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Unveiling transient protein-protein interactions that modulate inhibition of alpha-synuclein aggregation by beta-synuclein, a pre-synaptic protein that co-localizes with alpha-synuclein

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Pathology in Parkinson's disease is linked to self-association of α -Synuclein (α S) into pathogenic oligomeric species and highly ordered amyloid fibrils. Developing effective therapeutic strategies against this debilitating disease is critical and β S, a pre-synaptic protein that co-localizes with α S, can act as an inhibitor of α S assembly. Despite the potential importance of β S as an inhibitor of α S, the nature, location and specificity of the molecular interactions between these two proteins is unknown. Here we use NMR paramagnetic relaxation enhancement experiments, to demonstrate that β S interacts directly with α S in a transient dimer complex with high specificity and weak affinity. Inhibition of α S by β S arises from transient α S/ β S heterodimer species that exist primarily in head- to- tail configurations while α S aggregation arises from a more heterogeneous and weaker range of transient interactions that include both head-to-head and head-to-tail configurations. Our results highlight that intrinsically disordered proteins can interact directly with one another at low affinity and that the transient interactions that drive inhibition versus aggregation are distinct by virtue of their plasticity and specificity.

Protein aggregation is the origin of a wide variety of human neurodegenerative diseases including Parkinson's, Alzheimer's, Huntington's and Creutzfeldt-Jakob disease^{1,2}. Parkinson's disease (PD), a highly debilitating illness, is the second most prevalent of the late onset neurodegenerative diseases and affects as many as 6 million people worldwide³. As life expectancy continues to increase, neurodegenerative diseases like Parkinson's are becoming increasingly common and a threat to global public health. The diagnostic hallmark of PD is a deposit called a Lewy body that is primarily composed of the pre-synaptic intrinsically disordered protein α -synuclein (α S). Fibril formation of α S is also implicated in other neurodegenerative diseases, including multiple system atrophy and dementia with Lewy bodies, referred to as synucleinopathies. Although the function of α S is not clearly defined it is thought to be involved in promoting SNARE complex assembly^{4,5}, in regulation of the synaptic vesicle pool^{6,7}, and in remodeling membranes⁸. The origin of α S pathology in neurodegenerative disease is clearly linked to the self-association of the intrinsically disordered α S monomers into pathogenic oligomeric species and highly ordered amyloid fibrils.

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Figure 1. Sequence alignment of α S and β S shows high similarity between proteins. Sequence differences are color-coded according to the hydrophobicity index shown in the bottom right (red-hydrophobic, blue-hydrophilic). Identical residues are shown by dots and deletions are shown by dashes. The line above the sequence shows the N-(blue), NAC (grey) and C-terminal regions (pink).

One approach to developing effective therapeutic strategies against this debilitating disease is to identify inhibitors of α S aggregation. Small molecule inhibitors of α S have been proposed^{9–13}. In addition, proteins such as heat shock proteins (Hsp40, Hsp70, Hsp90, α B-crystallin)^{14,15}, and the intrinsically disordered β -synuclein (β S), a homologue of α S with which it co-localizes have been shown to interfere with α S assembly. A number of studies have established a neuroprotective role for β S^{16–24}. Masliah and co-workers have shown that β S is expressed at similar levels as to α S in the central nervous system. However the ratio of β S to α S at the mRNA level is significantly decreased in diseased brains, suggesting a regulatory role within the synuclein family²⁴. *In vivo* it has been shown that over-expression of α S with β S in mouse models significantly decreases the number of plaques formed²⁵ and that intracerebral injection of the lenti- β S virus reduces the formation of α S inclusions in transgenic mice²⁶. *In vitro*, it has been shown that the presence of β S with α S slows its aggregation^{16–18,25–27}. Despite the fact that β S has a very similar sequence to α S, it does not form fibrils on its own^{16,28–30}, but may form aggregates whose toxicity is debated^{31,32}. The *in vivo* data clearly suggest that β S plays an important regulatory role in inhibition of α S pathology but at this stage there is no molecular information about the nature, location and specificity of the protein-protein interactions that initiate the inhibition of α S by β S.

