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Membrane Dynamics of γ -Secretase with the Anterior Pharynx-Defective 1B Subunit

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Abstract

The four-subunit protease complex γ -secretase cleaves many single-pass transmembrane substrates, including Notch and β -amyloid precursor protein to generate $A\beta$, central to Alzheimer's disease. Two of the subunits, anterior pharynx-defective 1 (APH1) and presenilin (PS), exist in two homologous forms, APH1-A and APH1-B, and PS1 and PS2. The consequences of these variations are poorly understood and could affect $A\beta$ production and γ -secretase medicine. Here, we developed the first complete structural model of the APH1-B subunit using the published cryo-EM structures of APH1-A (PDB: 5FN2, 5A63 and 6IYC). We then performed all-atom molecular dynamics simulations at 303 K in a realistic bilayer system to understand both APH1-B alone and in γ -secretase without and with substrate C83 bound. We show that APH1-B adopts a 7TM topology with a water channel topology similar to APH1-A. We demonstrate direct transport of water through this channel, mainly via Glu84, Arg87, His170, and His196. The apo and holo states closely resemble the experimental cryo-EM structures with APH1-A, however with subtle differences: The substrate-bound APH1-B- γ -secretase was quite stable, but some TM helices of PS1 and APH1-B rearranged in the membrane consistent with the disorder seen in the cryo-EM data. This produces different accessibility of water molecules for the catalytic aspartates of PS1, critical for $A\beta$ production. In particular, we find that the typical distance between the catalytic aspartates of PS1 and the C83 cleavage sites are shorter in APH1-B, i.e. it represents a more closed state, due to interactions with the C-terminal fragment of PS1. Our structural-dynamic model of APH1-B alone and in γ -secretase suggests generally similar topology but some notable differences in water accessibility which may be relevant to the protein's existence in two forms and their specific function and location.

Keywords: γ -secretase; Alzheimer's disease; APH1-B; molecular dynamics, membrane protein

Introduction

Alzheimer's disease (AD), the major neurodegenerative disease that affects tens of millions of people worldwide and causes gradual impairment of memory, cognition, and identify, is linked to deposits of aggregated amyloid- β peptides ($A\beta$) in senile plaques.[Blennow et al., 2015; Selkoe and Hardy, 2016] The $A\beta$ peptides are formed by cleavage of the β -amyloid precursor protein (APP) by β -secretase, giving first the C99 fragment, and then γ -secretase, giving $A\beta$ peptides of different lengths.[Esch et al., 1990; Golde et al., 1992; Julia and Goate, 2017; Takami et al., 2009; Vassar et al., 1999] Of the two most predominant isoforms, the shorter 40-residue $A\beta_{40}$ has natural functions[Kepp, 2017; Lee et al., 2004a; Smith et al., 2002] and is less toxic than $A\beta_{42}$, which has two more hydrophobic residues, often forms oligomers and aggregates.[Cheung et al., 2006; Hardy and Higgins, 1992] and could be a molecular culprit of AD.[Sun et al., 2016; Tang and Kepp, 2018]

γ -secretase is an integral membrane aspartyl protease complex comprised of four discrete protein subunits, nicastrin, presenilin (PS1/PS2), anterior pharynx-defective 1 (APH-1A/B), and presenilin enhancer 2 (PEN-2)[Kimberly et al., 2003; Sato et al., 2007]. The membrane protease is a major player in membrane protein turnover as it catalyzes intramembrane hydrolysis of more than 100 single-pass TM substrates with diverse cellular functions.[Haapasalo and Kovacs, 2011] Mutations in the catalytic subunit PS1/PS2 and in the substrate APP are the main causes of early-onset familial AD, suggesting that disease somehow correlates with changes in APP processing.[Luukkainen et al., 2019] Most of these mutations tend to lower enzyme activity and increase the ratio of formed $A\beta_{42}/A\beta_{40}$, a tendency that significantly correlates with the clinical onset of the disease in mutation carriers.[Sun et al., 2016; Tang and Kepp, 2018] This ratio could cause disease either by toxic gain of function of $A\beta_{42}$ [Cheung et al., 2006; Hardy and Higgins, 1992; Pauwels et al., 2012] or by loss of natural function of $A\beta_{40}$ [Kepp, 2016] or other substrates of γ -secretase.[Shen and Kelleher, 2007] For these reasons, the processing efficiency of γ -secretase is central to understanding AD and

therapeutic efforts to modulate it are ongoing.[Crump et al., 2013; Golde et al., 2013; Imbimbo and Giardina, 2011]

Among the subunits of γ -secretase, APh-1 is a 7TM protein that aids the trafficking and assembly of the enzyme complex.[Francis et al., 2002; Goutte et al., 2002; Sun et al., 2015] In humans, APh-1 is encoded by two homologous genes APh-1A and APh-1B, and APh-1A includes two alternatively spliced transcripts forming APh-1A(L-large) and APh-1A(S-small).[Gu et al., 2003; Lee et al., 2002] APh-1A and APh-1B associate with PS1/2, NCT and PEN-2 to produce active γ -secretase complexes with diverse biochemical and physiological properties and locations.[Araki et al., 2006; Serneels et al., 2009; Shirotani et al., 2004] Despite this heterogeneity, all structural studies and most biochemical studies have so far concentrated on APh-1A as the preferred model of γ -secretase and A β production. Understanding how APh-1B affects the structure, dynamics and function of the complex is thus of substantial interest.

APh-1A has been suggested to form a water channel of the membrane-bound enzyme complex, but whether APh-1B does the same is unknown.[Aguayo-Ortiz and Dominguez, 2019] APh-1B γ -secretase seems to play a special role in processing of the membrane-bound signaling molecule neuregulin-1(NRG-1).[Dejaegere et al., 2008; Fazzari et al., 2014] Inactivation of APh-1B- γ -secretase in a mouse AD model may improve some AD-related features without any Notch-related side effects,[Serneels et al., 2009] whereas low expression of APh-1B may cause neurodevelopmental phenotypes in rats.[Coolen et al., 2006] Importantly, APh-1B has been reported to increase the A β_{42} /A β_{40} ratio without modifying ϵ -cleavage position.[Lessard et al., 2015] These various studies suggest that APh-1B does not work identically to APh-1A in the protein complex.

