Mechanism of Tripeptide Trimming by γ-Secretase

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Abstract

The membrane-embedded γ-secretase complex processively cleaves within the transmembrane domain of amyloid precursor protein (APP) to produce 37-to-43-residue amyloid β-peptides (Aβ) of Alzheimer's disease (AD). Despite its importance in pathogenesis, the mechanism of processive proteolysis by γ-secretase remains poorly understood. Here, mass spectrometry and western blotting were used to quantify the efficiency of the first tripeptide trimming step (Aβ49→Aβ46) of wildtype (WT) and familial AD (FAD) mutant APP substrate. In comparison to WT APP, the efficiency of this first trimming step was slightly higher for the I45F, A42T and V46F APP FAD mutants, but substantially diminished for the I45T and T48P mutants. In parallel with biochemical experiments, all-atom simulations using a novel Peptide Gaussian accelerated molecular dynamics (Pep-GaMD) method were applied to investigate tripeptide trimming of Aβ49 by γ-secretase. The starting structure was active γ-secretase bound to Aβ49 and APP intracellular domain (AICD), as generated from our previous study that captured activation of γ-secretase for the initial endoproteolytic cleavage of APP (Bhattarai *et al., ACS Cent Sci*, 2020, 6:969-983). Pep-GaMD simulations captured remarkable structural rearrangements of both the enzyme and substrate, in which hydrogen-bonded catalytic aspartates and water became poised for tripeptide trimming of Aβ49 to Aβ46. These structural changes required a positively charged N-terminus of endoproteolytic coproduct AICD, which could dissociate during conformational rearrangements of the protease and Aβ49. The simulation findings were highly consistent with biochemical experimental data. Taken together, our complementary biochemical experiments and Pep-GaMD simulations have enabled elucidation of the mechanism of tripeptide trimming by γ-secretase.

Significance statement

Production of amyloid β-peptide (Aβ) of Alzheimer's disease (AD) from its precursor protein requires a series of proteolytic events carried out by the membrane-embedded γ-secretase complex. Mutations in the substrate and enzyme that produce Aβ cause hereditary AD and these mutations affect tripeptide trimming of initially formed long Aβ peptides by γ-secretase. Little is known about the structural mechanism of this trimming process. Here we have developed a molecular dynamics model for the first step of trimming (Aβ49 to Aβ46) by γ-secretase. Conformational changes in the enzyme-substrate complex to set up this trimming step require the presence of N-terminally charged endoproteolytic cleavage co-product. Computational effects of AD-causing mutations in Aβ49 on the trimming process were validated through biochemical experiments.

Keywords: amyloid precursor protein (APP), proteolysis, Alzheimer's disease (AD), familial AD (FAD), Peptide gaussian accelerated molecular dynamics (Pep-GaMD), mass spectrometry (MS).

Introduction

Alzheimer's disease (AD) contributes to more than 80% of all dementia cases(1). Deaths related to AD in the United States increased by 89% between 2000 and 2014, and more than 6.2 million Americans are affected with AD in 2021 (www.alz.org). AD is characterized by deposition of longer amyloid β-peptides (Aβ) in the form of plaques in the brain cerebrum. The amyloid β-precursor protein (APP) is processed by γ-secretase to produce Aβ peptides. β-secretase first sheds the APP extracellular domain to produce transmembrane peptide C99, followed by processive proteolysis by γ-secretase to produce Aβ peptides of varying lengths(2). Membrane-embedded γ-secretase is a multi-domain aspartyl protease with presenilin as the catalytic subunit. γ-Secretase is considered "the proteasome of the membrane", with more than 100 known substrates, including APP and the Notch family of cell-surface receptors(3, 4). The location of the proteolysis and the number of cleavages within the APP transmembrane domain by γ-secretase determines the length of final Aβ products and the likelihood to form plaques.

Of the many transmembrane substrates, processive proteolysis of APP by γ-secretase is the most studied. γ-Secretase first carried out endoproteolytic (ε) cleavage of C99 peptide near the cytosolic end, producing Aβ49 and Aβ48 peptides and their respective AICD co-products (AICD50-99 and AICD49-99, respectively)(5). These initially formed long Aβ peptides are then cut every 3-4 residues from their C-termini to release tri- or tetrapeptides co-products. The two general pathways of γ-secretase processive proteolysis are Aβ48→Aβ45→Aβ42→Aβ38 and Aβ49→Aβ46→Aβ43→Aβ40(6, 7), producing Aβ42 and Aβ40 as their dominant products, respectively. Among these two, the longer Aβ42 peptide is more prone to aggregate and form plaques(8). Moreover, early-onset familial AD (FAD) APP mutants can bias the enzyme to produce longer Aβ peptides that are pathological and cause AD(9, 10). The trimming of APP

substrate by γ-secretase enzyme is dictated by active site S1', S2' and S3'subpockets that respectively bind to P1', P2' and P3' substrate residues(9).

Critical gaps remain in understanding the mechanism of intramembrane processive proteolysis by γ-secretase. Recently reported cryo-EM structures of γ-secretase bound to Notch and APP substrates provided valuable insights into the structural basis of substrate recognition of the enzyme(11, 12). However, artificial structural constraints were included that could affect the enzyme-substrate interactions. Molecular dynamics (MD) simulations have proven useful in understanding the structural dynamics of γ-secretase, notably the enzyme-substrate interactions (13-17). Recently, we computationally restored the wildtype (WT) enzyme-substrate co-structure and applied all-atom simulations using the Gaussian accelerated molecular dynamics (GaMD) method to build the first dynamic model of γ-secretase activation(18). GaMD is an enhanced sampling technique that works by adding a harmonic boost potential to smooth the potential energy surface and reduce system energy barriers(19). Our GaMD simulations captured the extremely slow motions underlying enzyme activation, with the two catalytic aspartates and a coordinated water molecule poised for proteolysis of APP at the ε cleavage site. We showed that the I45F and T48P FAD mutations in APP enhanced the ε cleavage of the amide bond between Leu49-Val50 compared with the WT APP. In comparison, the M51F mutation in APP shifted the ε cleavage to the adjacent Thr48-Leu49 amide bond, changing the proteolysis from the Aβ49 to the Aβ48 pathway. Despite these advances, the detailed atomistic mechanism of processive proteolysis by γ-secretase remains elusive. This is consistent with γ-secretase being a well-known slow-acting enzyme (k_{cat} for APP ε proteolysis ~ 2 per hour), making it difficult to capture the dynamic transitions comprising large energy barriers in MD simulations. Hence, despite its importance in

the pathogenesis of AD, the mechanism of processive proteolysis (tripeptide trimming) by γ-secretase remains poorly understood.

Here, we report the first dynamic model of tripeptide trimming of Aβ49 to Aβ46 (ζ cleavage) by γ-secretase. Extensive all-atom simulations using a novel Peptide GaMD (Pep-GaMD) method(20) captured the slow dynamic molecular transition from the ε to ζ proteolytic cleavage step. In Pep-GaMD, a boost potential is applied selectively to the essential potential energy of the peptide to effectively model its high flexibility and accelerate its dynamic motions(20). In addition, another boost potential is applied on the protein and solvent to enhance conformational sampling of the protein and facilitate peptide binding. Pep-GaMD has been demonstrated on binding of three model peptides to the SH3 protein domains. Independent 1-μs dual-boost Pep-GaMD simulations have captured repetitive peptide dissociation and binding events, which enable calculation of peptide binding thermodynamics and kinetics. The calculated binding free energies and kinetic rate constants agreed very well with the available experimental data(21).

