Review

Toward the structure of presenilin/γ-secretase and presenilin homologs

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A B S T R A C T

Presenilin is the catalytic component of the γ-secretase complex, a membrane-embedded aspartyl protease that plays a central role in biology and in the pathogenesis of Alzheimer’s disease. Upon assembly with its three protein cofactors (nicastrin, Aph-1 and Pen-2), presenilin undergoes autoproteolysis into two subunits, each of which contributes one of the catalytic aspartates to the active site. A family of presenilin homologs, including signal peptide peptidase, possess proteolytic activity without the need for other protein factors, and these simpler intramembrane aspartyl proteases have given insight into the action of presenilin within the γ-secretase complex. Cellular and molecular studies support a nine-transmembrane topology for presenilins and their homologs, and small-molecule inhibitors and cysteine scanning with crosslinking have suggested certain presenilin residues and regions that contribute to substrate recognition and handling. Identification of partial complexes has also offered clues to protein–protein interactions within the γ-secretase complex. Biophysical methods have allowed 3D views of the γ-secretase complex and presenilins. Most recently, the crystal structure of a microbial presenilin homolog has confirmed a nine-transmembrane topology and intramembranous location and proximity of the two conserved and essential aspartates. The crystal structure also provides a platform for the formulation of specific hypotheses regarding substrate interaction and catalysis as well as the pathogenic mechanism of Alzheimer-causing presenilin mutations. This article is part of a Special Issue entitled: Intramembrane Proteases.

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1. Introduction

Among the more surprising findings in biochemistry has been the discovery of proteases that carry out hydrolysis within the hydrophobic environment of the lipid bilayer. These intramembrane-cleaving proteases (i-CLiPs) include the zinc-containing site 2 protease (S2P) family, the rhomboid family of serine proteases, and the presenilin family of aspartyl proteases.
family of aspartyl proteases. All three families have their active sites embedded within the membrane and cleave within the transmembrane domains of their respective substrates. While at first it may seem strange for hydrolysis to occur within membranes, no biochemical or biophysical rules need be violated. Indeed, other membrane proteins serve as channels for water and ions, creating microenvironments by which polar and charged species can pass through the membrane without directly encountering the hydrophobic tails of membrane lipids.

The first crystal structures of i-CLiPs, of rhomboids [1–5] and an S2P [6], provided insight into how water can access the membrane-embedded active sites, revealing pores or cavities by which water can reach the catalytic residues without contacting membrane lipids. At the same time, these structures suggested means by which substrate transmembrane domains might enter the active site through a lateral gating mechanism. Results from biochemical experiments guided the interpretation of the mechanistic meaning of the crystal structures, and the structural results in turn suggested hypotheses that could be tested by further biochemical experiments (e.g., [7]). Thus, the interplay between biochemistry and structural biology has proven quite powerful in the study of rhomboid serine i-CLiPs and S2P metallo i-CLiPs.

Presenilin-type aspartyl I-CLiPs, on the other hand, had been resistant to crystallographic analysis until most recently. A new structure from an archaeal presenilin-like protease is consistent with much of what has been learned of this class of membrane-embedded enzyme from molecular and biochemical experiments and will no doubt provide a platform from which to design further biochemical experiments toward understanding the workings of these proteases. Below I review the discovery of the presenilin family of I-CLiPs followed by molecular and biochemical evidence (e.g., [8,9]). Thus, the interplay between biochemistry and structural biology has proven quite powerful in the study of rhomboid serine i-CLiPs and S2P metallo i-CLiPs.

Presenilin is cut into two pieces, an N-terminal fragment (NTF) and a C-terminal fragment (CTF) [32,33], the formation of which is contained moieties typically found in aspartyl protease inhibitors [27,28]. These findings led to the identification of two conserved transmembrane aspartate residues in the multi-pass presenilin that are critical for γ-secretase cleavage of APP (Fig. 1), suggesting that presenilins might be the responsible aspartyl proteases [29–31].

