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Reconstitution and functional characterization of the FtsH protease in lipid nanodiscs

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ABSTRACT

FtsH is a membrane-bound protease that plays a crucial role in proteolytic regulation of many cellular functions. It is universally conserved in bacteria and responsible for the degradation of misfolded or misassembled proteins. A recent study has determined the structure of bacterial FtsH in detergent micelles. To properly study the function of FtsH in a native-like environment, we reconstituted the FtsH complex into lipid nanodiscs. We found that FtsH in membrane scaffold protein (MSP) nanodiscs maintains its native hexameric conformation and is functionally active. We further investigated the effect of the lipid bilayer composition (acyl chain length, saturation, head group charge and size) on FtsH proteolytic activity. We found that the lipid acyl chain length influences AaFtsH activity in nanodiscs, with the greatest activity in a bilayer of di-C18:1 PC. We conclude that MSP nanodiscs are suitable model membranes for further *in vitro* studies of the FtsH protease complex.

1. Introduction

The vast majority of proteins must fold into precise three-dimensional conformations in order to gain functional activity. However, in the cellular environment, proteins are prone to misfolding, causing the formation of aggregates and other toxic species [1]. To avoid these problems, cells are equipped with protein quality control machines, consisting of molecular chaperones and proteases [2,3]. Proteases are responsible for the removal of misfolded and non-functional proteins from the cell. In general, proteolysis in bacteria is mediated by energy-dependent AAA+ (ATPases associated with various cellular activities) proteases, that use ATP hydrolysis to unfold, translocate, and degrade protein substrates [4,5]. Various types of AAA+ proteases have been characterized in bacteria, including ClpXP, ClpAP, HslUV, Lon, and FtsH [4].

In *E. coli*, FtsH is the only membrane-bound and the only essential AAA+ protease [6]. FtsH is anchored to the cell membrane *via* its N-terminal domain by two transmembrane segments, while the C-terminal cytosolic part consists of the ATPase and the proteolytic domains [7]. The N-terminal domain of FtsH is involved in membrane localization, oligomerization, and its proteolytic activity against membrane proteins [8–10]. The ATPase domain unfolds and translocates substrates into a proteolytic chamber. In the cell membrane, FtsH forms a hexameric structure that is crucial for its catalytic activities [9,11,12]. Besides its function in the degradation of misfolded proteins, FtsH also serves a quality control function, recognizing various types of substrates including LPS biosynthesis machines and alternative sigma factors [13,14].

Recently, we studied the structure and function of *Aquifex aeolicus* FtsH (AaFtsH) in LMNG detergent micelles and found that AaFtsH is functionally active in detergents and has a very high structural flexibility [15]. Although detergent systems have proven useful in the study of protein structures, they have disadvantages in terms of stability and suitability for some biophysical and biochemical techniques [16]. In an attempt to study the function of FtsH in a more native environment, here we used lipid nanodiscs as model membranes. In contrast to detergents that form micelles around the protein, nanodiscs provide membrane proteins with a biologically relevant membrane environment. Lipid nanodiscs are usually formed by amphiphilic molecules, such as polymers (*e.g.*, styrene maleic acid [17,18]) or proteins (*e.g.*, membrane scaffold protein (MSP) [19,20]) wrapping around a lipid bilayer with a discoidal shape. MSP nanodiscs are wrapped with two copies of the MSP protein, which can stabilize membrane protein-lipid complexes [19,20]. MSP nanodiscs diameters range between 9 and 17 nm, depending on the length of the MSP [21]. Other model membranes like bicelles and liposomes have previously been used to study FtsH [22,23]. Nanodiscs have several advantages over bicelles and liposomes, in terms of stability, size homogeneity, control of lipid composition, and control of

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membrane protein oligomeric state [24,25]. In this work, we reconstituted the full-length AaFtsH complex into nanodiscs. We characterized the proteolytic activity of AaFtsH reconstituted in nanodiscs of varying particle sizes and lipid compositions.