In this study, we have combined biochemical experiments, including matrix-assisted laser desorption/ionization - time-of-flight) mass spectrometry (MALD-TOF MS), liquid chromatography - tandem mass spectrometry (LC-MS/MS) and western blotting, with Pep-GaMD enhanced sampling simulations to elucidate the mechanism of tripeptide trimming of Aβ49 by γ-secretase. Our Pep-GaMD simulation findings of the WT and five FAD mutants (I45F, A42T, V46F, I45T and T48P) of Aβ49 bound to the γ-secretase were highly consistent with quantitative biochemical analysis of their specific proteolytic products, providing important mechanistic insights into tripeptide trimming by the enzyme.

Results

Probing ζ cleavage of WT and FAD-mutant Aβ49 by γ-secretase in biochemical experiments.

To compare the ζ cleavage of the WT and FAD mutants of APP by γ-secretase, we used an *in vitro* cleavage assay using purified γ-secretase and recombinant APP-based substrate C100-FLAG, which contained the C99 APP C-terminal fragment with an N-terminal start methionine and a C-terminal FLAG epitope tag. Efficiency of the cleavage of substrate Aβ49 to products Aβ46 and tripeptide was calculated by measuring concentrations of Aβ49 production and Aβ49 degradation. To quantify Aβ49 production by ε cleavage of APP substrate, levels of co-products AICD 50-99 were determined using a combination of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and quantitative western blotting.

First, AICD produced in the assay was immunoprecipitated with anti-FLAG antibodies and detected by MALDI-TOF MS (**Figure 1A**). For the WT, A42T, V46F and I45T APP substrate, the signal intensities corresponding to AICD 49-99 and AICD 50-99 show higher level of AICD 49-99 than AICD 50-99. However, for mutants I45F and T48P APP substrate, signal intensities show higher level of AICD 50-99 than AICD 49-99. This suggest I45F and T48P favor production of Aβ49 rather than production of Aβ48 while A42T, V46F and I45T favor production of Aβ48 rather than production of Aβ49.

The same reaction mixtures were subjected to quantitative western blotting with anti-FLAG antibodies (**Figure 1B**), where standards of known concentrations of C100-FLAG were also run to make a standard curve, plotting band intensity against concentration of FLAG-tagged C100. From this standard curve, the concentration of total AICD-FLAG product produced in the enzyme reaction was quantified (**Figure 1C**). Quantification of the total AICD reveal increased

total AICD production for V46F mutant substrate and decreased total AICD production for A42T, I45F, I45T, and T48P mutant substrates compared to AICD production for the WT. The concentration of AICD 50-99 was calculated using the total AICD level determined by quantitative western blot and the ratio of AICD 49-99 to AICD 50-99 determined from MALDI-TOF MS. (**Figure 1D**). The calculated concentrations of AICD 50-99 thus provided the level of production of co-product Aβ49. Aβ49 production is slightly increased for I45F mutant while for all other mutants A42T, V46F, I45T and T48P decreased Aβ49 production is observed compared to Aβ49 production of the WT.

To determine the degradation of Aβ49, we calculated and quantified trimming product tripeptide ITL. The mixtures from the cleavage assay were subjected to LC-MS/MS analysis to detect tripeptides. All substrate constructs studied produced ITL except for T48P mutant which produced IPL due to the replacement of T with P. For quantification of these tripeptides production, standard curves of each peptide were generated by plotting the concentration of synthetic peptide against the integrated areas of the three most abundant ion fragments from MS/MS (**Figure S17**). The ITL and IPL peptide generated in the γ-secretase cleavage was monitored and quantified. (**Figure 1E**). The quantification of the trimming product (ITL or IPL) or the Aβ49 degradation reveal decrease in Aβ degradation for A42T, V46F, I45T and T48P. For I45F, Aβ49 degradation is similar to that of the WT. Concentration of both Aβ49 production as well as Aβ49 degradation was used to calculate the percent efficiency (**Figure 1F**). For, all constructs cleavage efficiency was close to 100 percent except for two mutants I45T and T48P, in which the cleavage efficiencies were decreased substantially, to 35% and 34%, respectively.

Activation of γ-secretase for tripeptide trimming of Aβ49 was captured in highly consistent Pep-GaMD simulations.

In parallel with the biochemical experiments, Pep-GaMD simulations were carried out on the g-secretase bound by the WT and the I45F, A42T, V46F, I45T and T48P mutants of Ab49. The active WT APP-bound g-secretase was obtained from our previous study(18) and the amide bond between Ab49 and AICD50-99 was cleaved as the new simulation starting structure **(Figure S1,** see details in **Methods)**. We initially performed dual-boost GaMD simulations on the g-secretase bound to Ab49 with AICD50-99 removed. However, even after running ~6 μs GaMD simulations, we could not effectively sample conformational transitions of the system for z cleavage of Ab49 to Aβ46 **(Figure S2)**. The distance between the enzyme Asp257 catalytic residue and substrate Val46-Ile47 amide bond presented a computational challenge for conformational sampling, with apparently high energy barriers to overcome. To address the challenge, we applied our recently developed Pep-GaMD(20) method, which selectively boosts the essential potential energy of the peptide substrate for significantly improved conformational sampling. We built three different Pep-GaMD simulation systems with g-secretase bound to Ab49 in the presence and absence of charged and neutral N-terminal AICD50-99 **(Figure S3)**. Spontaneous activation of g-secretase for z cleavage of Ab49 was observed during 600 ns Pep-GaMD simulations **(Figures S4** and **S5** and **Movie S1)**. The enzyme activation for z cleavage was characterized by coordinated hydrogen bonding between the enzyme Asp257 and carbonyl oxygen of substrate Val46. The catalytic aspartates were at a distance of ~7-8 Å between their Cγ atoms, which could accommodate a water molecule for nucleophilic attack of the carbonyl carbon of the scissile amide bond **(Figure S4)**. The water molecule formed hydrogen bonds with both catalytic aspartates and was at ~4 Å distance away from the carbonyl carbon of substrate Val46 residue. The activated g-secretase conformation

was well poised for cleavage of amide bond between Val46 and Ile47 for z cleavage of the Ab49. In the charged N-terminal AICD system, we observed AICD50-99 dissociation in addition to enzyme activation for z cleavage **(Figure S10 and Movie S6)**. The AICD50-99, initially located near the Ab49, slowly moved downwards to the intracellular PS1 pocket and then dissociated completely from the enzyme. Meanwhile, the AICD50-99 transitioned from b-sheet to a loop/un-structured conformation during the Pep-GaMD simulations **(Figure S10 and Movie S6)**. In comparison, the g-secretase systems bound to APP in the absence and presence of neutral N-terminal AICD50-99 could not sample enzyme activation for z cleavage and AICD50-99 dissociation (**Figure S5E** and **S5F**). This showed that the presence of charged N-terminal AICD50-99 was crucial for the enzyme activation for z cleavage of Aβ49.