Presenilin has nine transmembrane domains and two conserved aspartates, in TMD 6 and TMD 7. Upon assembly with other members of the γ-secretase complex, presenilin undergoes autoproteolysis within the large loop connecting TMD 6 and TMD 7 to form an N-terminal fragment (NTF, copper) and C-terminal fragment (CTF, gold). Each presenilin subunit contributes an aspartate to the active site. APP is a type I integral membrane protein that undergoes ectodomain shedding by β-secretase, with retention of a C-terminal fragment in the membrane that is subsequently cleaved by γ-secretase in at least two sites: a γ site to release the Aβ peptide to the luminal/extracellular space and an ε site to release the APP intracellular domain (AICD) into the cytosol.
3. Discovery of SPP and other presenilin homologs

The concept of presenilin as the catalytic component for γ-secretase was considerably strengthened when signal peptide peptides (SPP) was found to be a similar intramembrane aspartyl protease. SPP clears remnant signal peptides from the membrane after their production by signal peptidase (Fig. 2). However, this process apparently also plays a role in immune surveillance, in which signal peptides from the major histocompatibility complex (MHC) type I are cleaved by SPP and the peptide products presented onto the cell surface as an indication to natural killer cells whether MHC synthesis is proceeding normally [66]. In addition, SPP is exploited by the hepatitis C virus for the maturation of its core protein, suggesting that this protease may be a suitable target for antiviral therapy [67]. See review by Jin Ye (“Roles of intramembrane proteases in virus infection and immunity”) in this special issue for further details.

SPP was identified by affinity labeling with a peptidomimetic inhibitor, and the protein sequence displayed intriguing parallels with presenilin [68]. SPP contains two conserved aspartates, each predicted to lie in the middle of the sixth or seventh transmembrane domain (Fig. 2), and the aspartate-containing sequences resemble those found in presenilins. Interestingly, prior to the identification of SPP, a computational search for presenilin-like proteins netted an entire family of so-called presenilin homologs (PSHs) [69]; however, it is not yet clear if all of these proteins have catalytic activity. Two homologs, SPP-like proteases, SPP2la and SPP2lb, have been found to cleave tumor necrosis factor receptor α [70] and the dementia-associated Briz2 protein [71], although the biological roles of these proteolytic events are unknown. See review by Voss et al. (“Mechanism, specificity and physiology of SPP and SPP-like proteases”) for a more detailed discussion.

SPP appears to be less complicated than γ-secretase. Expression of human SPP in yeast reconstituted the protease activity, suggesting that the protein has activity on its own and does not require other mammalian protein cofactors [68]. This has been confirmed by the expression of various SPP orthologs in *E. coli* and purification of active enzyme to homogeneity [72]. Moreover, unlike presenilins, SPP is not processed into two pieces. Thus, SPP and other presenilin-like proteases that function as single polyprotein chains may be more tractable enzymes for shedding light on γ-secretase structure and function. Indeed, this has turned out to be the case (see below).

4. Topology of presenilins and other γ-secretase components

The topology of PS1 had been a subject of some controversy and confusion, with evidence for 6, 7 or 8 transmembrane domains (TMDs). This issue finally appeared to be settled using insertion of glycosylation sequences into possible loop regions and near the C-terminus, showing that presenilin-1 contains nine TMDs [73,74], with the N-terminus in the cytosol and the C-terminus exposed to the luminal/extracellular space (Fig. 1). The final three transmembrane segments had been the difficult ones to confirm, one reason apparently being their interdependence. TMD7 is a relatively short hydrophobic domain, with one of the conserved aspartates in the middle. Incorporation of this TMD has been shown to require TMD8 [74]. This concept of more hydrophobic regions pulling in less hydrophobic regions is emerging as a theme in membrane protein insertion and folding [75]. Another study suggested that presenilin forms a ring-like structure [76], with TMD1 proximal to TMD8. Crosslinking of wild-type cysteines in PS1 likewise suggested TMD1 and TMD8 proximity [77]. The nine-transmembrane topology and proximity of TMD1 and TMD8 are also findings consistent with a new crystal structure of a presenilin-like protease (see later). The apparent topology of SPP and its homologs likewise determined through insertion of glycosylation sites in loops and at the C-terminus, also resemble that of presenilins, placing the key aspartates in the same relative position to each other in the membrane [78]. The orientation of the aspartate-containing transmembrane domains of SPP and its homologs, however, is opposite that of presenilins (Fig. 2), consistent with the fact that SPP cleaves signal peptides: presenilin cleave type I integral membrane proteins, oriented with N-to-C termini going from...
extracellular/luminal to cytosolic, while signal peptides have the opposite orientation.