With the advancements in cryogenic-electron microscopy (Cryo-EM) structures, γ -secretase has been characterized at near atomic resolution in both the apo state (PDB: 5A63, 5FN2, 5FN3, 5FN4 and 5FN5) and as substrate-bound holo states (6IYC and 6IDF).[Bai et al.,

2015b, 2015a; Sun et al., 2015; Yang et al., 2019; Zhou et al., 2019] These structures have opened new avenues for a mechanistic understanding of AD. All of these structures involve the APH-1A subunit, APH-1B not yet structurally characterized. We also note that the cryo-EM structures represent compositions on a film, which structurally resemble a mixture of the states in lipid and water, whereas the real protein complex acts dynamically at physiological temperature in a complete membrane. Both the membrane and the temperature work together to expand the protein's conformation states, as recently shown by direct comparison of the experimental and simulated ensembles at high and low temperature, and with and without membrane.[Mehra et al., 2020] Molecular dynamics (MD) simulations are thus essential to understand the physiological temperature dynamics of this important protein complex in realistic membrane models on the background of the experimental structural constraints, as now intensely pursued in several dedicated computational chemistry labs.[Aguayo-Ortiz et al., 2018; Aguayo-Ortiz and Dominguez, 2018; Dehury et al., 2019a, 2019b, 2019c; Dominguez et al., 2016; Hitzenberger and Zacharias, 2019a; Kong et al., 2015; Mehra et al., 2020; Petit et al., 2019; Somavarapu and Kepp, 2017]

We present here the first structural models, obtained from homology modeling, of both APH-1B alone and APH-1B- γ -secretase in both the apo- and substrate-bound states. These structural models were then embedded in complete water-bilayer systems and studied in triplicate by all-atom MD simulations. To the best of our knowledge, this is the first study of the structure and dynamics of APH-1B both alone and inside γ -secretase. Our study notably identifies the conformational dynamics of γ -secretase with APH-1B inside a realistic membrane model at relevant temperature, identifies a water conductance channel in the structures, and quantifies several different conformational states of APH-1B- γ -secretase as compared to the APH-1A isoform.

Computational methods

Molecular Modeling of APH-1B alone

The three-dimensional structure of APH-1B subunit is not known. However, APH-1A and APH-1B (257 amino acids, UniProtKB ID: Q8WW43) share ~57% sequence identity. To model APH-1B, we used BLASTp[Altschul et al., 1997] to search for experimental protein structures in the protein data bank (PDB) as optimal templates for homology modeling. As expected, BLAST suggested the cryo-EM structures of γ -secretase APH-1A subunits (5FN2, 5A63 and 6IYC) as the most reliable templates with high query coverage. The templates feature the same APH-1A subunit and very similar topology (C α -RMSD of 0.32 Å (5FN2/5A63), 0.59 Å (5A63/6IYC), and 0.67 Å (5FN2/6IYC)). We used the evaluation criteria of Modeller version 9.23[Webb and Sali, 2017] to characterize the structures by their least discrete optimized protein energy (DOPE). The structures were then optimized using the loop model protocol in Modeller and the Galaxy refine tool.[Heo et al., 2013] The model with least C α -RMSD vs. the templates was selected and validated by SAVES version 5.0 (<https://servicesn.mbi.ucla.edu/SAVES/>) and Molprobity.[Chen et al., 2010] Based on the model validation statistics (**Table S1**), APH-1B structures used to build different forms of γ -secretase were chosen as described in further detail below.

Constructing a model of γ -secretase with APH-1B subunit

We developed four systems for all-atom molecular dynamics simulation i.e., one of APH-1B alone in a membrane and three γ -secretase states with APH-1B in a membrane (apo-state with both catalytic aspartates deprotonated, apo-state with protonated Asp-257, and C83-bound).

For the apo state, we used the cryo-EM structure of γ -secretase resolved at 4.2 Å resolution (PDB ID: 5FN2) as the initial template.[Bai et al., 2015a] The missing side-chains of the

structure were built using the WHAT-IF server.[Hekkelman et al., 2010] The long intracellular loop 2 (264 to 377 amino acids of PS1) bridging TM6 and TM7 of the PS1 component was not included due to the lack of available structural information; since we are mainly interested in APH-1A/1B differences, the absence of these structural data in PS1 should be less critical to our work. Then the complete γ -secretase model was developed upon structural superposition and coordinate transfer of each subunit using PyMOL version 2.0, (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC.), replacing APH-1A with our modeled APH-1B subunit. The APH-1B- γ -secretase complex was then embedded in a POPC bilayer using CHARMM36m.[Huang et al., 2016].

We simulated two distinct γ -secretase models (apo forms), one with both catalytic residues (Asp257 and Asp385 of PS1) deprotonated, and the other with protonated Asp257. Previous pK_a calculations of both experimental and simulated structures have shown that these are the only likely protonation states, with deprotonation being most prevalent at neutral pH.[Mehra et al., 2020] BIOVIA Discovery Studio Visualizer (Dassault Systèmes BIOVIA, BIOVIA DSV, 4.5, San Diego: Dassault Systèmes, 2019) was used to remove steric clashes before merging the model with the membrane-solvent system. The arrangement of γ -secretase with respect to the lipid bilayer was obtained from the Orientation of Proteins in Membranes (OPM) server.[Lomize et al., 2012] For the substrate-bound model, the initial 3D coordinates were adopted from 6IYC.[Zhou et al., 2019] After removal of hetero-atoms, the mutations Q112C and D385A in the catalytic subunit PS1 were converted to the wild type using PyMOL, as these are necessary artifacts of the experimental protocol required to keep the substrate inside the protein.

