Published in final edited form as:

J Am Chem Soc. 2018 April 04; 140(13): 4656–4665. doi:10.1021/jacs.8b00832.

Folding-Degradation Relationship of a Membrane Protein Mediated by the Universally Conserved ATP-dependent Protease FtsH

Yiqing Yang^{†,||}, Ruiqiong Guo^{†,||}, Kristen Gaffney^{‡,||}, Miyeon Kim[†], Shaima Muhammednazaar[†], Wei Tian[§], Boshen Wang[§], Jie Liang[§], and Heedeok Hong^{*,†,‡}

[†]Department of Chemistry, Michigan State University, East Lansing, MI 48824, USA

[‡]Department of Biochemistry & Molecular Biology, Michigan State University, East Lansing, MI 48824, USA

[§]Department of Bioengineering, University of Illinois at Chicago, Chicago, IL 60607, USA

Abstract

ATP-dependent protein degradation mediated by AAA+ proteases is one of the major cellular pathways for protein quality control and regulation of functional networks. While a majority of studies of protein degradation have focused on water-soluble proteins, it is not well understood how membrane proteins with abnormal conformation are selectively degraded. The knowledge gap stems from the lack of an *in vitro* system in which detailed molecular mechanisms can be studied as well as difficulties in studying membrane protein folding in lipid bilayers. To quantitatively define the folding-degradation relationship of membrane proteins, we reconstituted the degradation using the conserved membrane-integrated AAA+ protease FtsH as a model degradation machine and the stable helical-bundle membrane protein GlpG as a model substrate in the lipid bilayer environment. We demonstrate that FtsH possesses a substantial ability to actively unfold GlpG, and the degradation significantly depends on the stability and hydrophobicity near the degradation marker. We find that FtsH hydrolyzes 380–550 ATP molecules to degrade one copy of GlpG. Remarkably, FtsH overcomes the dual-energetic burden of substrate unfolding and membrane dislocation with the ATP cost comparable to that for water-soluble substrates by robust ClpAP/XP proteases. The physical principles elucidated in this study provide general insights into membrane protein degradation mediated by ATP-dependent proteolytic systems.

Graphical abstract

*Corresponding Author: honghd@msu.edu.

^{||}**Author Contributions:** These authors equally contributed to this work.

Supporting Information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Detailed experimental procedures and additional figures including analysis of the orientation distribution of FtsH in vesicles, GlpG activity, description of GlpG constructs for *in vivo* and *in vitro* degradation, analysis of Western blotting data, validation of the fluorescence-based *in vitro* degradation assay, validation of the steric trapping strategy, data for unfolding kinetics of GlpG, description of the computational strategy to calculate dislocation free energy, ATPase activity of FtsH with bound substrates, analysis of ATPase and degradation assays, and folding parameters of GlpG and various model substrates. (Figures S1-S10 and Table S1-S6) (PDF)

ORCID: Heedeok Hong. 0000-0003-0118-5193

Notes: The authors declare no competing financial interest.

Introduction

ATP-dependent proteolysis is an essential cellular process to degrade damaged or misfolded proteins and to modulate the concentration of regulatory proteins¹. This process is carried out by AAA+ proteases including ClpXP, ClpAP, Lon, HslVU, FtsH and 26S proteasomes, in which hexameric AAA+ ATPase binds substrates possessing degradation markers, and actively unfolds and translocates them into a protease chamber for degradation². Current knowledge on ATP-dependent proteolysis is largely limited to degradation of water-soluble proteins with insufficient understanding for membrane proteins. This discrepancy is mainly due to the lack of a proper *in vitro* system for detailed analysis of degradation mechanisms. Also, inherent difficulties in studying membrane protein folding have hindered investigation of the quantitative relationship between folding and degradation.

FtsH family proteins are widely conserved AAA+ proteases localized in the inner membranes of bacteria and mitochondria, and in the thylakoid membranes of chloroplasts³. They generally consist of an N-terminal transmembrane (TM) domain, and aqueous C-terminal AAA+ and Zn²⁺-metalloprotease domains, which assemble into a hexamer for function^{4,5} (Figure 1a). In their respective membranes, FtsH proteases serve as major protein quality inspectors by degrading misassembled or damaged membrane proteins as well as short-lived water-soluble enzymes and transcription factors⁶. In *E. coli*, disruption of the gene encoding FtsH induces the envelope stress response and leads to a severe growth defect⁶. In yeast, defects in FtsH orthologs cause impaired assembly of the respiratory chain and alter mitochondrial morphology^{7,8}. In plant, FtsH in chloroplasts mediates turnover of the photo-oxidized core proteins of the photosynthetic reaction center and unassembled subunits in the electron transfer chain^{9,10}. In human mitochondria, mutations in FtsH orthologs paraplegin and AFG3L2 are implicated in severe neurological disorders such as spastic paraplegia and spinocerebellar ataxia^{11,12}.

Compared to other AAA+ proteases, FtsH is known to possess unique features in recognition of substrates. FtsH is the weakest at hydrolyzing ATP and unfolding water-soluble model proteins¹³. While ClpXP and ClpAP rapidly unfold and degrade stable water-soluble proteins¹⁴⁻¹⁶, FtsH cannot degrade them^{17,18}. FtsH degrades a misfolded variant of the membrane protein diacylglycerol kinase (DGK), but cannot degrade wild-type or a thermostable variant *in vivo*¹⁸. Therefore, it has been proposed that FtsH lacks robust unfoldase activity, which allows for selective degradation of unstable or misfolded proteins. These findings also suggest that FtsH degrades proteins depending on their spontaneous unfolding, and ATP hydrolysis is mainly used to translocate unfolded substrates¹⁸.

To understand how FtsH degrades membrane proteins, several key questions need to be answered. First, although FtsH is known to lack the ability to actively unfold water-soluble substrates, the relationship between the intrinsic folding properties of a membrane substrate (e.g., stability and unfolding rate) and its degradation has not been quantitatively studied. To define the folding-degradation relationship, it is necessary to establish a model membrane substrate with defined structure and folding properties that can be quantitatively characterized. Second, to degrade membrane substrates, FtsH dislocates their hydrophobic TM segments from the bilayer to the proteolytic active site in the cytosol using ATP hydrolysis¹⁷. Single-molecule force spectroscopy and thermodynamic analysis indicate that dislocating a single TM helix from the bilayer to water in an unfolded form requires an enormous free energy cost (50–100 kcal/mol)^{19,20}. With the proposed weak unfoldase activity, how can FtsH drive such a vastly unfavorable process? Third, the proposed weak ATPase and unfoldase activities have been mainly derived from *in vitro* studies in micellar solution^{13,18}. However, does the micellar condition fully support native FtsH activity?

To answer these questions, we reconstituted membrane protein degradation mediated by FtsH of *E. coli* using the stable helical-bundle membrane protein GlpG of *E. coli* as a model substrate in the bilayer environment provided by bicelles. Bicelles are aqueous lipid-detergent assemblies in which bilayer fragments are edge-stabilized by certain detergents²¹. Measuring the stability and unfolding rate of GlpG directly under native conditions was enabled by steric trapping²², a novel method for studying membrane protein folding. We demonstrate that contrary to the previous proposals, FtsH can actively unfold the thermodynamically and kinetically stable membrane protein GlpG and the degradation is significantly influenced by the unfolding energy landscape and the hydrophobicity of the substrate. FtsH efficiently utilizes ATP hydrolysis to degrade membrane proteins, overcoming the dual energetic burden of substrate unfolding and membrane dislocation.

Experimental Section

Preparation of FtsH

Full-length FtsH was cloned into pET21a vector with a C-terminal His₆-tag. FtsH was expressed in *E. coli* C43 (DE3) strain. Procedures for purifying FtsH are described in Supporting Methods.

ATPase Activity Assay

ATP hydrolysis by FtsH was measured by an enzyme-coupled assay in 20 mM HEPES (pH 7.8), 10 mM MgCl₂, 0.4 mM ZnCl₂, 0.1% BME, 100 mM KCl, 15% glycerol, 0.5 mM NADH, 10 mM phosphoenolpyruvic acid (PEP), and 5 units/ml of pyruvate kinase and lactic dehydrogenase at 37°C. ATP hydrolysis coupled to the oxidation of NADH was monitored using OD_{340nm} on a microplate reader (M5e, Molecular Devices). Procedures of data analysis are described in Supporting Methods.

Preparation of Bicelles

15% (w/v) stock of DMPC/DMPG/CHAPS (lipid-to-detergent molar ratio, $q = 2.8$) bicelles were prepared by hydrating DMPC/DMPG (molar ratio = 3:1) mixture with water and adding 20% (w/v) CHAPS to reach the desired q value.

