Molecular dynamics simulation reveals that switchable combinations of β -sheets underlie the prion-like properties of α -synuclein amyloids.

Hiroki Otaki^{*1}, Yuzuru Taguchi^{*2}, and Noriyuki Nishida²

1 Center for Bioinformatics and Molecular Medicine, Nagasaki University Graduate School of Biomedical Sciences,
Nagasaki, Japan

2 Division of Cellular and Molecular Biology, Nagasaki University Graduate School of Biomedical Sciences,
Nagasaki, Japan

* The authors equally contributed to the work

*To whom correspondence should be addressed:

Hiroki Otaki, otaki@nagasaki-u.ac.jp, or Yuzuru Taguchi, ytaguchi@nagasaki-u.ac.jp

Division of Cellular and Molecular Biology,
Department of Molecular Microbiology and Immunology,
Nagasaki University Graduate School of Biomedical Sciences
Basic Medical Science Building
1-12-4 Sakamoto, Nagasaki 852-8523, Japan
TEL: +81-95-819-7059 / FAX: +81-95-819-7060

Abstract

Diversity of prion strains is one of the most mysterious traits of prions because they are mere aggregates of abnormally-folded forms of single protein species, prion protein (PrP^{Sc}), without genome. Although the strain-specific properties are hypothesized to be enciphered in the strain-specific structures of PrP^{Sc} instead of nucleotide genome, specifically what structure can code the information remains an enigma due to the incompatibility of PrP^{Sc} with structural analyses. Although the strain diversity was regarded as unique to prions, recently other disease-associated amyloids of α -synuclein (α Syn) or tau are also reported to have “strains”. As detailed structures of α Syn amyloid are already identified and the properties of mutant α Syn associated with familial Parkinson’s diseases, e.g. A53T, H50Q, and G51D, have been characterized, structure-phenotype relations of this type of amyloid could be investigated by using the α Syn amyloid as a model. Here we intensively investigated the mutant α Syn amyloids by molecular dynamics simulation to characterize influences of mutations on the structures of homo- or hetero-oligomer stacks of the amyloid. The simulations revealed directionality of the amyloid stack, remote effects of the mutations on distant β -sheets, existence of at least two switchable interfaces/amyloid cores, and distinct effects of hetero-oligomerization depending on mutation types. Collectively, those findings implied a possible mechanism of the strain diversity of the amyloids which have multiple in-register parallel β -sheets side-by-side, and support the view that their prion-like properties are inherent in the characteristic structures. We expect that the notion is also applicable to PrP^{Sc}.

Introduction

Prion disease is a unique group of neurodegenerative disorders which are caused by conformational abnormality of prion protein (PrP) and used to be mistaken as viral infections because of very high infectivity, diverse clinicopathological pictures suggestive of existence of many virus strains, strain-specific host ranges, and species barriers along with new-host adaptations [1]. Since a prion in reality consists solely of PrP in its abnormal isoform (PrP^{Sc}) [1], the mechanism how mere aggregates of a single protein acquire the virus-like properties has been a long-standing challenge to the current biology. For example, regarding strain diversity of prions, an enormous body of evidences have indicated that strain-specific pathogenic information is enciphered in strain-specific structures of PrP^{Sc} [2], but specifically what structures could code the information is unsolved yet, partly because detailed structures of PrP^{Sc} have not been identified due to its incompatibility with high-resolution structural analyses. Based on currently available data, PrP^{Sc} is hypothesized to be either in-register parallel β -sheet amyloid [3] or β -solenoid [4].

Many neurodegenerative diseases including Alzheimer's disease (AD) and Parkinson's disease (PD) are also caused by amyloid formation of their respective responsible proteins, e.g. A β peptide in AD, α -synuclein (α Syn) in PD, and tau protein in tauopathies. Interestingly, those disease-associated amyloids are often in-register parallel β -sheet amyloids [5][6][7][8] and have "prion-like" properties in common albeit lesser extents than those of authentic prions, suggesting a possibility that the properties are inherent in the in-register parallel β -sheet structures: for example, seeding efficiencies by an amyloid can be inefficient in heterologous reactions where substrates and template molecules are distinct in primary structures [9], and clinicopathological pictures of familial PD vary depending on mutations [10], reminiscent of species barriers and strain diversity of prions, respectively. Those similarities among the amyloids imply a possibility that knowledge from investigation on one amyloid is applicable to all the others.

If the prion-like properties are inherent in the structures of the in-register parallel β -sheet amyloids, structural investigation of those amyloids can provide a clue to elucidate the prion-like properties. Although they also share the similar difficulties in structural analyses as PrP^{Sc}, high-resolution structures are available for some amyloids including A β peptide [6], brain-derived tau protein [8], and *in vitro*-formed α Syn fibrils [7], which were determined by solid-state NMR (ssNMR) and/or cryo-electronmicroscopy. We thought that molecular dynamics (MD) simulation of in-register parallel β -sheet amyloids could provide an insight into their intrinsic properties which are responsible for the prion-like properties. We were particularly interested in the α Syn amyloid (PDB ID 2n0a)[7], because of its relatively large size and the "Greek-key" conformation with multiple β -sheets intricately interacting one another. α Syn is a cytoplasmic protein consisting of 140 residues, well-known as the main component of Lewy bodies which are characteristically observed in Parkinson's disease and dementia with Lewy bodies [11], or as the precursor of the "non-amyloid- β component" (NAC) fragments of amyloid plaques associated with AD [12]. While the physiological micelle-bound conformation has two large α -helixes in the N-terminal-side domain and disordered C-terminal-side domain [13], in α -synucleinopathies including PD, α Syn are found in amyloid forms: the conformations seem to be associated with the clinicopathological pictures, like prions [10]. The relations between the structures of the α Syn amyloids and the clinicopathological pictures are well demonstrated by the variation in clinical features among familial PD associated with mutations of α Syn, e.g. A53T, G51D and H50Q [10], although the molecular mechanisms how those mutations precipitate amyloid formation leading to development of the

characteristic diseases are unsolved. α Syn is also suitable for *in-vitro* experiments because the recombinant proteins can be easily purified and form amyloid *in vitro* which exerts toxicity on cultured cells or animals [14][15]; those features enable efficient assessment of consistency between the *in silico* findings and the *in vitro* behaviors of the protein. We therefore utilized α Syn for investigation of general properties of in-register parallel β -sheet amyloids, and as a potential surrogate model of PrP^{Sc}. Since the detailed structures of the α Syn amyloid was available, we tested the validity of the hypothesis that the prion-like properties are inherent in the in-register parallel β -sheet structures in *in silico* settings.