MD simulations

The atomic details and compositions of all the studied systems are summarized in **Table S2**. We used the CHARMM-GUI membrane builder[Jo et al., 2008] to insert the proteins into a

hydrated, equilibrated bilayer composed of 2-oleoyl-1-palmitoyl-sn-glycero-3-phosphocholine (POPC) molecules in the upper and lower leaflet. Sodium and chloride ions were added to neutralize the system, reaching a final concentration of approximately 150 mM. Each of the above-described systems were simulated by three independent MD simulations of 500 ns using GROMACS version 2018.5[Abraham et al., 2015] applying the CHARMM36m force field and the TIP3P water model. The input systems were first subjected to energy minimization, equilibration, and then production simulation using the GROMACS input scripts generated by the CHARMM-GUI. Each system was energy minimized using 5,000 steps of steepest descent, followed by 1 ns of NVT (constant particle number, volume, and temperature) and NPT (constant particle number, pressure, and temperature) equilibrations. The van der Waals interactions were evaluated using a switching cutoff at 10–12 Å, whereas the long-range electrostatic interactions were calculated using the particle-mesh Ewald procedure. The temperature and pressure were held at 303.15 K and 1 bar, respectively.

During the production simulations, an NPT ensemble was used with semi-isotropic pressure coupling via the Parrinello–Rahman barostat method, whereas the Nose–Hoover thermostat was used to maintain a temperature of 303.15 K. A leapfrog integration scheme was used, and all covalent bonds were constrained. We used a time-step of 2 ps during both NPT equilibration and production MD simulations.

Analysis of MD Trajectories

All simulations were analyzed using the inbuilt GROMACS tools and FATSLiM tools.[Buchoux, 2017] The 2-dimensional plots were generated with XMGRACE (<http://plasma-gate.weizmann.ac.il/Grace/>), whereas structures were rendered using PyMOL. Deuterium order parameters, the density of the membrane components, and the area per lipid head group were computed using FATSLiM. Principal component analysis (PCA) was employed to identify the large-scale collective motions of main-chain atoms of each system

209 using the last 300 ns trajectories of each simulation, since equilibration typically takes 1-200 ns
210 for these large membrane-embedded protein complexes, i.e. the first 1-200 ns may contain
211 artefacts of the non-equilibrated starting models.[Dehury et al., 2019b; Somavarapu and Kepp,
212 2017] Clustering analysis was performed with the *gmx cluster* utility toolkit and a cut-off of 0.2
213 nm to select the most representative structures of each simulated ensemble, using the last 300
214 ns of each trajectory.

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Results and discussion

Sequence and structure differences between APH-1A and APH-1B

Pair-wise sequence alignment using MultAlin (**Figure 1A**) shows that APH-1A and APH-1B share 75% sequence similarity and 57% identity. The N-terminal is more conserved than the C-terminal, and there are variations in the loops connecting the TM segments. As described in methods, we used the APH-1A structures solved by cryo-EM (5FN2, 5A63 and 6IYC) as templates to construct a complete model of the human APH-1B subunit. Based on Procheck[Laskowski et al., 1993], 97.8% (221) of the residues of our model were in the most favored regions and 2.2% (5 residues) were in additional favored regions, with no residues in disallowed regions of the Ramachandran plot (**Figure 1B**), indicating the high quality of our proposed model. These results were confirmed by Molprobit[Chen et al., 2010], giving 99.2% residues in favored regions with no residues having poorly described rotamers and bonds. Similarly, 81.4% of the residues had 3D-1D scores > 0.2 as calculated by Verify 3D,[Lüthy et al., 1992] and the overall quality score of 94.6 from ERRAT[Colovos and Yeates, 1993] shows that the modeled structure is very realistic by any of the normal structural assessment standards.

The 7TM helical topological architecture of our modelled APH-1B with conserved (red) and variable (white) regions is shown in **Figure 1C**. The Gly122, Gly126, and Gly130 residues (**Figure 1A and 1D**) in TM4 constitute a part of the membrane GxxxG motif, which is essential for stable association of APH-1A(L) with the other subunits by helix-helix interactions and highly conserved.[Araki et al., 2006; Lee et al., 2004b] Structural superimposition of our APH-1B model with APH-1A from 5FN2, 5A63, and 6IYC (**Figure 1D**) gave C α -RMSD values of 0.47, 0.37, and 0.46 Å, respectively, i.e. our proposed model fully respects the structural constraints from the experimental structures. There are some minor differences in the loops bridging the TMs (**Figure 1D**) as expected from the different sequences (**Figure 1A**). Whether

these differences become more pronounced upon full equilibration of the model by MD is analyzed below.

All-atom dynamics of APH-1B in a realistic membrane structure

Since APH-1B as well as γ -secretase are membrane proteins, understanding their structures and dynamics in the context of a membrane is essential, with membrane proteins typically adopting distinct conformational states in membranes that may contribute to protein activity.[Corradi et al., 2019; Zhuang et al., 2014, 2016] Our APH-1B systems were comprised of 240 POPC lipids, 18,611 water molecules, and 49 Na⁺ and 56 Cl⁻ ions. In order to assess whether our protein-lipid-water system is realistic, we computed various membrane properties, including the deuterium order parameters, the local density of the membrane components, and the distribution of area per lipid for each system (**Figure S1** and **S2**). The computed membrane order parameters compare excellently with the experimental data for equivalent protein-membrane systems.[Zhuang et al., 2016] In particular, the deuterium order parameters with the splitting near the lipid head-group closely resemble experimental values. Artificial changes in the simulated membrane structure would be revealed from the density profiles, but our values are close to experiment, indicating that the overall membrane structure integrity is maintained (**Figure S1**). The average lipid per unit area of ~ 0.62 nm resembles that expected for CHARMM36m simulations.[Lyubartsev and Rabinovich, 2016]

The dynamical stability of our systems were monitored by computing the backbone RMSD relative to the starting structure across the full 500 ns of each triplicate simulation, as shown in **Figure 2A**. The systems reached stable conformational ensembles after ~ 150 ns and maintained a horizontal trend with an average RMSD of ~ 2.8 – 3.2 Å. The variation in RMSD implies somewhat different conformational states of APH-1B being sampled, as explored further below. The root-mean square fluctuations (RMSF) of each C α -atom of APH-1B averaged over the trajectories are displayed in **Figure 2B**. The TMs (3-26: TM1, 31-57: TM2,

65-104: TM3, 113-138: TM4, 156-183: TM5, 186-204: TM6 and 213-229: TM7) displayed reduced flexibility, enforced further by their embedding in the membrane, whereas the loops bridging TM2 -TM3 and TM4-TM5 were highly flexible. We note the general very good agreement between the three independent simulations indicating that the obtained dynamics of APH-1B are reproducible and significant.

