

Short Review

**Quality control of Photosystem II: the molecular basis for the action of FtsH protease and the dynamics of the thylakoid membranes**

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**Abstract**

The reaction center-binding D1 protein of Photosystem II is damaged by excessive light, which leads to photoinhibition of Photosystem II. The damaged D1 protein is removed immediately by specific proteases, and a metalloprotease FtsH located in the thylakoid membranes is involved in the proteolytic process. According to recent studies on the distribution and organization of the protein complexes/supercomplexes in the thylakoid membranes, the grana of higher plant chloroplasts are crowded with Photosystem II complexes and light-harvesting complexes. For the repair of the photodamaged D1 protein, the majority of the active hexameric FtsH proteases should be localized in close proximity to the Photosystem II complexes. The unstacking of the grana may increase the area of the grana margin and facilitate easier access of the FtsH proteases to the damaged D1 protein. These results suggest that the structural changes of the thylakoid membranes by light stress increase the mobility of the membrane proteins and support the quality control of Photosystem II. (159 words)

**Keywords:** Photosystem II; light stress; FtsH protease; grana; thylakoid

**Contents**

1. Introduction
2. Damage and degradation of the D1 protein under light and heat stresses
3. Molecular structure and general function of the FtsH protease
4. Specific roles of FtsH proteases in cyanobacteria and chloroplast thylakoids under light or heat stress
5. Assembly and distribution of FtsH proteases within spinach thylakoid membranes
6. Light- or heat-induced unstacking of spinach thylakoids

## 1. Introduction

The D1 protein that binds the reaction center of Photosystem II (PSII) is easily damaged by illumination with strong and prolonged light [1-5]. Reactive oxygen species (ROS), in particular singlet oxygen ( $^1\text{O}_2$ ) molecules, produced by the photochemical reaction in PSII are responsible for the damage [1-3, 6-12]. The damage reduces PSII activity and is believed to be the major cause for photoinhibition under high light conditions [1-3, 6, 13]. The damaged D1 protein is immediately removed from the PSII complex by proteolysis following transport from the grana core to the grana margins [14, 15]. Spatial rearrangement of other subunit proteins of the PSII complex and the associated light-harvesting complexes may also take place in the grana [6, 16-20]. After these steps, a new copy of the D1 protein is inserted co-translationally into the D1-depleted PSII complex through thylakoid-bound ribosomes [3-5, 21-23]. Phosphorylation and dephosphorylation of PSII proteins are involved in the PSII repair cycle. By kinases STN8 and STN7, PSII complex and the light-harvesting chlorophyll-protein complexes (LHCII) are phosphorylated in light [24, 25]. The phosphorylation retards degradation of the D1 protein by proteases [26]. After migration of the damaged PSII complex from the grana to the grana margins and stroma thylakoids, a phosphatase dephosphorylates the PSII complex, which initiates degradation of the damaged D1 proteins by Deg proteases and/or FtsH proteases [27, 28]. The presence of such a finely coordinated repair cycle of the photodamaged D1 protein in the thylakoid membranes ensures that the PSII system maintains its activity even under severe light stress.

Similar damage and degradation processes of the D1 protein were also seen under moderate heat stress when spinach thylakoids were incubated at 40 °C for 30 min in the dark (hereafter heat-inactivation of PSII) [29, 30]. In both photoinhibition and heat-inactivation, the protease systems work for swift repair of PSII in the thylakoid membrane, and also possibly in the stroma and in the thylakoid lumen [6]. Deg proteases and FtsH proteases are likely to be the proteases responsible for the degradation of the D1 protein [29, 31-38]. However, there are still many important questions to be answered in the degradation mechanism of the D1 protein. For example, where the damaged D1 protein is degraded in the thylakoids and how the proteases recognize the damaged D1 protein among the many proteins and protein complexes in the highly crowded thylakoids represent major unanswered questions. In this review article, we describe the outline of this attractive subject and present our recent research progress to solve these questions.