To understand the mechanism by which the intrinsically disordered protein β S interacts with α S, we use NMR to map the monomer-monomer interactions that lead to inhibition or promotion of aggregation. Despite the importance of these interactions, the molecular details are extremely difficult to obtain due to their transient nature and low population. Paramagnetic relaxation enhancement experiments (PRE) offer an excellent tool for characterization of weak and transient interactions because they are able to probe states that exist at low populations (even 0.5–5%) and exhibit short life times (250–500 μ s)^{33,34}. Here we use inter-chain NMR PRE experiments to identify and characterize weak transient complexes of α S and β S³⁵. We show that α S homo-dimers sample a heterogeneous range of population distributions, including head- to- head and head- to- tail configurations, while α S/ β S hetero-dimers exist primarily in head- to- tail configurations.

NMR inter-chain PRE titration experiments previously used on folded proteins³⁶ are applied here to intrinsically disordered proteins. These experiments allow us to obtain residue specific dissociation constants to inform us about the specificity and affinity of dimer interactions in the different regions of the transient disordered complexes. Our results show that the hetero-dimer transient head- to- tail interactions between α S and β S are approximately 5 times stronger than the interactions observed in the homo-dimer α S species suggesting that these α S/ β S interactions create a kinetic trap which delays or inhibits the formation of α S fibrils. The novel insight presented in this paper not only defines residue specific contacts between two intrinsically disordered proteins but also links the homo and hetero-complexes with distinct pathways that lead to aggregation versus inhibition.

Results

β S inhibits α S fibril formation in a dose dependent manner. α S and β S are part of the synuclein family. These can be described as: the N-terminus that contains KTKXGV repeats and forms helices at membranes³⁷, the non-amyloid- β S component (NAC) region, and the highly acidic and solubilizing C-terminus (Fig. 1). α S and β S have similar sequences, particularly at the N-terminus, but very different fibrillation and oligomerization properties (Fig. 1). The N-terminus for all synucleins is highly conserved, with only 6 substitutions between α S and β S sequences. In contrast, the C-terminus is the least conserved region with more prolines and more negatively charged residues with a net charge of -12 in α S and -15 in β S. β S has an 11 residue deletion in the NAC region that was thought to be important

Figure 2. Aggregation inhibition of α S by β S as monitored by ThT and TEM. (A) ThT fibrillation assay curves of: α S alone (blue), β S alone (blue), α S+ β S 1:1 mixture (violet), α S+ β S 1:5 mixture (magenta). Experimental conditions: 10 mM MES, pH 6, 70 μ M protein. The experiment was performed in triplicate at 37°C with shaking with teflon beads and average values were plotted with associated error bars. The buffer conditions matched the conditions of NMR PRE experiments. Negatively stained electron micrographs (scale 100 nm) of end products of the ThT fibrillation assay of: (B) α S (C) β S (D) α S co-incubated with β S, ratio 1:1.

in preventing fibril formation but substitution of this region into β S does not recover full fibrillation potential of α S^{28,29}.

Similarly to α S it has been established that the physiological form of β S and its pathological mutants *in vivo* are N-terminally acetylated³⁸. All experiments in this study are performed on the acetylated forms of the protein that we will refer to as α S and β S. All previous characterization of β S was performed on non-acetylated protein^{16,17,28,39}, therefore we use NMR and other biophysical approaches here to determine whether N-terminal acetylation affects the conformation or oligomerization state of the protein. NMR and other biophysical techniques including dynamic light scattering (DLS) and circular dichroism (CD) show that acetylated β S, similarly to acetylated α S, is primarily monomeric and unfolded (Supplementary Fig. 1 & 2). Secondary structure propensities indicate the formation of a transient N-terminal helix relative to the non-acetylated form of β S similarly to α S (Supplementary Fig. 1 & 2). In addition, the C-terminus of acetylated β S is more extended than the C-terminus of acetylated α S consistent with previous results on non-acetylated protein⁴⁰. Electrospray ionization mass spectroscopy (ESI-MS) experiments (Supplementary Fig. 1) show that acetylated β S can populate an extended and a compact form with a higher population of extended conformation relative to acetylated α S.