To ensure that the simulated structures remain realistic, we compared the most representative ensemble structures from cluster analysis to the experimental APH-1A structure (5FN2) (**Figure 2C**). The C α -RMSD values of 2.54, 2.17 and 1.97 Å are very reasonable since our model represents free APH-1B alone in a membrane, whereas the experimental structure represents APH-1A in the γ -secretase in a chemical composition somewhere between water and a lipid system, on a sample film. The APH-1B loops are dynamic, as expected.

Active water channel in APH-1B

The electrostatic surface potential for the experimental APH-1A (5FN2) and for our three ensemble-representative APH-1B structures (**Figure S3**) indicates different charge distributions near TM5 and adjacent loops. A large cavity is formed adjacent to TM2 and TM3, which may be due to the flexibility of the loops in C-terminal TM segments. The obtained cavities with variable interior space resemble different states of a membrane solvent channel, with indications of both an open and more closed structure (**Figure S3**).

To understand the obtained APH-1B ensemble better, we performed PCA on the last 300 ns of each trajectory, with the eigenvalues of first two eigenvectors (EVs) and the projection of the movements of main-chain atoms summarized in **Figure S4A-S4B**. Simulation 1 (blue) represents a more open conformational ensemble, whereas simulation 2 (red) is in-between and simulation 3 is the least variable (most compact) state. To understand these variations, we generated porcupine plots for top EVs containing the major movements (**Figure S4C**). Consistent with the RMSF analysis, most TMs were rigid, and the loop bridging TM4-TM5

was flexible. We also identify a significant inward motion of the C-terminal of TM2 and N-terminal of TM3, (these two TMs are somewhat separated from the remaining TMs), which correlates with the opening and closing of the cavity in the membrane.

We note that the cryo-EM structure of the APH-1A subunit within γ -secretase (PDB: 5FN3 solved at 4.1 Å) already indicates a central cavity similar to small molecule/ligand recognition sites in G-protein coupled receptors (GPCR).[Sun et al., 2015] In addition, a recent study has shown that the central region of APH-1A (mostly occupied by TM3) possesses lower hydrophathy due to the polar residues in the cavity.[Aguayo-Ortiz et al., 2018] Our obtained APH-1B cavities are thus not unusual, yet manifest very clearly. Crystallographic GPCR structures and MD simulations indicate that the polar central cavity enables entry of water molecules into the membrane, which is usually excluded.[Lee et al., 2016] These water molecules play important regulatory and activation roles in GPCRs.[Angel et al., 2009] To understand if this is the case also for APH-1B, we inspected our ensemble-representative structures and identified multiple water-containing sites within the membrane/TM region. As for GPCRs, we find that the water-containing cavity of APH-1B crosses the middle part of the membrane and almost reaches the intracellular space. It is well-known that water influx into GPCR central cavities allows the occasional transport of water molecules from one cellular compartment to another[Lee et al., 2016; Tomobe et al., 2017] and we thus consider this analogy for APH-1B to be of interest.

More specifically, we found that a variable number (average ~ 37) of water molecules crossed APH-1B and formed a continuous water channel within the membrane-protein system (**Figure S5 and S6**). We observed multiple events of complete transfer of water molecules at the studied time scale (200–500 ns) both from the extracellular to the intracellular space and vice-versa. Close inspection of the MD snapshots revealed that TM3, TM4, TM5, and TM6 interact with the water molecules during translocation (**Figures S5-S6**). The water molecules interacted particularly with the polar residues Glu84, Arg87, His170, and His196. Interestingly,

both APH-1A and APH-1B harbors strongly conserved histidines His170 and His196, which is consistent with a possible conserved role in water transportation across the bilayer.

A water channel is likely to be regulated by conformational changes of the membrane-protein system. To understand such effects, we monitored the dynamic changes in the helix tilt angles of TM2, TM4, TM5 and TM6, which were found to vary considerably (**Figure S7**). Since lipid headgroups play a vital role in membrane protein function,[Bogdanov et al., 2008] we analyzed the APH-1B residues that consistently formed hydrogen bonds with the POPC headgroups (**Figure S8**). We observed that positively charged arginine and lysine as well as cysteine at the cytoplasmic edge of the TM helices hydrogen bonded with the headgroups.

Dynamics of γ -Secretase complex with APH-1B

Whereas the results above concerned the free APH-1B in a membrane, in the following we discuss our analogous results for the complete APH-1B- γ -secretase system. The structure, stability, and functions of a membrane protein depend on its orientation in the membrane, the spatial distribution of amino acid residues within the membrane protein, and the lipid composition.[Bondar and White, 2012; Lomize et al., 2006, 2012; Ng et al., 2014] This also applies to γ -secretase, whose lipid environment is known to affect the biological activity.[Aguayo-Ortiz et al., 2018] γ -secretase tends to remain active in a membrane composed solely of phosphatidylcholine (PC) lipids, but the substrate cleavage activity is modulated by the membrane structure and composition.[Osenkowski et al., 2008] Thicker membranes favor the production of A β ₄₀, while thinner membranes favor A β ₄₂ production,[Osenkowski et al., 2008; Winkler et al., 2012] in good agreement with the Fit-Induce-Stay-Trim (FIST) model, where more compact, hydrophobic, tight “grapping” of the substrate in the semi-open state, controlled by TM2, TM3, TM6, and TM9 of PS1, leads to stabilized enzyme-substrate interactions, longer retention and extended cleavage of C99 to shorter A β .[Somavarapu and Kepp, 2016, 2017] Accordingly, more carbons in the lipid chains

increase the activity of γ -secretase and reduce the $A\beta_{42}/A\beta_{40}$ ratio,[Holmes et al., 2012] which correlates with the clinical onset of familial AD.[Sun et al., 2016; Tang and Kepp, 2018]

For these reasons, the ambient temperature dynamics in a realistic membrane model, as can be obtained from MD simulations, are thus an important supplement to the cryo-EM structural data needed to understand the structure-dynamics-function relations, in particular in relation to substrate binding and cleavage of C83 to produce $A\beta$.[Mehra et al., 2020]

To ensure that the complex membrane-water-protein ensembles are realistically described, we computed the order parameters (S_{cd}), membrane properties, area per lipid distribution of the sampled lipid bilayers as a necessary first quality control (**Figure S9** and **S10**). All properties were in agreement with experimental membrane properties and MD simulations of equivalent membrane-protein systems.[Zhuang et al., 2014, 2016] Thus, we have produced a realistic high quality model of APH-1B not only in close structural agreement with the cryo-EM data for APH-1B, but also with a realistic membrane system. This model is the first of APH-1B- γ -secretase to our knowledge.