The Greek-key conformation of α Syn amyloid has been analyzed in many *in silico* analyses including MD simulation. For example, Xu *et al.* assessed influences of pathogenic mutations on the amyloid [16], and postulated that the mutations switch preferred amyloid conformations of the substrate α Syn between the Greek-key conformation and a superpleated β -structure, whose distribution patterns of β -sheets were mutually rather distinct. Here, we devised a few points in MD simulation of α Syn amyloids for reliable and informative results. First, we used a ten-molecule stack of α Syn for MD simulation to characterize behaviors of molecules at different positions/layers in the stack: the molecules at the both ends of the stack, i.e. the Chains A and J (**Fig 1A, right panel**), are predictable to be most unstable, while in contrast those in the middle of the stack would be stable; then how the stability of the peptides transit from the middle to the end? We thought that ten layers would be thick enough to address the question. Second, we set the sampling time length at 400 ns per run for reliable evaluation of the stabilities of amyloid stacks and the influences of mutations on the structures; besides, each simulation was repeated three to five times for statistic evaluation to reduce influences of stochastic factors. Moreover, since most of the mutant α Syn-associated familial PD patients are heterozygous for the mutant allele, we analyzed hetero-oligomer A53T amyloids, which combine A53T mutant and wild-type α Syn at various ratios, to characterize the influences of the mutant molecules on the wild-type in the same stack.

To our surprise, the relatively small and simple α Syn amyloids exhibited behaviors reminiscent of characteristic properties of prions, particularly strain diversity and species barriers. The results seemed to support our view that the prion-like properties of amyloids are inherent in the in-register parallel β -sheet structure, a rather singular status where many molecules take the same conformations and intimately interact. We expect that the knowledge is applicable to other disease-associated amyloids as well, to help address their unsolved problems derived from in-register parallel β -sheet structures.

Material & Methods

Modeling Structure

We used the Greek-key conformation of the α Syn amyloid (PDB ID 2n0a) for the starter. Since our interest was in the general properties of in-register parallel β -sheet amyloids rather than α Syn itself, we chose to truncate the N- and the C-terminal disordered regions, namely 1-35 and 100-140, to reduce calculation burdens. Then, to minimize the electrostatic interactions at the N- and C-termini, we modified them with acetylation and methylation using AmberTools16 [17], respectively. We ascertained that behaviors of the “capped 36-99” were not very different from those of either “uncapped 36-99” or “capped 20-110” (data not shown). For simplicity of description, we numbered the β -sheets from β 1 to β 7 (**Fig 1A, middle panel**), and refer to each layer from top to bottom as the Chain A to J (**Fig 1A, right panel**). For modeling of the mutant α Syn including A53T, G51D, and H50Q, we used

SCWRL4 [18]. Modeled amyloid was solvated with a rhombic dodecahedron water box with minimum wall distance of 12 Å. Na⁺ and Cl⁻ ions were randomly placed to neutralize the system and yield a net concentration of 150 mM NaCl. Protonation status of the histidine at the codon 50 was fixated as N^δ-H tautomer (HID form in AMBER naming convention) in all the simulations.

MD simulation

For all the MD simulation, we used GROMACS software [19] with AMBER ff99SB-ILDN force fields [20] for the αSyn amyloids and the TIP3P water model [21]. The system was minimized for 5,000 steps with steepest descent method, followed by 2,000 steps with conjugate gradient method. During the minimization, heavy atoms of the amyloids were restrained with a harmonic potential with the force constant of 10.0 kcal/mol•Å². After the minimization, the temperature of the system was increased from 0 to 310 K during 1 ns simulation with the restraints. Next, 1 ns of equilibration run was performed by gradually reducing the restraints from 10.0 kcal/mol•Å² to zero and subsequent equilibration was performed in the NPT ensemble for 2 ns at 310 K and 1 bar. Production runs were carried out for 400 ns in the NPT ensemble at 310 K and 1 bar (**Suppl Fig S1A**). We used velocity-rescaling scheme [22] and Berendsen scheme [23] for thermostat and barostat, respectively. The LINCS algorithm [24] was used to constrain all bonds with hydrogen atoms, allowing the use of 2 fs time step. Electrostatic interactions were calculated with the Particle-mesh Ewald method [25]. The cutoff length was set to 12 Å for Coulomb and van der Waals interactions. The Verlet cutoff scheme [26] was used for neighbor searching. We have carried out 5 MD simulations for the wild-type amyloid and the homo-oligomer A53T amyloid and 3 MD simulations for the other mutants. For each production run, trajectory snapshots were saved at every 10 ps.

Analyses

For the analysis of the MD simulations, the first 100ns of each trajectory was discarded except for calculation of root-mean-square deviation (RMSD). Thus, 30,001 snapshots (trajectory from 100 to 400ns) were used for the analysis. The RMSD for backbone atoms of every chain was calculated using *gmx rms* program in GROMACS package. The ssNMR structure was used as the reference structure. Although we first tried ordinary root-mean-square fluctuation (RMSF) using *gmx rmsf* program in GROMACS for evaluation of the structural stabilities of each peptides, it proved not very suitable for the αSyn amyloid because the deviations in the β1-β2 regions were so large as to mask the deviations in the other regions (**Supplementary Movie**). We therefore decided to utilize the local backbone B-factor suggested by Fuchs et al [27]. The local backbone B-factor is calculated via multiple local alignments to all backbone atoms including hydrogen of the residue of interest instead of global alignment for usual RMSF. The local backbone B-factor is evaluated as the mass-weighted B-factor of the atom groups used for the alignment. We also use the ssNMR structure as the reference for alignment and *cpptraj* [28] in AmberTools for calculation. Hydrogen bond pattern was characterized according to their occupancy in the trajectory with geometric criteria using *H-bond* plugin in VMD [29]. A hydrogen bond is assigned if the distance between donor (D) and acceptor (A) is less than 3.2 Å and the angle A-D-H is less than 30°. The secondary structure content during the simulations was calculated using DSSP program [30][31] and *gmx do_dssp* program in GROMACS. Hydrophobic contact was analyzed using *PyInteraph* [32]. The hydrophobic contact is assigned if the distance between the centers of mass of side-chains is less than 5 Å [33]. The results of the hydrogen bond

analysis and hydrophobic contact analysis were visualized using Cytoscape [34]. All molecular structures for α Syn amyloids were drawn using UCSF Chimera [35] and simulation movie was generated using VMD [29].

Short MD runs

We noticed that certain effects of mutations were already present at the very starting point of the product run of MD simulation and would remain unchanged or even accentuated over the subsequent sampling time, suggesting that the effects manifest in the pre-run phase. In such cases, we exploited it and repeated short MD simulation 50 times to exclude a possibility that the apparent effects of the mutation actually just resulted from probability fluctuation. We carried out 5 ns of production runs from the common minimized structure according to the same procedure as the 400 ns simulations (**Suppl Fig S5A**).