Free energy profiles were calculated from Pep-GaMD simulations to characterize the activation of γ-secretase for ζ cleavage of the Aβ49 substrate, for which the distance between the enzyme catalytic aspartates and the distance between the enzyme protonated Asp257 and substrate residue Val46 were selected as reaction coordinates **(Figure 1G-1L, S5 and S8)**. In the WT Aβ49, three low-energy conformational states were identified from the free energy profile, including "Final", "Intermediate" and "Initial" **(Figure 1G and S5A)**. In the "Final" conformational state, the aspartates were ~7-8 Å apart to accommodate the water molecule in between. The substrate Val46 maintained a distance of ~3 Å from the active site Asp257 to form hydrogen bond in the Final active state. In the "Initial" state, the substrate Val46 was distant (~8-9 Å) from the active site Asp257, while the inter-aspartate distance was ~6-7 Å. The "Initial" state represented the active state for the ε cleavage of APP. In the "Intermediate" state, the aspartates remained ~6-7 Å apart, while the Aβ49 peptide was at a distance of ~6 Å from the protonated Asp257 **(Figure 1G)**.

In the I45F mutant system, two low-energy conformational states, “Initial” and “Final”, were identified from the free energy profile of Pep-GaMD simulations **(Figure 1H, S5B and S8B and Movie S3)**. Two out of three Pep-GaMD simulations could capture the activation process, as the Asp257 could form stable hydrogen bond with Val46 as reflected in the distance time course plot (**Figure S5B**). The “Final” state in the free energy profile represented the active conformation of the enzyme for ζ cleavage of the scissile amide bond between Val46 and Ile47 APP residues. In the “Final” conformational state, the two catalytic aspartates were ~7-8 Å apart, and APP Val46 was ~3 Å distance away from the protonated aspartate. In the “Initial” state, the substrate Val46 was further away from the catalytic aspartate (~6-7 Å). The aspartates were ~7-8 Å distance away from each other **(Figure 1H)**.

In the A42T mutant APP system, four low energy conformational states were identified from the free energy profile **(Figure 1I, S5C and S8C and Movie S4)**. Mutation of Ala42 to Thr42 caused the enzyme-substrate complex to be more flexible and sample a larger conformational space. In addition to the “Initial” and “Final” states, two new “Inhibited-1” and “Inactive” conformational states were identified for the A42T mutant system. The catalytic aspartates were ~4-5 Å (too close) apart in the “Inhibited-1” state and 13 Å away (too far) in the “Inactive” state. In the “Inhibited” state, the catalytic aspartates could not accommodate a water molecule between them and hence was inhibited from proteolytic activation. APP Val46 was ~4-5 Å from the protonated Asp257 in this inhibited state. In the “Inactive” state, the aspartates were ~13 Å apart and thus too far to form the dual hydrogen bonds with the water in between them, even though the Asp257 could form a hydrogen bond with the Val46 carbonyl oxygen. This hindered activation required for ζ cleavage.

In the V46F mutant system, two low-energy conformational states were identified, including "Inhibited-2" and "Final" **(Figure 1J, S5D and S8D and Movie S5)**. Like other γ-secretase systems, the "Final" state corresponded to the active conformation of the enzyme poised for ζ cleavage of Aβ49. Moreover, the "Inhibited-2" state had the two aspartates at proximity (~4-5 Å) between the Cγ atoms and unable to accommodate a water molecule in between for enzyme activation. APP substrate was ~10 Å away from active site Asp257 in the "Inhibited-2" state.

Furthermore, Pep-GaMD simulations on I45T and T48P mutant Aβ49-bound γ-secretase were carried out **(Figure 1K-1L, S5G-S5H and S8G-S8H)**. Both of these mutant systems were not able to activate the enzyme for ζ cleavage, being consistent with the experimental results where the ζ cleavage efficiency dropped about one third compared to that of the WT. In the Pep-GaMD free energy profile of the I45T mutant system, one "Intermediate" low energy conformational state was identified. This "Intermediate" state was the same low-energy conformation as the one in the WT system. Likewise, in one of the three Pep-GaMD production simulations of T48P system, the hydrogen bond distance was maintained between APP Val46 and the protonated Asp257 for certain time **(Figure S5H)**. However, in the free energy profile, we could identified two low-energy conformational states, including "Inhibited-1" and "Initial", but not the "Final" active state **(Figure 1L)**. The "Inhibited-1" state resembled the one identified in the A42T mutant system. The "Initial" conformational state was the same as the one identified in the WT, I45F and A42T systems. These Pep-GaMD simulation findings were consistent with the biochemical experiments, verifying the I45T and T48P systems as negative controls.

Conformational changes in activation of γ-secretase for tripeptide trimming of Aβ49

We calculated root mean square fluctuations (RMSFs) of γ-secretase bound by the WT and FAD-mutant APP from Pep-GaMD simulations **(Figure S6 and Movie S2)**. In the WT Aβ49-bound γ-secretase, the TM2, TM6, TM6a and C-terminus of TM9 helix were flexible in the catalytic PS1 subunit. The Pen-2 subunit exhibited high fluctuations with ~ 3 Å RMSF. Helices α1, α2, α5, α12, α17, and TM domain of nicastrin were also flexible during the Pep-GaMD simulations. Structural clustering was performed on Pep-GaMD snapshots of the system using hierarchical agglomerative algorithm in CPPTRAJ(22) (see **Methods**). The top-ranked cluster was selected as the representative "Final" active conformation for the ζ cleavage of Aβ49. The starting structure from ε cleavage of APP was obtained as the "Initial" active conformation. The catalytic PS1 of the "Final" conformation was compared to that of the "Initial" conformation in **Figure 2A**. Relative to the "Initial" conformation, the substrate helical domain tilted by ~50 degrees in the "Final" conformation **(Figures 2A and 2B)**. Residue Leu49 in the substrate C-terminus moved downwards by ~5 Å **(Figures 1B and 4A and S7)**. The last residue in a helical conformation in the "Final" state of Aβ49 was Thr43 whereas it was Ile45 in the "Initial" state. In transition from the "Initial to the "Final" conformational state, two substrate residues, Val44 and Ile45 unwound their helical conformation and changed to a turn/loop conformation. Residues Thr43 and Ile45 were in similar positions in the "Initial" and "Final" active conformations relative to the membrane perpendicular axis (**Figure S7**). In comparison, the substrate C-terminal Leu49 moved downwards by ~5 Å while straightening the C-terminal loop **(Figures 2B and S7B)**.

At the enzyme active site, the catalytic Asp385 did not have significant movement during substrate peptide trimming (**Figure 2C**). In comparison, the protonated catalytic Asp257 moved by ~3 Å towards the substrate. Asp257 moved forward to a form hydrogen bond with the carbonyl oxygen of the scissile amide bond between the substrate residues Val46 and Ile47. Similarly, TM3

moved outwards by ~2 Å **(Figure 2D)** and TM6a moved downwards by ~2 Å **(Figure 1E)**. Flexibility in these helices involved important FAD mutation sites including Tyr154, His163, Ala164, Leu166, Trp165, Ser169, Ile168, Tyr256, Ala260, Leu262, Cys263, Pro264, Pro267, Arg269, Val272 and Leu271 (www.alzforum.org). Trp165 and His163 from TM3 and Arg269 from TM6a showed significant movements in their side chains. With a major part of C-terminus of APP absent (as AICD dissociates, see next section) the β2 loop at N-terminus of TM7 moved away from the APP by ~5 Å in the Final state as compared to the Initial state **(Figure 2F)**. FAD mutation residues in the β2-TM7 region including Arg377, G378, L383 and G384 showed flexibility in the simulations. In particular, residue Arg377 reoriented its side chain in the "Final" conformational state.