Figure 3 displays the backbone RMSD values for the simulated γ -secretase systems. The backbone RMSD of APH-1B-apo- γ -secretase with deprotonated Asp257 and Asp385 increased until 200 ns but then stabilized and averaged ~ 3.5 Å for the three independent simulations (**Figure 3A**). The RMSD of apo- γ -secretase with protonated Asp257 averaged to ~ 3.8 Å for the three simulations (**Figure 3B**; simulation 2 had a distinct compact state). In contrast, the C83-bound APH-1B- γ -secretase was consistently less variable (**Figure 3C**). After ~ 150 ns, both the apo and holo-states displayed stable evolution, and the RMSD remained close to the experimental structures 5FN2 (RMSD < 4 Å) and 6IYC (RMSD < 3 Å), in agreement with previous simulations of APH-1A-apo- γ -secretase[Somavarapu and Kepp, 2017] and C99-bound APH-1A- γ -secretase[Dehury et al., 2019b]. The RMSD of each subunit (**Figure S11**) shows that nicastrin displayed higher RMSD typical of the large inward-outward movement relative to the membrane as reported in many other studies.[Aguayo-Ortiz et al., 2017;

Aguayo-Ortiz and Dominguez, 2018; Bai et al., 2015a; Somavarapu and Kepp, 2017] PEN2 varied in its conformation space for the apo- and holo-states, consistent with an rearrangement of PS1 upon substrate binding already evident from the cryo-EM data.[Zhou et al., 2019] Other subunits displayed stable ensembles after 200 ns, and APH-1B was clearly less structurally variable in the C83-bound state, i.e. substrate binding dampens APH-1B dynamics. The radius of gyration (R_g) and solvent accessible surface area (SASA), which monitor the shape and compactness of the ensembles (**Figure S12**), were largely constant with minor changes during the initial 200 ns. The obtained R_g values were 39.6–40.2 Å for the apo- γ -secretase systems and 39.9–40.6 Å for the substrate-bound state.

To compare our structures quantitatively with the experimental cryo-EM structures for APH-1A- γ -secretase, we extracted the top-ranked structure from cluster analysis of the last 300 ns of each simulated trajectory (**Figure S13**) and structurally aligned the C α -atoms of these structures with those of the cryo-EM structures for apo- γ -secretase (5FN2[Bai et al., 2015a] with APH-1A) system, the RMSD values were 2.54, 2.17 and 1.97 Å for the triplicate simulations (**Figure S13A**). Apo- γ -secretase with protonated Asp257 gave 2.43, 3.68, and 2.46 Å, respectively (**Figure S13B**); the large value of 3.68 Å arose from an open PS1/PS2 state as seen previously[Dehury et al., 2019b, 2019a; Somavarapu and Kepp, 2017]. C83-bound APH-1B- γ -secretase compared to 6IYC[Zhou et al., 2019] gave RMSD values of 1.81, 2.32, and 1.37 Å (**Figure S13C**). Considering the resolution of the cryo-EM structures (5FN2: 4.2 Å and 6IYC: 2.6 Å), our MD simulated models thus obey the experimental topology and provide an accurate basis for exploring the all-atom membrane-protein dynamics at physiological temperature.

We compared the representative ensemble structures from clustering analysis with the experimental apo (5FN2) and holo (6IYC) states (**Figure 4**). We observed complete loss of β -strand of C83 in all systems, consistent with findings that this strand is lost in membranes at normal temperature but prevails at low temperature.[Mehra et al., 2020] In one simulation, we

observed retention of the two β -strands in PS1 (one extended from TM6 and the other with TM7). In other systems, both these strands were converted to turns, indicating that they are mostly flexible and undergo coil-turn-strand transitions at ambient temperature, as shown previously.[Mehra et al., 2020] Close inspection of the APH-1B subunit as compared to APH-1A revealed that the TM regions are essentially similar except for the small C-terminal JM helix. Furthermore, we observed minor differences in the arrangement of TM helices in APH-1B probably due to the sequence-distinct loops connecting these TMs (**Figure 1A**).

Residue-specific dynamics of γ -secretase with APH-1B

To understand the residue-wise dynamics, we plotted the C α root-mean-squared forces (RMSF), which resemble qualitatively (but not quantitatively[Caldararu et al., 2019]) crystallographic B-factors[Sun et al., 2019], for each subunit of the complexes (**Figure 5**). As anticipated, the RMSF of nicastrin-ECD displayed numerous high peaks as typical of loops, while the TM helix (Ser665–Phe698) was distinctly rigid. The extracellular residues of nicastrin Phe240–Glu245 probably contribute to substrate recognition[Petit et al., 2019] and to the dynamic stability of γ -secretase-A β assemblies.[Szaruga et al., 2017] In the C83-bound holo enzyme, we observed very little fluctuations in this region as compared to both the apo systems. The extra-cellular hydrophilic loop 1 (HL1) bridging TM1 and TM2 in PS1, potentially involved in substrate gating,[Takagi-Niidome et al., 2015] displayed notably differential fluctuations in the apo and holo states. HL1 also constitutes a binding pocket for γ -secretase modulators[Cai et al., 2017] which may thereby affect differently the apo and substrate-bound state.[Zhou et al., 2019]