Statistical analyses

For analysis of statistical differences between two groups, we used the two-tailed Student t-test of the Excel2013 (Microsoft Corporation, Redmond, WA). A p-value less than 0.05 was considered as statistically significant.

Results

MD simulation of wild-type α Syn amyloid

First, we ascertained stability of the stack of wild-type α Syn [α Syn(WT)] amyloid in the MD-simulation environment (**Fig 1B**). The α Syn(WT) amyloid molecule remained mostly stable over 400 ns of sampling time albeit relative instability in the two loop regions of the stack-end molecules, the Chains A and J, as anticipated (**Fig 1B, brackets**). Interestingly, the Chains A and J showed different degrees of instability: the loop encompassing the residues 56-62 (**Fig 1B, larger bracket**) and the adjacent β -strands, β 3 and β 4, of the Chain A were more disordered than those on the Chain J side (**Fig 1B, arrow**). Stabilities of the stack-end molecules seemed varied also between the five runs (**Suppl Fig S2A**).

Analysis of β -sheet propensity revealed more details of the differences between the chains. The stack-end Chain A and J had the most unstable β -sheets as indicated by the low average β -propensity values (**Fig 1C**) and large standard deviation (SD) values (**Fig 1D**). In addition to the β -sheet propensity analysis, we also analyzed local B-factors to quantify regional mobility [36] and found them consistent with the images of MD simulation (**Fig 1E**). The Chains C-H showed constantly high β -propensity and low SD values in most β -sheets, corroborating structural stability of the middle layers. The heatmaps also corroborated differences in structural stabilities between Chains A and J: The average β -sheet propensities of the β 3 and β 4 regions were lower in the Chain A than in the Chain J (**Fig 1C, red circles**) along with higher B-factor values (**Fig 1E, red circles**), whereas the β 7 region of the Chain J showed lower β -propensity values than the Chain A (**Fig 1C, black circle**). These distribution patterns of high β -sheet propensity could be important determinants of properties of the amyloid because the stack-end molecules are theoretically the immediate interfaces for the amyloid to interact with other cognate amyloidogenic proteins or non-amyloidogenic proteins. We therefore mainly focus on the stack-end molecules, the Chains A, B, I and J, from here on.

MD simulation of homo- and hetero-oligomer A53T amyloid

After the characterization of behaviors of the α Syn(WT) amyloid (WT amyloid) in the MD simulation, we assessed influences of a representative familial PD-associated substitution mutation A53T [10]. The homo-oligomer (homo-A53T) amyloid of the mutant α Syn with A53T substitution [α Syn(A53T)] apparently behaved similarly to WT amyloid, except for a tendency to lose β -sheet structures in β 7 of the Chains A and B (**Fig 2A, arrow**). Analysis of β -sheet propensity and local B-factor values confirmed the remote effects of the A53T substitution on the β 7 region, which is too distant for the residue 53 to directly interact: average β -sheet propensity values of homo-A53T amyloid were obviously lower in the β 7 region of the Chains A, B, I, and J than those of WT (**Fig 2B, red circle and bracket**), along with higher SD values suggesting poorer reproduction of β -sheet structure (**Fig 2C, bracket**) and larger local B-factor particularly in the Chain A and B reflecting local structural instability (**Fig 2D, red circle**). The A53T substitution reduced β -sheet propensity values of the β 7 regions of the Chain A and B similarly (**Fig 2E**), and the differences from those of WT in five independent runs were statistically significant (**Fig 2F, right column**). The propensity values of β 7 of the Chain J also tended to be lower than those of WT, but the differences were not significant. Although less prominent than β 7, the A53T substitution also affected the β 5 region: the β -sheet propensities of the residue 74 of the Chain A, B, I and J tended to be lower in the homo-A53T than in WT, while those of the residue 72 oppositely tended to be higher (**Fig 2F, left column**). Those alterations in the β 5 prompted us to analyze hydrogen bonds and hydrophobic effects of the amyloid, because β 3 and β 5 were close enough to directly interact (**Fig 3**). As expected, hydrogen bonds were newly formed between the substituted Thr53 and Gly73 or Val74 (**Fig 3A, red lines**), which reminisced the speculative model by Rodriguez *et al.* [37]. A noteworthy fact is that they were “inter-layer” bonds bridging a Thr53 with the Gly73 of one-upper layer, e.g. Thr53 of the Chain B and Gly73 of the Chain A (**Suppl Fig S3**). To the contrary, hydrophobic effects between Ala53 and Val74 in the WT are absent in the homo-A53T amyloid (**Fig 3B**). The diagrams also revealed interactions between β 5 and β 7, e.g. hydrogen bonds between Gln79 and Ala89 or hydrophobic effects connecting Val77 and Ala90, and those interactions could affect the structures of β 7 when β 5 is affected by the A53T mutation.

Given the discernible differences between WT- and homo-A53T amyloids, we analyzed hetero-oligomer amyloids containing both α Syn(WT) and α Syn(A53T) at various ratios. Since most familial-PD patients are heterozygous for the pathogenic mutations, expressing both wild-type and mutant α Syn, this could appropriately recapitulate the *in vivo* circumstances. Which phenotype predominates when they are mixed in a stack? To address the question, we analyzed the following hetero-oligomer A53T (hetero-A53T) amyloids: α Syn(A53T) at (1) Chains A and J (AJ), (2) Chains A, B and J (ABJ), (3) Chain A only, (4) Chain J only, (5) Chains A and B (AB), (6) Chains I and J (IJ), (7) Chains A to C (ABC), (8) Chains H to J (HIJ), and (9) Chains A, B, C, D and E (ABCDE). As a result, all the hetero-A53T amyloids seemed more similar to WT amyloid than homo-A53T in terms of the β -sheet propensity values of β 7 in the Chains A and B (**Fig 4A and 4B**), i.e. the unique instability of homo-A53T amyloid was suppressed by the hetero-oligomerization. The backbone Local B-factors were also consistent with the results of the β -sheet propensity values (**Suppl Fig S4**). Interestingly, hetero-A53T amyloids with α Syn(A53T) on the Chain-A side, e.g. AB-, ABC- and ABCDE-amyloid, tended to have higher β -sheet propensity values in β 3 of the Chain A and B than WT particularly in the residues 52-54 (**Fig 4C**), suggesting that they are not simple phenocopies of the WT amyloid. The newly-formed hydrogen bonds between Thr53 and Val52 may contribute to this stabilization of β 3 (**Suppl Fig S3**). Besides, characteristics of the homo-A53T were also hinted in β -sheet

propensity values at the residue 74 of $\beta 5$, particularly in those with α Syn(A53T) clustered on the Chain-A side, again (**Fig 4D, compare with “A53T”**). To our surprise, even a single mutant in an amyloid stack could affect properties of all the chains in the stack, as seen in the small variations in the β -sheet propensity values of Gly93-Phe94 of the Chain A in the “J only” (**Fig 4E**). “IJ” and “HIJ” showed the similar tendencies. Possibly, the numerous hydrogen bonds and salt bridges between the chains facilitate conduction of motions over a long range across the stack, so that all the chains share the motion irrespective of the primary structures.