Lipid and Protein Diffusion Assays in Bicelles

To test lipid diffusion in bicelles, 3% bicelles containing fluorescently labeled lipids (NBD-DPPE and rhodamine-DPPE; a lipid mole fraction of 0.01 for each) were mixed with 3% unlabeled bicelles at a 1:35 volume ratio in 20 mM HEPES (pH 7.5), 15% glycerol (v/v), 80 mM KCl and 10 mM MgCl₂. FRET signal, *i.e.*, the ratio of fluorescence intensity of NBD at 530 nm to that of rhodamine at 590 nm, was monitored over time at 37°C. To test protein diffusion in bicelles, GlpG labeled with NBD or rhodamine was incorporated together into the bicelles by direct injection to the final concentration of each labeled proteins at 74 μM. GlpG diffusion in bicelles was measured in the same manner as in lipid diffusion.

GlpG Degradation Assay *In Vivo*

All *in vivo* constructs contain the TM domain of GlpG (residues 87-276) with N- or C-terminal degradation marker *and* FLAG or HA epitope, which were cloned into pBAD/HisA vector. *E. coli* AR3289 (+*ftsH*) and AR3291 (−*ftsH*) strains were transformed with each GlpG plasmid. LB containing 100 μg/mL of ampicillin was inoculated with a single colony, which were grown for 18 hr at 37°C for AR3289 and 30°C for AR3291. When OD_{600nm} reached 1.5-1.7 (AR3289) and 0.8-1.0 (AR3291), GlpG expression was induced by addition of 0.2% (w/v) arabinose at 37°C for 30 min. Then protein synthesis was blocked by addition of 300 μg/mL spectinomycin. To monitor degradation over time, samples were incubated at 37°C and an aliquot of each sample was taken at subsequent time points. Spun-down cells were resuspended with protein sample buffer and run on SDS-PAGE. GlpG degradation was monitored by Western blotting against HA or FLAG epitope.

Preparation of GlpG for *In Vitro* Degradation

GlpG variants fused to N-terminal maltose binding protein followed by a TEV cleavage site were cloned into pET30a vector. The resulting plasmids encoded the proteins with an N-terminal His₆-tag. Based on this construct, a degradation marker was fused to the N-(Dps_N or YccA_N) or C-terminus (108 or SsrA) of GlpG. Cysteine mutant G172C was generated for thiol-reactive NBD labeling. Detailed procedures are described in Supporting Methods.

GlpG Degradation Assay *In Vitro*

Degradation of NBD-labeled GlpG variants by FtsH was measured in 3% DMPC/DMPG/CHAPS bicellar solution ($q = 2.8$; 2 μM FtsH; 20 mM HEPES, pH 7.5, 100 mM KCl, 15% glycerol, 0.1% BME, and 0.4 mM ZnCl₂) with an ATP regeneration system (5 unit/ml pyruvate kinase and 10 mM PEP). GlpG degradation was initiated by addition of 2 mM ATP and monitored by quenching of NBD fluorescence at $\lambda_{\text{emission}} = 545$ nm with $\lambda_{\text{excitation}} = 485$ nm on a microplate reader over time. The net fluorescence change induced by GlpG degradation was obtained by subtracting NBD fluorescence with ATP from that without ATP. Data analysis is described in Supporting Methods.

Steric Trapping of GlpG

Detailed procedures are described in Supporting Methods.

Results

Bicelles enhance ATPase activity of FtsH and allow facile protein diffusion

To establish an ideal *in vitro* system for quantitative investigation of FtsH function, we first optimized its ATPase activity by testing various lipid conditions, *i.e.*, micelles, bicelles and liposomes (Figure 1b, Table S1 and Figure S1a). In detergent Triton X-100, the maximal ATP hydrolysis rate per FtsH hexamer ($k_{\text{cat,ATP}}$) was $51 \pm 2 \text{ min}^{-1} \text{ FtsH}_6^{-1}$, and the ATP concentration required for half-maximal velocity ($K_{\text{m,ATP}}$) was $400 \pm 50 \mu\text{M}$ from Michaelis-Menten kinetics. Surprisingly, in the large neutral (DMPC/CHAPS) and negatively charged (DMPC/DMPG/CHAPS) bicelles (disk diameter $\approx 30 \text{ nm}^{23}$), $k_{\text{cat,ATP}}$ substantially increased by 3.3 and 2.5 fold, respectively, relative to that in Triton X-100, and $K_{\text{m,ATP}}$ decreased by half. FtsH reconstituted in liposomes composed of *E. coli* or DMPC/DMPG lipids displayed higher overall ATPase activity than in Triton X-100 (Figure 1b). To quantify ATP hydrolysis rate in the liposomes, we analyzed the orientation distribution of FtsH inserted in the liposomes using limited proteolysis (Figure S1b). >90% of FtsH was oriented with the AAA+ and protease domains outside, accessible to ATP added to the outside of the liposomes. ATPase activity was negligible in detergent CHAPS. Thus, enhanced ATP hydrolysis in bicelles appears mainly due to the interaction of FtsH with the bilayered lipid region rather than with detergents. Notably, $k_{\text{cat,ATP}}$ in native *E. coli* lipids was similar to that in DMPC/DMPG/CHAPS bicelles. Many AAA+ proteases exhibit moderate positive cooperativity in ATP hydrolysis with Hill coefficients ($n_{\text{H,ATP}}$) of 1.4–2.0^{24–27}. In contrast, FtsH lacked cooperativity ($n_{\text{H,ATP}} = 0.9\text{--}1.0$) in all tested lipid environments, implying, either negligible coupling among the AAA+ subunits or a 1:1 binding stoichiometry between ATP and FtsH hexamer.

We further tested bicelles for the ability to mediate facile diffusion and mixing of the lipid and protein components (Figure 1c), which is crucial for quantitative analysis of enzyme function. We employed a FRET-based mixing assay developed for studying membrane fusion²⁸. First, to test lipid diffusion, bicelles containing NBD (donor)- and rhodamine (acceptor)-labeled lipids were prepared, in which NBD fluorescence was highly quenched by rhodamine due to FRET. Upon mixing of the fluorescent bicelles with the bicelles without fluorescent labels, NBD fluorescence rapidly increased within a minute to the level of the homogeneously mixed state. We similarly tested protein diffusion. The model substrate GlpG labeled with either NBD or rhodamine was incorporated together in bicelles, and then mixing of the fluorescent protein-bicelle complex with the bicelles without fluorescent labels was monitored by FRET. We observed rapid protein diffusion in a similar timescale to that of lipid diffusion. This result demonstrates that a bicelle is not a discrete lipid assembly but subject to spontaneous fusion and dissociation, enabling facile mixing of lipid and protein components throughout the entire hydrophobic phase in solution.

It would be ideal to study FtsH function in pure lipid bilayers. However, in liposomes, each of which is regarded as a discrete assembly, it is inherently difficult to control substrate-to-

enzyme ratio, and their relative orientation and mixing. Thus, we chose the negatively charged DMPC/DMPG/CHAPS bicelles as a lipid medium because of their ability to support ATPase activity similar to native *E. coli* lipids as well as to allow rapid protein mixing.

Model substrate GlpG is highly thermostable in bilayers

To quantitatively study the folding-degradation relationship of membrane proteins mediated by FtsH, we chose the TM domain of the site-specific intramembrane protease GlpG of *E. coli*²⁹ as a model substrate (Figure 2a). We chose GlpG because: (1) it is a monomeric sixhelical bundle membrane protein with structural information available from >25 PDB entries (www.rcsb.org); (2) its folding has been extensively studied using various methods including thermal and SDS denaturation, singlemolecule force spectroscopy and steric trapping in dodecylmaltoside (DDM) micelles and DMPC/CHAPSO bicelles (Table S2)^{22,30-32}.

We tested thermostability of GlpG in DMPC/DMPG/CHAPS bicelles and *E. coli* lipids employed in this study. GlpG was resistant to thermal inactivation and aggregation up to 85°C (Figure S2). As a control, we measured the thermostability in DDM. The onset temperature of thermal denaturation (65–70°C) agreed well with the reported value³⁰. Taken together, GlpG is a highly thermostable membrane protein in the bilayer environments.