## **2. Damage and degradation of the D1 protein under light and heat stresses**

The D1 protein plays a central role in PSII [12]. The D1 protein ligates the most important and photoactive components of the reaction center. However, because of its important location and function in PSII, the D1 protein is damaged rather easily when anomalous photochemical reactions take place under strong illumination conditions. Singlet oxygen ( $^1\text{O}_2$ ) and hydroxyl radicals ( $\text{HO}^\bullet$ ), which are typical ROS, are produced around PSII when this system is exposed to excessive light [2, 3, 6, 39, 40]. This is the so-called acceptor-side photoinhibition of PSII. Endogenous cationic radicals such as  $\text{P680}^+$  or  $\text{Try Z}^+$ , which are the oxidized primary and secondary electron donors of PSII, may also be produced when PSII has the impaired donor side illuminated. This explains the mechanism of the donor-side photoinhibition of PSII. More recently, it was also suggested that lipid peroxidation induced by strong illumination accelerates the damage to the D1 protein by producing  $^1\text{O}_2$  [41].

Heat-inactivation of PSII induced by incubation of the thylakoids at 40 °C for 30 min in the dark is quite similar to the acceptor-side photoinhibition. Here, the damage appears only with the D1 protein and not with other PSII subunits [29]. The cleavage of the protein leaves a fragment of the D1 protein, the molecular mass of this fragment is similar to that observed in the acceptor-side photoinhibition. Heat-inactivation of PSII depends on the presence of oxygen, and under aerobic conditions the protein cleavage is inhibited by the addition of Na-ascorbate [42]. These results suggest that the heat-inactivation of PSII is not due to simple heat-induced denaturation of the protein, but due to oxidative damage to the protein. To support this hypothesis, it was found by EPR spin trapping that  $^1\text{O}_2$  is produced during the heat-inactivation of PSII [42].

The proteases responsible for the degradation of the photo- or heat-damaged D1 protein have attracted significant attention in this field. The best candidate responsible for this degradation of D1 may be the metalloprotease FtsH [14, 29, 35, 43-46]. Historically, the Deg protease was first proposed as a protease candidate [31, 37, 38, 47], but there has been controversy over the exact roles of Deg and FtsH in the proteolytic process of the photodamaged D1 protein [32, 48-50]. The discussion on this issue is ongoing with newly obtained data. For the degradation of the D1 protein in the heat-inactivated PSII, it has been proposed that the same FtsH protease that functions in the proteolysis of the photodamaged D1 protein is involved [29].

## **3. Molecular structure and general function of FtsH proteases**

FtsH proteases play an important role in quality control of both prokaryotic and eukaryotic cells. The *ftsh* gene encoding the FtsH protease was first described with *E.coli* [51-53]. The gene encodes a 71 kDa polypeptide, and its homologs have been identified in other bacteria, cyanobacteria and mitochondria, and chloroplasts of eukaryotes [54-56]. In *E.coli*, FtsH has been shown to be involved in the degradation of the heat shock transcription factor  $\sigma^{32}$  and other membrane proteins to maintain cell viability [57-59]. In addition to its function as a protease, FtsH was shown to work as a chaperone involved in protein assembly and folding [60]. FtsH proteases belong to the ATPase associated with various cellular activities (AAA) protein family [61]. The main hydrophilic portion of FtsH consists of the AAA ATPase with the conserved Walker A and B motifs, a  $Zn^{2+}$ -metalloprotease domain, and second region of homology (SRH) motifs (Fig. 1). Each subunit has two transmembrane helices, and the helices are connected with a loop. Stimulation of the protease activity by zinc ions indicates that FtsH belongs to the family of zinc metalloprotease. The arginine residue at the C-terminus of the SRH motif, the so-called 'arginine finger', is crucial for ATP hydrolysis [54, 62]. The AAA family proteins assemble into oligomers, often hexamers, and form a ring-shaped structure with a central pore that has the catalytic site. FtsH forms a hexameric ring structure [63, 64] and can initiate proteolysis depending on the length of the tail of the target substrate from both the N- and C-terminus [65]. Furthermore, FtsH can work both as an exoprotease and an endoprotease [66]. This bidirectionality in the processive digestion makes it possible for FtsH to promptly degrade unnecessary membrane proteins. FtsH genes exist not only in bacteria but also in photosynthetic organisms, such as cyanobacteria and higher plants [67-69]. An FtsH homolog in higher plants was first found by an immunological analysis with an antibody against *E. coli* FtsH protease, which was shown to cross-react with a protein in spinach thylakoid membranes [70]. Whereas FtsH in *E. coli* is encoded by a single gene, 12 *ftsh* genes have been identified in *Arabidopsis thaliana*. Of these genes, nine are located in the chloroplasts (FtsH1, 2, 5-9, 11 and 12), while the remaining three (FtsH3, 4 and 10) are in the mitochondria [55, 67, 70, 71]. In the thylakoid membranes, there are four FtsH proteases (FtsH1, 2, 5 and 8).