Short MD runs confirm the singularity of the homo-A53T amyloid

The low β -sheet propensity of $\beta 7$ in homo-A53T amyloid was likely to be a unique feature attributable to the A53T substitution. However, there still was a possibility that they just resulted from probability fluctuation. As we noticed that the $\beta 7$ region of the homo-A53T was already disordered at the very starting point and would remain unchanged or even accentuated over the subsequent sampling time, we attempted to address the possibility by repeating short MD simulation runs (**Suppl Fig S5A**). As anticipated, the diminution of β -sheet propensities in the $\beta 7$ region of homo-A53T amyloid reproducibly occurred in the very initial step of the simulations (**Fig 5A, “0 ns”**). As the hetero-A53T(ABCDE) was similar to WT amyloid as described, we increased the ratio of α Syn(A53T) and performed short-MD simulations to identify when the characteristics of the homo-A53T emerge (**Fig 5B**). Surprisingly, none of the hetero-A53T amyloids was similar to the homo-A53T amyloid, including even the one with the mutant only at the Chain J, i.e. hetero-A53T(A-I) (**Fig 5B; Suppl Fig S5B, S6**).

MD simulation of homo-H50Q and homo-G51D amyloids

Unlike the homo-A53T amyloid, the homo-oligomer (homo-H50Q) amyloid of α Syn with H50Q substitution [α Syn(H50Q)] was rather similar to the WT amyloid in terms of apparent stability of β -sheets on the Chain-A side (**Fig 6A**). The β -sheet propensity and the local B-factor values were also similar to those of WT, although the $\beta 7$ region of the Chain J seemed slightly unstable than that of WT (**Fig 6B and 6C, bracket**). On the other hand, the local B-factors in the $\beta 3$ region were smaller than those of WT (**Fig 6C, red box**). Characteristically, there were inter-chain hydrogen-bond formation between the substituted Gln50, i.e. so-called a glutamine ladder (**Suppl Fig S7**), possibly contributing to the stability of $\beta 3$ (**Fig 6E, left column**). Unlike A53T, H50Q did not affect β -sheet propensity values of $\beta 5$, and those of the $\beta 7$ region of the Chain J were only slightly smaller than those of WT (**Fig 6D and 6E, right columns**). As for the hetero-oligomer amyloids, while hetero-H50Q(ABC) amyloid was very similar to homo-H50Q amyloid or WT (**Suppl Fig S8**), hetero-H50Q(ABCDE) amyloid occasionally showed substantial deformity in $\beta 6$ and $\beta 7$ particularly on the Chain-J side (**Suppl Fig S9A, arrows**), which is also reflected in the low β -sheet propensity values of $\beta 7$ (**Suppl Fig S9B, red box**). The present study indicates that α Syn(H50Q) can behave like α Syn(WT), or even advantageously in the amyloid formation process because of the glutamine ladder. Regardless, the age of onset of the H50Q-associated familial PD is relatively late [10] and, besides, pathogenicity of H50Q mutation itself is recently questioned [38]. Possibly, destabilization by hetero-oligomerization with α Syn(WT) might be responsible.

In MD simulation of α Syn amyloid with G51D substitution [α Syn(G51D)], the $\beta 3$ region of homo-oligomer (homo-G51D) amyloid was severely disordered, with the peptides dissociating from one another (**Fig 7A, arrow; Fig 7B, 7D & 7E, red boxes**). The unique influence of the mutation was predictable because the substituted

aspartate residues would align side-by-side with short intervals in the in-register parallel β -sheets and their negative charges would make the peptides mutually repel. In contrast to the severely disordered β 3, the regions encompassing β 5 to β 7 seemed as stable as those of the WT amyloid (**Fig 7B, 7D & 7E, black boxes**). On the Chain-A side, the β -sheet propensities of the β 7 regions were only slightly lower than those of the WT amyloid (**Fig 7E and 7F, right column**), while they tended to be higher than those of WT on the Chain-J side, although not statistically significant. The β 5 region of the homo-G51D showed much lower β -sheet propensities at the residues 72 and 73 than those of WT (**Fig 7F, right column**).

MD simulation of homo-(G68E+V95G), homo-S87N & homo-(A53T+S87N) amyloids

Species barriers are one of characteristic features of prions. Efficiencies of cross-seeding reactions, i.e. seeding reactions between amyloidogenic proteins with non-identical primary structures, can be equivalent to the species barriers in interspecies transmissions of prions. We were therefore interested in influences of mutations which are reported to greatly affect the cross-seeding efficiencies of α Syn amyloids, derived from ovine α Syn or mouse α Syn [39][9].

Human α Syn mutants with either of the two ovine residues, G68E or V95G, are not cross-seeded by fibrils of α Syn(WT) [39]: Gly68 and Val95 were located in the turn between β 4 and β 5 and at the C-terminal part of β 7, respectively. Interestingly, not only the cross-seeding by human α Syn fibrils, fibril formation of sheep α Syn itself seems to be rather inefficient. In MD simulation of the homo-oligomer amyloid combining both G68E and V95G [homo-(G68E+V95E)], the global structures were unexpectedly stable (**Fig 8A, left panel**). Nevertheless, certain signs of incompatibility could be recognized, like reduced β -sheet propensity values in the distal β 7 of the Chain A and B, and β 4 of the Chain A (**Fig 8B, black circles**), accompanied by larger local B-factors suggesting local structural instabilities (**Fig 8C, black circles**).

Luk et al. systematically created various human-mouse chimeric α Syn and investigated their cross-seeding efficiencies by *in vitro* fibril formation and *in vivo* propagation in mice [9] and demonstrated that A53T, S87N and their combination differentially affect the cross-seeding efficiencies. In MD simulation of the mutant α Syn with S87N substitution alone [α Syn(S87N)], the homo-oligomer amyloid of α Syn(S87N) (homo-S87N) seemed rather stable with apparent stabilization of β 3, β 4, and the intervening loop (**Fig 8A, middle, bracket**). This was interesting because Asn87 cannot directly interact with those regions nor β 5 through which β 3 could be indirectly affected. We analyzed hydrogen bonds and found that an Asn87 formed an inter-layer hydrogen bond with the carbonyl oxygen of the Gly84 of one-up layer, e.g. the Asn87 of the Chain D and the Gly84 of the Chain C (**Fig 8D**). Those interactions across the loop encompassing 84-87 probably stabilize the local structures around the loop including β 6 and proximal β 7, and their stabilization could subsequently stabilize the interacting/flanking regions like β 2, β 3 and distal β 5, and so on.