Changes in secondary structures of the WT and FAD-mutant Aβ49 during tripeptide trimming

Secondary structures of the WT and FAD-mutant Aβ49 bound to γ-secretase were recorded during the Pep-GaMD simulations and plotted in **Figures 3 and S11**. Changes in secondary structures of Aβ49 during ζ cleavage were compared to that of APP substrate ("Initial" active conformation) during ε cleavage from our previous study(18) **(Figure S12)**. The unwinding of the Aβ49 C-terminus during ζ cleavage was observed in the secondary structure plot. During the ε cleavage, the C-terminus of the WT APP substrate could maintain helical conformation up to Ile45/Val46 **(Figure S12)**. In comparison, WT Aβ49 was helical up to Thr43 in the C-terminal region **(Figure 3A and 1B)**. About 2-3 residues unwound near the ζ cleavage site to expose the scissile amide bond between Val46 and Ile47 to the catalytic aspartates and the coordinated water for activation. A new helix was formed for residues Ser26 to Ala30 in the Aβ49 during the transition from ε to ζ

cleavage in the WT system **(Figure 3A and S12)**. With the 50-degree tilt of Aβ49 peptide in the space between TM2 and TM3, the N-terminus is exposed to the hydrophobic lipid bilayer **(Figure S13)**. This helped the N-terminal loop to transition to a α-helical conformation. Such new membrane helical conformation was formed in the I45F, A42T and V46F mutant systems as well **(Figure 3A-3D)**. A turn/unstructured conformation at residues Ala30-Ile31 separated these two helices. In addition, the N-terminus of Aβ49 lost its interactions with the hydrophobic loop 1 (HL1) because of the tilting away from this loop.

Similarly, secondary structures changes were recorded for the I45F, A42T and V46F Aβ49 mutant systems **(Figure 3B-3D and S14-S15)**. Like the WT, the I45F and A42T Aβ49 mutants maintained helical conformation up to Thr43 at the N-terminus during the Pep-GaMD simulations. C-terminal residues after the Thr43, which included the ζ cleavage site bond between Val46 and Ile47, were observed mostly in a turn/unstructured conformation. This allowed the catalytic aspartates and water to approach the scissile amide bond for forming coordinated hydrogen bonds required for this cleavage. Likewise, bands of new helix formation were observed in the secondary structure plots from Asn27 to Ile31 and from Asp23 to Lys28 for I45F- and A42T-mutant Aβ49 systems, respectively **(Figure 3B and 3C)**. The new helix formed was due to its exposure to the hydrophobic lipid membrane. V46F Aβ49 was observed to be the most dynamic in terms of secondary structure changes **(Figure 3D)**. A band of helix was observed between Gly29 to Thr43, with a turn conformation formed between at Leu34 – Val36. Thr43 to Ile47 transitioned between helix and turn conformations during the Pep-GaMD simulations of the V46F Aβ49. Like the WT and other mutant systems, new N-terminal helix formation was observed at residues Phe20 to Gly25 in the V46F mutant APP. The hydrophobic lipid environment helped these residues transition from turn to a helical conformation in the V46F mutant APP.

Active-site subpockets formed in γ-secretase for tripeptide trimming.

The "Final" active conformational state of Aβ49-bound γ-secretase was further analyzed for the P1', P2' and P3' substrate residues at the ζ cleavage active site and the respective S1', S2' and S3' subpockets in which they reside(9). The S1' subpocket constituting the P1' residue in the WT Aβ49 was formed by residues from PS1 TM6a helix, β1 loop, TM3 helix and TM7 N-terminal region **(Figures 2 and 4A)**. The residues that formed the subpocket are listed in **Table S1**. The S2' subpocket occupied the P1' substrate residue constituted residues from PS1 TM6 helix, TM6a helix, PAL motif of TM9 helix, β1 and β2 loop region. Moreover, the P3' residue constituting S3' subpocket was formed by residues from PS1 TM6 helix, TM6a helix and β1 loop. In reference to Aβ49, S1' and S3' pockets were located on the same side (TM6a and TM3 helices) whereas the S2' pocket was located on the opposite side (TM6 and TM9 helices).

Similarly, the "Final" active conformational states of the I45F and A42T mutant Aβ49 bound γ-secretase systems had the same subpockets formed at the active site during the ζ cleavage as that of the WT system **(Figure 4B-4C and Table S1)**. In the I45F and A42T "Final" active conformation, the S1' and S3' subpockets occupied by the respective P1' and P2' substrate residues constituted the residues from PS1 TM6 helix, TM6a helix, TM7 helix, β1 and β2 loop region. In comparison, the S3' subpocket was located on the opposite side in reference to the Aβ49 constituting residues from PS1 TM6 helix, TM6a helix, PAL motif of TM9 helix, β1 and β2 loop. Furthermore, in the V46F "Final" active conformation, locations of the S1' and S2' subpockets constituting the respective P1' and P2' Aβ49 substrate residues were different as compared to that of the WT system **(Figure 4C and 4D)**. The S1' pocket occupied by the P1' residue of Aβ49

constituted residues from TM6 helix, TM6a helix and TM2 helix **(Table S1)**. The S2' subpocket occupied by the P2' residue of the V46F mutant in the "Final" active state was the same as the S1' subpocket in the "Final" active state of the WT, I45F and A42T systems **(Figure 4C and 4D)**. Moreover, the S3' subpocket comprising the P3' substrate residue in the V46F mutant was the same as the one of the WT, I45F and A42T systems (**Figure 4E**).

Discussion

Current AD treatments ease symptoms, but none has been clearly demonstrated to slow or halt disease progression. While the molecular cause of AD remains poorly understood, the hallmark pathological criteria for AD diagnosis is the deposition of amyloid-β (Aβ) plaques in the brain(23). Aβ peptides are products of processive proteolysis by γ-secretase. Dominant missense mutations in the substrate (APP) and the enzyme (presenilin component of γ-secretase) cause early-onset FAD, and these mutations result in deficient carboxypeptidase trimming of initially formed long Aβ peptides to shorter secreted forms (24-26). Yet the mechanism of processive proteolysis of APP by γ-secretase is unknown. Recent reports of cryo-EM structures of γ-secretase bound to APP and Notch substrates as well as to γ-secretase inhibitors and modulators revealed details of the structural basis of substrate recognition as well as enzyme inhibition and modulation(12, 27). Regardless, static conformations of the enzyme cannot explain the underlying mechanism of enzyme activation and substrate processing. Essentially nothing is known about the dynamic mechanism of processive proteolysis by γ-secretase.

