]

The aggregation pathways of many of the disease implicated IDPs, *in vitro*, can be described by a general mechanism of amyloid formation. IDP partners interact and nucleate directly from monomers to form primary nuclei, which can immediately undergo elongation, secondary nucleation and fragmentation to proliferate and grow.^[20–22] These molecular processes occur during all three phases (lag phase, growth phase, plateau phase) of the detected macroscopic aggregation profile, with rates and activities that vary with time.^[23] Several factors have been found to modulate the rate of α S aggregation, including pH, temperature, post-translational modifications, agitation, salt concentration, surfaces, and chaperone proteins.^[20, 21, 24, 25] Post-translational modifications may play a role in disease progression, with hyperphosphorylation of Ser129^[26] and small ubiquitin-related modifiers (SUMOylation) at K96 and K102 found in LBs.^[27] Various small molecules, peptides, and proteins have been shown to inhibit α S at various stages of the aggregation process. The antibiotic rifampicin was shown to inhibit fibril formation and disaggregate existing fibrils *in vitro*,^[28] while a mouse model of MSA showed marked reductions in monomeric, oligomeric, and S129 phosphorylated forms of α S with rifampicin treatment.^[29] Nortriptyline, an antidepressant, was found to delay the onset of fibril formation *in vitro* and showed some efficacy *in vivo* in protecting from α S neurotoxicity, and was suggested to bind directly to the α S monomer.^[30] The natural product squalamine reduced α S aggregation both *in vitro* and *in vivo*, by displacing α S from the surfaces of lipid vesicles.

[31] Small heat shock proteins (Hsps) have been found in LBs, and may play roles in directing α S folding and aggregation. Although the interactions between α S and Hsps was found to be weak and transient,[32] several (HspB8, Hsp27, Hsp70, α -crystallin) were found to prevent or reduce the formation of mature α S fibrils *in vitro* and provide neuroprotection *in vivo*.^[32, 33]

Targeting an intrinsically disordered protein with a small molecule, peptide or a second IDP differs from the traditional approach to drug design, in which the drug molecule typically targets a well-defined protein fold. Instead, for targeting an IDP, the promiscuity of interactions and the ensemble of interconverting IDP conformers must be considered,^[34] a field which is still in development.^[35] Interestingly, a few examples of direct IDP-IDP interactions have been identified recently, including the histone H1/prothymosin- α high affinity complex^[36] and the 4.1G/NuMA proteins,^[37] bringing up the possibility of utilizing a second IDP to target α S monomers in the earliest stages of aggregation. The remainder of this review will focus on the intrinsically disordered β S and its interactions with α S, its own potential cytotoxicity, and its potential utility as a therapeutic intervention to inhibit α S aggregation.

3. The Curious Case of β -Synuclein: Natural Inhibitor or Cytotoxic Homologue?

The IDP sequences of α S and β S contain ~78% sequence similarity and are described by an N-terminal region that contains several imperfect KTKEGV repeats, a hydrophobic NAC region, and a highly negatively charged C-terminal region (Fig. 1). Despite their similarities, the sequences differ significantly in the NAC region due to a 11-residue deletion, and β S contains more negatively charged residues and prolines than α S in the C-terminal region (Fig. 1). Studies of average secondary structure by circular-dichroism (CD) and hydrodynamic radius indicated that β S adopts a more unfolded conformation relative to α S at neutral pH.^[24] NMR measurements of the residual structural ensembles of these two IDPs in solution also found that β S had a reduced propensity for α -helical secondary structure in the N-terminal region compared to α S, while the C-terminus of β S adopted extended conformations.^[17] Unlike α S, β S does not aggregate to form fibrils under normal physiological conditions;^[24] instead, fibrillation can be induced under a variety of conditions, including acidic pH,^[38] metal ions and certain pesticides,^[39] and in the presence of lipid vesicles at elevated temperatures.^[40] We have recently found that a single-residue mutation located between the N-terminal and NAC domains (E61A) is sufficient to remove the pH-dependence of β S fibril formation, and allows it to form fibrils at a rate comparable to α S fibril formation at neutral pH.^[38] Despite its sequence similarity to α S, wild-type β S is not known to contribute to any of the synucleinopathies in humans, and instead has been found to provide some neuroprotective effects *in vivo*,^[9, 41] and acts as an inhibitor of α S aggregation *in vitro*.^[9, 10, 24, 42–44] However two missense mutations of β S, V70M and P123H, have been linked to DLB,^[45] and P123H β S can produce neurodegeneration in a transgenic mouse model;^[46] these have been proposed as “toxic gain-of-function” mutations that may occur in sporadic or familial synucleinopathies.^[47] The conformational ensemble of P123H β S was found to be more α S-like (i.e. more flexible), which allows for this