MD simulation of mutant α Syn combining A53T and S87N [α Syn(A53T+S87N)], which has the identical primary structure with the residues 36-99 of mouse α Syn, was completely unexpected. The β 5-7 regions of the Chain J were so severely disordered as to dissociate from the stack (**Fig 8A, red arrows**), while the Chain-A side was rather calm (**Fig 8A, right "A-side"**). Correspondingly, β -sheet propensity values of the Chain J were extensively diminished from the β 5 to the β 7 regions (**Fig 8B, red box**) and the local B-factor values in the region were also high (**Fig 8C, red box**). Possibly, the structural strains posed by A53T substitution, which manifested in

$\beta 7$ of the Chains A and B in the homo-A53T, were “redirected” toward the Chain J in the homo-(A53T+S87N) amyloid due to the inter-layer interactions by S87N substitution fixating the loop 84-87 (**Suppl Fig S10**). Since inefficient cross-seeding of mouse α Syn by human α Syn preformed fibrils indicates that the conformation of the human α Syn amyloid is not very compatible with mouse α Syn [9], the drastic destabilization of the Chain J of homo-(A53T+S87N) amyloid might correspond to the incompatibility of mouse α Syn observed in the experiments. Whether the mutually distinct properties of homo-A53T, homo-S87N, and homo-(A53T+S87N) amyloids in the MD simulation milieu have biological significance is very intriguing and to be investigated in the future.

Discussions

The MD simulation of WT and mutant α Syn amyloids successfully revealed unique intrinsic properties of each which possibly affect amyloid formation reactions. One challenge was the subtleness of the structural alterations caused by mutations under the conditions of MD simulation. Even the stacks of α Syn(G68E+V95G) or α Syn(A53T+S87N), which are reportedly inefficiently cross-seeded by human α Syn amyloids and should be relatively incompatible with the conformation [40][39], showed the unexpectedly small structural aberrations in the limited sampling time frame. Regardless, by repetition of runs to circumvent stochastic factors, certain signs of the incompatibility manifested as substantial local destabilization in the relevant regions. From this view point, the unique destabilization of $\beta 7$ over two layers, the Chains A-B, by A53T seemed to be consistent as a manifestation of substantial incompatibility with the Greek-key conformation. Another limitation is that the investigated proteins are “artificial proteins” derived from human α Syn by truncation of the unstructured N-terminal and the C-terminal moieties. Since those regions could contribute to amyloid-formation efficiency or strain-specific properties of α Syn amyloids [14][9], the findings on the truncated molecules cannot be directly extrapolated to the actual α Syn amyloids observed *in vitro* or *in vivo*. We think that the present model is still legitimate as a structural model of an in-register parallel β -sheet amyloid in a Greek-key conformation, and that identification of the most incompatible regions of mutant amyloids would be informative about their preferred/compatible amyloid forms. Actual biological relevance of the findings need to be ascertained by *in vitro* experiments with recombinant proteins in the future.

Asymmetry between the Chain-A and the Chain-J sides of the stack

MD simulation proved to be particularly advantageous in investigations of behaviors of the stack-end molecules, e.g. Chain A and J. They should be important for properties of the amyloid like growth rates of the amyloid fibrils and even the repertoire of non-amyloid proteins they interact, because they are the interfaces which directly interact with the cognate proteins or non-amyloidogenic proteins [41]. Since the behaviors of the stack-end molecules are not recognizable to conventional structural analysis methods, MD simulation can greatly contribute to amyloid researches. A noticeable finding here regarding the stack-end molecules was the apparent asymmetry in structural stabilities between Chain-A and Chain-J sides of the stacks, which reminded that elongation of α Syn fibrils often shows directionality [42]. What mechanism underlies the asymmetry? One possibility is twisting of parallel β -sheets. MD simulation of the α Syn amyloids demonstrated that all the β -sheets incessantly moved in a vibratory manner in narrow spaces, except for $\beta 1$ and $\beta 2$ which tended to freely twist and fan (**Supplementary Movie**). Considering the intrinsic twisting tendency [43], it is conceivable that the other β -sheets also share the tendency to twist like $\beta 1$ and $\beta 2$. Although they are not allowed to twist freely due to the compact packaging in the

Greek-key conformation, even small twists can cause differences in the distances of β -sheets between on the Chain-A side and on the Chain-J side, and consequently differences in structural stabilities because hydrogen bonds or hydrophobic effects are greatly affected by the distances. Another possibility stems from a fact that all the β -strands of a layer of the stack are not necessarily on the same flat plane, as seen in a tau amyloid [8] or an A β amyloid [5]. In the case, inter-layer interactions of the β -strands can contribute to the asymmetry because obviously the stack-end molecules lack the interactions in distinct ways. In this regard, the severe destabilization of the Chain J of the homo-(A53T+S87N) is interesting because both the A53T and the S87N mutations newly formed inter-layer hydrogen bonds, which possibly modulate “vertical” positional relations and/or the twisting tendencies of the bonded β -sheets. Whether the directionality of amyloids contribute to cross-seeding/strain barriers is to be investigated.

Remote effects of the mutations

The contrasting remote influences of A53T and G51D on the stability of the β 7 region, in spite that both the mutations are in the β 3 region, were most interesting in this study. Given the existence of hydrogen bonds and hydrophobic effects between β 3, β 5 and β 7 as revealed by the MD simulation, alterations in those interactions seem the most likely mechanism of the remote effects. In addition, the intrinsic twisting tendency of β -sheets could modulate the interactions because those properties of β -sheets affect each other [43]. The extra inter-layer hydrogen bonds of homo-A53T amyloid between Thr53 and Gly73 and/or Val74 suggest more intimate β 3- β 5 interactions (**Fig 3**), which also explain the alterations of β -sheet propensities in the residues of β 5. In contrast to the augmented β 3- β 5 interactions in homo-A53T amyloid, the interactions were obviously disrupted in homo-G51D amyloid due to the severely-disordered β 3. The relatively stable β 7 regions of homo-G51D amyloid suggest that the disruption of the β 3- β 5 interactions is advantageous for the structural stability of β 7, as if the three β -sheets of the WT amyloid are in balance, i.e. β 3- β 5 vs β 5- β 7. A mutation in a Greek-key-type amyloid can affect structural stability of a remote region through successive interactions between β -sheets; this notion could be generalized to any amyloid with a similar architecture.