Addition of acetylated β S to acetylated α S inhibits fibril formation in a dose dependent manner, consistent with previous findings for the non-acetylated forms of the proteins (Fig. 2A)¹⁶. As the concentration of β S is increased we observe a significant change in the rate of the elongation phase and in the total ThT intensity as well as a small change in the lag phase. Previous work has shown that ThT can be used as a semiquantitative method to estimate relative amounts of fibril formed, although caution should be applied in interpreting ThT intensities^{41,42}. In this case, the inhibition of fibril formation as shown by the ThT experiments is supported by Transmission Electron Microscopy (TEM) data which shows significant changes in fibril morphology of α S/ β S relative to fibrils of α S alone (Fig. 2B–D). β S does not form fibrils and the α S/ β S mixture forms significantly fewer and shorter fibrils than α S alone as seen in TEM (Fig. 2C,D).

Mapping of residue specific transient interactions in α S/ α S homo- and α S/ β S hetero-dimer complexes using NMR inter-chain PRE experiments. ThT fluorescence experiments and TEM data (Fig. 2) have established that β S alters the aggregation kinetics of α S, however there has been no evidence to date of a direct interaction between these proteins. To determine the existence of, and to characterize transient inter-chain interactions between homo-dimers (α S/ α S, β S/ β S) and hetero-dimers (α S/ β S, β S/ α S), inter-chain NMR paramagnetic relaxation enhancement experiments were performed. In this experiment, NMR blind ¹⁴N-MTSL labeled protein is mixed with NMR visible (¹⁵N) unmodified protein and broadening of signal is limited to residues on the NMR visible chain that interact with the MTSL labels on the NMR blind chain (Supplementary Fig. 3). Signal broadening therefore reflects interactions between the NMR blind and NMR visible protein^{33–35,43}.

Figure 3. Contact maps of $\alpha S/\alpha S$ homo-dimers and $\alpha S/\beta S$ hetero-dimers show distinctive interaction profiles. Contact maps of transient dimers, shown as heat maps where each strip represents the color coded value of the residue-specific inter-chain paramagnetic relaxation enhancement rate (PRE rate- $H_N\Gamma_2$) induced by the proximity of the MTSL label to the residues in the indicated protein. Contact maps (A–D): figures A–D show four heat maps for all possible permutations of the spin label and NMR detectable chains of αS and βS : (A) ^{14}N - αS -MTSL/ ^{15}N - αS , (B) ^{14}N - αS -MTSL/ ^{15}N - βS , (C) ^{14}N - βS -MTSL/ ^{15}N - αS , (D) ^{14}N - βS -MTSL/ ^{15}N - βS . Contact maps show the PRE values colored in accordance with the legend; residues that do not exhibit interactions are colored blue and interactions higher than 12 Hz are colored red. Each strip on the contact maps corresponds to the spin label (y-axis). In one strip there are bins which correspond to the residue number on the NMR visible chain (x-axis). (E,F) Schematic representations of possible interactions of homo $\alpha S/\alpha S$ and hetero- $\alpha S/\beta S$ synuclein dimers. (E) $\alpha S/\alpha S$ corresponds to interactions from the contact map (A), (F) $\alpha S/\beta S$ corresponds to contact map (B,C); no homo-dimer for βS is shown as we observe no inter-chain interactions between βS chains. HS in the schematic stands for interactive “Hot Spot”. The hot spot size is defined based on the interactivity of the regions as shown in Supplementary Fig. 4A. Hot spot encompasses residues 38–45.

