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Coupling of the Non-Amyloid-Component (NAC) Domain and the KTK(E/Q)GV Repeats Stabilize the α -Synuclein Fibrils

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Abstract

The aggregates of α -synuclein (α S) are a major pathological hallmark of Parkinson's disease (PD) making their structure-function relationship important for rational drug design. Yet, the atomic structure of the α S aggregates is unavailable, making it difficult to understand the underlying aggregation mechanism. In this work, based on available experimental data, we examined plausible molecular structures of α S(20/30–110) fibrils for the first time by employing computational approaches. The optimized structure was used to investigate possible interactions with aggregation inhibitors. Our structural models characterize the essential properties of the five-layered fold of the α S fibril. The distribution of the β -strands and the topology of the five β -strands in the relatively stable models are in good agreement with experimental values. In particular, we find that the KTK(E/Q)GV repeat motifs significantly stabilize the α S fibrils. The charged residues within each repeat prefer exposure to the solvent in order to further stabilize the inter-layered interactions by salt-bridges. The organization of the repeat K⁵⁸T⁵⁹K⁶⁰E⁶¹Q⁶²V⁶³ between the β 2 and β 3 layers significantly affects the stability of the non-amyloid-component (NAC) domain. The coupling between the NAC domain and the KTK(E/Q)GV repeats indicates that both regions can be potential binding sites for inhibitor design. The distinct binding modes of chemical agents that alter α S aggregation highlight the potential of our models in inhibitor design.

Graphical Abstract

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Author contributions

LX, BM designed and simulated structures. LX, BM, RN wrote the paper. All authors have given approval of the final version of the manuscript.

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Keywords

alpha-synuclein; Parkinson's disease; fibril structure; molecular dynamics; drug design

1. Introduction

α -synuclein (α S) is a 140-residue protein that is primarily expressed at presynaptic terminals of neuronal brain cells [1, 2]. The aggregation of α S is closely related to several neurodegenerative diseases including Parkinson's disease (PD) [1, 3]. Recently it was found that α S was present in aggregates of the Krabbe disease and apparently prone to fibrillization in the presence of psychosine [4]. α S adopts different conformations under various environments. Under physiological conditions, α S is characterized as an intrinsically disordered protein, exhibiting a random coil conformation in aqueous solution [3]. In the presence of the membrane, α S displays an α -helical secondary structure as observed from solution NMR structure of micelle-bound α S [5, 6]. Full-length α S can be divided into three distinct domains, the N-terminal domain (residues 1–60) which is amphipathic and responsible for binding to phospholipids [7]; the non-amyloid- β component (NAC) domain (residues 61–95) which is highly hydrophobic and assumed to be essential for α S aggregation [3, 8], and the acidic negatively charged C-terminal domain (residues 96–140) believed to be critical for the chaperone-like activity of α S [9, 10]. The self-assembly of α S is accompanied by conformational transitions [11]; however, the molecular mechanism remains largely unknown partly due to the lack of molecular structures of α S aggregates.

The structural features of α S fibrils have been extensively studied experimentally [12–18]. These studies reveal that both the N-terminal (~20 residues) and C-terminal (~35–40 residues) are highly dynamic and unfolded, whereas ~70 residues have been identified in the ordered fibril core. The folded fibril core of α S shows similar in-register parallel β structures as those observed in the fibrillar structures of amyloid β associated with Alzheimer's disease [19]. In an earlier study based on site-directed spin labeling and electron paramagnetic resonance spectroscopy, a five-layered model of the α S fibril has been derived by the Langen group. There are five β -strands connected by four loops in this folding pattern (β 1-loop- β 2-loop- β 3-loop- β 4-loop- β 5) [17]. Later, a similar fibril model was proposed by the Riek group based on NMR experimental studies of α S(30–110) [13]. The five β -strands involve residues 37–43, 52–59, 62–66, 68–77, and 90–95, respectively (Fig. 1). The assignments of β -strands in Riek's model are largely in agreement with the study of Heise et al. [12] and twisting assemblies of α S in the study of Melki group [16]. The propensity of β 1 and β 2 is in line with a β -hairpin of α S(35–56) trapped by a β -wrap protein where two β -

strands (V37–K43, V48–T54) are connected by a turn (T44–G47) [20]. The β -strand propensity of residues 68–77 is also consistent with previous experimental results which identified residues 71–82 as essential for the α S filament assembly [21]. However, due to different experimental conditions and sample preparation as well as the polymorphic property of the α S fibril structures, an exact consensus from the different experiments cannot be achieved. For example, both studies of Heise and Melki group demonstrate that different strains display distinct distribution of β -sheets along α S sequence [12, 16]. In the NMR study of Wu et al., the C-terminal region (residues 100–140) shows a high β -strand propensity at low temperature (263 K) [18]. In addition, another NMR study at 310 K indicates that the N-terminal of α S fibrils assume a certain extent of β -sheet structures [15].