Here, we have addressed the issue by combining novel Pep-GaMD enhanced sampling simulations and biochemical experiments. Different γ-secretase systems bound to WT and FAD-mutant Aβ49 substrate were investigated to understand the first trimming step, ζ cleavage. Four γ-

secretase systems—bound to the WT, I45F, A42T and V46F Aβ49 in presence of charged N-terminal AICD—underwent activation for ζ cleavage during 600 ns of the Pep-GaMD simulations. This was consistent with biochemical experiments, as these mutant systems showed equal or increased efficiency for the Aβ49 → Aβ46 proteolytic step (ζ cleavage). In comparison, the γ-secretase complex bound by I45T and T48P FAD mutant Aβ49 showed little or no sample activation. Furthermore, the Aβ49-bound γ-secretase in the absence of AICD was not able to sample the "Final" active state for ζ cleavage of the substrate **(Figure S9A)**, similarly for γ-secretase bound to WT Aβ49 in the presence of neutral N-terminal AICD **(Figure S9B).** This highlighted the importance of AICD and its N-terminal charge in facilitating processive proteolysis by γ-secretase. Following ε cleavage, both the C-terminus of Aβ49 and N-terminus of AICD at the active site could be exposed to water molecules and thus charged at biological pH 7 (as carboxylate and ammonium, respectively). The charged state likely aided movement toward the polar aqueous environment and away from the hydrophobic transmembrane interior of the PS1 active site. Indeed, the AICD with charged N-terminus could dissociate from PS1 in the Pep-GaMD simulations, while the charged C-terminus of Aβ49 helped prepare for the next cleavage during processive proteolysis by γ-secretase.

During ζ cleavage activation, two residues unwound from the C-terminus of the Aβ49 helix, changing to a turn conformation. This was observed in the time courses of the substrate secondary structures as well. Unlike the helical conformation, the loop/turn conformation facilitated exposure of the scissile amide bond to the catalytic aspartates and the coordinated water molecule. In parallel, relative positions of Thr43 and Ile45 residues of the "Initial" active and "Final" active states in reference to the membrane were similar whereas the C-terminal residue Leu49 moved downwards by ~5 Å. Moreover, the helical domain of Aβ49 tilted by ~50 degrees.

Thus, tilting of the helical domain and unwinding of C-terminal helix in the substrate apparently facilitated the proteolytic progression from ε to ζ cleavage by γ-secretase. Helix unwinding was accompanied by straightening of the C-terminal loop/turn and downward movement of the terminal residue Leu49. Similarly, the β-sheet conformation between the APP C-terminus and the β1 loop was broken as ε cleavage product AICD50-99 dissociates. This caused the β1 loop to move away from the APP C-terminus by ~5 Å. This region has been suggested to be important for substrate recognition and proteolytic processing(12). Similarly, γ-secretase inhibitors (GSIs) and transition state analogs (TSAs) bind to this region(28). Pep-GaMD could show important atomistic dynamism of this region during the enzyme activation for ζ cleavage.

Pep-GaMD captured the enzyme activation for ζ cleavage for γ-secretase systems bound to WT and three FAD-mutant (I45F, A42T and V46F) APP substrates. The low-energy "Final" active conformation was identified in the Pep-GaMD free energy profiles of all these systems. However, each PMF profile representing different enzyme systems was different in terms of distinct low-energy states and the conformational space sampled by the enzyme during ζ cleavage. The I45F and V46F mutant systems sampled two low-energy conformations with the I45F system being the least conformationally dynamic **(Figure 1H and 1J)**. Three and four low-energy states were identified from free energy profiles of the WT and the A42T mutant systems, respectively, with the A42T mutant system being more dynamic **(Figure 1G and 1I)**. Each system had its own set of conformations and a distinct activation pathway. This suggested that the enzyme is very dynamic, consistent with its ability to cleave over 100 different substrates(29). Hence, the Pep-GaMD simulations remarkably captured the slow molecular transitions in atomistic detail.

In the "Final" active state of γ-secretase poised for the ζ cleavage, subpockets were formed in the active site that were different than that formed for the ε cleavage **(Figure 4 and S16)**. This

finding was consistent with the observation that the C-terminus of Aβ49 during ζ cleavage did not form a β-sheet conformation with the PS1 TM6a β-2 region, instead adopting a loop conformation **(Figure 1F)**. The locations of S1', S2' and S3' subpockets formed for ζ cleavage were the same for different γ-secretase systems bound to the WT and -mutant Aβ49 except for the V46F mutant system. The locations of the active site subpockets formed for ζ cleavage were compared to those formed for ε cleavage **(Figure S16)**. The S2' subpocket constituting the T48 P2' residue formed for ζ cleavage was the same as the S1' subpocket constituting the V50 P1' subpocket formed for ε cleavage. In contrast, the ζ cleavage S3' subpocket constituting the L49 P3' residue was the same as the ε cleavage S3' subpocket constituting the L52 P3' residue. The S1' subpocket constituting the I47 P1' residue for ζ cleavage and the S2' subpocket constituting the M51 P2' residue for ε cleavage had their own unique location in their respective Final active states. Regardless, S1'/S3' and S2' subpockets were located on opposite sides in both of the "Initial" and "Final" active states.

In summary, the Pep-GaMD simulation findings were highly consistent with mutagenesis and mass spectrometry experiments, which quantified AICD50-99 (and therefore Ab49) production as well as the ζ-cleaved tripeptide product of these APP FAD-mutant systems. Overall, our combined Pep-GaMD simulations and biochemical experiments have advanced understanding of the mechanism of processive proteolysis by γ-secretase.

Materials and Method Summary

γ-Secretase assay was performed with purified γ-secretase and C100-Flag substrates. The reaction mixture was subjected to MALDI-TOF mass spectrometry to detect AICD-FLAG species, Same reaction mixture was western blotted with Anti Flag antibodies along with C100 standard to quantify the total AICD species. The reaction was also subjected to LC-MS/MS to detect

tripeptides generated in the reactions. Pep-GaMD simulations was performed on the γ-secretase activation for ζ cleavage of Aβ49. Active APP-bound γ-secretase was taken from the previous study(18) and the amide bond between Aβ49 and AICD50-99 was cleaved as the starting structure. The simulation systems of γ-secretase bound by the WT and the I45F, A42T, V46F, I45T and T48P mutants of APP are summarized in **Table 1**. The CHARMM36m(30) parameter set was used for the protein and POPC lipids. Initial energy minimization and thermalization of the γ-secretase complex followed the same protocol as used in the previous GaMD simulations of membrane proteins.(31, 32) The Pep-GaMD implemented in the GPU version AMBER 20(30) was then applied to perform the simulations using the dual-boost scheme, which included 10 ns short cMD simulation used to collect potential statistics for calculating the Pep-GaMD acceleration parameters, 50 ns equilibration after adding the boost potential and finally multiple independent 600 ns production runs with randomized initial atomic velocities. The simulation frames were saved every 0.2 ps for analysis. The VMD(33) and CPPTRAJ(22) tools were used to visualize and analyze the Pep-GaMD trajectories. The *PyReweighting* toolkit(34) was applied to reweight Pep-GaMD simulations for free energy calculations by combining all simulation trajectories for each system. Details of the γ-secretase assays, mass spectrometry, Western blotting, Pep-GaMD method, system setup, simulation protocol, energetic reweighting, and simulation analysis are provided in **Supporting Information**.

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Competing Interests Statement

There is no competing interest.

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Table 1: Summary of Pep-GaMD simulations performed on different systems of γ-secretase bound by Aβ49 and AICD50-99 peptides. [a]N_{atoms} is the total number of atoms in the simulation systems. [b]ΔV_{avg} and [c]$\sigma_{\Delta V}$ are the average and standard deviation of the Pep-GaMD boost potential, respectively.