The reported topologies of Aph-1 and Pen-2 [79,80] are as shown in Fig. 3. Insertion of glycosylation sites in predicted loop regions and near termini, in combination with selective permeabilization of the plasma membrane and immunofluorescence microscopy, supported a seven-TMD topology for Aph-1, with N-terminus in the lumenal/extracellular space and the C-terminus in the cytosol [81]. The topology of Pen-2 was deduced using a similar strategy: the N-linked glycosylation sites present in the N- and C-terminal domains of Pen-2 were utilized, whereas a site in the hydrophilic region connecting the two predicted transmembrane domains, presumably a loop, was not [80]. This topology was confirmed using epitope tags near the N- and C-termini. The topology of Nicastrin, in contrast, has never been seriously in question, as it has every indication of being a type I integral membrane protein, with a single transmembrane domain, an N-terminal ectodomain in the extracellular/luminal space and short C-terminal tail in the cytosol. Consistent with this notion is the extensive glycosylation of the N-terminal ectodomain, which is increased upon assembly into the full γ-secretase complex [82–86]. Tryptic digestion experiments further suggested that assembly of nicastrin into full complexes leads to a conformational change [87]. Mature, fully glycosylated nicastrin is protected from trypsin degradation, even as other γ-secretase components are digested.

5. Subunit interactions and stoichiometry

γ-Secretase is so far unique among intramembrane proteases in being composed of several different proteins: all the others apparently work alone as single proteins. Coexpression, RNA interference, and the identification of assembly intermediates suggest the order in which these four subunits come together [61,88,89], and partial dissociation of the protease complex with detergent offers a model for how these subunits interact [88]. Assembly of the γ-secretase complex begins in the endoplasmic reticulum soon after translation and membrane insertion. Nicastrin and Aph-1 assemble into a subcomplex, with nicastrin remaining in an immature, hypoglycosylated form [90]. Whether presenilin and Pen-2 interact with each other first before assembly with the nicastrin/Aph-1 subcomplex is unclear, but knockdown of Pen-2 does lead to a Nicastrin/Aph-1/Presenilin subcomplex in which presenilin remains as a holoprotein [61,88,91]. Assembly of all four components results in presenilin autoproteolysis [29,92] into NTF and CTF subunits, nicastrin maturation through glycosylation, and active
γ-secretase. Pen-2 is also required to stabilize the presenilin subunits [93].

Partial dissociation of the γ-secretase complex using the nonionic detergent dodecyl-β-D-maltoside (DDM) followed by 2D PAGE analysis revealed how the γ-secretase components are arranged in the active protease complex [94]. As expected, nicastrin interacts with Aph-1, but PS1 NTF was found to interact with Pen-2 as well as with PS1 CTF, and nicastrin and Aph-1 together can also interact with PS1 CTF. A Pen-2/PS1 holoprotein complex was also observed. Other studies have confirmed the PS1 NTF/Pen-2 interaction and pinpointed transmembrane domain 4 of PS1 as the site of contact with Pen-2 [95,96]. Crosslinking studies corroborate these interactions [97]. Bifunctional chemical crosslinking reagents were used to covalently connect proximal subunits within endogenous human γ-secretase complexes. Crosslinked subunits included PS1 NTF and CTF, PS1 NTF and PEN-2, and NCT and APH-1. PS1 CTF and APH-1 was also observed, providing further information on the nature of the full, active complex. Site-directed mutagenesis with co-immunoprecipitation experiments further suggested that the C-terminal region of PS1 interacts with the N-terminal region of the nicastrin TMD [98,99]. Taken together, these findings have led to the model for the γ-secretase complex shown in Fig. 4.