These experimental studies greatly enhanced the understanding of the structure and function of α S fibrils. A partial structural model of NAC (E⁶¹–V⁹⁵) has been proposed recently by computational modeling and simulation [22]. To elucidate the molecular mechanism of α S misfolding and aggregation we aim to construct atomic models of α S fibrils using computational methods based on reported NMR secondary and tertiary structures. We focus on the fold of full-length α S fibrils not only because there are reported experimental results available, but also because there are six familial PD mutations (A30P [23], E46K [24], H50Q [25], G51D [26, 27], A53T [28], and A53E [29]) that have been identified in the N-terminal region. These point mutants can affect membrane binding and aggregation kinetics of α S [30–34]. It is thus important to include the N-terminal region in the structural model. In particular, we carefully examine the role of the imperfect repeat of the KTK(E/Q)GV motif in the stabilization of α S fibrils. There are six such repeats in both the N-terminal and NAC region, which has been suggested to be crucial for lipid binding and aggregation [35–38]. Since both NAC and these repeat are important for α S aggregation, it is also interesting to examine their coupling effect within α S fibrils. Here we propose the first atomic structural models of α S(20/30–110) fibrils, which provide insights into the polymorphic behavior of α S fibrils, the organization of these repeats in the context of fibrils, and the role of the coupling of NAC and these repeats in the stability of α S fibrils.

2. Results and Discussion

2.1 The organization of charged repeats is crucial for the stability of α S fibril models

In the first five-layered α S pentamer structure (M1) (Fig. 2A), we tested the position of residues K⁴³, K⁴⁵, and E⁴⁶ between β 1 and β 2 layers. The possibility of forming E³⁵–K⁵⁸ salt-bridges to stabilize both β 1 and β 2 was examined by positioning K⁶⁰ between β 2 and β 3.

However, after 20-ns MD simulation, the structure collapsed. The layer of β 3 moves far away from β 4 indicating unfavorable interactions between these two layers. The turn/loop connecting β 1 and β 2 becomes disordered possibly due to the competing electrostatic interactions between K⁴³ and K⁴⁵ with E46. Similarly, the loop containing E⁵⁷, K⁵⁸, E⁶⁰, and K⁶¹ also becomes disordered. As a result, no stable salt-bridge was observed during the MD simulations. This structural model has the highest GBMV energy (–8054 kcal/mol, Fig. 3) among all structures investigated, implying it is the least stable α S fibril model. Also, it

was noted that this fibril model has the lowest β -strand content in its NAC domain (~17%, Fig. 4).

In the second fibril model (M2) (Fig. 2B), we check the possibility of exposing both K⁴³ and K⁴⁵ to the solvent in order to eliminate the electrostatic repulsion present in M1 but still keep E⁴⁶ inside the fibril layer. To increase the interaction between different layers, E³⁵ and K⁵⁸, E⁶¹ and K⁸⁰ initially form salt-bridges, as well as E⁵⁷ and K⁶⁰. Different from M1 where H⁵⁰ was oriented to β 3, to examine the interactions between β 2 and β 3 in this model, H⁵⁰ was oriented to β 1. After 20-ns MD simulation, this model is even less stable than the M1 model, although the interactions between β 1 and β 2, and between β 3 and β 4, seem more favorable than those in M1, possibly due to the introduction of inter-layered salt-bridges. The separation of β 2 from β 3 could indicate that H⁵⁰ should be oriented to β 3 instead of β 1 even though this model has a relatively lower GBMV energy (–8212 kcal/mol) than M1, which suggests that inter-layered salt-bridges contribute to the stability of α S fibril structures. In addition, the β -strand in the NAC domain increased to ~25%, indicating that the interaction between E⁶¹ and K⁸⁰ may stabilize NAC.

To further investigate the role of salt-bridges in the loop containing K⁴³, K⁴⁵ and E⁴⁶ as observed in the M2 structure, we examined the M3 model (Fig. 2C) where K⁴³ and E⁴⁶ form a salt-bridge but not K⁴⁵, which was kept exposed to the solvent. After 20-ns MD simulation, β 2 also moves away from β 3 as observed in M2. However, the introduction of the salt-bridge between K⁴³ and E⁴⁶ seems not to be able to stabilize this loop. The structures of β 1 and β 2 display a globular conformation instead of extended β -strands. Compared to M2, such results imply that the formation of the salt-bridge between K⁴³ and E⁴⁶ inside the fibril layer will not contribute to the stability of α S fibril structures. As a consequence, this model has a comparative GBMV energy (–8208 kcal/mol) to that of M2. The results are also consistent with the familial variant of E46K promoting amyloid formation [39–41]. If the M3 model contribute to α S fibril formation, the E46K mutation should destabilize the α S fibril. Interestingly, this fibril model has the highest percentage of β -strand in the NAC domain (~28%).