NMR PRE experiments were performed on all four combinations of possible homo- and hetero-dimers ($\alpha S^{15N} \alpha S^{MTSL}$, $\alpha S^{15N} \beta S^{MTSL}$, $\beta S^{15N} \alpha S^{MTSL}$, $\beta S^{15N} \beta S^{MTSL}$) to determine whether there is evidence for direct residue specific transient interactions and to establish the location of the interactions. The results of Fig. 3 are striking as they show immediately that the four combinations of possible hetero and homo-dimers have different transient interaction profiles (Fig. 3 and Supplementary Fig. 4). There is evidence for interactions between the homo-dimer complexes of αS , and the hetero-dimer complexes of αS and βS ; interactions between βS are essentially non-existent. We have introduced four spin labels along the sequence at positions 11, 44, 90 and 132 for αS and 11, 44, 80, 134 for βS to probe interactions in the N, NAC and C-terminal regions. Experimental results are presented as heat maps (Fig. 3A–D), where each strip shows color-coded values of residue-specific inter-chain paramagnetic relaxation enhancement rates (PRE rate- Γ) induced by the proximity of the MTSL label to another chain. Strong interactions (>12 Hz) are defined relative to the lack of interactions otherwise observed in the βS homo-dimer contact maps. Under the conditions of the experiment, αS and βS do not form fibrils or oligomers; therefore dimer detection arises as a result of low populations of dimers existing in equilibrium with the monomer precursor (for αS lack of higher order species in the sample has been confirmed by ESI-IMS-MS experiments⁴⁴ and for hetero-species this has been confirmed with ESI-MS).

αS populates a heterogeneous range of transient complexes while $\alpha S/\beta S$ hetero-complexes sample primarily head-to-tail non-propagating interactions. The heat map for the $\alpha S/\alpha S$ homo-dimer shows strong interactions between the N-terminal αS -MTSL labeled positions A11 and T44 with the N-terminal region 36 to 44, and the C-terminal region 124 to 140 (Fig. 3A). Earlier studies

by our group³⁵ on α S showed transient inter-chain interactions between the N- and C- termini (N-C), but by increasing the number and positions of the MTSL spin labels we now observe new inter-chain interactions between the N-termini showing that α S can populate multiple dimer configurations. In noticeable contrast, the α S/ β S hetero-dimers show strong interactions between the N-terminal α S-MTSL labeled positions A11 and T44 and the C-terminus from residues 105 to 134 and extremely weak N-N terminal interactions between 37 and 41 (Fig. 3B). According to the heat map, the hetero-dimer interactions between the N-terminus of α S and the C-terminus of β S appear to be more extensive and stronger (residues 105–134 compared to residues 124–140 in α S/ α S homo-dimers) (Fig. 3A–C) than those in α S. β S shows extremely minimal interactions with itself supporting the view that β S does not form fibrils (Fig. 3D).

A schematic representation of the dominant homo and hetero-dimer interactions shows that both N-N and N-C configurations are sampled by the α S homo-dimers while only N-C terminal interactions are sampled in the α S/ β S hetero-dimers (Fig. 3E,F). The favorable interactions of the N-terminal hydrophobic region of α S with itself (N-N), and with the C-terminus of α S and β S suggest that they act as an aggregation initiation region. The highly interactive N-terminal region encompassing residues 38–45 is referred to as ‘hot spot’ region (Suppl. 4A). In the α S homo-complexes, the interactions detected by N- and C-terminal probes show symmetry, implying that the interactions we observe are not experimental artifacts. The NMR PRE data show that α S homo-dimers can sample a heterogeneous range of populations, including head- to- head and head- to- tail configurations while α S/ β S hetero-dimers sample only head- to- tail dimers. This suggests that the hetero-dimers have a more limited range of conformational preferences for the dimer species.

Aggregation versus inhibition of α S by β S is due to a balance between specificity and affinity of transient interactions. The inter-chain NMR PRE experiments described above are powerful as they provide us with direct evidence for the existence of transient interactions and allow us to pinpoint the specific residues involved in these encounter complexes, however the specificity and affinity of the interactions remains unknown. We extend an earlier approach^{36,45} designed for folded proteins to transient encounter complexes of IDPs. We obtain residue-specific equilibrium dissociation constants (K_D) by performing titrations of ^{15}N labeled α S with MTSL-labeled ^{14}N - α S. K_D s are obtained by fitting the intermolecular transverse ^1H relaxation rates to a titration curve (see methods) (Fig. 4).