FtsH efficiently degrades stable GlpG *in vivo*

FtsH is known to processively degrade a substrate starting from either N- or C-terminus by binding an unstructured tail (>20 residues) with diverse sequences as a degradation marker^{34,35}. To establish GlpG as model substrate, we chose four well-characterized degradation markers which are preferentially recognized by FtsH or other AAA+ proteases, and examined their ability to induce GlpG degradation *in vivo*. To test specific degradation by FtsH, we employed the *E. coli* strain with intact chromosomal *ftsH* (+*ftsH*) and the strain with disrupted *ftsH* (–*ftsH*)³⁶.

As C-terminal degradation markers (Figure 2b and Figures S3a–b), we tested the FtsH-specific 108 tag (GlpG-108) and the universal SsrA tag (GlpG-SsrA)^{37,38}. Time-dependent degradation of the whole protein was monitored by Western blotting using FLAG epitope at the N-terminus. GlpG-108 was efficiently degraded in +*ftsH* (half-life, $T_{1/2} \approx 25$ min) but not in –*ftsH*. Degradation of GlpG lacking a marker was negligible. Thus, FtsH degraded GlpG by recognizing the 108 tag. GlpG-SsrA was degraded in +*ftsH* at a similar rate to GlpG-108. However, GlpG-SsrA was also slowly degraded in –*ftsH* generating two large fragments (~22 kD and ~14 kD; MW of the full length = 26 kD), implying the involvement of other proteases in recognition of the SsrA tag.

To test GlpG degradation by N-terminal markers, we employed the N-terminal tails (residues 1–23) of *E. coli* proteins YccA (YccA_N-GlpG) and Dps (Dps_N-GlpG) as degradation markers (Figure 2c), which are known to be specifically recognized by FtsH and ClpXP, respectively^{34,39}. Degradation of GlpG was monitored using HA epitope at the C-terminus. Two Asp residues (DD) were added after the epitope to suppress unwanted degradation from the C-terminus⁴⁰ (Figure S3c). GlpG variants with the N-terminal markers were gradually

degraded in *+ftsH* ($T_{1/2} \approx 35$ min for YccA_N-GlpG and $T_{1/2} \approx 1$ hr for Dps_N-GlpG) (Figures S3d–e), while stable in *–ftsH*.

Although FtsH had been thought to degrade only unstable proteins because of its proposed weak unfoldase activity¹⁸, the thermostable membrane protein GlpG was efficiently degraded by FtsH *in vivo*. It should be noted that GlpG with the Dps_N-tag, which is known as a ClpXP-specific marker, was degraded depending on FtsH.

Reconstitution of FtsH-mediated degradation of GlpG in bicelles

To quantitatively study the mechanism of FtsH-mediated degradation of membrane proteins without involvement of unknown cellular factors, we reconstituted the degradation using purified FtsH and GlpG in the bicelles optimized for ATPase activity and protein diffusion. GlpG variants with the fused degradation markers retained the activity level of wild-type (WT), indicating that the markers did not affect the native conformation (Figures S4a–b). For precise and efficient measurement of degradation rates, we developed a fluorescence-based assay using the environment-sensitive fluorophore NBD conjugated to GlpG (Figure 3a and Figures S4c–d).

In bicelles, we successfully recapitulated FtsH-mediated degradation of GlpG (Figures 3b–c) observed *in vivo*. At the substrate-to-enzyme molar ratio of 10, FtsH degraded GlpG with the C-terminal 108- and SsrA-tags ($T_{1/2} \approx 50$ min) in a comparable timescale as *in vivo* ($T_{1/2} \approx 25$ min) in the presence of ATP. GlpG lacking a marker was not degraded, indicating that the degradation was specifically induced by the markers. Interestingly, GlpG with the N-terminal markers (YccA_N- and Dps_N-tag) were degraded significantly slower than those with the C-terminal markers. There was no noticeable cleavage of FtsH by GlpG, which is a site-specific protease (Figure S4e). Degradation rate of GlpG-108 was enhanced in bicelles by ~3 fold relative to that in micelles (Figures S4f–g), demonstrating that bicelles support not only ATP hydrolysis but also substrate degradation superior to micelles.

Degradation rate of GlpG is determined by the location of the marker not by its sequence

Next, we carried out kinetic analysis of GlpG degradation in bicelles by measuring the degradation rate as a function of substrate concentration (Figure 3d and Figures S4h–l). Because both enzyme and substrate were constrained in bicelles, the substrate concentration was expressed as a mole fraction in the protein-bicelle complex (X_{GlpG}). Fitting of degradation rates to a Michaelis-Menten equation yielded $K_{\text{m,deg}} = 3.0\text{--}3.7 \times 10^{-5} X_{\text{GlpG}}$ for GlpG-108 and GlpG-SsrA, and $K_{\text{m,deg}} = 6.3\text{--}9.0 \times 10^{-5} X_{\text{GlpG}}$ for YccA_N-GlpG and Dps_N-GlpG (Table S3), indicating that the C-terminal markers bound FtsH slightly tighter than the N-terminal markers. Remarkably, the degradation rate was largely determined by the position of the marker rather than by its amino acid sequence. We observed a similar maximum degradation rate, $k_{\text{cat,deg}} \approx 2.5\text{--}2.7 \times 10^{-1} \text{ min}^{-1} \text{ FtsH}_6^{-1}$ for GlpG-108 and GlpG-SsrA while $k_{\text{cat,deg}} \approx 1.6 \times 10^{-1} \text{ min}^{-1} \text{ FtsH}_6^{-1}$ for YccA_N-GlpG and Dps_N-GlpG. GlpG with the C-terminal markers were degraded more rapidly than that with the N-terminal markers by ~1.7 fold.

The strong dependence of the degradation rate on the position of the marker may have stemmed from the different efficiency of FtsH to process GlpG variants that are engaged in

the opposite directions. However, previous *in vivo* studies indicate that membrane substrates lacking stable tertiary or quaternary interactions (*e.g.*, YccA, the SecY subunit and the TM fragment of proW) are rapidly degraded by FtsH with $T_{1/2} < 8$ min regardless of the position of the marker, much faster than GlpG ($T_{1/2} \approx 25\text{--}60$ min)^{34,41,42}. Also for other AAA+ proteases including ClpXP, ClpAP and proteasomes, the translocation rates of substrates are not significantly affected by the direction of pulling^{43,44}. Therefore, our result raises a possibility that certain differences in the folding properties between the N- and C-terminal regions of GlpG may have caused the difference in the degradation rate.

Steric trapping to measure spontaneous unfolding rate of GlpG

Our findings that FtsH efficiently degrades stable GlpG and that the degradation rate strongly depends on the location of the marker raise important mechanistic questions regarding the folding-degradation relationship of membrane proteins: (1) Can FtsH actively unfold a membrane substrate? (2) How do the intrinsic folding properties of the membrane substrate influence its degradation?

To determine if FtsH can actively unfold membrane substrates, it is necessary to measure the spontaneous unfolding rate (k_U) of GlpG under the same bicellar condition for measuring degradation and compare the measured k_U to the ATP-driven unfolding rate. Here we employed the novel steric trapping strategy developed for studying membrane protein folding^{22,45}. Steric trapping couples unfolding of a doubly-biotinylated protein to competitive binding of bulky monovalent streptavidin (mSA) (Figure 4a). This method is advantageous because: (1) k_U and thermodynamic stability (ΔG_U) can be measured directly under native bilayer conditions without using perturbants such as chemical denaturants and pulling force; (2) trapping of the unfolded state depends on transient unfolding of the region between two specific biotin labels separated at a close distance in the folded state. Thus, it allows for measuring the local unfolding rate depending on the position of the biotin pair.

Using steric trapping, k_U can be determined by shifting the reaction flux dominantly towards unfolding upon addition of a molar excess of mSA-WT with a high biotin-binding affinity (Figure S5a)⁴⁵⁻⁴⁷. The apparent unfolding rate ($k_{U,app}$) is asymptotic as a function of mSA concentration, the maximum value of which corresponds to k_U (Figure S5b). We have identified two pairs of optimal biotinylation sites, 95C/172C and 172C/267C²² (Figure 4b). After biotinylation, each biotin pair approximately covers the N-terminal (N-subdomain, probed with the biotin pair 95/172_N-biotin₂) or C-terminal half (C-subdomain, probed with the biotin pair 172/267_C-biotin₂) of GlpG²² (the thiol-reactive biotin derivative employed in this study is described in Figure S5c).

FtsH actively unfolds GlpG for degradation

To test if FtsH can actively unfold GlpG during degradation, we determined k_U in bicelles using GlpG activity as an unfolding readout at a 20-times molar excess of mSA-WT (Figure 4b and Figures S5d-e). The C-subdomain unfolded slowly with $k_{U,app} = 2.7 \pm 0.7 \times 10^{-4} \text{ min}^{-1}$. The N-subdomain unfolded even slower by ~ 1.5 fold ($k_{U,app} = 1.8 \pm 0.5 \times 10^{-4} \text{ min}^{-1}$). Increase of the mSA concentration up to a 30-times molar excess did not significantly change $k_{U,app}$, indicating $k_{U,app} \approx k_U$ (Figure S5f).