We observed large movements of the N-terminal of TM2 and TM9, in good agreement with our previous findings.[Dehury et al., 2019b; Somavarapu and Kepp, 2017] The APH-1B and PEN2 subunits displayed more or less the same trends in RMSF for the apo and holo states. The dynamics properties of the substrate C83 have been illustrated in **Figure S14**. The

hydrophilic loop 2 (HL2) bridging TM6 and TM7 is missing in the experimental apo structures and in the present models, whereas the C83-bound conformation harbors two β -strands in PS1, β 1 comprised of Ile287–Tyr288–Ser289 extended from TM6a, and β 2 involving five residues of TM7 (Gly378–Gly382). The C-terminal fragment of C83 harbors the TM helix and a β -strand Val721–Lys725, which displayed very distinct fluctuation in one simulation (**Figure S14B**). This fluctuation correlated with a loose conformation of C83 inside γ -secretase, i.e. the pathogenic “open” state of PS1 that produces less active, imprecise cleavage and longer A β peptides according to the FIST model.[Dehury et al., 2019b; Somavarapu and Kepp, 2017; Tang et al., 2018]

Characterizing the ensembles by principal component analysis

To capture global motions in various γ -secretase complex systems we performed PCA on the last 300 ns of each trajectory (**Figure S15-S17**). The most pronounced motions depicted by the top eigenvectors (**Figure S16-S17**) quantify the large breathing mode of the nicastrin ECD in both the apo- and holo-states. The second principal component (**Figure S17**) indicated a different but correlated opening and closing motion that changes the size of the cavity formed by the nicastrin ECD and the TMDs, in good agreement with previous studies.[Aguayo-Ortiz et al., 2017; Elad et al., 2015; Lee et al., 2017] This motion is probably responsible for the guiding of substrate molecules towards the intramembrane cleavage site of γ -secretase.[Fukumori and Steiner, 2016]

Additional dynamics were observed in the TM helices of PS1, with TM2 and the directly connected N-terminal region of TM3 being highly mobile in both apo and substrate bound states (**Figure S16 and S17**). These dynamic motions largely explain the static low resolution (disorder) in the experimental cryo-EM structures 5FN2[Bai et al., 2015a] and 6IYC[Zhou et al., 2019], and has been repeatedly found by MD simulations.[Dehury et al., 2019a, 2019b,

2019c; Mehra et al., 2020; Somavarapu and Kepp, 2017] We also note that the loops in C-terminal region of APH-1B displayed some movements in all the studied γ -secretase states.

To better understand the two major motions of the two nicastrin ECD lobes relative to PS1 in apo and holo γ -secretase, we measured the distances and angles formed between amino acids Leu121 (small lobe), Val328 (large lobe) of ECD and the active site residue Asp257 of PS1 (**Figure S18** and **Table S3**). The Val328–Asp257 distances differed significantly in the apo and holo states, and were on average larger in the substrate-bound state, consistent with variations also implied by the cryo-EM structures of γ -secretase.[Bai et al., 2015a; Zhou et al., 2019] Equally, the distance between Leu121 and Asp257 of PS1 was on average longer in the C83-bound state, in particular for one conformation (blue trajectory in **Figure S18**).

Functionally important dynamics of APH-1B- γ -secretase

To understand the functionally relevant motions of γ -secretase with APH-1B, we measured the C α -C α distance between terminal end residues of the substrate gating loop HL1, the distance between the catalytic Asp257 and Asp385 residues of PS1, and the PAL motif terminal residue distance (**Table S4**). The apo states displayed HL1 distances above 2 nm and in some cases close to 3 nm, whereas the holo states displayed more compact HL1 conformations with distances below 2 nm (**Figure S19A**). The motions are arguably the basis for the static ordering of these parts of the protein upon binding to substrates seen in the cryo-EM data.[Yang et al., 2019; Zhou et al., 2019]

PS1 can adopt distinct states with the catalytic Asp257 and Asp385 either relatively close or somewhat farther apart, i.e., closed, semi-open and open states of γ -secretase.[Aguayo-Ortiz et al., 2017; Dehury et al., 2019b; Hitznerberger and Zacharias, 2019b] These states can explain C83 processing and the production of different types of A β , according to the FIST (fit-induce-stay-trim) model,[Somavarapu and Kepp, 2017; Tang et al., 2018] where the semi-open state is innocent and most active, the closed state does not fit the substrate and is inactive, and the open

state gives imprecise cleavage, diverse products, and less activity due to lower substrate affinity and earlier release, i.e. looser “grapping” by the “fist”. This open state is argued to be favored by pathogenic PS1 mutations by destabilizing the membrane protein’s hydrophobic packing,[Mehra and Kepp, 2019; Somavarapu and Kepp, 2016, 2017] explaining their lower activity and higher A β ₄₂/A β ₄₀ ratios in assays.[Sun et al., 2016] In contrast, γ -secretase modulators may contribute their binding affinity to stabilize the enzyme-substrate-modulator complex, increase substrate residence time, trimming, and production of shorter A β . [Tang et al., 2018]

We found that protonation of Asp257 in APH-1B-apo- γ -secretase produced a short Asp-Asp distance (~ 9.2 Å; vs. 8.0 Å in 5FN2) whereas for C83-bound APH-1B- γ -secretase it averaged to ~ 10.4 Å, comparing well to the experimental 6IYC (10.6 Å). In the obtained open state the distance averages to 1.2 nm with a broader Asp-Asp distribution, fully consistent with the previous studies[Dehury et al., 2019a, 2019b; Somavarapu and Kepp, 2017] that led to the structural-dynamic support for the FIST model (**Figure S19B**).

The PAL motif (Pro433-Ala434-Leu435) in PS1 plays a vital role in substrate recruitment.[Sato et al., 2008] The Ca-Ca distance between Pro433 and Leu435 implied a distinct extension of this motif in the deprotonated apo state as compared to both the apo- γ -secretase with Asp257 protonated and the holo state (**Figure S19C**). Mutation of Leu432 affects the PAL motif structure and eliminates the catalytic activity of the enzyme,[Yang et al., 2019; Zhou et al., 2019] and thus the dynamic changes with substrate binding and protonation could be of relevance, although the pH-dependency of γ -secretase is a largely unexplored topic at this point.

Dynamics of catalytic aspartate - cleavage site interactions

The distance between the catalytic aspartates of PS1 and the actual cleavage sites of the substrate (in this case C83) is also important to understand the specific cleavage mechanism.