NMR PRE titration experiments and data analysis is complex as K_D s are anticipated to be weak due to the disordered nature of the monomers. The titration curves are grouped according to their profiles and three different types of patterns emerge (Fig. 4A–C). Representative examples of titration curves arising from interactions between α S-44-MTSL and residues 38–41 of the N-terminus (Fig. 4A), and residues 125–140 of the C-terminus (Fig. 4B), show that they are distinct from one another. The titration curves for the N-N interactions show a linear dependence between PRE values and concentration indicating non-specific interactions at this site, while interactions with the C-terminal 125–140 exhibit non-linear titration curves suggesting specific interactions. The range of K_D values in this group is between $K_D \sim 500 \mu\text{M}$ (range 90–1200 μM) using the data analysis described in methods. While we observe both non-specific (N-N) (Fig. 4A) and specific interactions (N-C) (Fig. 4B) in homo- α S complexes, the hetero-complexes α S/ β S (Fig. 4C) have only specific N-C interactions. The titration profiles of α S-44-MTSL with β S in the region 115–134 have a narrower range of K_D values ($K_D \sim 100 \mu\text{M}$, range 40–350 μM) than those associated with the K_D values of α S-44-MTSL α S with its own C-terminus ($K_D \sim 500 \mu\text{M}$, range 90–1200 μM) suggesting more uniform behavior across this region and higher specificity and affinity by approximately 5 fold (Supplementary Tables 1&2). The differences between the strengths of the interactions and the range of residues over which the interactions are occurring is seen clearly in the 3D plots where the interaction regions in α S are more rugged while the interactions between α S and β S are smoother, more uniform and extend over a wider range of residues (Fig. 4D,E).

Discussion

β S plays a role in the inhibition of α S aggregation but the mechanism by which this occurs and the stage in the aggregation pathway at which β S first interacts with α S has been unknown. We demonstrate that the monomer species of α S and β S interact directly with one another at specific sites suggesting that inhibition may begin at the very earliest stages of the fibril formation process. The molecular interactions and affinities obtained in the NMR PRE experiments described here support the view that early stages of aggregation versus inhibition may be due to a balance between conformational heterogeneity, specificity and affinity. Early stages of aggregation in α S may be promoted by sampling or searching conformational structures with weak transient affinities, including both non-specific head- to- head and weak specific head- to- tail dimers (Fig. 5A). In contrast early stages of inhibition may be favored by sampling only head- to- tail interactions with higher affinity and specificity within the α S/ β S hetero-complex (Fig. 5B).

Head- to- tail interactions exist in both the homo- and hetero-dimer complexes, which strongly suggests that they may play a regulatory role on α S folding or misfolding. In light of the fact that the fibril state of α S assembles into in-register parallel cross- β -structure^{46,47}, the NMR data suggests that head-to-tail α S/ β S dimers would have to undergo conformational rearrangement to reach the final fibril form. This may thereby delay or inhibit the kinetics of fibril formation. We propose that the α S homo-complexes are more aggregation prone than α S/ β S complexes for two reasons: first, α S can sample