2. Materials and methods

2.1. Materials

Lipids were purchased from Avanti Polar Lipids. The following lipids were used in this study: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylglycerol (POPG), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), 1,2-dimyristoleoyl-sn-glycero-3-phosphocholine (di-14:1 PC), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (di-14:0 PC), 1,2-dipalmitoleoyl-sn-glycero-3-phosphocholine (di-16:1 PC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (di-16:0 PC), 1,2-dioleoyl-sn-glycero-3-phosphocholine (di-18:1 PC), and 1,2-dierucoyl-sn-glycero-3-phosphocholine (di-22:1 PC). *n*-Dodecyl β-D-maltoside (DDM) and Lauryl Maltose Neopentyl Glycol (LMNG) were purchased from Anatrace. Plasmids MSP2N2 [21], MSP1E3D1, and MSP1D1 [25] were obtained from Addgene. HisTrap-5 mL column, Superose 6 10/300 GL column, and centrifugal filters were purchased from GE Healthcare. All other chemicals were purchased from Sigma-Aldrich.

2.2. Protein expression and purification

Plasmids containing *Aquifex aeolicus* FtsH (AaFtsH) were transformed into *E. coli* strain C43 and were expressed as described previously [15,26]. Cells were harvested at 3500 *g* for 25 min at 4 °C and disrupted using a French press. Cell debris was removed by centrifugation at 20,000*g* for 15 min and membranes were isolated at 125,000*g* for 3 h. Membranes were solubilized in 20 mM Tris-HCl pH 8.0; 100 mM NaCl; 1% (w/v) *n*-Dodecyl β-D-maltoside (DDM) for 2 h at 4 °C. The insoluble material was removed by centrifugation at 125,000*g* for 1 h at 4 °C. The sample was purified by affinity chromatography, using a HisTrap column. AaFtsH fractions were eluted in 20 mM Tris-HCl pH 8.0; 500 mM NaCl; 0.02% (w/v) DDM; 200 mM imidazole. AaFtsH fractions were combined and concentrated using a 50 kDa centrifugal filter before reconstitution into nanodiscs. Membrane scaffold proteins MSP2N2, MSP1E3D1, and MSP1D1 were expressed and purified as described previously, according to a protocol established by Sligar and co-workers [20,21,25]. Cells were resuspended in 20 mM Tris-HCl pH 8.0; 300 mM NaCl and were disrupted using a French press. The lysate was cleared by centrifugation at 40,000*g* for 1 h. The protein was purified using a HisTrap column. MSP fractions were eluted in 20 mM Tris-HCl pH 8.0; 300 mM NaCl; 400 mM imidazole. The fractions were combined and concentrated using a 10 kDa centrifugal filter.

2.3. Reconstitution of AaFtsH in MSP nanodiscs

The lipids were dried overnight and solubilized in 20 mM Tris-HCl pH 8.0 and 1% DDM. For nanodisc formation, the purified AaFtsH, MSP, and lipids were mixed at a molar ratio of 1:1:100 for MSP2N2 and 1:1:75 for MSP1E3D1. The reconstitution mixture was incubated for 1 h at near the phase transition temperatures (T_m) of the lipids (Tables S1 and S2). Reconstitution with DPPC (di-C16:0 PC) is done at 37 °C, di-C14:0 PC and di-C22:1 PC at 25 °C, and other lipids at 4 °C. The detergent was then removed by overnight incubation with Bio-beads. Biobeads were removed and the reconstituted mixture was centrifuged at 20,000*g* for 15 min. The nanodisc sample was concentrated and loaded into a Superose 6 10/300 GL SEC column pre-equilibrated with 20 mM Tris-HCl pH 8.0; 150 mM NaCl; 5% glycerol. The concentration of AaFtsH nanodiscs was estimated on SDS-PAGE gels, using detergent-solubilized AaFtsH of known concentration as standard. Empty nanodiscs without AaFtsH were prepared using the same protocol.

2.4. SEC-MALS analysis

The molecular weight of AaFtsH and the lipid content in nanodiscs were determined by size exclusion chromatography coupled to multi-angle light scattering (SEC-MALS). SEC-MALS measurements were performed on an Agilent HPLC system with an autosampler, connected to a Wyatt DAWN-HELEOS instrument. The data were processed using the protein conjugate analysis program within ASTRA software (Wyatt Technology). Input for refractive index increment, dn/dc, was 0.185 mL/g for protein [27] and 0.135 mL/g for lipids [28]. The nanodisc samples were run over a Superose 6 10/300 GL SEC column in 20 mM Tris-HCl pH 8.0; 150 mM NaCl at room temperature.