The above results suggest that the repeat K⁴³T⁴⁴K⁴⁵E⁴⁶G⁴⁷V⁴⁸ may not form stable salt-bridges inside the fibril layer; as such, we constructed the M4 model where K⁴³, K⁴⁵ and E⁴⁶ are exposed to the solvent (Fig. 2D). The organization of the remaining repeats is the same as that in the M3 structure except that H⁵⁰ was oriented to β 3. This model was also used to examine the effect of another charged repeat K⁵⁸T⁵⁹K⁶⁰E⁶¹Q⁶²V⁶³ on the stability of α S fibril. After 20-ns MD simulation, the five-layer fold of the α S fibril structure is retained. The repeat K⁴³T⁴⁴K⁴⁵E⁴⁶G⁴⁷V⁴⁸ adopts primarily a loop conformation; however, the β -strand of β 2 disappears, which could be attributed to the unfavorable electrostatic interactions in the repeat involving E⁵⁷, K⁵⁸, K⁶⁰, and E⁶¹. It could also be due to the interactions between β 1 and β 2. The calculated GBMV energy (–8159 kcal/mol) suggests that it is less stable than M2 and M3, although this model could maintain the fold of the α S fibril structures. In addition, the β -strand content (~22%) in the NAC of this model is also slightly lower than that of M2 (~25%) and M3 (~28%).

Based on M4, we further examined model M5 (Fig. 2E) where both E⁵⁷ and K⁵⁸ were exposed to the solvent, so that initially there is no salt-bridge between E⁵⁷ and K⁶⁰. Only K⁶⁰ was kept inside the layer as in M4. We observed that the β -strand of β 2 remains relatively stable during 20-ns MD simulation, suggesting that the presence of such salt-bridge between E⁵⁷ and K⁶⁰ could affect the stability of the β 2 strand. However, the presence of K⁶⁰ inside the fibril core has a significant effect on the fold of the α S fibril as β 2 separates from β 3. We speculate that the absence of counter ions results in strong electrostatic interactions between adjacent K⁶⁰ in different β layers, and the interactions between β 2 and β 3 are not able to counteract such effect. Other possibility is that neighboring charged residues like K⁸⁰ could also disrupt the interactions of β 2 and β 3. Consequently, the M5 model has a relatively high GBMV energy (−8095 kcal/mol, Fig. 3). Unlike M3 where E⁵⁷ interacts with K⁶⁰, the β -strand of NAC in this model (~19%) is lower than that of M3 (~28%), implying that electrostatic interactions have a more significant effect on the stability of the NAC domain.

2.2 The relatively stable models characterize the essential secondary and tertiary structures of the α S fibril as observed experimentally

In M5, we examined the possibility that K⁸⁰ could interact repulsively with K⁶⁰ and then destabilize the interactions between β 2 and β 3, although it initially forms a salt-bridge with E⁶¹. M6 was used to explore the possibility that K⁸⁰ establishes a salt-bridge with E⁸³ inside the fibril core (Fig. 5). To increase the hydrophobic interactions between β 4 and β 5, the orientation of β 4 was reversed, leading T⁷⁵ and A⁷⁸ to have opposite orientation to that shown in M5. The fold of α S fibril was maintained after 20-ns MD simulation. We then extended the simulation to 40 ns and found that this model structure is relatively stable. However, this fibril model has a higher GBMV energy (−8172 kcal/mol) even than M2 (−8212 kcal/mol, Fig. 3) where the fibril fold collapses. Note that the percentage of β -strand in the NAC domain of M6 is comparable (~23%) to that of M2 (~25%).