As for the stoichiometry of the γ-secretase complex, this has been a matter of some controversy, with discrepancies in the reported size of the complex and in the number of presenilin molecules per complex. Sizes of 100–150 KDa to 2 MDa have been reported [35,36,62,83,100,101], and several studies suggested that two presenilins reside at the catalytic core of the protease complex [102–104]. However, rigorous biochemical and biophysical experiments have shown that isolated, active complexes contain only one of each component [105]. Differential epitope tagging of PS1 demonstrated that one PS1 molecule does not co-immunoprecipitate with another. A similar approach demonstrated the same for Aph-1. Quantitative western blotting, using epitope-tagged standards of similar size, showed that one PS1 molecule does not co-immunoprecipitate with another. This finding that only one presenilin molecule is required for γ-secretase activity has mechanistic implications, as it means that the protease complex contains only one pair of conserved transmembrane aspartates and therefore one active site. This is important, because the enzyme displays three different types of proteolytic activities: (1) an autoproteolytic function in which full-length presenilin is cleaved within the TMD6-TMD7 loop to NTF and CTF to activate the complex for cleaving other substrates [29,92], (2) an endoproteolytic function of type I integral membrane protein substrates to release substrate intracellular domains [15], and (3) a carboxypeptidase function, in which the remaining fragment, containing most of the transmembrane domain, is trimmed roughly every 3 amino acids until the fragment is finally released [111]. For APP, initial cleavage at the ε site to release AICD also forms either a 48- or 49-residue Aβε, which contains almost all of the transmembrane domain [112]. Subsequent processive proteolysis from the C-terminus of these long Aβε peptides ultimately leads to release of the 38–43-residue secreted forms of Aβε [113]. Similar processive proteolysis of the corresponding fragment of the Notch receptor apparently also occurs, and this is likely to be true of other γ-secretase substrates as well [114,115].

Evidence supports two pathways for Aβ42 production depending on the exact site of initial ε cleavage: Aβ49 → Aβ46 → Aβ43 → Aβ40 and Aβ48 → Aβ45 → Aβ42 → Aβ38, findings particularly supported by the detection of the corresponding tri- and tetrapeptide products [111]. Alzheimer-causing presenilin mutations can alter the initial site of ε cleavage to increase formation of Aβ48 over Aβ49 [116], thereby increasing the ratio of secreted Aβ42/Aβ40, which is critical in the aggregation propensity of the peptide [117]. Recent evidence also shows that the carboxypeptidase function of γ-secretase is reduced by such disease-causing mutations [118,119]. These findings appear to resolve a long-standing controversy about how presenilin mutations cause Alzheimer's disease, with one side suggesting that reduction of γ-secretase proteolytic function is what ultimately causes synaptic dysfunction and neurodegeneration [120] and another camp suggesting that a toxic gain of function, an increase in Aβ42/Aβ40, is responsible [9]. These ideas may be synthesized by considering that the specific reduction of the carboxypeptidase function by the presenilin mutations results in the increase in the proportion of longer, more aggregation-prone neurotoxic forms of Aβ42 [121–123].

**Fig. 4.** Model for subunit interaction within the γ-secretase complex and substrate interaction. Presenilin NTF (copper), presenilin CTF (gold), Pen-2 (red), Aph-1 (blue), and nicastrin (green). Substrate APP CTF (yellow) is shown interacting at the interface between presenilin NTF and CTF via its transmembrane domain and with nicastrin via its N-terminus. Substrate then enters the internal active site (pink star) for proteolysis to Aβ42 and AICD.

6. Multiple proteolytic functions of γ-secretase

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Inhibitors of γ-secretase and of SPP have served as important probes for protease structure and function. Transition-state analog inhibitors converted to affinity labeling probes tagged both PS1 NTF and PS1 CTF [38,39], suggesting that the active site lies at the interface between the two presenilin subunits, consistent with each subunit contributing one of the two conserved aspartates required for proteolytic function. Transition-state analog inhibitors have also provided evidence for a large S1 pocket [124] and loose sequence specificity [27,125,126]. Such probes have been further used to show that the active site conformation of γ-secretase is altered by Alzheimer-causing PS1 mutations [127,128]. Gold-coupling of a transition-state analog inhibitor has also confirmed the presence of one active site per protease complex [129].

Interestingly, affinity isolation of the γ-secretase complex using an immobilized transition-state analog inhibitor revealed co-purification of an endogenous APP substrate, suggesting that substrate can bind to the enzyme complex even when the active site is occupied by an inhibitor [63]. The existence of an initial substrate docking site on γ-secretase makes sense given the intramembranous nature of the hydrolytic reaction. The active site, with two aspartates and water, is predicted to reside in the interior of the complex so as not to come in thermodynamically unfavorable contact with the hydrophobic lipid tails of the membrane. The transmembrane substrate can move within the bilayer in only two-dimensions and would therefore be expected to first interact on the outer surface of the protease complex before enter in whole or in part into the internal active site.