We measured the minimum of these distances from the last 300 ns trajectories of the holo states, as summarized in **Figure 6**. The ϵ -cleavage occurs via two different pathways either at Thr719-Leu720 or at Leu720-Val721 leading to the formation of A β ₄₈ or A β ₄₉. [Bolduc et al., 2016; Wolfe, 2020]. We observed differential distributions in the distance between the catalytic Asp257 and cleavage site residues, with some below 4 Å and some above 8 Å, whereas the distance was generally very short (below 4 Å) in case of Asp385. Thus, the ensembles mainly represent states that are ready to cleave via water binding to Asp385, but later cleavage via Asp257 will then probably occur after loss of the tripeptide fragment. [Bolduc et al., 2016; Takami et al., 2009] The open state arguably enables both of these cleavage pathways to proceed whereas the semi-open state favors the A β ₄₀ pathway.

The intermolecular hydrogen-bond dynamics of C83, nicastrin and PS1 (**Figure S20**) indicate a distinct interaction between C83 and PS1 in simulation 3, with a higher number of hydrogen bonds typical of the more compact semi-open state of γ -secretase. We also measured the hydrogen bonds formed between C83 with the lipid molecules and water molecules in each trajectory of the substrate bound conformations of γ -secretase complex (**Figure S20**). A well-positioned water molecule near the two aspartates is required to cleave the substrate. [Singh et al., 2009] As evident from **Figure S20**, fewer hydrogen bonds are formed between lipids and C83, while, ~44 hydrogen bonds existed between C83 and water (**Figure S20**). We observed different accessibility of water to the substrate in the three simulations (~50 in simulation 1, ~70 in simulation 2, and ~26 in simulation 3) by the catalytic aspartates in each C83-bound γ -secretase state (**Figure S20**). These differences may be attributed to the distinct binding states, with the more open state enabling more water molecules to access the catalytic site. To understand the interaction of C83 by γ -secretase with APH-1B, we computed the intermolecular contacts of the structural ensembles obtained from clustering analysis (**Table S5**). In simulation 1, we observed most hydrogen bonds and least in the case of simulation 3. A number of residues in the loop bridging TM6a and the strand β 1 interacted consistently with

the positively charged triplet lysine anchor motif in C83, consistent with the known role of this polybasic region in anchoring substrate molecules[Xu et al., 2016]. We also found several electrostatic interactions that aid the positioning of the substrates inside the pocket of catalytic PS1.[Yan et al., 2017]

Differences between γ -secretase with APH-1A and APH-1B

The most important question of our work is arguably whether, and if so, how, APH-1B affects γ -secretase differently than APH-1A, in particular in relation to the catalytic PS1 subunit. To understand this, we measured the deviations in the TM tilt angles of PS1 and APH-1B. We compared the present data to the MD data of C83 bound γ -secretase with APH-1A subunit from our recently published study[Mehra et al., 2020] that used similar setup, making the two studies directly comparable. We observed surprising differences in PS1 and APH-1B in the apo deprotonated-state (**Figure S21**): The distributions of the tilt angles in TM2, TM3 and TM6 of PS1 differed notably between the APH-1A and APH-1B systems. With APH-1B, TM2, TM3 and TM5 of PS1 varied substantially in apo- γ -secretase, whereas the C83-bound γ -secretase with APH-1A and APH-1B differed in terms of TM2, TM3, TM5 and TM6 (**Figure 7**). These differences indicate that APH-1A and APH-1B could favor the open, closed, and semi-open states of PS1 differently, which would affect activity.[Somavarapu and Kepp, 2017]

A recent MD study has shown that APH-1A (inside γ -secretase complex: 5FN3) can act as a channel apart from providing stability to γ -secretase complex.[Aguayo-Ortiz and Dominguez, 2019] The trajectories obtained in the present work also identify a very persistent channel architecture of APH-1B (**Figures S22-S23**) with many events of water molecules crossing the bilayer near TM3, TM4, TM5 and TM6 of APH-1B. In this respect, the two homologous proteins thus appear similar.

553

554 **Conclusions**

555 The intramembrane aspartyl protease complex γ -secretase, containing nicastrin, PEN-2,
556 PS1/PS2, and APH-1A/1B, plays a major role in AD and actively cleaves more than a hundred
557 membrane-bound helical substrates, including C99 to produce the infamous A β peptides of
558 variable length. Although all structural and computational work has focused so far on γ -
559 secretase with APH-1A, different γ -secretase complexes containing APH-1A or APH1-B
560 subunits display heterogeneous biochemical and physiological properties.[Araki et al., 2006;
561 Serneels et al., 2009] APH-1A/B interacts with presenilin and affects the processing of
562 substrates, thus modulating the A β ₄₂/A β ₄₀ ratio.[Acx et al., 2014; Lessard et al., 2015]
563 Accordingly, it is important to understand the structure and dynamics also of γ -secretase with
564 the APH-1B subunit, which was the aim of the present work.

565 From our simulated homology models of APH-1B alone and in complex with γ -secretase
566 both with and without substrate (C83) bound, we find that APH-1B resembles APH-1A in its
567 ability to transport waters across the bilayer and adopting the same 7TM topology with minor
568 variation in the JM helix at the C-terminal end. The water molecules inside the APH-1B cavity
569 formed interactions with several polar residues including notably Glu84, Arg87, His170, and
570 His196. The conserved histidines His170 and His196 have been implicated to play decisive
571 role in water transportation across the bilayer in APH-1A in previous work, and our work on
572 APH-1B supports these findings.[Aguayo-Ortiz and Dominguez, 2019] Further studies seem
573 necessary to understand the role of the water-containing cavity in APH-1 in the context of the
574 proteolytic activity of γ -secretase.

575 We conducted multiple all-atom MD simulations of γ -secretase with APH-1B subunit in a
576 complete POPC lipid bilayer to understand the physiological temperature membrane dynamics
577 of the protein complex, using the cryo-EM structures as templates.[Bai et al., 2015a; Zhou et
578 al., 2019] Our structures after equilibration are in excellent agreement with the experimental

structures both differ in some parts of APH-1B which also mostly correlate with major sequence variations in the two homologous forms of APH-1A/B. We observed that the protonation of the catalytic aspartates of PS1 affect the distance between the aspartates and the size of the catalytic pocket, which could potentially affect substrate cleavage, although the pH-dependency of γ -secretase activity has not yet been studied to our knowledge. Fluorescence lifetime imaging indicates that PS1 becomes more compact upon binding to APH-1B compared to APH-1A.[Serneels et al., 2009] Our dynamic ensembles support these findings and trace them to differences in the loops and TM helices.