To quantify the degree of acceleration of GlpG unfolding by FtsH, we decomposed the degradation process represented by $\tau_{\text{cat,deg}}$ (substrate lifetime, $1/k_{\text{cat,deg}}$) into three sequential sub-processes^{17,48}: ATP-driven unfolding ($\tau_{\text{cat,U}}$) and membrane dislocation ($\tau_{\text{cat,dislocation}}$) by the AAA+ domain, and proteolysis ($\tau_{\text{cat,proteolysis}}$) by the protease domain (*i.e.*, $\tau_{\text{cat,deg}} = \tau_{\text{cat,U}} + \tau_{\text{cat,dislocation}} + \tau_{\text{cat,proteolysis}}$). Although we were not able to determine the lifetime of each sub-process, it is evident that $\tau_{\text{cat,deg}} > \tau_{\text{cat,U}}$. Next, we compared $\tau_{\text{cat,deg}}$ obtained with the N-terminal marker to τ_{U} (lifetime of spontaneous unfolding, $1/k_{\text{U}}$) obtained from steric trapping at the N-terminal biotin pair, and $\tau_{\text{cat,deg}}$ with the C-terminal marker to τ_{U} from steric trapping at the C-terminal biotin pair (Figures 3d and 4b). By this comparison, $\tau_{\text{U}}/\tau_{\text{cat,deg}} \approx 800\text{--}900$ regardless of the position of the degradation marker. Because $\tau_{\text{cat,deg}} > \tau_{\text{cat,U}}$, we conclude that FtsH accelerated GlpG unfolding at least 800 fold (*i.e.*, $\tau_{\text{U}}/\tau_{\text{cat,U}} > 800$), clearly demonstrating the substantial ability of FtsH to actively unfold GlpG. It is surprising because FtsH has been known to degrade substrates depending on spontaneous unfolding¹⁸.

Substrate stability and hydrophobicity contribute to degradation rate

To determine how intrinsic folding properties of a membrane substrate influence the degradation, it is necessary to obtain G°_{U} , k_{U} and activation energy of unfolding ($E_{\text{a,U}}$) (*i.e.* the unfolding energy landscape) of the substrate, and examine the correlation between each folding property and the degradation rate. Due to slow unfolding, however, it was difficult to measure G°_{U} of GlpG using steric trapping under the same bicellar condition for measuring degradation. Instead, because GlpG unfolded faster in micelles by 40–100 fold, we were able to describe the unfolding energy landscape in DDM micelles using G°_{U} that has been previously determined²², and k_{U} and $E_{\text{a,U}}$ obtained in this study (Figures 5a–b and Figure S6). Notably, we obtained an highly asymmetric unfolding energy landscape in micelles (Figure 5b), *i.e.*, the N-subdomain possessed higher kinetic ($E_{\text{a,U}} \approx 8$ kcal/mol) and thermodynamic ($G^{\circ}_{\text{U}} \approx 1$ kcal/mol) stability than the C-subdomain.

Having the tools to analyze the unfolding energy landscape and degradation in hand, we hypothesized that the degradation rate of GlpG depends on the unfolding kinetic barrier and stability of the region to which a degradation marker is attached, analogous to the suggestion from degradation studies of water-soluble proteins by ClpAP and ClpXP¹⁴. To test this, we made an amino acid substitution on GlpG to modify the unfolding energy landscape and investigated how the modification would change the degradation rate. We chose M100A substitution in the internal packing region (Figure 5a), which destabilizes the N- and C-subdomains by G°_{U} WT-M100A = 2.5 and 2.8 kcal/mol, respectively, in micelles²². $E_{\text{a,U}}$ of the two sub-domains similarly decreased by ~ 5 kcal/mol (5–10 fold increase in k_{U}) (Figure 5b). Also in bicelles, the substitution accelerated unfolding by 35–50 fold relative to WT for both subdomains (Figure 5c). Although the residue M100 is located at the N-terminal TM1 helix, the structural perturbation by the substitution was propagated throughout the protein²². Indeed, this destabilizing substitution increased $k_{\text{cat,deg}}$'s of both variants with the N- and C-terminal markers (GlpG_{M100A}-108 and YccAN-GlpG_{M100A}) by ~ 1.5 fold (Figure 5d and Table S3).

Based on the asymmetric unfolding energy landscape and the sensitivity of the degradation rate to the stability change, we reasoned that the slow degradation of GlpG with the N-terminal degradation markers (Figure 3d) was caused by the higher conformational stability of the N-subdomain. However, from the hydrophobicity analysis of GlpG using the membrane depth-dependent hydrophobicity scale^{49,50}, and Hessa-von Heijne and Wimley-White scales determined near the center of the bilayer^{51,52} (Figure 5e), we noticed that the N-terminal TM1 helix was much more hydrophobic than the C-terminal TM6 helix by 4–10 kcal/mol. This fact raises a possibility that, in addition to the conformational stability that resists unfolding, the hydrophobicity of the TM segment near the marker that would resist membrane dislocation may also control degradation. To test this possibility, we designed a quadruple substitution on the lipid-exposed residues in TM6 (A259L/A263L/V267W/L270F) near the C-terminal 108 tag (GlpG_{LLWF}-108 hereafter) to increase its hydrophobicity to the comparable level to that of TM1 (Figures 5a and 5e). This variant possessed the activity and stability similar to WT (Figures S7a–b). If the hydrophobicity dominantly determines the degradation rate, GlpG_{LLWF}-108 would be degraded slower than GlpG_{WT}-108 but at a similar rate to GlpG with the N-terminal marker (YccA_N-GlpG_{WT}). Indeed, $k_{cat,deg}$ of GlpG_{LLWF}-108 decreased but only half way to the level of YccA_N-GlpG (Figure 5f). Thus, the dependence of the degradation rate on the position of the degradation marker appears to be caused by the combined effect of the stability and hydrophobicity of the substrate region near the marker.

In summary, we found that the conformational stability and hydrophobicity of membrane substrates are important factors that control degradation. Nonetheless, the large changes in the unfolding rate (30–50 fold in bicelles) and the hydrophobicity (~4 kcal/mol) led to only moderate changes in the degradation rate. Probably, substantial unfoldase activity of FtsH elucidated in this study dampened the sensitivity of the degradation rate to the changes in these folding properties. *In vivo*, M100A and LLWF substitutions induced similar changes in the degradation rate to those observed *in vitro* (Figure S7c).

Free energy cost to dislocate GlpG

To obtain a quantitative measure of the hydrophobic nature of GlpG and the energetic cost of its membrane dislocation, we calculated the total free energy of dislocation ($G_{dislocation}$) using the depth-dependent hydrophobicity scale (Figure S8 and Table S4). We assumed that the tertiary structure of GlpG is completely unraveled in the bilayer before dislocation while the helical content of each TM segment is maintained. $G_{dislocation}$ was defined as the free energy change from GlpG whose tertiary structures are unraveled in the bilayer to its random coil form in water. The calculated $G_{dislocation}$ was +360 kcal/mol, indicating that FtsH overcomes an enormous free energy cost to dislocate GlpG. Studies using single-molecule force spectroscopy report that $G_{dislocation}$ for a single TM helix ranges from 50 to 100 kcal/mol²⁰. Our calculated $G_{dislocation}$ (~60 kcal/mol/TM helix) reasonably agrees with this result.

Total ATP cost to degrade GlpG

Finally, to understand how efficiently FtsH utilizes ATP hydrolysis to degrade membrane substrates, we quantified the total number of ATP molecules hydrolyzed during GlpG degradation in bicelles using the equation⁴⁸:

$$\text{Total ATP cost} = k_{\text{cat,ATP}} \times \tau_{\text{cat,deg}}$$

$k_{\text{cat,ATP}}$ denotes the maximal ATP hydrolysis rate per FtsH₆ with bound substrate, and $\tau_{\text{cat,deg}}$ indicates the substrate lifetime ($1/k_{\text{cat,deg}}$). These parameters were obtained from Michaelis-Menten analysis of ATP hydrolysis and degradation of GlpG-108 and YccA_N-GlpG (Figure S9, Tables S3 and S5). The total number of ATP molecules hydrolyzed by FtsH hexamer to degrade a single copy of GlpG was 380 ± 30 for GlpG-108 and 550 ± 60 for YccA_N-GlpG.