Figure 4. Residue specific binding affinities for transient α S/ β S hetero-complexes show higher specificity and affinity than α S/ α S homo-complexes. (A–C) Representative examples of titration curves arising from PRE titration profiles from α S-44-MTSL to 15 N- α S and 15 N- β S. Illustration of how the K_D is obtained from fitting the PRE titration data as a function of the molar ratio of 14 N- α S-T44C-MTSL labeled to 15 N- α S (A,B) and to 15 N- β S (C). (A) α S residues 38–41 show a linear dependence between PRE values and concentration indicating non-specific interactions at these sites, (Residues colors: 38-black, 39- blue, 40-pink, 41-green), (B) α S residues 125–140 exhibit non-linear titration curves indicating specific interactions. (Residues colors: 125-orange, 130-blue, 135-pink, 137-green, 139-black), (C) β S residues 109–134 interact strongly and more uniformly with α S-44-MTSL (Residues colors: 115-green, 120-orange, 121-blue, 129-pink, 131-black). Shaded areas indicate the range of possible K_D values. Titration profiles for α S/ β S show a higher degree of saturation consistent with more specificity and higher affinity than those for α S/ α S (D,E) 3D representation of titration curves in the C-terminus (D) 15 N- α S C-terminus titrated with T44C-MTSL labeled 14 N- α S (E) 15 N- β S C-terminus titrated with T44C-MTSL labeled 14 N- α S titration. The x-axis shows the C-terminal residues of both α S and β S after sequence alignment, the z- axis depicts the PRE values, the y- axis the ratio of T44C-MTSL labeled 14 N- α S to 15 N sample concentration. The surface is colored using a rainbow palette, where low $H_N\Gamma_2$ values are red and the highest values are purple (according to legend below the plot).

head- to- head interactions that are potentially aggregating promoting while α S/ β S does not sample these; second, the non-propagating head- to- tail α S/ α S complex has weaker, lower affinity interactions relative to the α S/ β S complex suggesting that the conformational rearrangement required for fibril formation may be more facile for the α S/ α S homo-complex.

Despite the fact that both the α S/ α S homo-dimer and the α S/ β S hetero-dimer sample head- to -tail interactions, there are notable differences in terms of the strength of the interactions and the range over which the interactions extend. For homodimers the interaction of the α S “hot spot” with the C-terminus of α S extends over ~ 15 residues, while the interaction of the α S “hot spot” with the C-terminus of β S extends over a broader range of residues from 105 to 134. In both cases the dimers are flexible and are able to probe a big surface area, possibly adopting multiple dimer conformations. In addition, the α S and β S C-termini are highly negatively charged and contain aromatic and hydrophobic residues suggesting that initial interactions are mediated by electrostatics, which are anchored and stabilized through the hydrophobic and aromatic interactions.

Using NMR PRE titration experiments we demonstrate that interactions between the C-terminus of β S and the N-terminus of α S are approximately 5 times stronger and more extensive than the interactions of

Figure 5. Schematic model of α S/ α S and α S/ β S transient interactions suggest a new molecular view of inhibition routes to α S aggregation. (A) α S/ α S homo-dimer interactions: head-to-head aggregation prone interactions at residues 36–44 co-exist with head-to-tail inhibitory interactions at residues 125–140. (B) α S/ β S hetero-dimer interactions: head- to- tail inhibitory interactions span a broader range of C-terminal residues 115–134 with higher affinity. (C) No interactions are observed between monomer chains of β S. Protein chains are color-coded according to the interactivity of the regions as shown in Supplementary Fig. 4. The NMR data show that α S homo-dimers can sample a heterogeneous range of populations including N-to-N and N- to C-terminal configurations while α S/ β S hetero-dimers sample only N- to C- terminal configurations with higher affinity and specificity. In light of the fact that the fibril form of α S forms β -strands that assemble into in register parallel β -sheets this would suggest that the N-C terminal dimers would have to undergo conformational rearrangement to reach the final fibril form, thereby delaying or inhibiting the kinetics of fibril formation.

the C-terminus of α S with its own N-terminus. This increased specificity and affinity may be attributed to two factors: a higher content of negative C-terminal charges thereby enhancing electrostatic interactions, and a higher proportion of proline residues thereby altering the conformational ensembles sampled by the C-terminus. NMR and ESI data indicate that the C-terminal of β S has a higher population of more extended species (Supplementary Fig. 1) and may therefore provide a more accessible surface area for interactions with the N-terminus “hot spot” of α S. These differences suggest that small changes in the binding surface, even in these highly dynamic IDP complexes, can lead to substantial changes in interactivity that can modulate the pathway of protein aggregation versus inhibition.