mutated β S to aggregate. Conformational differences of IDP monomers which effect their aggregation propensities have also recently been identified with the tau protein.^[48] The normal inhibitory properties of β S were also lost with the P123H mutation, and suggests that the extended structure of the proline-rich C-terminus is important for inhibiting α S fibril formation.^[49]

Recently, the notion that wild-type β S may itself cause neurodegeneration has been proposed in the literature. Overexpression of β S in cultured primary cortical neurons led to cell loss and signs of metabolic impairment, but to a lesser extent than overexpressing α S neurons.^[50] A further *in vivo* rat model showed slower signs of neurodegeneration upon infection with β S containing adeno-associated virus (AAV) vectors consistent with the cultured neurons, but eventually reached the same level of dopaminergic cell loss as α S (8 weeks β S vs. 2 weeks α S).^[50] When expressed in *Saccharomyces cerevisiae* (yeast), β S was found to form toxic cytosolic inclusions in a similar manner to α S.^[51] Even more interestingly, when β S and α S were co-expressed in yeast the cytotoxicity increased, even though hetero-dimers of α S and β S were found to exist, which were previously implicated as an important step in inhibition of α S aggregation by β S.^[10] Upregulation of SUMOylation machinery in yeast provided a protective effect to β S toxicity.^[52] These results warrant more study in the future to determine the basis of β S toxicity in yeast, and whether it is applicable to the synucleinopathies in humans.

4. The Interactions Between α -Synuclein and β -Synuclein

The interactions between β S and α S have been found to occur at three levels: through the formation of hetero-dimers (i.e. monomer-monomer interactions), through the formation of hetero-oligomers, and by influencing secondary nucleation processes (e.g. competition for binding on surfaces).

Monomer-monomer Interactions Between β S and α S

Mapping the interactions between IDPs is an inherently difficult task due to their transient nature, and is amenable to characterization by only a few experimental techniques.^[12, 53] To investigate monomer-monomer interactions between α S and β S, one approach has been to use solution-state NMR paramagnetic relaxation enhancement (PRE) measurements to probe weak and transient inter-chain interactions.^[54] In the PRE experiment, a paramagnetic probe is incorporated into the sequence of one protein that is not isotopically labeled with $^{15}\text{N}/^{13}\text{C}$ (NMR-invisible), and its effect on the relaxation parameters of another $^{15}\text{N}/^{13}\text{C}$ isotopically labeled protein (NMR-visible) is recorded. For synuclein, to probe the interaction between α S and β S monomers, cysteine mutations to residues 11, 44, 90 and 132 on α S and 11, 44, 80, and 134 on β S were introduced in order to incorporate a paramagnetic nitroxide spin label (MTSL); then NMR spectra were recorded of combinations of wild-type ^{15}N labeled α S or β S (NMR-visible) and ^{14}N MTSL labeled α S or β S (NMR-invisible) protein.^[10] This allowed for the observation of α S/ α S, β S/ β S and α S/ β S interchain interactions. It was found that α S homo-dimer interactions exhibit both transient head-to-head and head-to-tail orientations, while α S/ β S hetero-dimer interactions were only found to have weak and transient head-to-tail oriented interactions. NMR PRE titration experiments found that