Discussion

Here, we present the first quantitative study that defines the folding-degradation relationship of a membrane protein mediated by a specific cellular degradation machine. A striking finding of this work is that FtsH substantially accelerates unfolding of a stable membrane protein (>800 fold) and carries out its membrane dislocation overcoming enormous free energy cost (~360 kcal/mol). This result redefines the unfolding capability of FtsH, shifting the long-standing paradigm that FtsH passively selects substrates depending on spontaneous unfolding. We also showed that the degradation of the membrane substrate is significantly influenced by the conformational stability and hydrophobicity. Hydrophobicity is a unique determining factor in degradation of membrane proteins, which may provide another layer of complexity in the degradation mechanism.

FtsH degrades membrane substrates more efficiently than water-soluble substrates

The weak unfoldase activity, which has long been regarded as a distinctive feature of FtsH¹⁸, is based on the observation that FtsH cannot degrade stable globular proteins such as DHFR, GFP and barnase which unfold slowly, whereas degrading fast unfolding Arc and its variants depends on their stability. Also, the correlation between the degradation rates of Arc variants by FtsH and their proteolysis rates by the energy-independent proteinase Arg-C suggested that FtsH cannot actively unfold a substrate^{18,53}. Thus, it is surprising that FtsH degrades GlpG *via* active unfolding although its conformational stability is comparable to or exceeds that of water-soluble proteins which cannot be degraded by FtsH (Table S2).

How can this discrepancy be explained? Structural and single-molecule studies of AAA+ enzymes suggest that during the cycles of ATP hydrolysis, AAA+ subunits undergo power-stroke motions applying pulling forces on the bound substrate to induce its unfolding and translocation^{54,55}. A similar principle has been suggested for FtsH⁵⁶. The cryo-EM image of Yta12, an FtsH ortholog in yeast, shows that the substrate entry pore formed by the AAA+ domains faces the membrane plane with a narrow gap of ~13 Å (Figure S10a)⁵. Under this spatial constraint, water-soluble and membrane proteins will be engaged with FtsH in very

different geometric arrangements through the flexible tails containing a degradation marker (Figure S10b–c). For water-soluble proteins, only the unfolded polypeptide chain, not a globular domain, can approach the gap. Thus, the tail bound to the entry pore will align the excluded globular domain at $\sim 90^\circ$ relative to the pore axis. For membrane proteins with known structure, TM helices are tilted from the bilayer normal at $\sim 20^\circ$ on average⁵⁷. Thus, the TM helices can be closely aligned along the pore axis. Although the pulling forces along the pore axis exert the same tension on the tails of the two types of proteins, a larger opposing frictional force would be generated at the deflection point near the pore for the water-soluble proteins aligned at $\sim 90^\circ$ than for the membrane proteins aligned at a smaller angle. Thus, the pulling forces would be more effectively applied on the membrane proteins than on the water-soluble proteins.

Another possible reason for the discrepancy is the difference in the spontaneous refolding rate (k_F) between the two types of proteins. Previous studies indicate that GFP that has been transiently unfolded by ClpXP can fold back to the native state when the ATP hydrolysis rate is low^{58,59}. The k_F 's of Barnase and DHFR in water, which cannot be degraded by FtsH, are ~ 300 and ~ 8 fold higher ($k_F \approx 12 \text{ s}^{-1}$ and 0.25 s^{-1}), respectively, than that of GlpG in neutral bicelles ($k_F \approx 0.04 \text{ s}^{-1}$)^{32,60,61}. Notably, unfolded DHFR can rapidly refold to a compact intermediate with $k_{F,U-I} \approx 6.7 \text{ s}^{-1}$ ⁶⁰. The k_F 's of these water-soluble proteins are comparable or higher than the rate of ATP hydrolysis on FtsH ($\sim 2 \text{ s}^{-1}$, Figure 1b), whereas that of GlpG is much lower. Therefore, even if barnase and DHFR can be unfolded by FtsH in a certain round of ATP hydrolysis, they possibly refold to the compact intermediate or native state before the next round of ATP hydrolysis. In contrast, once GlpG unfolds, it is likely to remain unfolded until the next ATP hydrolysis occurs, entering the subsequent dislocation and degradation stages.

Our result seems hard to reconcile with the result that FtsH cannot degrade the membrane protein DGK *in vivo*¹⁸. DGK forms a tightly packed trimer with high kinetic stability^{62,63}. It does not unfold by steric trapping up to 30 days in micelles⁴⁵, much slower than GlpG ($\tau_U \approx 2$ days). Thus, it is likely that the kinetic barrier of DGK unfolding is beyond the unfolding capability of FtsH.

Folding-degradation relationship of membrane proteins

Previous studies on degradation of water-soluble proteins by ClpXP and ClpAP have shown that the pulling forces generated by ATP hydrolysis act primarily on the local structure adjacent to a degradation marker^{14,44,64}. Thus, degradation strongly depends on local conformational stability as well as unfolding cooperativity of the whole structure⁵⁹. Here we demonstrate that the same principle can be applied to degradation of membrane proteins. For GlpG, we observed a strong correlation between the degradation rate and the local stability of the region to which a degradation marker was attached (Figure 3d). Then, if one subdomain is unraveled by FtsH, what will happen in the other? Previously, we have shown that the perturbation of the native tertiary interactions near the N- or C-terminus is readily propagated throughout the protein²². Thus, once FtsH actively unravels either subdomain with its substantial unfoldase activity, subsequent unfolding of the rest would not experience much resistance.

Through analysis of various intrinsic folding properties of GlpG, we revealed the role of the conformational stability and hydrophobicity in determining the degradation of a membrane protein. Recently, the conformational stability has been shown to be a key determinant for the cellular trafficking efficiency of the human membrane protein PMP22⁶⁵. In yeast, the presence of a mitochondrial FtsH ortholog increases the threshold hydrophobicity level of a TM helix for its retention in the inner membrane⁶⁶. Our systematic study dissecting the effects of the two physical forces further supports these observations.

A three-step model for membrane protein degradation

Based on our findings, we suggest a three-step model for FtsH-mediated membrane protein degradation (Figure 6). In the first “engagement” step, the degradation marker on a membrane substrate binds to the entry pore of the AAA+ hexamer. Although we only tested well-characterized markers, the substrate-enzyme affinity does not seem to strongly depend on the detailed sequence of the marker (Figure 3d). Thus, any flexible cytoplasmic tails on native membrane substrates or the tails that are exposed upon protein misfolding, chemical or enzymatic cleavage, subunit dissociation or premature truncation of translation may serve as degradation markers^{2,34,35}. In the second “unfolding” step, FtsH actively unravels the tertiary structure of the bound substrate in the membrane using ATP hydrolysis. If the membrane substrate is highly stable, unfolding would require multiple power-stroke motions, during which the substrate may undergo “slipping-release” events without unfolding⁴⁸. Also, as noted earlier, the transiently unfolded substrate may refold before the next pulling occurs. Thus, it is likely that the engagement and unfolding steps are reversible. In the third “dislocation and degradation” step, FtsH dislocates the unfolded substrate from the membrane to the protease chamber for reinsertion of the TM segments may repeatedly occur during pulling events.

Degradation of GlpG requires the hydrolysis of a total of 380–550 ATP (1.7–2.5 ATP/residue). Although it appears energetically wasteful, the ATP cost per residue is comparable to that of other AAA+ proteases in degradation of water-soluble substrates (0.2–6.6 ATP/residue, Table S6). To degrade water-soluble substrates, ATP hydrolysis is used to unfold them in water and translocate the unfolded chains into the similarly aqueous proteolytic chamber. Surprisingly, for membrane substrates, FtsH carries out the energetically expensive dislocation step ($G \approx 360$ kcal/mol) with a similar ATP cost.

To further understand the mechanisms of membrane protein degradation, it is necessary to determine which step is rate-determining and how the total ATP cost is distributed between the unfolding and dislocation steps. Extensive mutational studies varying the stability and hydrophobicity in a wide range and assessing their correlation with degradation rates and ATP consumption are under progress.