Although M6 indicates that the fold of the α S fibril structures could be maintained in the presence of inter-layered salt-bridges, such a model structure is energetically less stable. Results from M1 to M3 suggest that charged residues (K⁴³, K⁴⁵, and E⁴⁶) in the first loop have a strong tendency to be exposed to the solvent. Taken together, the results of M4 to M6 lead us to conclude that charged residues in all three loops prefer to be exposed to the solvent. We then examined a model M7 (Fig. 6A) that satisfies all the inter-layered distance restraints, and the stabilization factors derived from M1 to M6. Initially, K⁴⁵ and E⁴⁶ interact with each other in the loop connecting β 1 and β 2 due to electrostatic attraction. Similarly, E⁵⁷ interacts with K⁵⁸ in the loop connecting β 2 and β 3. In addition, E³⁵ in β 1 may participate in the salt-bridge to further stabilize the interactions between β 1 and β 2. Similarly, E⁶¹ and K⁸⁰ form a salt-bridge, with K⁶⁰ also possibly involved. Two independent 40-ns MD simulations were performed (referred as M7a and M7b thereafter) with the same initial conformation but different velocity distribution. Both fibril structures could maintain the α S fibrils fold after 40-ns simulation (Fig. 6B and 6C). Their GBMV energies suggest that both models converged with similar energies, with M7a (−8313 kcal/mol) is slightly more favorable than M7b (−8288 kcal/mol, Fig. 3). The two models also display similar

percentages of β -strand in their NAC domains (~24% and 25% for M7a and M7b, respectively).

Based on the above three relatively stable fibril structures (M6, M7a, and M7b), we examined the change of the secondary structures during the MD simulations (Fig. S1 and Fig. S2). The average β -strand content of these pentamers is about 39%, 36%, and 33% for M6, M7a, and M7b, respectively; that of the inner core is about 39%, 38%, and 37% for M6, M7a, and M7b, respectively, which is close to the β -strand population (~43%) determined by Riek's group [13]. The distribution of the β -strand motif for these models was also calculated and summarized in Fig. 7 and Fig. S2. All fibril models display similar distributions of five β -strands as derived from solid-state NMR experiments. The first β -strand comprises residues ~37–41 as indicated by NMR data, however, both M6 and M7b also show a short β -strand consisting of T⁴⁴–E⁴⁶, which seems too short to be identified experimentally. Solid-state NMR data suggests that the second β -strand comprises residues ~52–58, whereas all models predict that it contains residues ~49–54. The layout of β 1 and β 2 in our models is in excellent agreement with the β -hairpin of α S(35–56) trapped by a β -wrap protein where two β -strands (V³⁷–K⁴³, V⁴⁸–T⁵⁴) are linked by a turn (T⁴⁴–G⁴⁷) [20]. However, it should be noted that β 2 lies in the layer involving E⁵⁷ and K⁵⁸. Although we have carefully examined the interactions especially of the salt-bridges in this loop, we cannot exclude other possible arrangements of these charged residues that could form stable β -strands. All three models characterize the third β -strand including residues ~62–66, which is in good agreement with the experimental result. As for the fourth β -strand, only M7a exhibits a short β -strand comprising residues 70–72, consistent with NMR data. Interestingly, residues 70–72 in our fibril M7a form a turn connecting β 3 and β 4. Such arrangement puts residues 72 and 73 in a loop, in agreement with the proposed five-layered β -sandwich by Riek group [13]. For the last β -strand that starts from A⁹⁰ and ends at V⁹⁵, a good agreement was obtained between the experimental result and both M6 and M7a.

The tertiary structure of these fibril models was characterized through calculation of the inter-layered distance and compared with available experimental results. However, because the ssNMR experiments were performed at a rather low temperature (273–283 K), and the α S fibril samples were in a dried gel-like state [13], direct comparison between our results from MD simulations performed in aqueous solution at 310 K and experimental data is not straightforward. Here, we compared the most important inter-layered residue distance and validated the consistency between our model structure with available experimental distance restraints [42]. A total of 20 unambiguous distance restraints with C α –C α distance within 6 Å were collected from the results of ¹³C-¹³C proton-driven spin diffusion spectra. As observed from one of the A β fibril structures (PDB ID: 2BEG) [19], the C α –C α distance between two β -layers varies from ~8.0–12.0 Å; therefore, a cutoff of 12.0 was used to evaluate the topology of our fibril models. The average C α –C α distance of the inner core structures was calculated and shown in Fig. 8.