residue specific K_d 's for the α S/ β S hetero-dimer were $\sim 100 \mu\text{M}$ (range 40–350 μM), while the α S/ α S homo-dimer K_d 's were $\sim 500 \mu\text{M}$ (range 90–1200 μM).^[10] Brown et. al. also found the interaction between the α S and β S monomers to be very weak. When equimolar amounts of β S monomer and α S monomer were incubated together in the presence of pre-formed α S seed-fibrils under quiescent conditions, no change in the aggregation behavior of α S was observed, suggesting that direct α S and β S interactions are extremely weak and indicating that β S does not interfere with α S fibril elongation.^[43] Homo-dimer interactions between β S monomers were not detected, indicating that any such interactions are extremely weak, consistent with β S's propensity to not form fibrils.^[10] The NMR data suggest that the sampling of transient dimer conformations promotes the very earliest stages of aggregation or inhibition. In this model, homotypic head-to-head interactions of α S dimers prefer aggregation, while the heterotypic head-to-tail interactions of α S/ α S and α S/ β S complexes prevent misfolding of α S by having to undergo conformational rearrangement to form the parallel arrangements of monomers in mature fibrils.^[55]

In a separate, more indirect approach to probe α S and β S interactions, a small library of domain-swapped α S/ β S chimeras were used to isolate the contributions of the N-terminal, NAC, and C-terminal sequence domains to the inhibitory interaction between α S and β S.^[56] Thioflavin-T (ThT) fluorescence assays showed the greatest degree of inhibition of fibril formation when α S was incubated in the presence of chimeras containing both the N-terminal and C-terminal primary sequence domains of β S simultaneously; having only the β S N-terminal or C-terminal domain (relative to a fixed NAC domain) resulted in a lesser degree of inhibition.^[56] This suggests that the inhibitory interactions are spread over multiple locations, at both the N- and C-termini, and may be cooperative. Taken together, these PRE and fluorescence measurements suggest that head-to-tail conformations, which exist in both the α S homo-dimer and α S/ β S hetero-dimer complexes and are mediated by interactions between the N- and C-termini, may provide a regulatory role in slowing down the conformational rearrangements needed to form aggregation-promoting head-to-head conformations.

A recent molecular dynamics and potential of mean force (PMF) computational study was conducted to better understand the strength of the association between α S and β S.^[57] Since there is no PDB structure of β S, the authors created a starting structure of β S by using the I-TASSER server, which aims to predict protein structures by using templates from the PDB in combination with iterative template fragment assembly simulations.^[58] The starting structure for β S that was created by the I-TASSER server is very similar to that of the micelle-bound α S (PDB ID: 1XQ8^[59]) that the authors chose to use as a starting point; namely, that there is a kinked α -helix in the N-terminus of each of the starting α S and β S structures. The authors found the atomic contact energy of the α S/ β S dimer to be more negative relative to the α S/ α S dimer, indicating a larger contact surface area of the heterodimer. The PMF calculations showed that the energy barrier for dissociation was twice as high for the α S/ β S hetero-dimer complex than for the α S/ α S homo-dimer. Taken together, the authors conclude that it is more favorable for α S to complex with β S to form hetero-dimers, rather than a second α S to form homo-dimers.^[57]

A second molecular dynamics study, again using structures derived from micelle-bound synuclein, found more favorable electrostatic energies of formation for α S/ β S head-to-tail hetero-dimer complexes relative to α S/ α S dimers, leading to the formation of stable α S/ β S non-propagating complexes.^[60] It was also observed that binding of a β S monomer to a preformed α S homo-dimer was stronger than the binding of an additional α S monomer to the homo-dimer. These experimental NMR and computational studies show the ability of β S to interact with and interrupt α S aggregation very early in the process, at the point where α S and β S monomers interact in both a solution state and in surface-associated conformations.