Implications in membrane protein quality control

Our findings have broad implications in the role of FtsH in membrane protein quality control. The substantial un-folding-dislocation ability discovered in this study will expand the range of potential substrates to: membrane proteins which are misfolded or aggregated with stable non-native interactions, membrane proteins which are folded but need to be

degraded for regulatory purpose or toxicity, and prematurely truncated translation products which need to be immediately degraded from the membrane. Several pieces of evidence support the need and existence of significant unfoldase activity of FtsH in bacteria, mitochondria and chloroplasts: (1) FtsH rapidly degrades the whole SecYEG translocase complex jammed with an inefficiently secreted protein⁶⁷; (2) FtsH in the thylakoid membranes mediates swift regeneration of the damaged photosynthetic reaction center by degrading the photo-oxidized core proteins^{9,68}; (3) Chaperone activity of FtsH has been reported which requires active unfolding⁶⁹. These quality control processes can be better understood by the FtsH activity redefined in this study.

Conclusions

We successfully reconstituted the membrane protein degradation mediated by the conserved AAA+ protease FtsH in the lipid bilayer environment. Using the stable membrane protein GlpG as a model substrate, we quantitatively defined the ATP-driven unfoldase activity of FtsH, and elucidated the relationship between its intrinsic folding properties and degradation. The physical principles obtained in this study would be generally applicable to other ATP-dependent proteolytic systems for membrane proteins. For example, in endoplasmic reticulum (ER)-associated degradation, membrane proteins are thought to be degraded by a similar logic, *i.e.*, misfolded membrane proteins are recognized by the ubiquitin ligase complexes in the ER membrane, dislocated by a membrane-associated AAA + enzyme, and finally targeted to the proteasome in the cytosol⁷⁰. Our work will provide useful knowledge for generating new hypotheses to elucidate the challenging biological problem of membrane protein degradation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Teru Ogura for sharing *E. coli* strains, David Weliky for critical reading of the manuscript, and Lee Kroos and Babak Borhan for sharing instruments. We also thank anonymous reviewers for their constructive comments that truly improved the quality of the manuscript.

Funding Sources. This work is supported by NIH grant R01GM118685 and the Hunt for a Cure foundation to H.H., and NIH grants R01GM079804 and R01GM126558 to J.L.

References

1. Gottesman S. Proteases and their targets in *Escherichia coli*. *Annu Rev Genet.* 1996; 30:465–506. [PubMed: 8982462]
2. Sauer RT, Baker TA. AAA+ proteases: ATP-fueled machines of protein destruction. *Annu Rev Biochem.* 2011; 80:587–612. [PubMed: 21469952]
3. Langer T. AAA proteases: cellular machines for degrading membrane proteins. *Trends Biochem Sci.* 2000; 25(5):247–251. [PubMed: 10782097]
4. Ito K, Akiyama Y. Cellular functions, mechanism of action, and regulation of FtsH protease. *Annu Rev Microbiol.* 2005; 59:211–231. [PubMed: 15910274]

5. Lee S, Augustin S, Tatsuta T, Gerdes F, Langer T, Tsai FT. Electron cryomicroscopy structure of a membrane-anchored mitochondrial AAA protease. *J Biol Chem*. 2011; 286(6):4404–4411. [PubMed: 21147776]
6. Shimohata N, Chiba S, Saikawa N, Ito K, Akiyama Y. The Cpx stress response system of *Escherichia coli* senses plasma membrane proteins and controls HtpX, a membrane protease with a cytosolic active site. *Genes Cells*. 2002; 7(7):653–662. [PubMed: 12081643]
7. Campbell CL, Tanaka N, White KH, Thorsness PE. Mitochondrial morphological and functional defects in yeast caused by yme1 are suppressed by mutation of a 26S protease subunit homologue. *Mol Biol Cell*. 1994; 5(8):899–905. [PubMed: 7803857]
8. Arlt H, Steglich G, Perryman R, Guiard B, Neupert W, Langer T. The formation of respiratory chain complexes in mitochondria is under the proteolytic control of the m-AAA protease. *EMBO J*. 1998; 17(16):4837–4847. [PubMed: 9707443]
9. Krynicka V, Shao S, Nixon PJ, Komenda J. Accessibility controls selective degradation of photosystem II subunits by FtsH protease. *Nat Plants*. 2015; 1:15168. [PubMed: 27251713]
10. Ostersetzer O, Adam Z. Light-stimulated degradation of an unassembled Rieske FeS protein by a thylakoid-bound protease: the possible role of the FtsH protease. *Plant Cell*. 1997; 9(6):957–965. [PubMed: 9212469]
11. Di Bella D, Lazzaro F, Brusco A, Plumari M, Battaglia G, Pastore A, Finardi A, Cagnoli C, Tempia F, Frontali M, Veneziano L, Sacco T, Boda E, Brussino A, Bonn F, Castellotti B, Baratta S, Mariotti C, Gellera C, Fracasso V, Magri S, Langer T, Plevani P, Di Donato S, Muzi-Falconi M, Taroni F. Mutations in the mitochondrial protease gene AFG3L2 cause dominant hereditary ataxia SCA28. *Nat Genet*. 2010; 42(4):313–321. [PubMed: 20208537]
12. Nolden M, Ehses S, Koppen M, Bernacchia A, Rugarli EI, Langer T. The m-AAA protease defective in hereditary spastic paraplegia controls ribosome assembly in mitochondria. *Cell*. 2005; 123(2):277–289. [PubMed: 16239145]
13. Koodathingal P, Jaffe NE, Kraut DA, Prakash S, Fishbain S, Herman C, Matouschek A. ATP-dependent proteases differ substantially in their ability to unfold globular proteins. *J Biol Chem*. 2009; 284(28):18674–18684. [PubMed: 19383601]
14. Lee C, Schwartz MP, Prakash S, Iwakura M, Matouschek A. ATP-dependent proteases degrade their substrates by processively unraveling them from the degradation signal. *Mol Cell*. 2001; 7(3):627–637. [PubMed: 11463387]
15. Kim YI, Burton RE, Burton BM, Sauer RT, Baker TA. Dynamics of substrate denaturation and translocation by the ClpXP degradation machine. *Mol Cell*. 2000; 5(4):639–648. [PubMed: 10882100]
16. Hoskins JR, Yanagihara K, Mizuuchi K, Wickner S. ClpAP and ClpXP degrade proteins with tags located in the interior of the primary sequence. *Proc Natl Acad Sci U S A*. 2002; 99(17):11037–11042. [PubMed: 12177439]
17. Kihara A, Akiyama Y, Ito K. Dislocation of membrane proteins in FtsH-mediated proteolysis. *EMBO J*. 1999; 18(11):2970–2981. [PubMed: 10357810]
18. Herman C, Prakash S, Lu CZ, Matouschek A, Gross CA. Lack of a robust unfoldase activity confers a unique level of substrate specificity to the universal AAA protease FtsH. *Mol Cell*. 2003; 11(3):659–669. [PubMed: 12667449]
19. Heyden M, Freites JA, Ulmschneider MB, White SH, Tobias DJ. Assembly and stability of alpha-helical membrane proteins. *Soft Matter*. 2012; 8(30):7742–7752. [PubMed: 23166562]
20. Preiner J, Janovjak H, Rankl C, Knaus H, Cisneros DA, Kedrov A, Kienberger F, Muller DJ, Hinterdorfer P. Free energy of membrane protein unfolding derived from single-molecule force measurements. *Biophys J*. 2007; 93(3):930–937. [PubMed: 17483176]
21. Sanders CR, Landis GC. Reconstitution of Membrane-Proteins into Lipid-Rich Bilayered Mixed Micelles for Nmr-Studies. *Biochemistry*. 1995; 34(12):4030–4040. [PubMed: 7696269]
22. Guo R, Gaffney K, Yang Z, Kim M, Sungsuwan S, Huang X, Hubbell WL, Hong H. Steric trapping reveals a cooperativity network in the intramembrane protease GlpG. *Nat Chem Biol*. 2016; 12(5):353–360. [PubMed: 26999782]