Multiple interfaces/amyloid cores and strain diversity

We think that the variable stability of the β 7 region of α Syn depending on the intimacy of β 3- β 5 interactions exemplifies an underlying mechanism of phenotypic diversity of amyloids, or prion strains. If each of β 3- β 5 and β 5- β 7 pairs independently functions as an interface/amyloid core of the amyloid, a substrate α Syn which favors intimate β 3- β 5 interactions would be preferentially incorporated into the amyloids with predominant β 3- β 5 amyloid core; oppositely, α Syn with inefficient β 3- β 5 interactions would be preferentially incorporated in the amyloids with predominant β 5- β 7, because blocking the pathway would facilitate reactions to the other pathway. Those two types of amyloids are therefore equivalent to two different “strains” with respective unique substrate preferences. From this view point, homo-A53T and homo-G51D amyloids are equivalent to the α Syn amyloids with β 3- β 5 amyloid core and β 5- β 7 core, respectively (**Fig 9, B and C**). Indeed, amyloids of α Syn(A53T) and α Syn(G51D) are likely to be different “strains” because of the clinicopathological pictures of the associated familial PD [10] and cross-seeding properties *in vitro* [44]. Detailed structures of their amyloid forms are unidentified yet, but the destabilization patterns observed in the MD simulations are informative of their preferred conformations.

Considering that amyloid formation of those mutant α Syn are not inhibited by the coexisting α Syn(WT) in the heterozygous familial-PD patients, conformations of the pathogenic amyloids of α Syn(A53T) or α Syn(G51D) could be ones which reconcile the preferences of α Syn(WT) and those mutants.

The balanced three β -sheets, β 3, β 5 and β 7, are characteristic of WT amyloid: possibly α Syn(WT) might be less efficient to form aggregates than α Syn(A53T) or α Syn(G51D) because it has to reconcile β 3- β 5 and β 5- β 7 interactions, unlike the mutants. If the balances between the β -sheets of the WT amyloids could be deranged even without mutations and the amyloid switches the phenotype to ones like homo-A53T or like homo-G51D amyloid, it could explain existence of multiple conformations of α Syn(WT) amyloids, e.g. “ribbon” and “string” which share many β -sheets [45], and also explain why α Syn(WT) is efficiently seeded by either preformed fibrils of α Syn(A53T) or α Syn(G51D) depending on conditions [42][44]. This modality of mechanism for strain diversity can generally occur to parallel β -sheet amyloids with more than two β -sheets aligned side-by-side, and can be operative also in some phenomena about prions (**Supplementary Discussion**).

Effects of hetero-oligomerization

The remote effects of the A53T substitution on the stability of β 7 was unexpected, and the suppression of the unique influences of A53T by hetero-oligomerization with the α Syn(WT) was another surprise. As discussed earlier, the unique pattern of destabilization of β 7 of homo-A53T amyloid as indicated by the low β -sheet propensity along with the large local B-factor values suggested incompatibility of α Syn(A53T) with the Greek-key conformation. α Syn(A53T) may actually prefer conformations with more intimate β 3- β 5 interactions, e.g. the speculative model postulated by Rodriguez et al. where the “pre-NAC” region containing Thr53 and the “NACore” around Gly73 tightly bind [37]. It is also reported that *in vitro*-formed α Syn(WT) fibrils and α Syn(A53T) fibrils are morphologically distinct [42]. The signs of the incompatibility of α Syn(A53T) with the Greek-key conformation was absent in any hetero-A53T amyloids that we tested, i.e. they were suppressed just by hetero-oligomerization with α Syn(WT). This notion helps address the question why A53T cause familial PD despite the incompatibility of α Syn(A53T) with the Greek-key conformation, without interfering with amyloid formation (**Fig 9C and D**). On the other hand, the destabilization of β 7 region observed in hetero-H50Q(ABCDE) demonstrated that hetero-oligomerization with α Syn(WT) can destabilize the amyloid stack depending on the mutation types. The MD simulation of the hetero-oligomers also implied a possibility that they can combine properties of WT and the mutant, e.g. higher β -sheet propensity values in the β 3 of hetero-A53T amyloids than WT. Those notions need to be considered in interpretation of cross-seeding and inter-species transmission of prions (**Supplementary Discussion**). Whether stability of hetero-oligomers corresponds to cross-reaction efficiency also needs further investigation in the future.

In summary, the present series of MD simulations of α Syn amyloids revealed the following: (1) structural asymmetry between the Chain-A and the Chain-J sides, (2) a mutation can affect stabilities of remote β -sheets through complex interactions between β -sheets, (3) existence of at least two potential interfaces/amyloid cores that can be switched by mutations, (4) the switchable interfaces possibly underlie the multiple α Syn-amyloid strains, and (5) unique properties of the homo-oligomer amyloid of mutant α Syn can be obscured by hetero-oligomerization with the wild-type. It was surprising that a stack of only ten peptides with moderately intricate conformation exhibit those properties reminiscent of complex phenomena about prions as discussed in detail in the **Supplementary**

Discussion. Those findings support our view that prion-like properties are inherent in the in-register parallel β -sheet structures. Thus, *in silico* studies are potentially powerful investigation tools of in-register parallel β -sheet amyloids. The present studies are valuable example of MD simulation of this type of amyloids with a relatively large size and complex architecture. Hopefully the knowledge from the studies are applicable to other amyloids including prion, A β peptide, and tau, and contribute to advance our understanding of their pathogenicity and pathophysiology.

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Figure 1

Fig 1. The Molecular dynamics (MD) simulation of the wild-type α Syn(WT) amyloid.

A. The starting "Greek-key" conformations of the α Syn amyloid [PDB ID: 2n0a (Tuttle *et al.*, 2016)] for the MD simulation: the left panel show the top view of the amyloid stack. For simplicity, the β -sheets are numbered from $\beta 1$ to $\beta 7$ (middle panel) and the ten chains are referred to as the Chain A to J, from top to bottom (right panel).

B. A representative image of WT amyloids after MD simulation for 400 ns, with the Chain A front side. Note that the stack-end molecules, Chains A and J, are substantially disordered particularly in the $\beta 3$ - $\beta 4$ regions (arrow), while the Chains B-I are rather stable. All the images of the five independent runs are presented in the Supplementary information (**Suppl Fig S2**).

C-E. Heat maps illustrating average β -sheet propensities (**C**), standard deviations (SD) of the β -sheet propensities (**D**), and the backbone local B-factor (Local-B) values (**E**), respectively. The values are based on five independent runs. The vertical axis represent residues from Gly36 to Gln99, and the horizontal axis corresponds to the Chain A to J (inset).