Designed peptides based on the transmembrane domain of APP and constrained in a helical conformation can potently inhibit γ-secretase, apparently by interacting with this docking site [130]. Conversion of these helical peptide inhibitors into affinity labeling reagents led to the localization of the substrate docking site to the presenilin NTF/CTF interface [127]. Transition-state analog inhibitors also bind directly to the NTF/CTF interface, but at a site clearly distinct from that of helical peptide inhibitors. These findings suggest a pathway for γ-secretase substrate from docking site to active site: upon binding to the outer surface of presenilin at the NTF/CTF interface, the substrate can pass, either in whole or in part, between these two presenilin subunits to access the internal active site (Fig. 4). Interestingly, extension of a ten-residue helical peptide inhibitor by just three additional residues resulted in a potent inhibitor [131] apparently capable of binding both docking site and active site [127], suggesting that these two substrate binding sites are relatively close. Coimmunoprecipitation experiments also show that nicastrin can interact with APP and Notch substrates at their N-termini, consistent with the observation that nicastrin has homology to aminopeptidase, but with an essential catalytic residue mutated [132,133]. Thus, nicastrin may also play a role in substrate recognition (Fig. 4). Another report, however, challenges this view [134].

Other compounds have been identified that have more subtle effects on γ-secretase activity. So-called γ-secretase modifiers reduce the production of aggregation-prone Aβ/42 and increase production of shorter, more soluble forms [135]. Other “Notch-sparring” γ-secretase inhibitors have been reported to inhibit Aβ production from APP with some degree of selectivity over Notch proteolysis [136–138]. Both types of compounds have the potential to be safer therapeutic agents targeting γ-secretase than broadly acting inhibitors. While some evidence supports the interaction of γ-secretase modulators with APP substrate [139], more compelling evidence favors interaction with the protease complex [110,140]. Affinity labeling indicates direct interaction of both modulators and Notch-sparring inhibitors with presenilin NTF [141–143], although one study found Pen-2 as the target of a novel class of γ-secretase modulators [144]. The latter study is called into question, however, by the use of a detergent (Triton X100) that completely disrupts the protease complex during the affinity labeling.

Taken together, these findings suggest that the γ-secretase complex, most notably presenilin NTF, contains a pocket that serves as the binding site of compounds that allosterically regulate the enzyme, rather than completely blocking access of all substrates. The specific identification of such allosteric binding sites, especially through co-crystallization, might allow rational, structure-based design of therapeutic candidates that target γ-secretase.

8. Cysteine crosslinking studies

Other structural features of the γ-secretase complex, specifically of the presenilin component, have been revealed by cysteine mutagenesis with crosslinking of chemical probes [145,146]. The generation of a cysteine-less version of presenilin that retains the ability to assemble with other complex members, to undergo endoproteolysis to NTF and CTF, and to process APP allowed incorporation of single cysteine residues at various sites near the key aspartates. Disulfide formation with biotinylated, thiol-containing reagents then provided information about the relative accessibility of these sites from the aqueous milieu, allowing the construction of a model in which water can funnel down to where the aspartates reside (the “catalytic pore” model).

In addition, simultaneous mutation of the two conserved transmembrane aspartates to cysteine and apparent intramolecular crosslinking provided the first evidence that these two aspartates are indeed in close proximity [149], which is required for them to coordinate and serve catalytic function. Using this same approach (cysteine mutagenesis and crosslinking), two recent studies suggest that TMD 9 serves as a gatekeeper for lateral entry of the substrate TMD [147,148], as this TMD appears to be flexible, close to the catalytic aspartate in TMD 6, and a site for inhibitor binding, including a helical peptide. Still another cysteine-crosslinking study suggests that TMD 1 is in direct contact with TMD 8 [77].

The cysteine mutagenesis/crosslinking approach was further used to study TMD 1 in detail. The results from these experiments suggested that TMD 1 is located in proximity to the catalytic motif in TMD 7 as well as to a Pro-Ala-Leu motif between TMD 8 and TMD 9 and directly faces the catalytic pore [149]. Competition experiments using known γ-secretase inhibitors suggested that the N-terminal region of TMD 1 functions as a subsite for substrate handling during proteolysis by γ-secretase. Intriguingly, binding of inhibitors affected water accessibility of residues at the membrane border of TMD 1, suggesting the possibility of a dynamic motion of TMD 1 during the catalytic process. The various conclusions drawn from all the cysteine mutagenesis/crosslinking studies are mostly supported by a new structure of an archael presenilin homolog (see below).