2.5. Negative staining electron microscopy

For negative staining electron microscopy (EM), the nanodisc samples were diluted and applied to a glow-discharged carbon-coated copper grid for 1 min. Excess fluid was removed and the samples were stained with 3% uranyl acetate for 1 min. Transmission electron microscopy was performed using a JEOL JEM-1400 plus equipped with a TVIPS TemCam-F416 camera.

2.6. Proteolytic activity assay

The proteolytic activity of AaFtsH in nanodiscs was assessed using β-casein [29] and resorufin-labeled casein as model substrates [30]. Before activity measurements, the concentration of AaFtsH reconstituted in nanodiscs of different lipid compositions was adjusted to a same level. β-casein was mixed with AaFtsH in protease buffer (50 mM Tris-HCl pH 8.0; 80 mM NaCl; 12.5 μM $ZnCl_2$; 5 mM $MgCl_2$) at 45 °C. Reactions were initiated by adding 5 mM ATP and were terminated after 0, 15, 30, and 60 min by adding SDS sample buffer. Samples were analyzed by SDS-PAGE gel and the amount of undegraded β-casein was quantified by densitometry. Protease assay using resorufin-labeled casein was performed in the same buffer condition at 45 °C, as described above. Reactions were terminated after 0, 15, 30, and 60 min by adding 5% trichloroacetic acid (wt/vol) and samples were centrifuged at 16,000*g* for 5 min. 400 μl of supernatant were mixed with 600 μL assay buffer (500 mM Tris-HCl pH 8.8). Protease activity was determined by measuring the absorbance at 574 nm.

3. Results

3.1. Purification and reconstitution of the full-length AaFtsH in MSP nanodiscs

AaFtsH with a C-terminal His-tag was overexpressed in *E. coli* cells and purified as previously described [15,26]. Purified AaFtsH was reconstituted into nanodiscs using the membrane scaffold protein MSP2N2 and POPC lipids. MSP2N2 is known to produce a nanodisc with a diameter of ~16 nm [25] and should be able to accommodate the 12 transmembrane helices of AaFtsH. A schematic picture of the reconstitution of AaFtsH into nanodiscs is shown in Fig. 1A. A AaFtsH:MSP2N2:POPC molar ratio of 1:1:100 was found to be optimal for minimum protein aggregation during AaFtsH reconstitution. Empty nanodiscs without AaFtsH were also prepared as control at a MSP2N2:POPC molar ratio of 1:150. Bio-beads were added to initiate the reconstitution by removing detergent from the system. After reconstitution, AaFtsH nanodisc samples were subjected to size exclusion chromatography (SEC).

For nanodisc preparation with AaFtsH, the SEC chromatogram displays two elution peaks at ~12 mL and ~15 mL (Fig. 1B). Nanodisc preparation without AaFtsH shows a single peak at ~15 mL. SDS-PAGE analysis revealed that AaFtsH co-elutes with MSP2N2 in the first elution peak representing AaFtsH nanodiscs. The second elution peak contains only the MSP2N2 protein, therefore representing empty nanodiscs