The accessibility of water molecules to the catalytic aspartates of PS1 was monitored by calculating the water molecules near the site along the trajectories. Many water molecules access the active site during the normal membrane dynamics, but the access is strongly regulated by the conformational states of the enzyme, and we notably see both open and compact states with respect to catalytic pocket size, controlled by the PS1 TMs, consistent with our previous findings for γ -secretase with APH-1A that underlie the FIST model.[Somavarapu and Kepp, 2016, 2017; Tang et al., 2018] APH-1B was markedly less structurally variable in the C83-bound state compared to the apo states, i.e. substrate binding dampens the motion of APH-1B in the same way as seen for APH-1A.

A notable difference between APH-1A and APH-1B, apart from the substantial dynamic differences in the loops where the sequences differ most, was the distribution of TM tilt angles in the apo and substrate-bound states, indicating different effect on the conformational states and perhaps stability of γ -secretase for the two APH-1 forms. In particular, we find that the typical distance between the catalytic aspartates of PS1 and the C83 cleavage sites are shorter in APH-1B, i.e. it represents a more closed state, due to interactions with the C-terminal fragment of PS1. Since both forms of γ -secretase occur physiologically, our findings should be important when understanding the physiological activity of the enzyme complex and when targeting it to modulate A β production as widely sought as a major AD drug strategy.

Supporting information

The data required to reproduce the present computational work and scrutinize details of the analysis are included in the file named “suppinfo.pdf”. Furthermore, the full representative structures from cluster analysis are available as PDB files for each independent simulation.

Competing interests statement

All authors hereby declare that they have no competing interests, neither financial nor non-financial, related to this work.

Data availability statement

The data that supports the findings of this study are available in the supplementary material of this article. Additional raw data (trajectory files, representative structures) are available from the authors upon reasonable request.

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Figure legends

Figure 1. Sequence and structural comparison of APH-1 subunits (APH-1 and APH1-B)

of γ -Secretase. (A) Sequence alignment of APH-1A (265 aa) and APH-1B (257 aa). The transmembrane helix region is marked by cyan encirclement and the GxxxG motif is marked with stars. (B) Ramchandran analysis of modelled APH-1B with 97.8% of residues in the most allowed region. (C) The overall 7TM architecture of APH-1B (conserved regions are shown in red and variable regions in white). (D) Structural overlay of our modelled APH-1B subunit (green) with the APH-1A subunits from cryo-EM structures (5FN2: light blue, 5A63: magenta and 6IYC: yellow), with the GxxxG motif encircled. The C- α RMSD of APH-1B with respect to APH-1A of 5FN2, 5FN3 and 6IYC are 0.47, 0.37, and 0.46 Å, respectively.

Figure 2. Structural dynamics of APH-1B alone in a lipid bilayer.

(A) Backbone RMSD of triplicate APH-1B simulations. Blue, orange and cyan lines represent simulation 1, 2, and 3. (B) C α -RMSF for each residue of APH-1B during the last 300 ns MD. The B-factors with the flipping regions are shown in putty format to the right, using PyMOL. (C) Experimental APH-1A from 5FN2 (green) superimposed with representative structures for simulation 1 (blue), 2 (orange), and 3 (cyan).

Figure 3. Simulated γ -secretase models with APH-1B in a lipid bilayer with zoomed view

of catalytic aspartates. (A) Apo- γ -secretase with APH-1B (with deprotonated Asp257 and Asp385) and the backbone RMSD of the complex simulations. (B) Apo- γ -secretase with protonated Asp257 with APH-1B and the backbone RMSD. (C) C83-bound APH-1B- γ -secretase and backbone RMSD. The small colored spheres represent the POPC head groups. Blue, orange and cyan lines represent simulation 1, 2, and 3 of each system.

Figure 4. Comparison of simulated γ -secretase (with APH-1B subunit and experimental cryo-EM structures 5FN2 and 6IYC (with APH-1A). (A) Superimposed view of the representative structures of apo- γ -secretase (deprotonated Asp257 and Asp385) with that of experimental 5FN2 structures. (B) Superimposed view of the representative simulated structures of C83-bound γ -secretase with APH-1B and experimental 6IYC (with APH-1A). The blue, orange and cyan colors represent simulation 1, 2, and 3; the experimental cryo-EM structures are shown in green.

Figure 5. C α RMSF analysis of each subunit of apo and holo states of γ -secretase with APH-1B. The blue, orange, and cyan lines represent the RMSF for C α -atoms of each subunit from simulations 1, 2, and 3. **Upper Panel:** C α -RMSF of Nicastrin, PS1, APH-1B and PEN2 in apo- γ -secretase with deprotonated Asp257 and Asp385 of PS1. **Middle Panel:** C α -RMSF of Nicastrin, PS1, APH-1B and PEN2 in apo- γ -secretase system with protonated Asp257. **Lower Panel:** C α -RMSF of Nicastrin, PS1, APH-1B, and PEN2 in C83-bound γ -secretase.

Figure 6. Distances between catalytic Asp257/Asp385 and C83 cleavage sites in γ -secretase with APH-1B. The distances shown are those between the two catalytic residues of PS1 and the cleavage sites Leu720-Val721 and Thr719-Leu720, computed using the *gmx mindist* utility toolkit from the last 300 ns of each trajectory.

Figure 7. Distribution of TM tilt angles of PS1 and APH1 in γ -secretase with APH-1A and APH-1B. The tilt angles of PS1/APH-1A are from our previous study.[Mehra et al., 2020] The tilt angles were computed using the C α coordinates of the TMs. **Left Panel:** Comparison of tilt angles of TM helices of PS1 in γ -secretase-C83 with APH-1A and APH-1B. **Right Panel:** Comparison of TM tilt angles of APH1 subunits in γ -secretase-C83 with APH-1A and APH-1B.