β S Interactions with α S Oligomers

The trending hypothesis for the IDP-misfolding diseases now favors an oligomer-centric model as the cause of neurodegeneration,^[61] with the oligomers causing membrane disruption or providing seeding activity for propagation of aggregation cell-to-cell.^[62] As the α S aggregation process continues, higher order oligomers of both an amorphous and ordered “proto-fibril” nature are formed, and it becomes crucial to understand the potential for β S to interact with α S oligomers and how this may affect the mechanism by which β S alters the kinetics of α S fibril formation. Single-molecule fluorescence measurements of α S/ β S interactions in a cell-free expression system showed that β S inhibits α S aggregation at the earliest stages, by preferentially incorporating into small oligomers.^[44] Two-color coincidence fluorescence assays, where α S is tagged with sGFP and β S is tagged with mCherry, showed that β S replaces α S in small oligomers as the ratio of β S: α S increases, suggesting that β S is able to shield α S/ α S homotypic interactions and inhibit further self-oligomerization.^[44] The ability of β S to inhibit aggregation of pathological mutations of α S, associated with familial forms of PD (A30P, G51D, E46K, H50Q, A53T), were also investigated. In the cell-free system, the mutants A30P and G51D were found to form small oligomers while E46K, H50Q and A53T formed larger aggregates and fibrils. Interestingly, β S was observed to efficiently inhibit aggregation of A30P and G51D mutants, while it did not efficiently interfere with aggregation of the other three mutants, although β S has previously been observed to delay the lag time of A53T proto-fibril and fibril formation.^[42] Taken together, these single-molecule fluorescence measurements provide evidence that β S interacts with early stage oligomers to inhibit aggregation of α S.

β S Influence on α S Surface Interactions

The normal physiological function of α S is not well understood, but it is thought to play a role in membrane remodeling and presynaptic vesicle release;^[63] during disease progression, α S oligomers are thought to disrupt cell membranes.^[64] Recent experimental evidence has provided several examples of α S and β S interactions with lipid membranes, [43, 60, 64–66] and both α S and β S have been found to interact with micelle and lipid-bilayer surfaces, with the N-terminal region adopting an α -helical secondary structure^[18, 67] and the charge composition and curvature of the membranes significantly affecting the degree of binding.^[66]

Assessment of the binding affinity of β S to lipid vesicles found that β S has a 5-fold lower affinity for DMPS (1,2-dimyristoyl-*sn*-glycero-3-phospho-L-serine) vesicles relative to α S, and does not show any lipid-induced increase in amyloid formation compared to the increase

in α S amyloid formation.^[43] However, more recently β S has been found to form DMPS lipid-induced protofibrils and mature fibrils when at an elevated temperature (60°C).^[40] In the former study, β S was found to decrease the rate of α S amyloid formation in a dose-dependent fashion, indicating that β S is inhibitory in the presence of lipid membranes.^[43] Further investigation determined that β S inhibited α S aggregation by competing for binding to the surface of the liposomes, and by a similar mechanism, β S also inhibited the auto-catalytic surface interactions^[21] of α S monomers by competing for binding to the surface of α S fibrils.^[43] The auto-catalytic surface interactions are described as a secondary-nucleation process, whereby already formed α S fibrils are able to template the nucleation of free monomers, which can then go on to form fibrils and further template nucleation and so on. This results in a significantly faster fibril growth profile relative to only growth by elongation of fibrils.^[20, 21, 68] It should be noted that the mechanism of inhibition proposed by Brown et. al. does not rely on direct interaction between α S and β S, but is rather a competition between α S and β S for binding sites on surfaces,^[43] and is therefore distinct from the inhibition mechanisms discussed in the previous sections which rely on direct α S and β S interactions.^[10, 44]