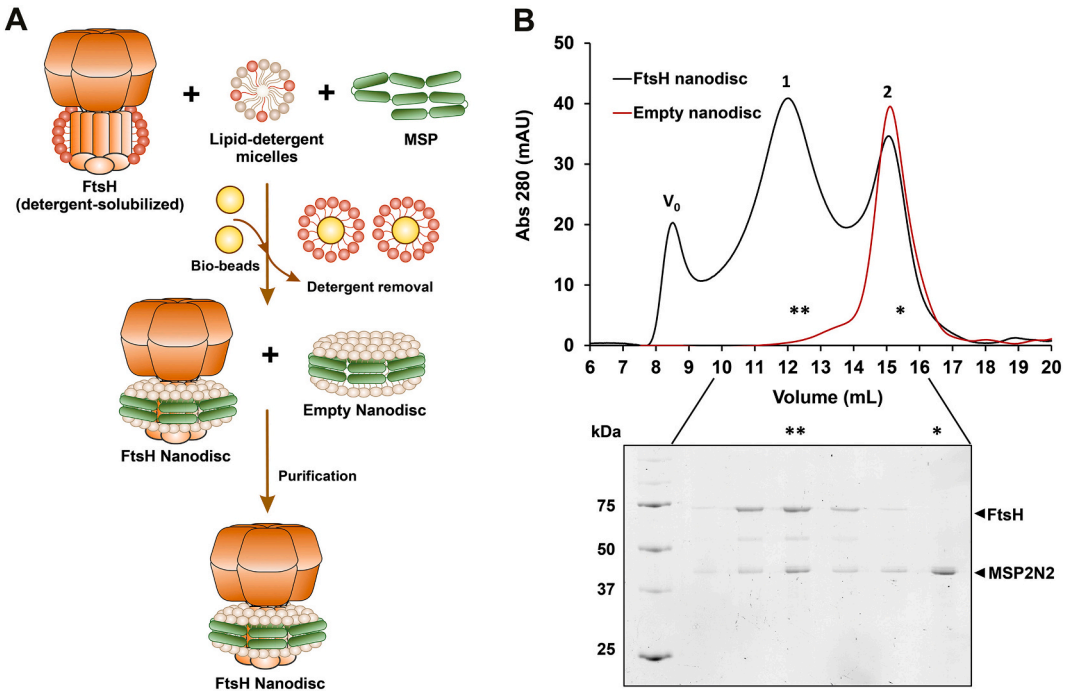

Fig. 1. FtsH nanodisc assembly. (A) Schematic representation of the reconstitution of FtsH into lipid nanodiscs. Detergent-solubilized FtsH was mixed with lipids and membrane scaffold proteins (MSPs). The formation of nanodiscs was initiated by the removal of detergent using bio-beads. (B) Size exclusion chromatography (SEC) profiles of FtsH nanodiscs (black line) and empty nanodiscs (red line). The chromatogram shows the absorbance at 280 nm. Void volume (V_o) and elution peaks (1 and 2) are indicated. The fractions from peak 1 and 2 were further analyzed by SDS-PAGE. Peak 2 contains empty nanodiscs (asterisk), while peak 1 contains FtsH nanodiscs because FtsH and MSP2N2 co-eluted from the SEC column (two asterisks). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 1B). The difference in elution volume between AaFtsH containing nanodiscs and empty nanodiscs likely occurs because of the large size of the cytosolic domain of AaFtsH, thus contributing to an increased hydrodynamic radius for the AaFtsH nanodiscs.

3.2. Characterization of AaFtsH MSP nanodiscs

To evaluate the oligomeric state of the AaFtsH complexes and their lipid content, nanodisc fractions were subjected to size exclusion chromatography combined with static light scattering (SEC-MALS). SEC-MALS accounts for the amount of lipid in nanodiscs and allows for determination of the molecular weight and the oligomeric state of proteins [31]. SEC-MALS experiments of AaFtsH nanodiscs resulted in an average molecular weight of ~515 kDa for the total protein content (Fig. 2A), which corresponds well to the molecular weight expected for two MSP2N2 (~45 kDa each) and one AaFtsH hexamer complex (~430 kDa). The average molecular weight of lipid determined by SEC-MALS was ~187 kDa, corresponding to a total of 246 POPC molecules per nanodisc. SEC-MALS experiments on empty nanodiscs revealed an average molecular weight of ~85 kDa for total protein corresponding to two MSP2N2 (theoretical mass of ~90 kDa), and a molecular weight of ~239 kDa for lipid corresponding to a total of 314 POPC molecules per nanodisc (Fig. 2B). The nanodisc fractions were further analyzed by negative stain electron microscopy. The images revealed particles with an average size of 16 nm as expected for AaFtsH and MSP2N2 nanodiscs (Fig. 2C and D).