In conclusion, our studies highlight that transient and weak interactions are important for protein recognition pathways of IDPs that lead to diseases such as amyloidosis where the proteins self-associate and propagate to highly ordered fibrils. Work by Radford *et al.*⁴⁸ have shown that weak interactions are also important for folded proteins in directing aggregation versus inhibition. By performing NMR inter-chain PRE titration experiments we have identified and characterized the strength and affinity of transient interactions between α S and β S, both IDPs. As IDPs are highly flexible, binding affinities are likely to behave in a non-cooperative manner across the protein⁴⁹. Our data support this view and highlight the variable nature of the binding affinities that result in aggregation promoting versus aggregation inhibiting configurations.

Knowledge about the distinct dissociation constants of different interaction regions provides a new framework for thinking about therapeutic intervention by providing direct information about which regions to target for small molecule intervention and about the conformational features that may be most effective at intervention. There have been some small molecule inhibitors that have targeted the N-terminus of α S but now based on our detailed molecular understanding of α S/ β S interactions we can optimize the surface interactions to design novel inhibitors^{10–12,50–52}. In addition, the powerful methods used here to identify the early stages of interaction of α S with β S can be extended to design inhibitors with biologics and can be applied even more widely to study other cross amyloid interactions, such as those between α S and amyloid- β -protein, or α S and tau, that have been shown to play a critical role in cross-seeding in neurodegenerative disease^{48,53–56}.

Methods

Mutagenesis, expression and purification. Cysteine mutants of α S (A11C, T44C) and β S (A11C, T44C, A80C, A134C), were prepared by site-directed mutagenesis using AccuPrime pfx from Invitrogen. To obtain N-terminal acetylated forms of α S and β S proteins, co-expression with the NatB plasmid N-Acetyltransferase B was performed, as described previously⁴⁴. Protein purification was performed according to previous protocols⁵⁷. Similarly, MTSL spin label conjugation to cysteine mutants was performed using previously established protocols³⁵.

PRE experiments/controls. All NMR PRE experiments were performed in 10mM MES, pH 6, without addition of salt and with 10% D₂O required for NMR experiments. NMR inter-chain PRE experiments were performed by mixing NMR blind ¹⁴N-MTSL labeled protein with NMR visible ¹⁵N unmodified protein³⁵. Samples were prepared as follows: lyophilized samples of ¹⁴N-MTSL-cysteine mutants or ¹⁵N non-modified proteins (α S or β S) were separately dissolved. Samples were passed through a 100 kDa filter to remove higher order oligomers, and then concentrated using 3 kDa filters to be able to dilute the sample to a final concentration of 250 μ M. Low sample concentration was chosen to minimize non-specific interactions. The total sample concentration was 500 μ M, with 250 μ M non-modified ¹⁵N protein and 250 μ M ¹⁴N-MTSL labeled protein. All combinations of proteins were mixed in order to see all possible interactions. Diamagnetic samples were prepared by reducing samples with 10 $\times$ excess of Ascorbic Acid and 5 $\times$ buffer exchange using 3 kDa cutoff filters from Millipore Inc. All the controls have similar patterns and are in the range of experimental error. Additionally, the pattern for the reduced diamagnetic control is consistent with the pattern for the mixture of ¹⁴N and ¹⁵N non-modified samples. The ¹H-¹⁵N HSQC of the cysteine mutants with the reduced spin label did not disrupt the HSQC pattern of α S and β S.

All ¹H-R₂ measurements of paramagnetic and diamagnetic (reduced) samples were acquired on a 600 MHz Varian at 15 °C using previously published pulse sequence and protocols^{33,35}. The inter-chain paramagnetic relaxation enhancement rate (PRE rate- Γ_2) is the residue specific difference of the ¹H-R₂ values of the paramagnetic and diamagnetic samples. Increased ¹H-R₂ relaxation rates on the ¹⁵N visible chain indicates that the NMR blind ¹⁴N -MTSL labeled protein is in the proximity of specific residues in the NMR ¹⁵N labeled visible chain. Para- and diamagnetic ¹H-R₂ were analyzed and processed using nmrpipe⁵⁸ and sparky⁵⁹. For all experiments 10 relaxation delays were used: 12, 32, 104, 12, 124, 64, 48, 94, 64, 20 ms. Two data points (12 ms, 64 ms) were repeated in the experiment to obtain good statistics for the error analysis. Errors of Γ_2 were calculated using error propagation, and errors were below 2 Hz. All of the interactions that were considered significant were at least 2 times higher than the mean value, and all of them were higher than the 3rd quartile and at least 8 Hz.