System	[a]N_{atoms}	Dimension (Å^3)	Simulation (ns)	[b]ΔV_{avg} (kcal/mol)	[c]$\sigma_{\Delta v}$ (kcal/mol)
WT Aβ49	254,233	152 x 123 x 146	600 x 3	106.9	14.0
WT Aβ49 - neutral AICD	254,334	152 x 123 x 146	600 x 3	133.9	11.1
WT Aβ49 - charged AICD	254,377	152 x 123 x 146	600 x 3	134.0	10.9
I45F Aβ49 - charged AICD	254,335	152 x 123 x 146	600 x 3	137.5	12.1
A42T Aβ49 - charged AICD	254,341	152 x 123 x 146	600 x 3	148.9	11.4
V46F Aβ49 - charged AICD	254,329	152 x 123 x 146	600 x 3	177.9	11.9
I45T Aβ49 - charged AICD	254,323	152 x 123 x 146	600 x 3	149.2	11.4
T48P Aβ49 - charged AICD	254,328	152 x 123 x 146	600 x 3	137.9	11.2

Figure Captions

Figure 1: Tripeptide trimming of the wildtype (WT) and FAD mutants of Aβ49 γ-secretase characterized by MS, western blotting and Pep-GaMD simulations. (A) MALDI-TOF MS detection of AICD 50-99 and AICD 49-99 products, (B) Anti-FLAG immunoblot of total AICD-FLAG levels. Purified C100-FLAG at a range of known concentrations was used to generate a standard curve, (C) Quantification of total AICD-FLAG levels from immunoblot by densitometry, (D) Quantification of both AICD 50-99 using total AICD levels determined from immunoblot and intensity ratios determined from MALDI-TOF MS, (E) Quantification of the ITL tripeptides generated from the first trimming step of the WT and FAD mutants of Aβ49, (F) Cleavage efficiency of the first trimming step. Grey dotted line denotes cleavage efficiency from WT APP substrate. (G-L) 2D free energy profiles calculated from the Pep-GaMD simulations of (G) the WT, (H) I45F, (I) A42T, (J) V46F, (K) I45T and (L) T48P Aβ49 bound to γ-secretase. The distances between the Cγ atoms of Asp257 and Asp385 in PS1 and between the hydroxyl oxygen of PS1 Asp257 and the carbonyl oxygen of Aβ49 Leu49 were selected as the reaction coordinates.

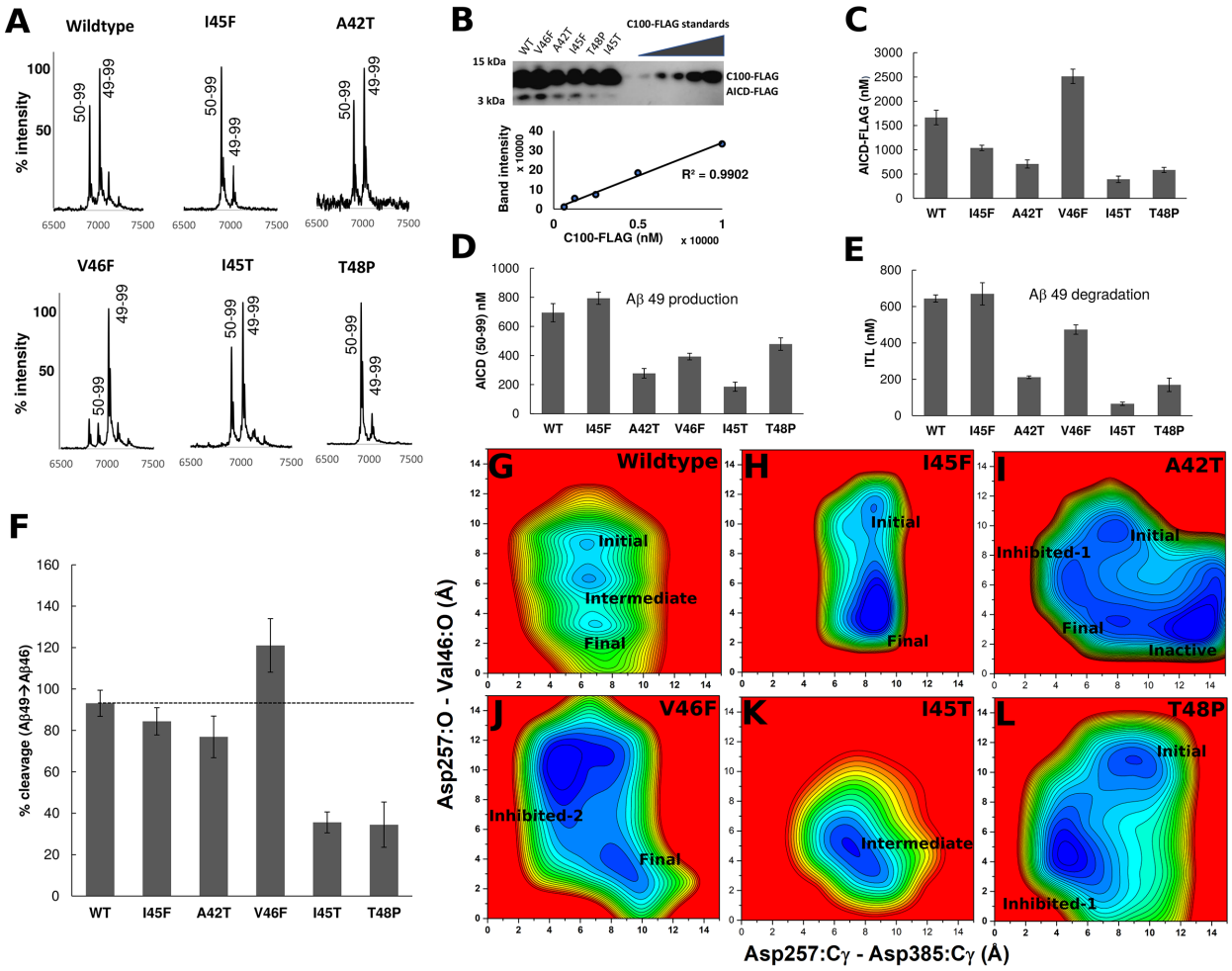
Figure 2: Conformational changes of the PS1 catalytic subunit and substrate during activation of γ-secretase for tripeptide trimming of Aβ49 in Pep-GaMD simulations. (A) Comparison of the Initial (active for ε cleavage, blue) and Final (active for ζ cleavage, red) conformations of the Aβ49-bound PS1. The enzyme activation for tripeptide trimming was characterized by coordinated hydrogen bonding between the enzyme Asp257, carbonyl oxygen of Aβ49 Val46 and a water molecule accommodated between the two aspartates poised for cleavage of the amide bond between Val46 and Ile47 residues. (B-F) Conformational changes of (B) Aβ49 substrate, (C) catalytic aspartates, (D) TM3, (E) TM6 and TM6a, and (F) β2 strand from the Initial to the Final conformational state. The helical domain of Aβ49 tilted by ~50º and residue Leu49 at

the C-terminus of Aβ49 moved downwards by ~5 Å. Protonated catalytic Asp257 moved ~3 Å towards the Aβ49 substrate. The enzyme TM3 moved outwards by ~2 Å and TM6a moved downwards by ~2 Å. The enzyme β2 strand (N-terminus of TM7) moved away from APP and closer towards the β1 strand (C-terminus of TM6a) by ~5 Å.