#### **4. Specific roles of FtsH proteases in the cyanobacteria and chloroplast thylakoids under light and heat stresses**

The importance of the FtsH proteases in oxygenic photosynthetic organisms is well acknowledged in the study of the cyanobacterium *Synechocystis* sp. PCC6803, where

four genes *slr1390*, *slr0228*, *slr1604* and *slr1463* were found to be homologous to the bacterial FtsH (FtsH1, 2, 3 and 4) [45, 69, 72]. Of the four homologs, FtsH1 and 3 are required for cell viability while FtsH2 and 4 are not essential for the survival of the cyanobacterium [68, 69]. Further studies, however, showed that a mutant lacking the FtsH2 is sensitive to light stress compared with the wild-type protein, and the photodamaged D1 protein was found to accumulate in the mutant thylakoids. A series of works using  $\Delta$ *slr0228* cells demonstrated that FtsH2 is involved in the primary cleavage of the photodamaged D1 protein [35, 45, 73]. It has been reported that FtsH2 (*slr0228*) proteases is also involved in the repair of damaged PSII by UV-B radiation [74]. In this proteolysis, it was demonstrated that the substrate D1 protein is degraded from the N-terminus. However, such a “processive” degradation of the D1 protein does not apply apparently in the degradation of the damaged D1 protein in higher plant chloroplasts under high light or high temperatures [29, 30, 39].

Actually there was a lengthy period confusion in the identification of the proteases participating in the primary and secondary cleavage of the damaged D1 protein. Initially Deg proteases, which are the other prokaryotic proteases found in cyanobacteria and higher plant chloroplasts, were the most promising protease candidate [31]. Through this and other related works, the D1 protein was suggested to be degraded first by Deg proteases [31], then further degraded by FtsH proteases [36, 37]. The FtsH proteases were then suggested as the proteases responsible for the primary cleavage of the D1 protein [35, 45]. More recently, the role of Deg proteases was reevaluated in the study of *Arabidopsis* Deg/FtsH double mutants, and a reversed order of the action of FtsH and luminal Deg was suggested [32, 75]. Here, we propose a hypothesis that the protease that operates initially is dependent on which site of the D1 protein is photodamaged first by the excessive light (Fig. 2). Photoinhibition of PSII under strong illumination conditions are caused by two mechanisms, namely the donor-side mechanism and acceptor-side mechanism [6]. The cleavage site of the D1 protein may depend on the mode of photoinhibition. The donor-side photoinhibition should cause damage to a lumen-exposed loop such as the AB loop [76, 77] and CD loop [77-80], whereas the acceptor-side photoinhibition is claimed to involve damage to the DE loop exclusively [78, 81-83]. As already discussed, the damage to the D1 protein and other PSII subunits are caused by the action of ROS and endogenous cationic radicals produced under light stress, and in the case of ROS produced by the acceptor-side photoinhibition in particular, the diffusion of the ROS within their lifetime should determine the actual site of the damage. Considering that ROS are produced near the PSII complexes, which are highly abundant in the grana regions of the thylakoids, the FtsH may function by