In all three models, there are at least 12 inter-layered residue distances that are less than 12 Å (Fig. 8), confirming that the proposed model structures are able to maintain the α S fibril fold. Due to the formation of a short β -strand in the region comprising residues 70–72, the distance between β 3 and β 4 layers is larger than 12 Å in M7a (Fig. 8B). In contrast, the

corresponding distances are all less than 8 Å in M7b, and the distance between G⁶⁷ of β3 and A⁷⁶ of β4 is even less than 6 Å (Fig. 8C). Also note that the initial distance restraints used to construct these model are all well preserved (Fig. 1), implying that such organization of the five β-strands of the αS fibril could provide favorable inter-residual interactions and such interactions could be further optimized during the MD simulations. In addition, the single-layered αS fibril has been suggested to have a diameter of ~20–35 Å, and a height of ~40 Å (vertical distance between β1 and β5) in its gel-like state. We projected the mass density along *x* and *y* direction respectively, from which we could estimate the diameter and height of the αS fibril models (Fig. S3). Using a threshold of 0.05 Da/Å³ to exclude the disordered regions, the dimension of our αS fibril structures is ~45–50 Å, and the height is ~50 Å. Note that such a geometry of αS in aqueous solution is generally larger than the experimental ssNMR data due to the different hydration effect. However, the above dimensions are close or in agreement with other atomic force microscopy (AFM) experiments where samples were prepared in solution (pH 7.5, 310 K). For example, based on observation by AFM, Carter's group suggested a dimension of 32–44 Å for αS protofilament [43], and the Anderson group obtained a height of 45–60 Å for αS protofilament [44]. In addition, the Bennati group used electron paramagnetic resonance spectroscopy to measure the height of the αS fibril, and obtained a distance of 40–50 Å between the external of β-strands [45]. (Here both protofilament and fibril terms refer to the building block of fibrils).

2.3 Effects of charged repeats on the stability of NAC

It has been shown that the NAC promotes αS aggregation, and the hydrophobic motif of V⁷¹–V⁸² can form filaments alone [21]. Based on our fibril models, we found that the hydrophobic NAC composed of three β layers (β3, β4, and β5) can preserve about ~23%–25% of β-strand conformation in the relatively stable structures (M6, M7, and M8). The presence of charged repeat KTK(E/Q)GV, however, has distinct effects on the stability of the NAC (Fig. 4). In particular, the arrangement of charged residues in K⁵⁸T⁵⁹K⁶⁰E⁶¹Q⁶²V⁶³ connecting β2 and β3 has the most significant effect on the stability of NAC.

We also found that the repeat K²¹T²²K²³Q²⁴G²⁵V²⁶ in the remote N-terminal may stabilize the NAC domain near to the C-terminal. To test if the secondary and tertiary structures of the above models are also stable in the presence of an additional K²¹T²²K²³Q²⁴G²⁵V²⁶ repeat, we extended the N-terminal of M7b to construct a fibril structure of αS(20–110) (M8, Fig. S4), and performed a 40-ns MD simulations under the same conditions. M8 displayed similar secondary structures as the fibril models of αS(30–110) (Fig. 7). However, the strand β5 comprises more ordered residues (residues 85–105) than M7b (residues 94–105) (Fig. 7), suggesting that the presence of the repeat K²¹T²²K²³Q²⁴G²⁵V²⁶ increases the stability of the NAC domain (residues 85–95 specifically), although the percentage of β-strand in the NAC of αS(20–110) fibril is comparable to that of αS(30–110) (M6 and M7) (Fig. 4). The tertiary structure of αS(20–110) is also similar to that of αS(30–110) (Fig. 8D), implying that the additional 10 residues have no significant effect on the five-layered topology. These results confirm that the atomic structure models presented here capture the characteristic features of αS fibrils independent of the sequence length.

Shaykhalishahi *et al.* found that introduction of an intramolecular disulfide bond in the double cysteine mutant G⁴¹C/V⁴⁸C will inhibit amyloid formation [46]. With the G⁴¹C/V⁴⁸C disulfide bond, the C α -C α distance range of cysteine disulfide bonds would be around 5.6 Å. However, the average G⁴¹/V⁴⁸ C α -C α distances from last 5-ns MD trajectories of the two stable fibril models are much longer, 12.8 Å and 15.9 Å for M7a and M7b, respectively. Clearly, the disulfide bond restriction would destroy the favorite turn conformation for the K⁴³T⁴⁴K⁴⁵E⁴⁶G⁴⁷V⁴⁸ repeat and thus inhibit α S amyloid formation.