Figure 3: Secondary structures of Aβ49 bound to γ-secretase calculated from the Pep-GaMD simulations. (A) The WT, (B) I45F, (C) A42T and (D) V46F systems of Aβ49. Plots for other simulations are plotted in **Figures S5 - S7**.

Figure 4: Active-site conformations of γ-secretase for tripeptide trimming of Aβ49 observed in the Pep-GaMD simulations. (A-D) Conformations of the substrate P1', P2' and P3' residues in the Final active conformations of the (A) WT (red), (B) I45F (pink), (C) A42T (green) and (D) V46F (cyan) Aβ49 systems. (E) Comparison of the PS1 active-site S1', S2' and S3' pockets that accommodate the WT and mutants of Aβ49.

Figure 1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Wildtype
I45F
A42T
V46F
I45T
T48P
% intensity
50-99
49-99
B
C100-FLAG standards
15 kDa
3 kDa
C100-FLAG
AICD-FLAG
Band intensity x 10000
R² = 0.9902
C100-FLAG (nM) x 10000
C
AICD-FLAG (nM)
D
AICD (50-99) nM
Aβ 49 production
E
ITL (nM)
Aβ 49 degradation
F
% cleavage (Aβ49→Aβ46)
G
Initial
Intermediate
Final
H
I
Inhibited-1
Inactive
J
Inhibited-2
K
L
Asp257:O - Val46:O (Å)
Asp257:Cγ - Asp385:Cγ (Å)

Figure 2

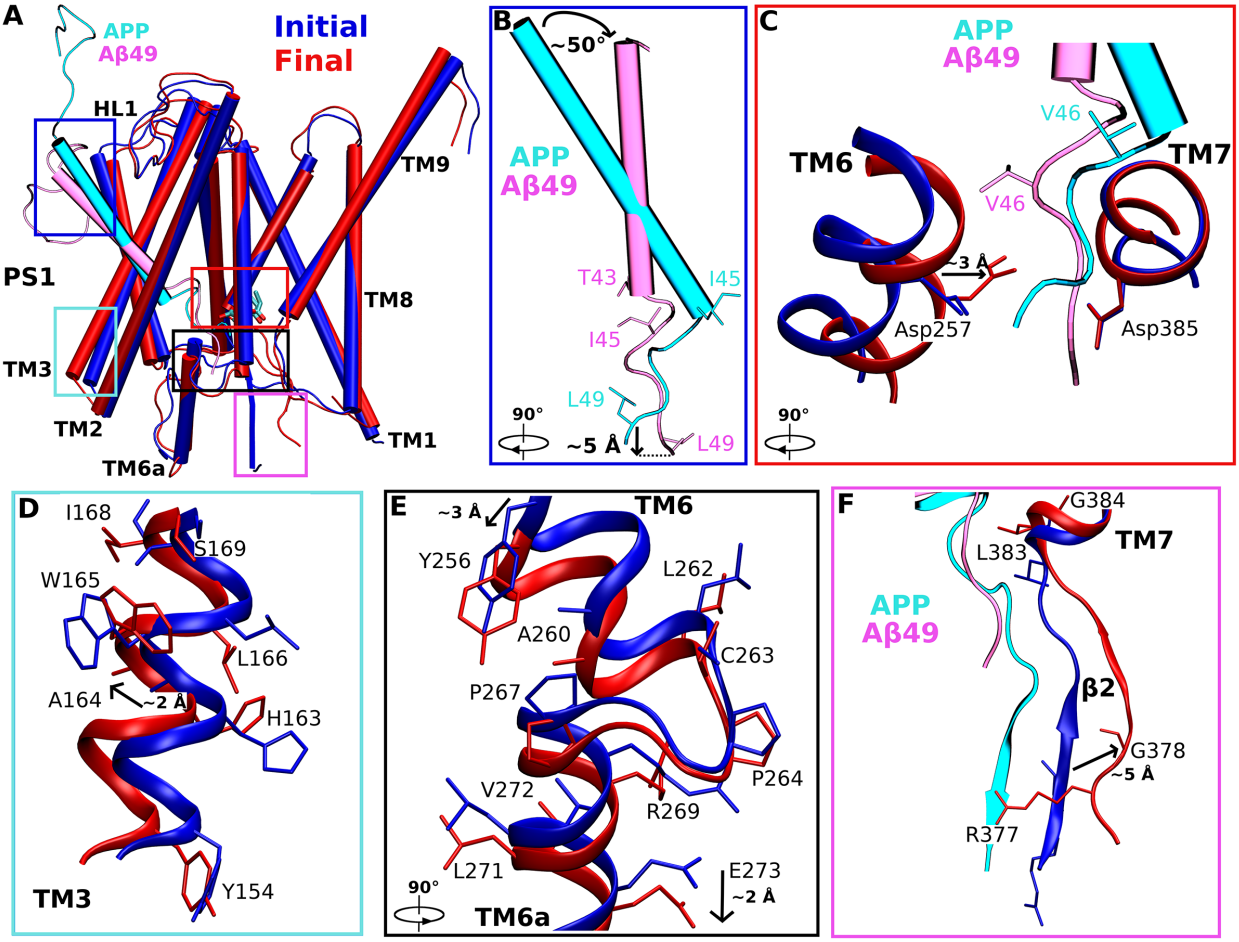
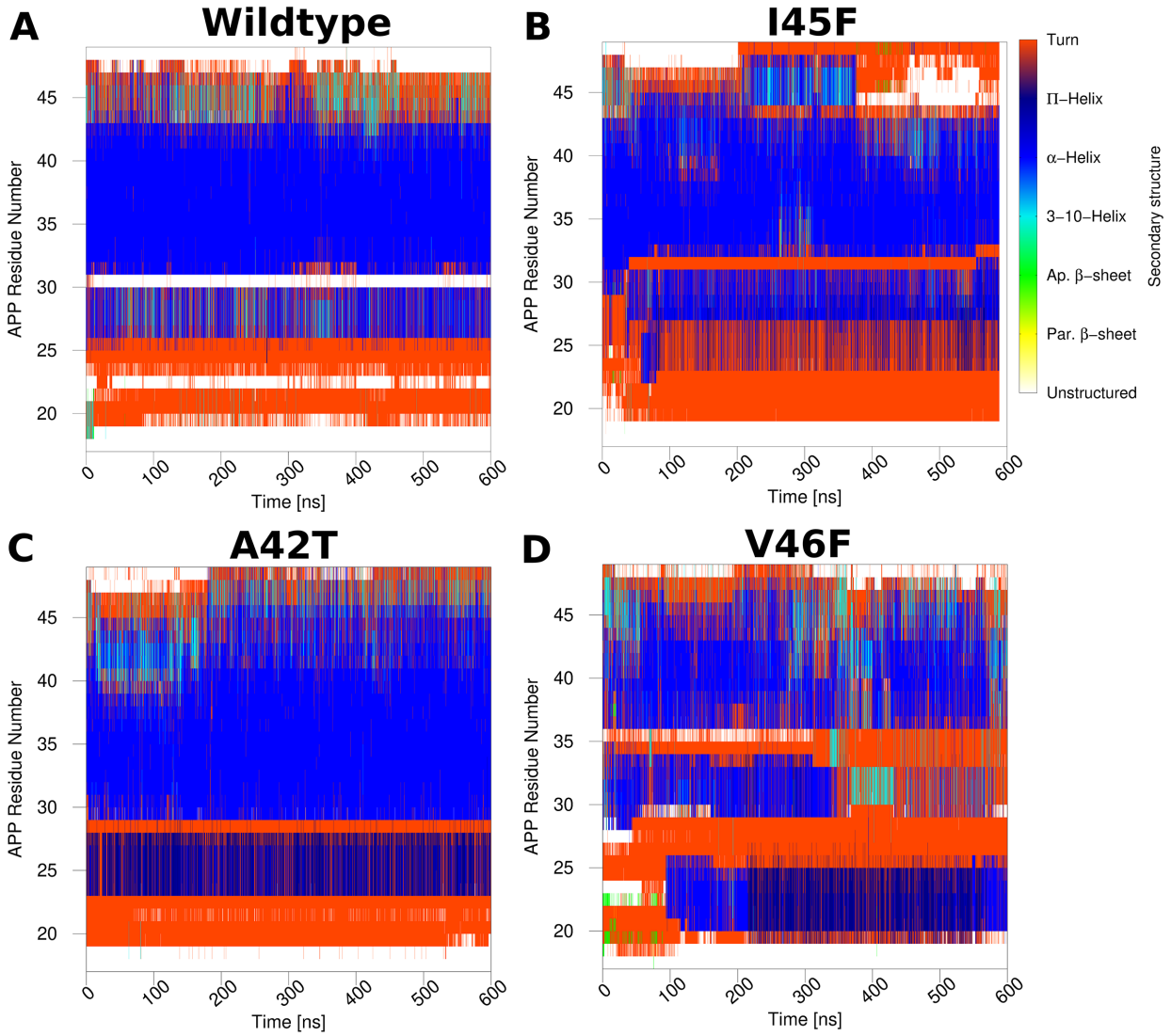