initially being excluded from the grana to avoid direct oxidative damage from the ROS in the grana regions. This may be the reason why we find most of the FtsH proteases in the stroma thylakoids and grana margins in non-stressed thylakoids. There is also an extrinsic PsbO protein associated with the luminal side of PSII [84], working to stabilize catalytic Mn in the oxygen evolution and also to scavenge the ROS in the lumen compartment [85, 86]. The PsbO protein may protect the proteases in the lumen from oxidative damage. However, if the donor-side photoinhibition of PSII takes place, the Deg proteases in the lumen may be inactivated by the strong oxidants produced at on luminal side of PSII. Thus when the donor-side photoinhibition is predominant, the FtsH proteases should play a primary role in the degradation of the damaged D1 protein. Considering these points, it is possible that proteolysis occurs at the site that is different from the damaged site. For example, the AB and/or CD loop of the D1 protein may be damaged by the donor-side photoinhibition, but the actual cleavage of the protein may take place on the stromal side of the protein by the action of stroma-exposed FtsH proteases.

It was shown previously the active role of FtsH proteases in the proteolysis of the D1 protein in the heat-inactivated PSII in spinach thylakoids [29]. The requirement of metals, in particular Zn, and ATP in the proteolysis support the role of FtsH. However, the trials to detect FtsH by mass spectroscopy in the fraction containing the proteolytic activity was not successful, and hence, direct evidence of the participation of FtsH in the degradation step of the D1 protein in the heat-inactivated PSII is still not available. Actually purification of FtsH from thylakoids is not an easy task because of the small content of the protease and difficulty in solubilizing the protease from membranes. The experiment using the cyanobacterial mutant lacking the *ftsh* gene (slr0228) (*Synechocystis* sp. PCC6803,  $\Delta$ FtsH2); however, showed that the FtsH protease is involved in the degradation of the D1 protein under moderate heat stress [87].

## **5. Assembly and distribution of FtsH proteases on spinach thylakoid membranes**

In a recent biochemical study using a strain of *Synechocystis* sp. PCC6803 expressing a GST-tagged FtsH, it was shown that FtsH2 forms a hetero-oligomeric complex with FtsH3 [88]. To obtain information about the details in the structure of FtsH2-GST/FtsH3 complex, transmission electron microscopy [58] and single particle analysis were performed. From these results, a three-dimensional hexameric model was constructed at a resolution of 26 Å, which provided insights into the advantages associated with FtsH protease preferably forming hetero-complexes over homo-complexes.

FtsH proteases in the thylakoids of higher plant chloroplasts exist as hetero-hexamers composed of type A (FtsH1/5) and type B (FtsH2/8) subunits at a ratio of 1:2 [71, 89-91]. In higher plant chloroplasts, the thylakoid membranes form grana stacks that occupy 80% of whole thylakoids, the remaining 20% being the stroma thylakoids [92]. The FtsH hexamer has a relative molecular mass of 400–480 kDa, and such a large hexameric FtsH is most likely to be located in the unstacked regions of the thylakoids. Because of the size of FtsH proteases (height ~6.5 nm), they are most likely excluded from the stacked grana core [93-95]. Another important reason for localizing FtsH outside the grana is dephosphorylation of the damaged D1 protein by a phosphatase. In a recent study on dephosphorylation of PSII core, a phosphatase PBCP was identified in the chloroplast stroma [27]. After dephosphorylation of PSII core proteins by PBCP at the grana margins, degradation of the damaged D1 protein by FtsH proteases starts.

Recently it was found that FtsH proteases form not only hexamers but also monomers and dimers in the stroma thylakoids [14]. This observation suggests that the monomers and dimers represent partially assembled forms of FtsH, which turn into active hexamers under high light (Fig. 3). It was reported that the hexameric FtsH proteases are abundant in the PSII-enriched membranes including the grana margins [14]. It is likely that the active hexameric FtsH proteases are located near the PSII complexes in the grana margins and involved in the rapid degradation of the damaged D1 protein. Indeed, strong light-induced and FtsH-dependent degradation of the D1 protein was shown to occur in the PSII membranes [14]. It was also noted that once FtsH forms a hexamer, it is relatively resistant to light stress and heat stress.