Inhibition of proteasomal degradation has been implicated as a contributing factor in Parkinson's disease, and there is evidence that α S binds to and inhibits a component of the 26S proteasome.^[69] Further, β S was shown to prevent proteasomal inhibition of α S by either interacting directly with α S monomers and aggregates or by competition with the binding interface on the proteasome.^[70]

5. Perspectives

While it is established that β S can reduce or inhibit the formation of α S fibrils *in vitro*, and is able to provide some neuroprotective effect *in vivo*, the mechanisms by which this arises are still not well understood. Here we have reviewed recent work on the different stages at which α S is inhibited by β S, and begin to provide a molecular description of the key interactions between α S and β S that arise in the early and later stages of α S assembly (Fig. 2). The aggregation of α S has been well characterized, and α S is known to adopt an intrinsically disordered conformation in solution (Fig. 2, *top row*). It can transiently form homotypic head-to-head interactions, leading to further aggregation, or off-pathway head-to-tail interactions. As more monomers are added to the initial aggregation nucleus, oligomers of a disordered, amorphous nature are formed; eventually a conformational change occurs, and the β -sheet content of these oligomers increases, transforming into "proto-fibrils." These proto-fibrils can undergo elongation to become mature amyloid fibrils, as well as participate in various secondary nucleation processes to seed new fibril growth. On the other hand, the aggregation process of β S has been less extensively characterized (Fig. 2, *middle row*). β S is intrinsically disordered in its monomeric state in solution, but no homotypic monomer-monomer interactions have been observed. It can form unstructured oligomers, but does not continue to aggregate to form fibrils unless mutations are introduced or non-physiologically neutral conditions are used.

The mechanisms of inhibition of α S by β S are just beginning to be understood and indicate that β S interacts with α S at several different levels in the aggregation pathway (Fig. 2,

bottom row). α S/ β S can form transient dimeric complexes of heterotypic head-to-tail interactions, form hetero-oligomeric complexes, and interact with the surface of α S fibrils to disrupt secondary nucleation processes. Further and more detailed characterization of the different intermediates along the aggregation pathways will help determine possible targets for therapeutic intervention. However, the difficulties of characterization here often stem from the inability to isolate and purify a specific oligomer out of a complex mixture of species along the aggregation pathway, or the amorphous nature of the oligomer itself, which preclude high resolution structural characterization. Further development and optimization of methods and techniques designed to purify and characterize this oligomer continuum, as well as methods to better define, simulate and model these ensembles, are needed in order to provide atomic-resolution detail on these important components of amyloid pathology, and bring light to one corner of this dark proteome. The studies reviewed here begin to provide a fundamental mechanistic understanding of how β S inhibits aggregation of α S, and highlight how interactions between β S and α S at the different stages of aggregation may create novel opportunities for developing therapeutic strategies to combat Parkinson's disease.

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Abbreviations:

IDP	intrinsically disordered protein
αS	alpha synuclein
βS	beta synuclein
PD	Parkinson's disease
DLB	dementia with Lewy bodies
MSA	multiple-system atrophy
LB	Lewy body
CD	circular dichroism
AAV	adeno-associated virus
PRE	paramagnetic relaxation enhancement
MTSL	<i>S</i> -(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl)methyl methanesulfonothioate
ThT	thioflavin T
PMF	potential of mean force
DMPS	1,2-dimyristoyl- <i>sn</i> -glycero-3-phospho-L-serine

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Figure 1.
Comparison of the aligned^[19] primary sequences of aS (*A*) and bS (*B*). Residues are colored by hydrophobic (gray), positively charged (blue), negatively charged (red), and polar-uncharged (green) residues.

Figure 2.

Summary of the species observed along the aggregation pathways of α S (*top row*), β S (*middle row*), and α S/ β S together (*bottom row*). α S can exist as a monomer, dimer, amorphous oligomer, proto-fibril, and mature fibril. β S exists primarily as a monomer, but can aggregate to form disordered oligomers. β S may delay α S aggregation by forming head-to-tail dimer complexes, hetero-oligomers, or by altering secondary nucleation properties.