A
APP
Aβ49
Initial
Final
HL1
TM9
PS1
TM8
TM3
TM2
TM6a
TM1
B
~50°
APP
Aβ49
T43
I45
I45
L49
L49
90°
~5 Å
C
APP
Aβ49
V46
TM6
TM7
V46
~3 Å
Asp257
Asp385
90°
D
I168
S169
W165
L166
A164
~2 Å
H163
TM3
Y154
E
~3 Å
TM6
Y256
L262
A260
C263
P267
P264
V272
R269
L271
E273
~2 Å
90°
TM6a
F
G384
TM7
L383
APP
Aβ49
β2
G378
~5 Å
R377

Figure 3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Wildtype
B
I45F
C
A42T
D
V46F
APP Residue Number
Time [ns]
Turn
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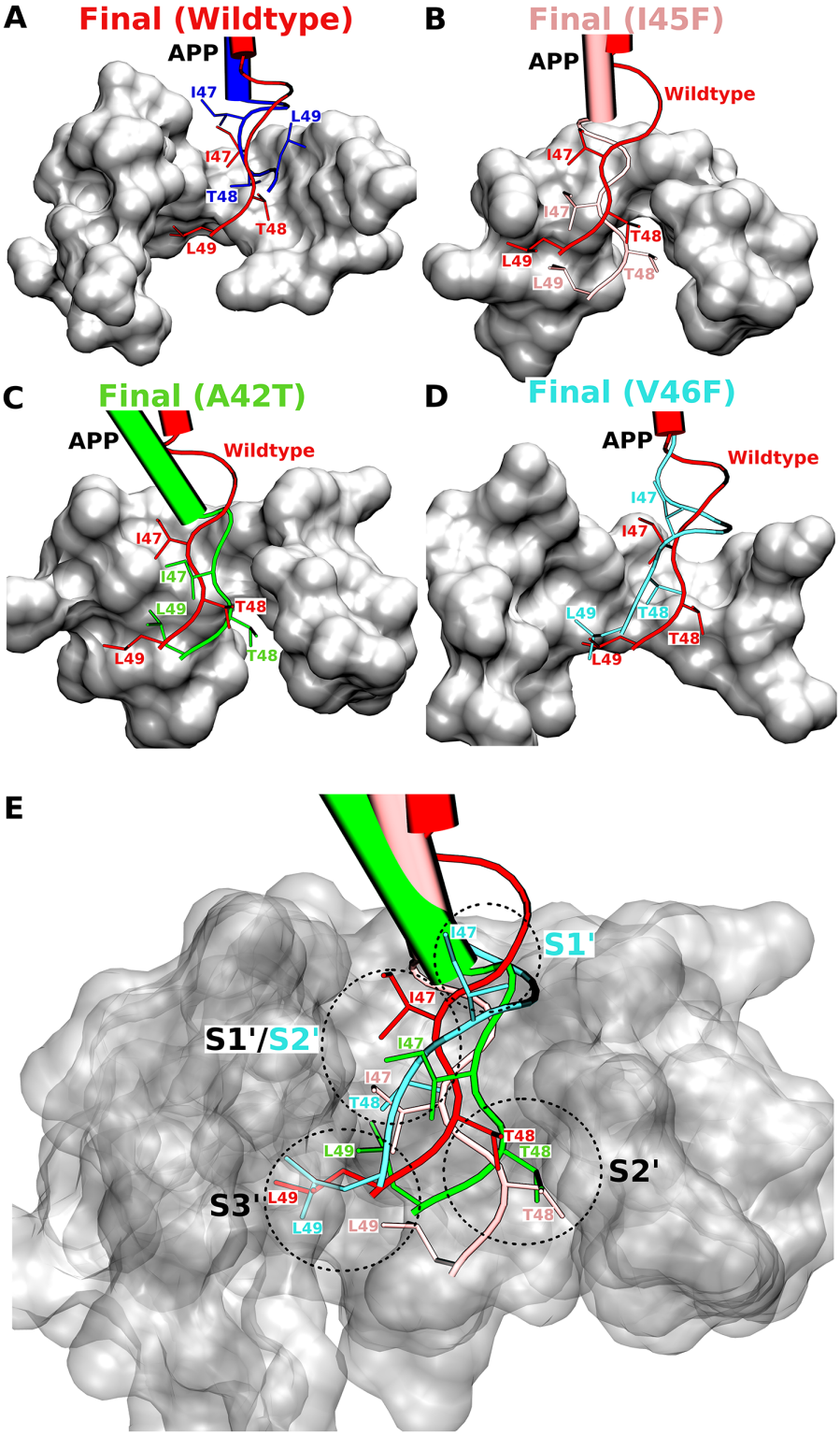
α–Helix
3–10–Helix
Ap. β–sheet
Par. β–sheet
Unstructured
Secondary structure

Figure 4


A
Final (Wildtype)
APP
I47
L49
I47
T48
T48
L49
B
Final (I45F)
APP
Wildtype
I47
I47
T48
L49
L49
T48
C
Final (A42T)
APP
Wildtype
I47
I47
L49
T48
L49
T48
D
Final (V46F)
APP
Wildtype
I47
I47
L49
T48
T48
L49
E
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S1'
I47
S1'/S2'
I47
I47
T48
T48
T48
L49
S2'
S3'
L49
L49
L49
T48