9. Electron microscopy

Several I-ClIPs that work as single polypeptide chains have now been crystallized and their structures elucidated at atomic resolution. This includes a presenilin-like protease (see below). However, a detailed structure of the entire γ-secretase complex is daunting, with its four different protein components (five after presenilin autoproteolysis to NTF and CTF subunits), a size of ~230 kDa, extensive glycosylation of nicastrin, and a grand total of 19 TMDs. Nevertheless, the purification of the γ-secretase complex [65] has allowed the first glimpses into its structure through electron microscopy (EM). Electron microscopy with uranyl acetate coating and single particle analysis revealed that the complex has a globular structure that at low resolution (10–15 Å) appears rather amorphous [150]. Another structure, elucidated in a similar manner but of much poorer resolution (~45 Å), has also been reported [151]. Orientation of the complex with respect to the membrane was established using lectin tagging of the highly glycosylated nicastrin ectodomain, and the dimensions were measured as ~75 × 120 × 75 Å. Despite the relatively low resolution, several important features can be gleaned. The first is a rather large low-density...
interior cavity of ~20–40 Å diameter that is presumably where the active site resides, a characteristic reminiscent of the proteasome. Another is the presence of two small openings that may be the site of entry for water. Finally, the complex contains a belt-like density ~60 Å high that is likely to be the transmembrane region.

More recently, an improved structure of γ-secretase was determined by cryo-EM at 12 Å resolution [106] (Fig. 5). Cryo-EM reveals a protein structure itself, rather than a surface of the protein structure coated by a heavy metal stain. The new cryo-EM structure reveals three smaller low-density interior regions, which do not coalesce to form a single chamber as observed in the negative stain structure [150]. The cryo-EM structure has better definition than the previous negative-stained structure, in that the ~4 nm thick transmembrane region and four extracellular density domains are resolved [106], and gave dimensions of ~80 × 90 × 85 Å. The structure also reveals an apparent groove along the membrane-exposed outer surface that may be the initial substrate docking site. An 18 Å cryo-EM structure showed similar dimensions (100 × 70 × 105 Å) [129]. However, in some respects this lower-resolution structure appears to be more compact and has a single central chamber that interfaces with a concave surface on the extracellular side. This cavity appears to be the site of labeling by a gold-coupled transition-state analog photoaffinity reagent, consistent with this apparently water-accessible cavity containing the active site. This same study reported the cryoEM structure of the PS1-nicastrin-Aph1 trimeric complex, which appeared to be quite similar to the full complex, suggesting that addition of the small (~10 kDa) Pen-2 subunit does not cause major conformational changes to the complex.

Ultimately, structure elucidation of individual subunits as well as subcomplexes, by NMR or crystallography (see below) may allow the piecing together of the entire γ-secretase complex with atomic level detail, using the lower resolution EM structures as a framework.

![Fig. 5. Structure of the γ-secretase complex at 12 Å resolution as determined by cryo-electron microscopy. Coloration represents different domains of the complex and is not meant to imply specific subunits. However, the large nicastrin ectodomain, identified with lectin, is located on the luminal/extracellular side. The thick red curved line in panel A identifies a groove on the outside of the membrane-spanning surface that may be the site of initial substrate binding. The structure reveals three openings, seen in the cutaway view in panel B, by which catalytic water may gain access to the active site.](image)

Such has been the case for other large and challenging macromolecular complexes, such as the ribosome.

# 10. NMR and crystal structures

A fuller understanding of the mechanism of γ-secretase and the nature of substrate recognition and handling requires higher resolution structural information. As a first step toward this goal, the solution structure of the human presenilin-1 CTF in SDS micelles was determined by NMR [152]. At ~18 kDa, this protein is just within the current size restrictions for protein structure determination by NMR. The human PS1 CTF was expressed using isotopically labeled amino acids under cell-free conditions, precipitated, and solubilized in SDS. In addition to NMR experiments to determine local conformation, paramagnetic relaxation enhancement was employed on monocysteine mutant versions of the PS1 CTF labeled with methanethiosulfonate to determine long-range distance restraints. Analysis revealed six α-helical regions, with three of these representing the core of the protein. Only one helical region, corresponding to TMD 8 in full-length PS1, showed typical transmembrane character. TMD 7, harboring one of the catalytic aspartates, was a shorter helix, with the aspartate at the N-terminus and predicted to be within the membrane. TMD 9 was severely kinked, due to the presence of a conserved proline at residue 455 (full-length PS1 numbering), with the N-terminal half perpendicular to TMD 7 and TMD 8 and the C-terminal half pointing toward the surface of the membrane.