## **6. Light- or heat-induced unstacking of thylakoids**

When the thylakoid membranes are subjected to light stress, structural changes of the thylakoid membranes occur [96]. Previously, it was reported that the stacked thylakoids became unstacked under strong illumination [97]. The photodamage to the D1 protein and generation of harmful hydroxyl radicals were remarkably stimulated in the stacked thylakoids, whereas this was not so dramatic in the unstacked thylakoids. According to these findings, it was proposed that unstacking of the thylakoid membranes under high light accelerates the lateral movement of the proteins, which supports rapid migration of the damaged PSII on the thylakoid membranes for repair [97]. Thus, it is likely that higher plants change the structure of the thylakoid membranes dynamically under light stress in order to protect the photosystems.

There is another report showing that phosphorylation of PSII complex is associated



with the structural changes of the thylakoid membranes and degradation of the damaged D1 protein [98]. By observation with transmission electron microscopy (TEM), it was shown that the thylakoid grana isolated from *stn7*×*stn8* and *stn8* mutant *Arabidopsis thaliana* exposed to high light have longer grana stacks and stroma thylakoids compared with the wild type. It was further hypothesized that phosphate groups unfold the tightly appressed thylakoid grana through electrostatic repulsion and facilitate the lateral protein diffusion from the grana to the stroma thylakoids [98]. A grana disc having a large diameter is inconvenient for repair of the damaged PSII because lateral protein diffusion of PSII subunits between the grana and stroma thylakoids is required for degradation of the D1 protein [99]. Therefore, it is suggested that although protein phosphorylation by STN7 and STN8 is not crucial for PSII repair cycle [24] they are needed to modulate the structure of grana under high light for efficient repair of PSII [18, 98, 100].

Recently it was reported that thylakoid swelling induced by strong light facilitates electron transport in the lumen mediated by plastocyanin and protein mobility in the thylakoids for the repair of PSII [95]. Strong light also caused lateral shrinkage of the grana, thereby decreasing the diameter of the grana [20]. This may help repair the photodamaged D1 protein by the repair system that is present in the grana margins and the stroma thylakoids. We need to know exactly what happens in the grana during light stress, using various techniques of electron microscopy. Our recent results obtained with spinach leaves using TEM showed that partial unstacking of the grana with outward bending of the stroma thylakoids at the ends of the grana takes place under strong illumination (unpublished data). This outward bending of the stroma thylakoids should increase the area of the grana margin significantly and help the swift repair of photodamaged D1 protein in the PSII complexes abundant in the grana. Furthermore, a light-induced increase in the fluidity of the grana regions was suggested [17, 20], and this fluidity change may also be brought about by partial thylakoid unstacking under light stress.

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## Figure legends

**Fig. 1.** Schematic representation of sequence of *Arabidopsis* FtsH2 protease. (A) The model of FtsH protease in thylakoid membrane. FtsH monomer has two transmembrane helices and a large hydrophilic region. FtsH protease forms a hexameric ring structure with a central pore. (B) Characteristics of the sequence of FtsH2 is shown as a horizontal bar with the transmembrane regions (TM), ATPase domain (AAA ATPase), second region of homology (SRH), Zn<sup>2+</sup>-metalloprotease domain (Protease) and coiled-coil region (Coil).

**Fig. 2.** Model of the photodamage and degradation of the D1 protein. The FtsH protease is an ATP-dependent zinc metalloprotease that has two transmembrane helices. FtsH1, 2, 5 and 8 exist in thylakoid membranes of chloroplasts. Deg protease is a periplasmic ATP-independent serine protease. Deg1, 5 and 8 are peripherally attached to the luminal side of thylakoid membrane. Deg2 and 7, and FtsH1, 2, 5 and 8 are located on the stromal side of thylakoid membrane. The transmembrane helices of the D1 protein are marked A to E. FtsH proteases and the Deg2 protease are involved in the cleavage of the D1-DE loop [31, 43]. The Deg7 protease participates in the primary cleavage of the photodamaged D1 protein [47]. Deg5 and 8 proteases are involved in the cleavage of the D1-CD loop [38]. The remaining Deg 1 protease possibly cleaves the D1-CD loop and downstream of the helix E [37].