2.4 Aggregation inhibitors block the NAC region

The design of potent inhibitors of α S aggregation provides promising therapeutic strategies. Recent experiments showed that epigallocatechin-3-gallate (EGCG), an abundant catechin in green tea, as well as small β -hairpin peptides, could prevent the formation of α S amyloids in different ways [47–49]. To understand the molecular mechanisms of the interactions between aggregation inhibitors and α S, we used the most stable structure obtained in this study to dock with several α S aggregation inhibitors. First, the EGCG molecule was blindly docked onto the most stable structural model of α S fibril (M7a). Similarly, peptides [48] including WW2 (sequence: KKLTWVWIP'GKWITVSA, P' = D-Pro), and cyclo-WW2 (sequence: cyclo- KKLTWVWIP'GKWITVSIP'P, P' = D-Pro), were also examined. Gasteiger charges were calculated for all structures. The Lamarckian genetic algorithm was applied to search the docking conformations of EGCG. Flexible and rigid docking experiments were performed for EGCG and β -peptides, respectively (the α S fibril structure is always kept rigid). AutoDock4 and AutoDockTools 4 were used to carry out the docking studies [50]. The distribution of the 100 top ranked conformations of EGCG and β -peptides relative to α S fibrils is shown in Fig. 9. We found that EGCG and β -peptides bind to different sites of the α S fibril. EGCG prefers binding to both N-terminal and NAC domain. A weak binding site in the C-terminal was also observed (Fig. 9A and 9B). In contrast, the β -peptides (WW2 and cyclo-WW2) interact primarily with both NAC and the C-terminal of α S fibril, and the binding of WW2 to the NAC seems particularly favorable (Fig. 9C–9F). The distinct binding modes of the EGCG and β -peptides indicate that they disrupt the formation of the α S fibril through different mechanisms, consistent with recent experimental observations [48].

In a membrane environment, α S has α -helix-rich conformation [51]. There are energy barriers for conformational transitions from α -helix-rich to β -strand-rich α S conformations. To estimate the energy difference between the α -helix-rich and the β -strand-rich conformations, we simulated a helical dimer of α S(30–110), with each monomer adopting α -helix conformation (the conformation on membrane) [51]. After 20-ns MD simulations, the dimer is still rich in α -helix structures (Fig. S5). Based on the last 5-ns trajectory we calculated the GBMV energy and compared with the value of dimer conformations extracted from trajectories of M7. The obtained conformational energy of a dimer in α -helix conformation is $\sim$ –3429 kcal/mol, lower than the dimer in a β sandwich conformation (–3152 kcal/mol and –3136 kcal/mol for dimers extracted from M7a and M7b, respectively). The above result suggests that the formation of α S fibril rich in β -strand needs to overcome a large energy barrier, and inhibitors of EGCG and β -peptides may modulate such process and redirect the aggregation pathway of α S.

3. Conclusions

Because the aggregates of α S are closely associated with PD, the study of the structure-function relationship of α S needs reliable structural models. With sufficient experimental data available, we probed possible fibrillar structures using computational approaches. Although many studies suggest that different α S strains have different structures, aggregation propensity, and levels of toxicity, one of these α S fibrils has been well investigated and its building block unit can adopt a topology with five-layered β -strands. However, the details of how these β -strands organize need to be elucidated, especially how the four imperfect KTK(E/Q)GV repeats arrange in the fibril structures, the interaction with membrane and the α S aggregation mechanism. Adopting a hierarchical strategy, we systematically examined the role of the salt-bridge formed between residues Lys and Glu within each repeat on the stability of model structures of α S(30–110) fibrils. The coupling of these repeats with the NAC domain was specifically investigated. Two obtained models (M6 and M7) are able to preserve the topology of β -strands, with M7a being more energetically stable. One interesting result is that all charged residues in these repeats prefer to be exposed to the solvent and form salt-bridges. The arrangement of charged residues in the repeat $K^{58}T^{59}K^{60}E^{61}Q^{62}V^{63}$ between the β_2 and β_3 layers is particularly important for the stability of the NAC domain. Two trajectories of M7 (M7a and M7b) were generated with the same initial conformation but different velocity distributions. Structural characterization suggests that they display somewhat different secondary and tertiary structures. The distribution of β -strand is in better agreement with experimental observations in M7a whereas the distance restraints of adjacent β layers are more consistent with ssNMR data in M7b model. Energetically, the M7a structure was very close to M7b. The stability of M7b was further validated in the context of the model structure α S(20–110) fibril, indicating it also could be a building block for the full-length α S fibrils. In summary, the proposed atomic structures of the α S fibrils not only provide valuable insights into the topology of the α S fibrils, but also offer reliable starting structures for investigations of the assembly mechanism of α S in aqueous or in membrane environments, and of the effect of familial mutations on the structure and aggregation kinetics. Our structural model can also be by examining their molecular interactions useful for rational design of molecules targeting α S aggregates.