Although this structure of the PS1 CTF was determined in SDS and in the absence of the other components of the γ-secretase complex, it is in general agreement with previous studies. The core structure, with three α-helical regions representing the predicted TMDs 7, 8 and 9, is consistent with the widely accepted nine-TMD structure of PS1 and with water-accessibility experiments carried out using cysteine scanning and labeling. While SDS is considered a denaturing detergent for soluble proteins, it has been used successfully to determine the NMR solution structure of other membrane proteins. Nevertheless, differences are to be expected between this NMR structure and the actual structure of the PS1 CTF in the context of the full, active γ-secretase complex. Indeed, some key differences are observed between the NMR structure and the corresponding region in the crystal structure of a microbial PS1 [153].

Yigong Shi and colleagues worked toward the crystal structure of a presenilin protease by first attempting to express, purify and crystallize eukaryotic PS1 orthologs [153]. When this failed, they turned to archaeal homologs, as these can display better solution properties more suitable for crystallization trials. The archaeon Methanoculleus marisnigri JR1 provided the presenilin homolog (mmPSH) with the best solution properties. This was fortunate, as mmPSH had already been identified as a presenilin homolog, expressed in and purified from bacteria, shown to cleave substrate (including one derived from APP) within the transmembrane domain, and found to be inhibited by certain γ-secretase inhibitors [154]. However, mmPSH still required engineering for further optimization for crystallization. Ultimately, five mutations were identified that together provided excellent properties for crystallization trials. These mutations were mostly in surface loops and did not affect protease activity, determined using the membrane protein Gurken as substrate [154]. Crystallization allowed structure determination at 3.3 Å resolution. It should be mentioned that the crystal structure of another polytopic aspartyl protease, an archaeal prefagellin protease called FlaK, has also been determined [155]. However, the evolutionary relationship between FlaK and presenilins is distant at best, the number and correspondence of transmembrane domains between FlaK and presenilins is different, and the active site aspartates of FlaK are found outside the membrane. Therefore, FlaK will not be further discussed here.

The mmPSH structure revealed nine transmembrane domains, consistent with the most accepted experimental findings, and the conserved aspartates in TMD 6 and TMD 7 (Asp162 and Asp220,
respectively, in mmPSH numbering) were in close proximity (Fig. 6). The N-terminal region with TMDs 1–6 forms a horseshoe-shaped structure which partially surrounds TMDs 7–9. Although the transmembrane aspartates are proximal, they are not close enough or oriented quite properly to together activate water for catalysis. Shi and colleagues speculate that interaction with substrate may lead to proper formation of the active site. However, it is also possible that the misalignment and slight spacing between the aspartates is an artifact of crystal packing. Indeed, mmPSH crystallized as a tetramer, and the biological relevance of this assembly is unclear. The two aspartates are at the bottom of a cavity exposed to the aqueous environment (corresponding to the cytosol in human presenilin), providing a reasonable explanation for how catalytic water accesses the intramembrane active site.

Another interesting and unexpected feature of the structure is the presence of a hole that runs the length of the transmembrane region, surrounded by TMDs 2, 3, 5 and 7 (Fig. 6). This hole is mostly hydrophobic and may be plugged up by a lipid molecule in the context of the membrane. Although the hole is large enough to accommodate small ions, and some evidence suggests that presenilin has properties of a calcium channel [156], this pore is highly hydrophobic and is unlikely to allow ions to traverse it. This hole, like the tetrameric arrangement, may be an artifact of crystal packing. Alternatively, this particular structure of mmPSH may represent one among a continuum of possible conformations, with the hole forming during the process of substrate interaction. Shi and colleagues hypothesize that substrate entry likely occurs between TMD 6 and TMD 9, which is consistent with cysteine scanning and crosslinking suggesting that TMD 9 may act as a substrate gate [147,148]. On the other hand, the accessibility of the active site from this trajectory seems quite hindered (Fig. 6). In contrast, the active site seems much more accessible between TMD 2 and TMD 6, where there is a relatively large gap. Interestingly, a TMD of another mmPSH molecule is packed into this gap. Thus, this transmembrane helix of the neighboring mmPSH molecule may occupy the initial substrate docking site, and this docking may lead to the creation of the hole by conformational adjustment of TMD 2 (and TMD 3 along with it). Together, TMD 2 and TMD 3 may act as a hinge, swinging open to accommodate substrate binding. Such ideas, while speculative, are now very testable with the new structure.