NMR PRE titration experiments. For NMR PRE titration experiments we used protocols described before^{36,45}. Spin label sample concentrations were reduced to low volume >32 μ L and were added to 350 μ L of 250 μ M ¹⁵N sample of either α S or β S; the changes in the sample volume were less than 10% of the overall sample volume. For α S titrations we used the following ratio of ¹⁴N- α S-44-MTSL labeled samples to ¹⁵N NMR visible samples: 0, 0.25, 0.5, 0.75, 1, 1.5, and in the case of β S we went up to ratio of 2. α S in this ratio exhibited shifts in the HSQC spectra, thus we removed this point from the analysis. We ran 6 data points ranging from 12–125 ms with the first point repeated twice for statistics. The PRE profiles during the titration did not show significant contributions from the non-interactive regions. The relaxation rates for the non-interactive NAC region for the titration ratio ¹⁵N/¹⁴N equal to 1.5 are well below 8 Hz.

NMR PRE titration fitting and analysis. Titration curves of ¹⁴N-MTSL labeled protein with ¹⁵N protein were fit using equation (1):

$$^1H_N\Gamma_2^{app} = \frac{1}{1 + xK_d^{-1}}\Gamma_2^{free} + \frac{xK_d^{-1}}{1 + xK_d^{-1}}\Gamma_2^{bound}, \quad (1)$$

where x is the concentration of ¹⁴N- α S (T44C-MTSL) in solution, Γ_2^{free} —represents paramagnetic relaxation enhancement for free protein, and Γ_2^{bound} represents the maximum observed saturation value. The fitting scheme was based on the papers by Bax⁴⁵ and Clore³⁶. We used a nonlinear regression model with three fitting parameters Γ_2^{free} , Γ_2^{bound} and K_d . The χ -squared statistic that measures the difference between the observed and predicted increase in ¹H_N Γ_2^{app} was optimized using the R statistics package minpack.lm, via the Levenberg-Marquardt algorithm⁶⁰. The resulting fit was robust to small changes in Γ_2^{free} , K_D , and Γ_2^{bound} and provided us with residue specific K_D values between ¹⁴N- α S-44-MTSL and ¹⁵N- α S or ¹⁵N- β S. The K_d calculations were performed for residues whose 5th titration point had PRE values higher than 15 Hz (Supplementary table 1&2). Results are summarized in the supplementary tables 1&2.

Thioflavin T (ThT) aggregation assays. The experimental set up for measuring time dependent fibril formation with ThT has been previously described⁵⁷. The conditions used for the ThT assay match

the conditions of the NMR PRE experiments described earlier. 5–10 mg of lyophilized α S and β S was dissolved in 10 mM MES, pH 6, and centrifuged for 10 min. at 14000 rpm to remove big oligomers, and purified using size exclusion chromatography (Superdex 75 GL 10/300, from GE Healthcare Life Sciences). Protein was subsequently concentrated using 3 kDa centrifugal units (Millipore Inc). The final concentration of α S and/or β S was 70 μ M. Addition of β S to α S was performed in multiples of 70 μ M. ThT fibrillation rates of the following samples were measured: (1) α S alone (2) β S alone; (3) ThT fluorescence of 1:1 the mixture α S + β S, and (4) ThT fluorescence of 1:5 the mixture of α S + 5x β S. Each condition was repeated 3 times in 37°C, with linear shaking in the presence of the Teflon beads. For each sample 3 curves are plotted on the figure to show the variability of the aggregation profiles.

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Author Contributions

M.J. designed, performed, analyzed experiments and wrote the manuscript, K.-P.W. developed the methodology for NMR PRE titration experiments, J.B. designed and supervised experiments and data analysis and wrote the manuscript.

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