C. Heatmaps of β -sheet propensity with value ranges 0-0.5 (left) and 0-1 (right). Note that the regions with high β -sheet propensities correspond to $\beta 1$ to $\beta 7$, and the stack-end molecules have relatively low values (arrowheads). Interestingly, color distributions were distinct between the Chain A and the Chain J sides (red circles).

D. The heatmap of SD of the β -sheet propensity revealed that the color distributions were distinct between the Chain A and J sides, particularly in the $\beta 5$ and $\beta 7$, with a tendency to be high at residues with relatively low β -sheet propensities.

E. The heatmap of the backbone local B-factors show relatively high values in the loop region between $\beta 3$ and $\beta 4$, and that the Chains A and J have distinct color distributions (arrowheads).

Figure 2

Fig 2. The homo-A53T amyloid is uniquely unstable in the β 7 region.

A. A representative image of the homo-oligomer (homo-A53T) amyloid of α Syn(A53T) after 400 ns of the sampling time. All the images of the five independent runs are presented in the **Supplementary Figure (Suppl Fig S2)**. The β 7 region of the Chain A and B are not in the β -sheet form (arrow).

B-D. Comparison of heatmaps of average β -sheet propensities (**B**), SD of the β -propensities (**C**), and backbone local B-factors (**D**) between WT and homo-A53T amyloids (the left and the right of each pair, respectively). The values are based on five independent runs. The vertical axis represent residues from Gly36 to Gln99, and the horizontal axis corresponds to the Chain A to J (inset). The red cross on the vertical axis indicates the position of the A53T substitution mutation.

B. Comparison of β -sheet propensities of WT and homo-A53T amyloids revealed that the β -sheet propensities of homo-A53T are substantially lower than the WT amyloid in the β 7 region of the Chains A and B (red circle, bracket).

C. SD of the homo-A53T amyloids are also higher in the β 7 region than that of WT (bracket), suggesting poorer reproducibility of the regional structures.

D. The local B-factors are higher in β 7 of the Chain A and B of homo-A53T amyloid (red circle), i.e. higher mobility of the chains in the region, consistent as local structural instability.

E. Bar graphs of β -sheet propensities of the β 7 regions (residues 88-99) of the stack-end Chains A, B, C, I and J, comparing the WT and the homo-A53T amyloids. The bars and the error bars represent the average values and the SD of five independent runs, respectively.

F. Dot plots of β -propensities of the β 5 regions (residues 70-75) and the β 7 region (residues 90-95) of the stack-end Chains A, B, I and J, comparing the WT and the homo-A53T amyloids. Asterisks, $p < 0.05$. Note that the β -propensity values in the β 7 of the Chain A and B of homo-A53T are similarly lower than those of WT.

Figure 3

Fig 3. Hydrogen Bond and Hydrophobic Networks: comparison between WT and homo-A53T amyloids.

A. Diagrams illustrating hydrogen bond networks of the WT and homo-A53T amyloids. Color code for each chain is presented between them. Residue 35-99 of α Syn are arranged from the bottom (arrow) of the circles in a counter-clockwise fashion, i.e. 36(A,B...J), 37(A-J), ... 99(A-J). Note that there are unique hydrogen bonds in the homo-A53T amyloid, e.g. T53-V74 and A69-G93, and the bonds involving A53T are high-lighted in red. A magnified view of a part of a circle is presented (**Suppl Fig S3B**) to show how the chains are arranged in the diagram.

B. Diagrams illustrating hydrophobic effects in the α Syn amyloids. Unlike the **Fig 3A**, the circles contain only residues contributing to hydrophobic effects. Color codes for each chain is the same as above. Note that a bundle of hydrophobic effects between the residues 53 and 74 in the WT amyloid is absent in the A53T amyloid.

Figure 4

Fig 4. The hetero-oligomers of α Syn(WT) and α Syn(A53T) are distinct from either homo-A53T or WT amyloid.

A. Heatmaps of average β -sheet propensities comparing WT, homo-A53T, and various hetero-A53T amyloids. The values of various hetero-A53T are averages of three independent runs, while those of WT and homo-A53T are averages of five runs. Note that the β -propensity profiles of the hetero-A53T amyloids are more similar to that of WT regarding the $\beta 7$ region, while those of homo-A53T are uniquely low (red box). The red cross indicates the position of the A53T substitution mutation.

B. Bar graphs of the β -sheet propensities of the $\beta 7$ regions of the stack-end Chains A, B, I and J, comparing WT, homo-A53T (red box), and various hetero-A53T amyloids. The bars and the error bars represent the average values and the SD of five (for WT and homo-A53T) or three (for hetero-A53T) independent runs, respectively.

C-E. Dot plots of the β -sheet propensities in $\beta 3$ (residues 52-55) (**C**), $\beta 5$ (73-75) (**D**) and $\beta 7$ (92-95) (**E**) regions of the stack-end Chains A and B comparing WT, homo-A53T and various hetero-A53T amyloids. Run numbers are the same as above.

Figure 5

Fig 5. The unique loss of β -sheet in the β 7 region of the homo-A53T amyloid occurs in the very early phase.

A. Heatmaps of β -sheet propensities calculated from 50 runs of the short-MD simulations, comparing those of WT and homo-A53T amyloids. Note that the low β -propensity in the β 7 of the homo-A53T amyloid is already present at the point of “0 ns”. The vertical axis represents the residues from 36 to 99, and the horizontal axis represents the Chain A-J (inset). The red cross indicates the position of the A53T substitution mutation.

B. Heatmaps of β -sheet propensities calculated from the short-MD simulations, comparing those of homo-oligomer amyloids and the hetero-oligomer amyloids in which α Syn(A53T) is the majority. Each heatmap represent longitudinal fluctuations in the average β -sheet propensity values over the production run of 5 ns for the Chain A, B or C. The horizontal axis represents the time of production run (inset). Not only the Chains A and B of the homo-A53T, even the Chain C show lower β -sheet propensities in the β 7 region than those of WT (compare the red boxes). The low β -sheet propensities in the β 7 region at the very early phase of simulation are unique to the homo-A53T amyloid and the hetero-oligomers could not acquire the phenotype, even when the ratio of α Syn(A53T) in the stack was increased up to the point only the Chain J is α Syn(WT) [“A53T(A-I)”].

Figure 6

Fig 6. Homo-H50Q amyloid is very similar to the WT amyloid.

A. A representative image of the homo-H50Q amyloid after 400 ns of sampling time. All the images from the three independent runs are presented in the **Supplementary Figure (Suppl Fig S7)**.