Next, we assessed the proteolytic activity of AaFtsH hexamers reconstituted in nanodiscs. AaFtsH nanodiscs were incubated at 45 °C in the presence of the β-casein substrate in a protease buffer, with and without ATP. Our data show that AaFtsH retains its proteolytic activity after extraction from *E. coli* membranes and insertion into lipid nanodiscs (Fig. 3). We further compared the activity of AaFtsH nanodiscs with AaFtsH in DDM and LMNG detergent. AaFtsH is equally active in nanodisc and DDM, but has a slightly higher activity in LMNG (Fig. S1).

3.3. Effect of lipid on AaFtsH activity

The lipid environment can affect the structure and function of membrane proteins [32] and has been shown to be important for membrane-bound proteases [33–35]. To investigate the effect of lipid bilayer properties on AaFtsH activity, we reconstituted AaFtsH in nanodiscs with different lipid compositions such as various lipid head groups and acyl chain lengths (Tables S1 and S2). First, we investigated the influence of the lipid head group composition by reconstituting AaFtsH into nanodiscs consisting of POPC:POPG (1:1, molar ratio) or POPC:POPE (1:1, molar ratio). PG is a negatively charged lipid that is often needed for optimal activity of membrane proteins [32]. PE is a zwitterionic lipid similar to PC, but PE has nonbilayer properties due to a smaller cross-sectional area of its head group [36,37]. In the case of AaFtsH, the addition of PG and PE into nanodiscs did not lead to any significant change in AaFtsH activity (Fig. 4A and B). We further investigated the effect of changing the lipid acyl chain length on AaFtsH activity. We reconstituted AaFtsH into nanodiscs consisting of unsaturated PC lipids of varied acyl chain length; di-C14:1 PC, di-C18:1 PC, and di-C22:1 PC. AaFtsH has the highest proteolytic activity in nanodiscs with di-C18:1 PC. AaFtsH nanodiscs with di-C14:1-PC and di-C22:1 PC have lower activities (Fig. 4C and D). We also reconstituted AaFtsH into nanodiscs consisting of saturated PC lipids; di-C14:0 PC, di-C16:0 PC. AaFtsH activity is higher in nanodiscs consisting of di-C16:0 PC compared to the activity in nanodiscs of di-C14:0 PC (Fig. 4E and F). Together, these data show that AaFtsH activity is sensitive to the lipid acyl chain length in nanodiscs. We next investigated the effect of lipid saturation by comparing AaFtsH activity in nanodiscs consisting of unsaturated PC (di-16:1 PC) and saturated PC (di-16:0 PC). We found that AaFtsH activity is not significantly affected by lipid saturation (Fig. S2).