4. Materials and Methods

The five-layered model structures of α S(30–110) pentamer were constructed using Chimera software [52]. The five β -strands were assigned according to the NMR results reported by the Riek group [13]. The loop connecting β_2 and β_3 contains only two residues (K^{60} and E^{61}) in the original NMR assignments, which makes the turn too narrow and residues in β_2 and β_3 may collide. We thus extend this loop involving residues 58–61. Similarly, we also extend the loop connecting β_3 and β_4 that consists of residues 68–73 in order to satisfy the tertiary contact between T^{64}/N^{65} of β_3 and A^{78} of β_4 . Other inter-layered distance restraints used to build the model are shown in Fig. 1. These restraints include residue pair $Y^{39}-A^{53}$, $H^{50}-N^{65}$, $N^{65}-A^{78}$, and $T^{75}-I^{88}$. Note that these restraints are unambiguously assigned between residues based on solid-state NMR (ssNMR) experiment [13]. Other inter-layer distance restraints are also available but not used to build the model structures and will be

checked later. We found that not all the “unambiguous” restraints can be applicable to MD simulation simultaneously. For example, ssNMR indicates that C α of Y³⁹ is within 6 Å of C α of A⁵³, V⁵⁵, and E⁵⁷, suggesting that the inter-layer distance (β 1 and β 2) is within 6 Å, if we apply these three distance restraints together, β 1 and β 2 will distort significantly. On the other hand, the normal C α -C α distance between two β -layers varies from ~8.0–12.0 Å. Therefore, we applied one distance restraints between each two adjacent layers, and check the other restraints later. Four of the six imperfect KTK(E/Q)GV motifs are present in α S(30–110). Except the K³²T³³K³⁴E³⁵G³⁶V³⁷ motif, the remaining three repeats (K⁴³T⁴⁴K⁴⁵E⁴⁶G⁴⁷V⁴⁸, K⁵⁸T⁵⁹K⁶⁰E⁶¹Q⁶²V⁶³, and K⁸⁰T⁸¹V⁸²E⁸³G⁸⁴A⁸⁵) have residues in two loops that connect β 2 and β 3, and β 4 and β 5, respectively. We thus constructed various models hierarchically in order to investigate the effect of a salt-bridge formed by Glu and Lys on the stability of the α S fibril models (Table 1). The effect of charged residues on the stability of the β -strand has been well recognized [53, 54], thus worth examining in the context of α S fibrils.

Molecular dynamics (MD) simulations have become a powerful tool in drug design and discovery [55–57]. Here we apply this approach to refine our structural models of α S fibril, which could be further used in future studies such as virtual screening of potential drugs targeting PD. All MD simulations were performed in the NPT ensemble using NAMD2 (v2.10) program [58]. Each model structure was represented by CHARMM27 force field with CMAP correction [59, 60], and solvated in a box filled with TIP3P water molecules [61]. The minimum distance between protein and the edge of water box is at least 15 Å. Each system was neutralized by adding a number of counter-ions. The Langevin piston method and Langevin dynamics were applied to control the pressure at 1 atm and the temperature at 310 K [62]. The Particle Mesh Ewald (PME) method was used to calculate the long-range electrostatics [63]. The hydrogen bonds were constrained by using the SHAKE algorithm [64]. The van der Waals interactions were calculated using a switching function with a twin cutoff of 10 Å and 12 Å. An integration time step of 2 fs was used and trajectory was saved every 10 ps. MD simulations were conducted in three successive steps. Each system was first energy minimized for 10000 steps, with the intermolecular hydrogen bonds of the β -strand harmonically restrained with a force constant of 20 kcal/mol. Then the system was heated to 310 K gradually with the same restraints. In the equilibration stage, the force constant was decreased to 10 kcal/mol, 5 kcal/mol, and eliminated entirely. In each step, the system was equilibrated for 2 ns. The production simulation for each system was run for 20–40 ns. If the fibril structures (M1–M6) are not able to maintain the five-fold β -sheet layer, no additional simulations were performed. Additional simulation as performed to verify stable structure (M7a and M7b).

The relative stability of each fibril model was evaluated in terms of the conformational energy calculated by the generalized Born using the molecular volume (GBMV) method implemented in CHARMM program (c37b2) [59, 65]. The standard parameters in the GBMV II algorithm were used. A single-point energy calculation with infinite cutoffs was performed after the structure was minimized 200 steps using the steepest descent method. For each system, the last 5-ns trajectory (500 frames) was used to obtain the average energy and other analyses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- α -synuclein amyloid structures were simulated based on experimental constraints.
- Optimized structure was used to investigate interactions with inhibitors.
- The KTK(E/Q)GV repeats strongly affect the stability of α -synuclein fibrils.
- EGCG and β -peptides modulate the aggregation of α -synuclein in different ways.