B-C. Comparison of heatmaps of average β -sheet propensities (**B**) and backbone local B-factors (**C**) between WT and homo-H50Q amyloids. The values are based on five runs for WT, and three for homo-H50Q. The vertical axis represent residues from Gly36 to Gln99, and the horizontal axis corresponds to the Chain A to J (inset). The red cross on the vertical axis indicates the position of the H50Q substitution mutation.

D. Dot plots of the β -sheet propensities in $\beta 5$ (70-75) and $\beta 7$ (90-95) regions of the stack-end Chains A, B, I and J, comparing WT and homo-H50Q amyloids. Run numbers are the same as above.

E. Bar graphs of β -sheet propensities of $\beta 3$ (residues 43-56) and $\beta 7$ (88-99) of the stack-end Chains A, B, I and J, comparing the WT and the homo-H50Q amyloids. The bars and the error bars represent the average values and SD, respectively. The values are based on three runs for homo-H50Q and five runs for WT.

Figure 7

Fig 7. The homo-G51D amyloid has severely disordered β 3 and stable β 7.

A. A representative image of the homo-G51D amyloid after 400 ns of sampling time. All the images from the three independent runs are presented in the **Supplementary Figure (Suppl Fig S11A)**.

B-D. Comparison of heatmaps of average β -sheet propensities (**B**), SD of the β -sheet propensity values (**C**) and backbone local B-factors (**D**) between WT and homo-G51D amyloids. The values of the homo-G51D are based on three independent runs. The vertical axis represent residues from Gly36 to Gln99, and the horizontal axis corresponds to the Chain A to J (inset). The red cross on the vertical axis indicates the position of the G51D substitution mutation. The parameters corroborate that the β 3 region of homo-G51D is severely deranged (red box). On the other hand, the β 7 region shows comparable β -sheet propensity profile along with relatively low SD values in the region (black box).

E. Bar graphs of β -sheet propensities of the β 7 regions (residues 88-99) of the stack-end Chains A, B, C, I and J, comparing the WT and the homo-G51D amyloids. The bars and the error bars represent the average values and SD, respectively. The run numbers are the same as above.

F. Dot plots of β -propensities of the β 5 regions (residues 70-75) and the β 7 region (residues 90-95) of the stack-end Chains A, B, I and J, comparing the WT and the homo-G51D amyloids.

Figure 8

Fig 8. Sheep- or mouse- α Syn-derived mutations which reportedly affect cross-seeding with human α Syn exert definite but limited effects under the simulation condition.

A-C. Representative images of the homo-(G68E+V95G) (**A**), homo-S87N (**B**) and homo-(A53T+S87N) (**C**) amyloids after 400 ns of simulation. All the images from the three independent runs for each are provided in the **Supplementary Figure (Suppl Fig S11B)**. As for the homo-(A53T+S87N) amyloid, image viewed from the Chain-J side is also presented, because it reproducibly exhibited severe instability in the β 4- β 7 region of the Chain J (red arrows), whereas the β 7 region of the Chain A was relatively stable despite the A53T substitution.

D-E. Heatmaps of the β -sheet propensities (**D**) and backbone local B-factors (**E**) of WT, homo-(G68E+V95G), homo-S87N and homo-(A53T+S87N) amyloids. The values are average of three runs for the mutants and five runs for WT. All the images from the three independent runs for each are provided in the **Supplementary Figure (Suppl Fig S11C-D)**. Comparison of those parameters showed that the structural derangement were limited to the vicinity of each mutation, in one to two stack-end layers (**black circles, red circles, and red box**). The red crosses and the black crosses on the vertical axis represent the positions of mutations of α Syn(A53T+S87N) and α Syn(G68E+V95G), respectively.

F. Diagrams illustrating hydrogen-bonds between Gly84 and Ser87, comparing those of WT and homo-S87N amyloids. The thickness of each arrow correlates with occupancy of the hydrogen bond. Note that inter-layer bonds are augmented in homo-S87N amyloid; specifically, the side chains of the residues 87 form hydrogen bonds with the backbone of the residues 84 one-layer up, e.g. from the residue 87 of the Chain G to the 84 of the Chain F. These inter-layer bonds could contribute to the stabilization of the loop region in the homo-S87N amyloid. The similar pattern of hydrogen bonds were also seen in the homo-(A53T+S87N) amyloid: All the diagrams of the five runs of WT and the three runs of homo-(A53T+S87N) amyloids are provided in **Supplementary Figure (Suppl Fig S10)**.

Figure 9

Fig 9. Schematic illustrations: A possible mechanism of strain propensity of α Syn amyloids, and what occurs in a cross-seeding reaction where unstable homo-oligomer and stable hetero-oligomer amyloids are produced.

A. The WT amyloid has balanced $\beta 3$, $\beta 5$ and $\beta 7$, to maintain the conformation.

B-C. In an α Syn amyloid with more intimate $\beta 3$ - $\beta 5$ interactions, like homo-A53T amyloid, the $\beta 7$ region is destabilized and the conformation would be inclined toward a distinct one compatible with the stable $\beta 3$ - $\beta 5$ structures (**B**). To the contrary, in an α Syn amyloid with less $\beta 3$ - $\beta 5$ interactions, like G51D amyloid, the $\beta 7$ region is more stabilized and the resultant amyloid would consist mainly of the "NAC" region (**C**). Since preferences for substrates would be distinct between those two types of amyloids, they can be regarded as two different strains.

D-E. Schematic illustrations postulating how stable hetero-oligomer and unstable homo-oligomer amyloids can affect the propagation in cross-seeding reactions, or heterologous transmissions of prions.

D. When seeds are present in an environment where heterologous substrates alone exist, e.g. interspecies transmission of prions, initially the stable hetero-oligomers can grow by incorporating the heterologous substrates, even if the conformation might not be completely the same as the original (top). However, after fragmentation of the grown amyloids, some still remain as stable hetero-oligomers containing the original nuclei, whereas others lose the original nuclei to become unstable homo-oligomers (upper middle). If the destabilized homo-oligomer lose the templating activity (white arrow), the oligomers cannot grow and are eventually degraded. On the other hand, if the destabilized homo-oligomers acquire a novel stable conformation suitable for the substrate along with templating activity, the new seeds would efficiently propagate, emerging as an "adapted strain".

E. When seeds are present in an environment where both homologous and heterologous substrates coexist, e.g. a host heterozygous for a pathogenic mutation in an amyloidogenic protein, the amyloid grows as a hetero-oligomer incorporating both types of substrates (top), and the fragmentation produces mainly hetero-oligomers which maintain the templating activity (bottom) to keep propagating as hetero-oligomer amyloids.