Next, we investigated the effect of nanodisc size on the activity of

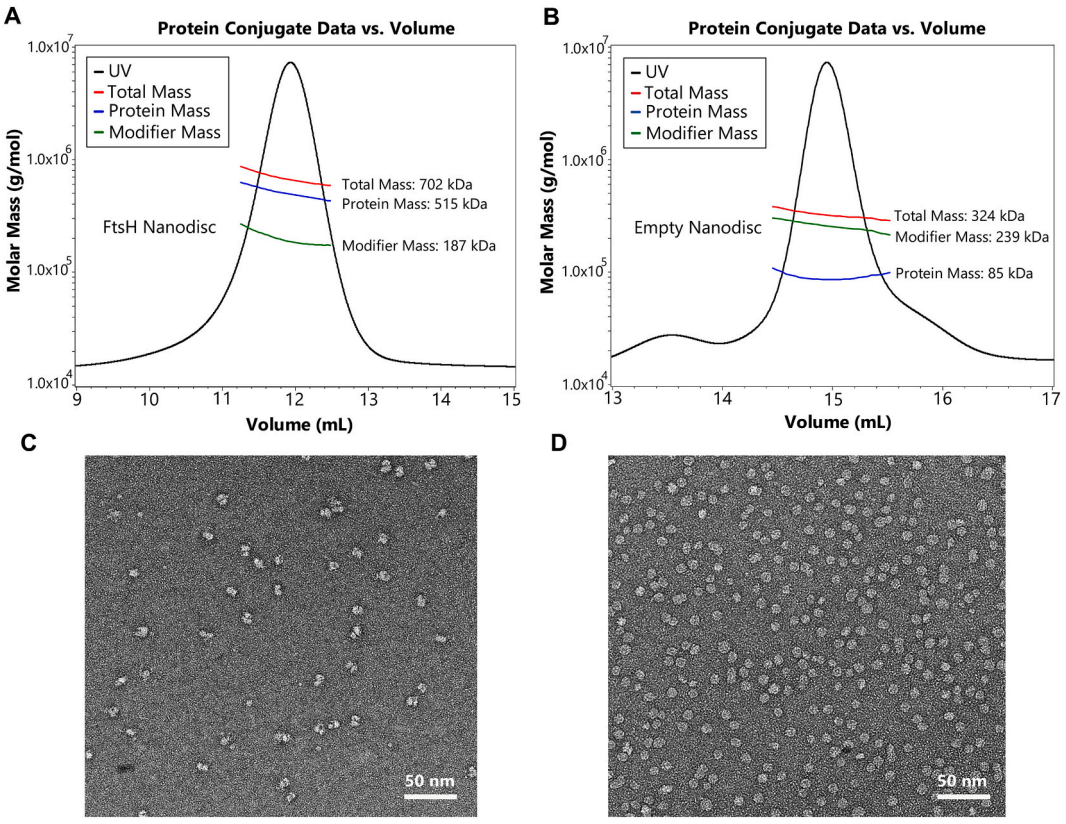

Fig. 2. FtsH nanodiscs characterization. (A) SEC-MALS analysis of FtsH nanodiscs and (B) empty nanodiscs. UV absorption chromatograms showing the distribution of the molar mass (g/mol) *vs.* retention volume (mL) for nanodisc complexes. The total calculated mass is shown as the red line across the elution peak, with the protein and modifier/lipid mass shown in blue and green respectively. The indicated mass values are the average molecular mass across the SEC peak calculated with the ASTRA software. (C) Negative stain electron microscopy image of the FtsH nanodiscs and (D) empty nanodisc samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

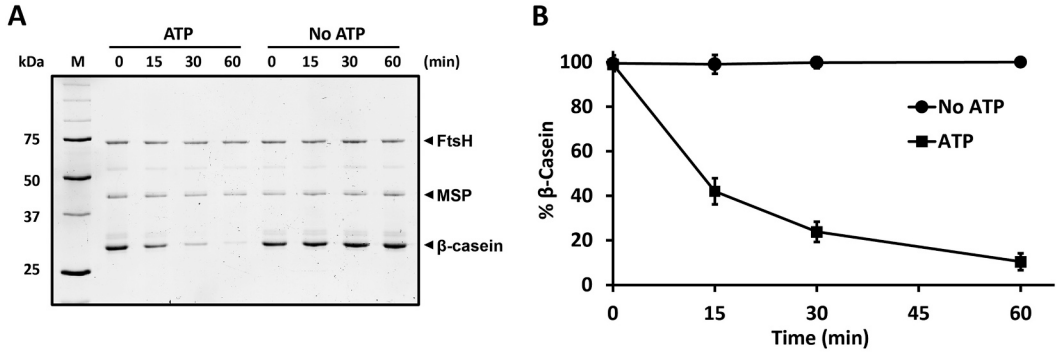

Fig. 3. Proteolytic activity of FtsH. (A) *In vitro* degradation of β-casein by FtsH reconstituted in nanodiscs in the presence or absence of ATP. Samples were taken at 0, 15, 30, and 60 min and analyzed by SDS-PAGE gels. (B) Kinetic plot of β-casein degradation from densitometric quantification of the SDS-PAGE gel. Data points are averages of independent replicates ($n = 3$).

AaFtsH. For this purpose, AaFtsH was reconstituted into smaller nanodiscs using MSP1E3D1 and MSP1D1, which according to their lengths, can form nanodiscs with ~12 nm and ~ 10 nm diameters, respectively [25]. For AaFtsH reconstitution with MSP1E3D1, the SEC chromatogram shows two elution peaks at around 12.5 mL and 15.5 mL. Nanodisc formation was confirmed by SDS-PAGE and SEC-MALS analysis (Fig. S3). Reconstitution of AaFtsH using MSP1D1 was not successful (data not shown), suggesting that a minimal diameter of 12 nm was necessary for proper insertion of AaFtsH transmembrane helices into the nanodiscs. We further compared the activity of AaFtsH in MSP2N2 and MSP1E3D1 nanodiscs. Proteolytic activity assays show that AaFtsH is equally active in both nanodiscs (Fig. S4), suggesting that, at least in this size range, the nanodisc size does not influence AaFtsH activity.