The sequences of mmPSH and human PS1 are 19.3% identical and 52.8% similar, and signature sequences, including the aspartate-containing regions in TMD 6 and TMD 7 are highly conserved. Thus, the structures of mmPSH and human PS1 likely share the same basic fold. Interestingly, mapping sites of Alzheimer-causing mutations in human PS1 onto the mmPSH structure reveals key regions where these mutations tend to be located. One is the active site cavity: residues in TMDs 1, 5, 6, 7, 8 and 9 contributing to the active site are mutated in familial Alzheimer’s disease in the corresponding residues of human PS1 (Fig. 7A). Another major site of Alzheimer mutations is the hydrophobic hole that traverses the membrane (Fig. 7B). TMDs 2, 3, 5 and 7 all contribute residues to this hole that are also sites of familial Alzheimer mutations. Many of the mutations, particularly in TMDs 2 and 5, are found specifically along the side of the helix of the TMD facing into this pore. A third major site of mutations is in TMD 1, again along one side of the helical TMD, but in this case the residues face outward toward the membrane (Fig. 7C). In human presenilins, these TMD 1 residues may interact with one of the other components of the γ-secretase complex, and mutations may alter activity by affecting the subunit interface.

The above discussion on Alzheimer PS1 mutations is offered as speculation and as one way to consider the mmPSH structure for formulating specific testable hypotheses. However, caution should be exercised in extrapolating from the mmPSH structure to PS1 structure–function relationships in the context of the full γ-secretase complex. First, mmPSH does not require other subunits for proteolytic activity, although a recent report demonstrates that under the right conditions, a human PS1 can have catalytic activity without its partner proteins [157]. Second, as pointed out earlier, γ-secretase displays three different proteolytic functions: an autoproteolytic function, an endoproteolytic function and a carboxypeptidase function, and it is this last function that is apparently altered by Alzheimer-causing presenilin mutations [118,119]. Whether mmPSH displays this carboxypeptidase function is unknown. If so, then it would be worthwhile to test the effects of human Alzheimer mutations in mmPSH on the trimming function versus the endoproteolytic function.

11. Conclusions and perspective

Since the initial discovery of presenilin as the catalytic component of γ-secretase in 1999, much has been learned about the structure of this novel intramembrane aspartyl protease and its interaction with cofactor proteins and substrates. In particular, a nine-TMD structure has been confirmed, with the transmembrane aspartates located in close proximity. Subcomplex formation, crosslinking and partial detergent dissociation have offered a model for subunit interactions. Cysteine scanning with crosslinking has provided information about proximity of specific residues and TMDs, water accessibility and inhibitor binding sites. Electron microscopy has revealed the first glimpses of the full protease complex, and NMR has provided a solution structure for the PS1 CTF. Most notable is the new crystal structure of a microbial presenilin homolog, which reveals for the first time the basic fold of this family of I-CLIPs and provides a platform for generating specific hypotheses regarding structure–function relationships.

Moving forward, it will be worthwhile to determine the structures of other presenilin homologs, including the SPP family. Determining the structure of a human presenilin, ideally in the context of the full γ-secretase complex, would also be highly worthwhile. This may be accomplished by crystallization of the entire complex, or more likely, by crystallizing individual components and subcomplexes and using the EM structures as the framework to piece together this 3D jigsaw puzzle. It will also be important to determine the nature of substrate interaction and how substrate accesses the active site. Finally, determination of inhibitor–protease complexes would allow rational drug design toward identifying potent and selective compounds that safely lower Aβ production by γ-secretase without unduly interfering with
cleft of critical substrates such as the Notch receptor. The hope is that these future milestones in the study of presenilin and the γ-secretase complex are years away, rather than decades.

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