23. Glover KJ, Whiles JA, Wu G, Yu N, Deems R, Struppe JO, Stark RE, Komives EA, Vold RR. Structural evaluation of phospholipid bicelles for solution-state studies of membrane-associated biomolecules. *Biophys J*. 2001; 81(4):2163–2171. [PubMed: 11566787]
24. Hersch GL, Burton RE, Bolon DN, Baker TA, Sauer RT. Asymmetric interactions of ATP with the AAA+ ClpX6 unfoldase: allosteric control of a protein machine. *Cell*. 2005; 121(7):1017–1027. [PubMed: 15989952]
25. Maurizi MR. ATP-promoted interaction between Clp A and Clp P in activation of Clp protease from *Escherichia coli*. *Biochem Soc Trans*. 1991; 19(3):719–723. [PubMed: 1783205]
26. Wohlever ML, Nager AR, Baker TA, Sauer RT. Engineering fluorescent protein substrates for the AAA+ Lon protease. *Protein Eng Des Sel*. 2013; 26(4):299–305. [PubMed: 23359718]
27. Yakamavich JA, Baker TA, Sauer RT. Asymmetric nucleotide transactions of the HslUV protease. *J Mol Biol*. 2008; 380(5):946–957. [PubMed: 18582897]
28. Duzgunes N, Allen TM, Fedor J, Papahadjopoulos D. Lipid mixing during membrane aggregation and fusion: why fusion assays disagree. *Biochemistry*. 1987; 26(25):8435–8442. [PubMed: 3442666]
29. Lemberg MK, Freeman M. Functional and evolutionary implications of enhanced genomic analysis of rhomboid intramembrane proteases. *Genome Res*. 2007; 17(11):1634–1646. [PubMed: 17938163]
30. Baker RP, Urban S. Architectural and thermodynamic principles underlying intramembrane protease function. *Nat Chem Biol*. 2012; 9:759–768.
31. Paslawski W, Lillielund OK, Kristensen JV, Schafer NP, Baker RP, Urban S, Otzen DE. Cooperative folding of a polytopic alpha-helical membrane protein involves a compact N-terminal nucleus and nonnative loops. *Proc Natl Acad Sci USA*. 2015; 112(26):7978–7983. [PubMed: 26056273]
32. Min D, Jefferson RE, Bowie JU, Yoon TY. Mapping the energy landscape for second-stage folding of a single membrane protein. *Nat Chem Biol*. 2015; 11(12):981–987. [PubMed: 26479439]
33. Wang Y, Zhang Y, Ha Y. Crystal structure of a rhomboid family intramembrane protease. *Nature*. 2006; 444(7116):179–180. [PubMed: 17051161]
34. Chiba S, Akiyama Y, Mori H, Matsuo E, Ito K. Length recognition at the N-terminal tail for the initiation of FtsH-mediated proteolysis. *EMBO Rep*. 2000; 1(1):47–52. [PubMed: 11256624]
35. Chiba S, Akiyama Y, Ito K. Membrane protein degradation by FtsH can be initiated from either end. *J Bacteriol*. 2002; 184(17):4775–4782. [PubMed: 12169602]
36. Ogura T, Inoue K, Tatsuta T, Suzaki T, Karata K, Young K, Su LH, Fierke CA, Jackman JE, Raetz CRH, Coleman J, Tomoyasu T, Matsuzawa H. Balanced biosynthesis of major membrane components through regulated degradation of the committed enzyme of lipid A biosynthesis by the AAA protease FtsH (HflB) in *Escherichia coli*. *Mol Microbiol*. 1999; 31(3):833–844. [PubMed: 10048027]
37. Herman C, Thevenet D, Boulloc P, Walker GC, D'Ari R. Degradation of carboxy-terminal-tagged cytoplasmic proteins by the *Escherichia coli* protease HflB (FtsH). *Genes Dev*. 1998; 12(9):1348–1355. [PubMed: 9573051]
38. Farrell CM, Grossman AD, Sauer RT. Cytoplasmic degradation of ssrA-tagged proteins. *Mol Microbiol*. 2005; 57(6):1750–1761. [PubMed: 16135238]
39. Flynn JM, Neher SB, Kim YI, Sauer RT, Baker TA. Proteomic discovery of cellular substrates of the ClpXP protease reveals five classes of ClpX-recognition signals. *Mol Cell*. 2003; 11(3):671–683. [PubMed: 12667450]
40. Gottesman S, Roche E, Zhou Y, Sauer RT. The ClpXP and ClpAP proteases degrade proteins with carboxyterminal peptide tails added by the SsrA-tagging system. *Genes Dev*. 1998; 12(9):1338–1347. [PubMed: 9573050]
41. Akiyama Y, Kihara A, Tokuda H, Ito K. FtsH (HflB) is an ATP-dependent protease selectively acting on SecY and some other membrane proteins. *J Biol Chem*. 1996; 271(49):31196–31201. [PubMed: 8940120]
42. Hari SB, Sauer RT. The AAA plus FtsH Protease Degrades an ssrA-Tagged Model Protein in the Inner Membrane of *Escherichia coli*. *Biochemistry*. 2016; 55(40):5649–5652. [PubMed: 27677373]

43. Berko D, Tabachnick-Cherny S, Shental-Bechor D, Cascio P, Mioletti S, Levy Y, Admon A, Ziv T, Tirosh B, Goldberg AL, Navon A. The direction of protein entry into the proteasome determines the variety of products and depends on the force needed to unfold its two termini. *Mol Cell*. 2012; 48(4):601–611. [PubMed: 23041283]
44. Olivares AO, Kotamarthi HC, Stein BJ, Sauer RT, Baker TA. Effect of directional pulling on mechanical protein degradation by ATP-dependent proteolytic machines. *Proc Natl Acad Sci U S A*. 2017
45. Jefferson RE, Blois TM, Bowie JU. Membrane proteins can have high kinetic stability. *J Am Chem Soc*. 2013; 135(40):15183–15190. [PubMed: 24032628]
46. Howarth M, Chinnapen DJ, Gerrow K, Dorrestein PC, Grandy MR, Kelleher NL, El-Husseini A, Ting AY. A monovalent streptavidin with a single femtomolar biotin binding site. *Nat Methods*. 2006; 3(4):267–273. [PubMed: 16554831]
47. Srisa-Art M, Dyson EC, de Mello AJ, Edel JB. Monitoring of real-time streptavidin-biotin binding kinetics using droplet microfluidics. *Anal Chem*. 2008; 80(18):7063–7067. [PubMed: 18712935]
48. Kenniston JA, Baker TA, Fernandez JM, Sauer RT. Linkage between ATP consumption and mechanical unfolding during the protein processing reactions of an AAA(+) degradation machine. *Cell*. 2003; 114(4):511–520. [PubMed: 12941278]
49. Lin M, Gessmann D, Naveed H, Liang J. Outer membrane protein folding and topology from a computational transfer free energy scale. *J Am Chem Soc*. 2016; 138(8):2592–2601. [PubMed: 26860422]
50. Tian W, Naveed H, Lin M, Liang J. GeTFEP: a general transfer free energy profile for transmembrane proteins. *BioRxiv*. 2017:191650.
51. Hessa T, Kim H, Bihlmaier K, Lundin C, Boekel J, Andersson H, Nilsson I, White SH, von Heijne G. Recognition of transmembrane helices by the endoplasmic reticulum translocon. *Nature*. 2005; 433(7024):377–381. [PubMed: 15674282]
52. Wimley WC, Creamer TP, White SH. Solvation energies of amino acid side chains and backbone in a family of host-guest pentapeptides. *Biochemistry*. 1996; 35(16):5109–5124. [PubMed: 8611495]
53. Burton RE, Siddiqui SM, Kim YI, Baker TA, Sauer RT. Effects of protein stability and structure on substrate processing by the ClpXP unfolding and degradation machine. *EMBO J*. 2001; 20(12):3092–3100. [PubMed: 11406586]
54. Maillard RA, Chistol G, Sen M, Righini M, Tan J, Kaiser CM, Hodges C, Martin A, Bustamante C. ClpX(P) generates mechanical force to unfold and translocate its protein substrates. *Cell*. 2011; 145(3):459–469. [PubMed: 21529717]
55. Glynn SE, Martin A, Nager AR, Baker TA, Sauer RT. Structures of asymmetric ClpX hexamers reveal nucleotide-dependent motions in a AAA+ protein-unfolding machine. *Cell*. 2009; 139(4):744–756. [PubMed: 19914167]
56. Bieniossek C, Niederhauser B, Baumann UM. The crystal structure of apo-FtsH reveals domain movements necessary for substrate unfolding and translocation. *Proc Natl Acad Sci USA*. 2009; 106(51):21579–21584. [PubMed: 19955424]
57. Bowie JU. Helix packing in membrane proteins. *J Mol Biol*. 1997; 272(5):780–789. [PubMed: 9368657]
58. Sen M, Maillard RA, Nyquist K, Rodriguez-Aliaga P, Presse S, Martin A, Bustamante C. The ClpXP protease unfolds substrates using a constant rate of pulling but different gears. *Cell*. 2013; 155(3):636–646. [PubMed: 24243020]
59. Martin A, Baker TA, Sauer RT. Protein unfolding by a AAA+ protease is dependent on ATP-hydrolysis rates and substrate energy landscapes. *Nat Struct Mol Biol*. 2008; 15(2):139–145. [PubMed: 18223658]
60. Swanwick RS, Shrimpton PJ, Allemann RK. Pivotal role of Gly 121 in dihydrofolate reductase from *Escherichia coli*: the altered structure of a mutant enzyme may form the basis of its diminished catalytic performance. *Biochemistry*. 2004; 43(14):4119–4127. [PubMed: 15065854]
61. Vu ND, Feng H, Bai Y. The folding pathway of barnase: the rate-limiting transition state and a hidden intermediate under native conditions. *Biochemistry*. 2004; 43(12):3346–3356. [PubMed: 15035606]