4. Discussion

To study the function of FtsH in a native-like environment, we have reconstituted the full-length *Aquifex aeolicus* FtsH complex into lipid nanodiscs. Reconstitution of AaFtsH in nanodiscs was confirmed by SDS-PAGE, SEC-MALS, and negative stain EM analysis. AaFtsH reconstituted in nanodiscs maintains its native hexameric conformation as confirmed by SEC-MALS analysis and is functionally active (Figs. 2 and 3). The negative stain EM images revealed homogeneous particles with an average size of ~16 nm as expected for AaFtsH, consistent with our

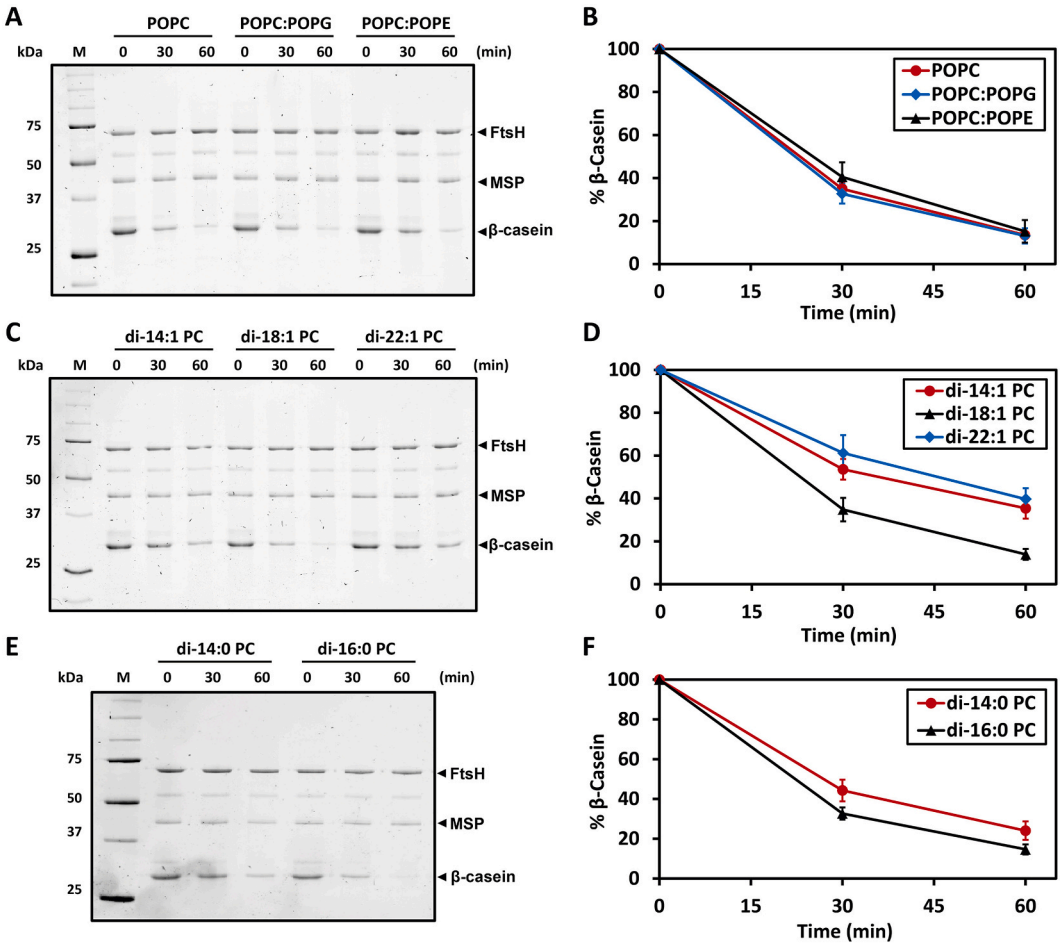

Fig. 4. Effect of lipid on FtsH activity. (A, B) *In vitro* degradation of β-casein by FtsH reconstituted in nanodiscs with different lipid head groups; POPC, POPC:POPG (1:1, molar ratio), and POPC:POPE (1:1, molar ratio). (C, D) *In vitro* degradation of β-casein by FtsH reconstituted in nanodiscs with mono-unsaturated PC lipids of varied acyl chain length; di-C14:1 PC, di-C18:1 PC, and di-C22:1 PC. (E, F) *In vitro* degradation of β-casein by FtsH reconstituted in nanodiscs with saturated PC lipids; di-C14:0 PC, and di-C16:0 PC. Samples were taken at 0, 30, and 60 min and analyzed by SDS-PAGE gels. The amount of β-casein was quantified by densitometry analysis. Data points are averages of independent replicates ($n = 3$).

previous report (Fig. 2) [15]. We compared the activity of AaFtsH reconstituted in nanodiscs with AaFtsH in detergent micelles. AaFtsH is equally active in nanodiscs and DDM, but has slightly lower activity than that of AaFtsH in LMNG (Fig. S1). This observation is likely related to the structural flexibility and substrate accessibility of the AaFtsH complex. The LMNG micelle could allow for greater flexibility and substrate accessibility, compared to lipid nanodiscs. However, this remains speculative, and determining the exact mechanism will require a comparison of high-resolution structures of AaFtsH in lipid nanodiscs and detergent micelles environment.

It has been proposed that protein substrates enter the translocation pore of FtsH *via* the space between the transmembrane domain and the cytosolic domain [15,38]. To evaluate how lipid bilayer properties might influence AaFtsH activity, we reconstituted AaFtsH in nanodiscs of different lipid compositions. The lipid membrane of *Aquifex aeolicus* is mainly formed by diacylglycerol phospholipids (DAGP), acyl ether glycerol phospholipids (AEGP), dietherglycerol phospholipids (DEGP) [39–41]. In addition to the presence of ether lipids, *Aquifex aeolicus* also contains many lipids with saturated fatty acids that enable their membranes to function at high temperatures, their alkyl and acyl chain lengths ranging from C14 to C22 [39]. We investigated the effect of the lipid acyl chain length, saturation, head group charge and size on AaFtsH proteolytic activity. The effect of the lipid head group compositions was studied by reconstituting AaFtsH into nanodiscs with 50% PG (negatively charged lipid) or 50% PE (nonbilayer lipid). These lipids are often needed for the optimal activity of membrane proteins [32,37]. We did not observe any significant changes in AaFtsH activity in nanodiscs with or without PG or PE (Fig. 4A and B). Thus, to our surprise, the presence of negatively charged lipid and nonbilayer lipid does not influence the function of AaFtsH. Interestingly, lipid acyl chain length influences AaFtsH activity in nanodiscs (Fig. 4C and D). According to the structure of AaFtsH in detergent micelles, the thickness of AaFtsH transmembrane domain is estimated to be ~40 Å [15], which is close to the thickness of di-C22:1 PC (~37 Å, Table S2) [42,43]. Surprisingly however, AaFtsH activity is lower in nanodiscs of di-C22:1 PC, indicating that soluble substrates have less efficient access to the active site of AaFtsH. The highest activity is achieved when reconstituting AaFtsH in nanodiscs formed by di-C18:1 PC, with a bilayer thickness of ~30 Å. Thus, the di-C18:1 PC likely provides the optimal bilayer thickness for AaFtsH to form the most accessible conformation for substrate entry. This finding also suggests another functional link between membrane association and the proteolytic activities of FtsH, in addition to the previously shown results [10,12].

In conclusion, we have successfully reconstituted full-length AaFtsH hexamers in MSP lipid nanodiscs in a functional state. Our study shows that the lipid bilayer thickness influences AaFtsH activity in nanodiscs, with the optimum activities in a bilayer of di-C18:1 PC. We believe that nanodiscs are a suitable model for further functional studies of the FtsH protease complex in a biologically relevant membrane environment.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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