Fig. 1.
The topology of the initial structural models of α S(30–110) fibril. The red region indicates the β -strand. The green spheres represent the distance restraints ($C\alpha$ distance $< 6.0 \text{ \AA}$) of inter-layered residues considered in the construction of model structures (Y^{39} - A^{53} , H^{50} - N^{65} , N^{65} - A^{78} , and T^{75} - I^{88}). The initial positions of charged residues in the loops are also shown.

Fig. 2. Structural models (M1–M5) of α S fibril. The starting (t=0 ns) and final (t=20 ns) conformations from MD simulations are shown.

Fig. 3.
Conformational energy calculated in terms of the GBMV method.

Fig. 4. Percentage of the β -strand in the NAC domain of each fibril model. Only the inner three chains of each pentamer were used for the calculation.

Fig. 5. Structural model of α S fibril (M6). The initial (A) and final (B) conformations from 40-ns MD simulations are shown.

Fig. 6. Structural model of α S fibril (M7). The initial (A) and final (B and C) conformations from 40-ns MD simulations are shown.

Fig. 7.
Comparison of the distribution of β -strand between ssNMR assignment and results of MD simulations of M6, M7, and M8.

Fig. 8.

Comparison of the inter-layered distance restraints between ssNMR data and results from MD simulations of M6 (A), M7a (B), M7b (C), and M8 (D). All the inter-layered distance restraints are extracted from the experimental results of Riek group [13].

Fig. 9. Results of docking of EGCG (A and B), WW2 (C and D), and cyclo-WW2 (E and F) onto α S fibril. Only the central three chains (chain I, chain II, and chain III) of α S fibril were used. The vertical dotted lines in B, D and F indicate the NAC part. The structure of α S fibril was shown in yellow and green, and the docking molecules were shown in cyan. A contact occurs if any atom of EGCG or the center of any residue of β -peptides is within 6 Å of the center of any residue of α S fibril.

Table 1

Summary of the orientation of charged residues in KTK(E/Q)GV motif. A hierarchical strategy was applied to optimize the structural model of α S(30–110) fibril. Based on M1–M5, K⁴³, K⁴⁵, and E⁴⁶ were found to expose to solvent. Further examination of M6 suggested that E⁵⁷, K⁵⁸, K⁶⁰, and E⁶¹ prefer to expose to solvent. The final model M7 integrates all these structural features. No stable salt-bridge was found in M7.

Model structure	Repeats involved	Side-chain orientation	Salt-bridge
M1		Interior: K ⁴³ and K ⁴⁵ ; Surface: E ⁴⁶ ; H ⁵⁰ towards β 3, and A ⁵³ towards β 1	
M2		Interior: E ⁴⁶ ; Surface: K ⁴³ and K ⁴⁵ ; H ⁵⁰ towards β 1, and A ⁵³ towards β 3	E ³⁵ -K ⁵⁸ ; K ⁴³ -E ⁴⁶
M3	K ⁴³ T ⁴⁴ K ⁴⁵ E ⁴⁶ G ⁴⁷ V ⁴⁸ , and K ⁵⁸ T ⁵⁹ K ⁶⁰ E ⁶¹ Q ⁶² V ⁶³	Interior: K ⁴³ and E ⁴⁶ ; Surface: K ⁴⁵ ; H ⁵⁰ towards β 1, and A ⁵³ towards β 3	E ³⁵ -K ⁵⁸ ; K ⁴³ -E ⁴⁶
M4		Surface: K⁴³, K⁴⁵, and E⁴⁶; H⁵⁰ towards β3, and A⁵³ towards β1	E ³⁵ -K ⁵⁸ , E ⁵⁷ -K ⁶⁰
M5		Surface: E ⁵⁷ and K ⁵⁸ ; Interior: K ⁶⁰	
M6	K ⁵⁸ T ⁵⁹ K ⁶⁰ E ⁶¹ Q ⁶² V ⁶³ , and K ⁸⁰ T ⁸¹ V ⁸² E ⁸³ G ⁸⁴ A ⁸⁵	Surface: E⁵⁷, K⁵⁸, and E⁶¹ ; Interior: K ⁶⁰	E ⁸⁰ -K ⁸³
M7/M8	K ⁴³ T ⁴⁴ K ⁴⁵ E ⁴⁶ G ⁴⁷ V ⁴⁸ , K ⁵⁸ T ⁵⁹ K ⁶⁰ E ⁶¹ Q ⁶² V ⁶³ , and K ⁸⁰ T ⁸¹ V ⁸² E ⁸³ G ⁸⁴ A ⁸⁵	Surface: K⁴³, K⁴⁵, E⁴⁶, E⁵⁷, K⁵⁸, K⁶⁰, E⁶¹, K⁸⁰, and E⁸³	