62. Koehler J, Sulistijo ES, Sakakura M, Kim HJ, Ellis CD, Sanders CR. Lysophospholipid micelles sustain the stability and catalytic activity of diacylglycerol kinase in the absence of lipids. *Biochemistry*. 2010; 49(33):7089–7099. [PubMed: 20666483]
63. Li D, Lyons JA, Pye VE, Vogeley L, Aragao D, Kenyon CP, Shah ST, Doherty C, Aherne M, Caffrey M. Crystal structure of the integral membrane diacylglycerol kinase. *Nature*. 2013; 497(7450):521–524. [PubMed: 23676677]
64. Cordova JC, Olivares AO, Shin Y, Stinson BM, Calmat S, Schmitz KR, Aubin-Tam ME, Baker TA, Lang MJ, Sauer RT. Stochastic but highly coordinated protein unfolding and translocation by the ClpXP proteolytic machine. *Cell*. 2014; 158(3):647–658. [PubMed: 25083874]
65. Schleich JP, Narayan M, Alford C, Mittendorf KF, Carter BD, Li J, Sanders CR. Conformational Stability and Pathogenic Misfolding of the Integral Membrane Protein PMP22. *J Am Chem Soc*. 2015; 137(27):8758–8768. [PubMed: 26102530]
66. Botelho SC, Tatsuta T, von Heijne G, Kim H. Dislocation by the m-AAA protease increases the threshold hydrophobicity for retention of transmembrane helices in the inner membrane of yeast mitochondria. *J Biol Chem*. 2013; 288(7):4792–4798. [PubMed: 23283966]
67. van Stelten J, Silva F, Belin D, Silhavy TJ. Effects of antibiotics and a proto-oncogene homolog on destruction of protein translocator SecY. *Science*. 2009; 325(5941):753–756. [PubMed: 19661432]
68. Jarvi S, Suorsa M, Tadini L, Ivanauskaite A, Rantala S, Allahverdiyeva Y, Leister D, Aro EM. Thylakoid-Bound FtsH Proteins Facilitate Proper Biosynthesis of Photosystem I. *Plant Physiol*. 2016; 171(2):1333–1343. [PubMed: 27208291]
69. Leonhard K, Stiegler A, Neupert W, Langer T. Chaperone-like activity of the AAA domain of the yeast Yme1 AAA protease. *Nature*. 1999; 398(6725):348–351. [PubMed: 10192337]
70. Carvalho P, Goder V, Rapoport TA. Distinct ubiquitin-ligase complexes define convergent pathways for the degradation of ER proteins. *Cell*. 2006; 126(2):361–373. [PubMed: 16873066]

Figure 1.

FtsH as a model degradation machine and bicelles as a model lipid medium. **(a)** Domain architecture and topology of FtsH in the *E. coli* inner membrane. TM: trans-membrane domain; PM: periplasmic domain. **(b)** Comparison of intrinsic ATPase activity of FtsH in 3% (w/v) Triton X-100, bicelles (neutral DMPC/CHAPS, molar ratio = 2.8:1; negatively charged DMPC/DMPG/CHAPS, molar ratio = 3:1:1.4), liposomes (*E. coli* phospholipids; DMPC/DMPG, molar ratio = 3:1) and CHAPS. The data were fitted to Michaelis-Menten (solid lines) and Hill (dashed lines) equations (see Supporting Methods). Data are represented as mean $\pm$ SEM ($n = 3$). **(c)** Diffusion of NBD- and rhodamine-labeled (*top*) lipids and (*bottom*) GpG in 3% DMPC/DMPG/CHAPS bicelles at 37°C monitored by FRET, *i.e.*, the intensity ratio of NBD fluorescence at 535 nm to rhodamine fluorescence at 595 nm. Dead time of mixing was $\sim$ 15 sec. (*Right*) NBD and rhodamine fluorescence spectra after the end of each mixing reaction.

Figure 2.

Degradation of GlpG variants *in vivo*. **(a)** Structure of the TM domain of GlpG (GlpG TM: residues 87–276, PDB code: 3B45³³) with the topology in the *E. coli* inner membrane. **(b–c)** *(Left)* GlpG constructs for testing the effect of the C- or N-terminal degradation markers on GlpG degradation. *(Right)* Time-dependent degradation of GlpG variants monitored by Western blotting in the *E. coli* *-ftsH* and *+ftsH* strains. Time 0 indicates the time when spectinomycin was added to block protein synthesis.

Figure 3.

GlpG degradation by FtsH in bicelles. **(a)** Principle of the fluorescence-based degradation assay for measuring degradation rates of GlpG *in vitro*. GlpG with an engineered Cys residue (G172C) was labeled with thiol-reactive environment-sensitive fluorophore NBD. Upon degradation, NBD fluorescence is quenched due to the transfer of NBD label from the bicellar phase to the aqueous phase. **(b)** (*Left*) Time-dependent degradation of GlpG variants (5 mM) by FtsH ([FtsH₆] = 0.5 mM) monitored by NBD fluorescence in 3% DMPC/DMPG/CHAPS bicelles at 37°C. Fluorescence intensity with 5 mM ATP at each time point was normalized to the intensity without ATP. (*Right*) Degradation of GlpG variants monitored by SDS-PAGE and Coomassie-blue staining. **(c)** Comparison of degradation of GlpG-108 monitored by NBD fluorescence and SDS-PAGE shown in **(b)**. **(d)** Kinetic analysis of FtsH-mediated degradation of GlpG with various degradation markers. Data are represented as mean ± SEM (*n* = 3).

Figure 4.

Steric trapping to measure spontaneous unfolding rate of GlpG. (a) Principle of steric trapping. When biotin tags are conjugated to two specific residues that are spatially close in the folded state but distant in the amino acid sequence, the first mSA binds either biotin label with intrinsic binding affinity, but due to steric hindrance, the second mSA binds only when native tertiary contacts are unraveled by transient unfolding. k_{on} : on-rate constant of mSA binding to biotin label; k_{off} : off-rate constant of mSA-biotin complex; k_U : spontaneous unfolding rate; k_F : refolding rate. (b) Unfolding kinetics of double biotin variants of GlpG measured by steric trapping in 3% DMPC/DMPG/CHAPS bicelles at 37°C. The N- and C-subdomains²² were color-coded in cyan and orange, respectively. GlpG activity with mSA relative to that without mSA was used as an unfolding readout. Errors designate $\pm$ STD from fitting.

Figure 5.

The conformational stability and hydrophobicity of GlpG control degradation. **(a)** The residues for amino acid substitutions to decrease the conformational stability (M100A) or increase the hydrophobicity of the C-terminal TM6 helix (A259L/A263L/V267W/L270F, designated as “LLWF”). **(b)** (Left) Arrhenius plot for measuring the activation energy of unfolding ($E_{a,U}$) of GlpG WT and M100A variant. Spontaneous unfolding rates (k_U) were measured using steric trapping in DDM at various temperatures. (Right) Unfolding energy landscape of GlpG WT and M100A variant in DDM including the thermodynamic stability²² (ΔG_U) and $E_{a,U}$. F, U and TS denote the folded state, unfolded state and transition state, respectively. **(c)** The effect of M100A substitution on k_U of GlpG measured by steric trapping in 3% DMPC/DMPG/CHAPS bicelles at 37°C. Errors designate $\pm$ STD from fitting. **(d)** The effect of M100A substitution on the degradation rates of GlpG with the C-terminal (the 108 tag) or N-terminal (the YccA_N tag) degradation marker. Data are represented as mean $\pm$ SEM ($n = 3-6$). **(e)** The whole-residue hydropathy plot of the GlpG TM domain, and M100A and LLWF variants using Hessa-von Heijne (HvH), Wimley-White (WW) octanol and Tian-Lin-Liang (TLL) depth-dependent hydrophobicity scales. **(f)** The effect of LLWF substitution on the degradation rate of GlpG with the 108 tag. The degradation rates of GlpG_{WT}-108 and YccA_N-GlpG_{WT} were also plotted for comparison. Data are represented as mean $\pm$ SEM ($n = 3$).

Figure 6.
Three-step model of FtsH-mediated degradation of membrane proteins.