**Fig. 3.** Assembly of FtsH protease and access to the D1 protein. The FtsH protease is located near the PSII complex; however, because of the size of its hydrophilic portion and the narrow width of the grana partition gap, the FtsH protease cannot interact with the D1 protein under normal conditions. Under light stress, monomerization of PSII dimers, dissociation of the associated light-harvesting complexes (LHCII) from the PSII core complexes and detachment of CP43 from the PSII complexes occurs. In parallel with these steps, thylakoid membranes show unstacking, which may dramatically improve the interaction between the damaged D1 proteins and FtsH proteases. The rapid degradation of the damaged D1 protein takes place in the grana region without long migration to the stroma thylakoids.

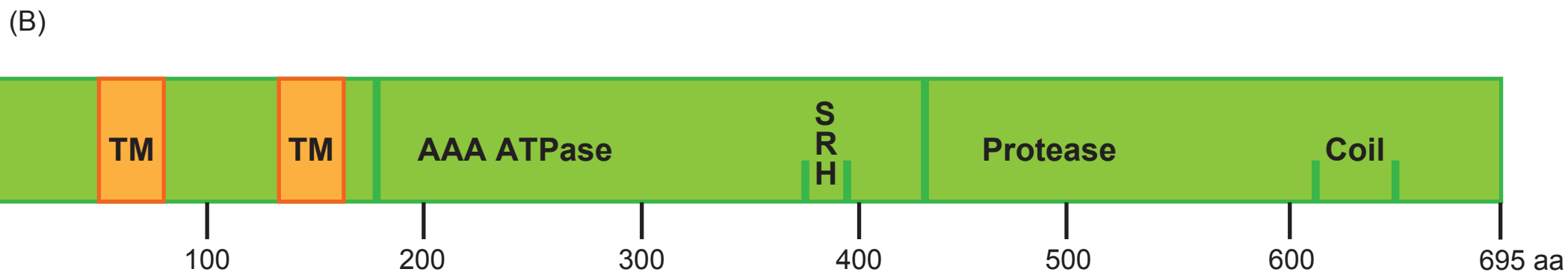
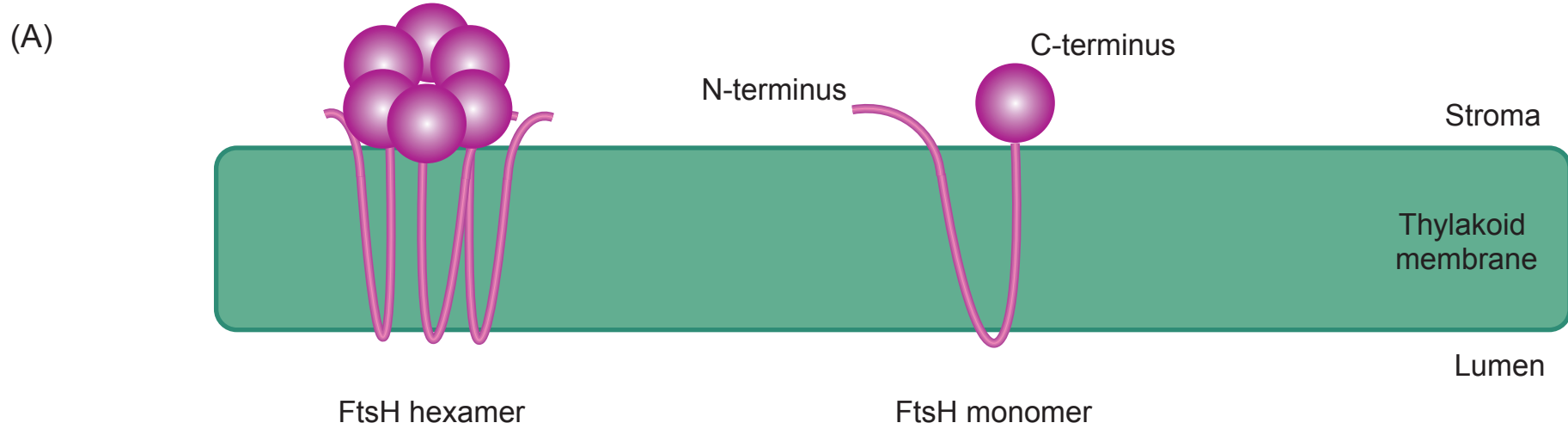


Fig. 1