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Lack of a Robust Unfoldase Activity Confers a Unique Level of Substrate Specificity to the Universal AAA Protease FtsH

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Summary

FtsH, a member of the AAA family of proteins, is the only membrane ATP-dependent protease universally conserved in prokaryotes, and the only essential ATP-dependent protease in *Escherichia coli*. We investigated the mechanism of degradation by FtsH. Other well-studied ATP-dependent proteases use ATP to unfold their substrates. In contrast, both in vitro and in vivo studies indicate that degradation by FtsH occurs efficiently only when the substrate is a protein of low intrinsic thermodynamic stability. Because FtsH lacks robust unfoldase activity, it is able to use the protein folding state of substrates as a criterion for degradation. This feature may be key to its role in the cell and account for its ubiquitous distribution among prokaryotic organisms.

Introduction

Intracellular proteases play an important role in diverse cell processes—cell division, signal transduction, and stress responses—by rapidly changing the levels of key regulators (Gottesman et al., 1997). This modulatory role is conducted principally by the ATP-dependent proteases, a class that includes the eukaryotic 26S proteasome and its structurally related prokaryotic counterparts. In the bacterium *E. coli*, five such ATP-dependent proteases have thus far been identified: ClpAP, ClpXP, HslUV, Lon, and FtsH (HflB). Among these, FtsH is the only essential protease in *E.coli* and is the only universally conserved ATP-dependent protease in bacteria (reviewed in Karzai et al., 2000). FtsH homologs also play important roles in the mitochondria and chloroplasts of eukaryotic cells (Langer, 2000); for example, mutation of a human FtsH ortholog causes a neurodegenerative disease (Casari et al., 1998).

FtsH is a membrane-anchored metallo-protease with its active site facing the cytoplasm. It contains a well-conserved 200 amino acid motif called the "AAA" motif (so named because the diverse functions of its member proteins are *A*TPase *a*ssociated with *a*ctivity [reviewed in Ogura and Wilkinson, 2001]). FtsH degrades both integral membrane and cytoplasmic proteins. FtsH performs a housekeeping function by degrading uncomplexed membrane proteins such as the SecY translocase (Kihara et al., 1995) and the F_0 component of ATP synthase (Akiyama et al., 1996). Among its known cytoplasmic substrates are three regulatory proteins: the heat shock sigma factor σ^{32}, which sets the transcription rate of the heat shock proteins including FtsH itself (Herman et al., 1995; Tomoyasu et al., 1995); LpxC, an enzyme involved in a key step in lipid metabolism (Ogura et al., 1999); and the activator λcII, which is the sensor responsible for determining whether λ phage chooses to lysogenize or lyse the infected bacterial cell (Shotland et al., 1997).

FtsH, like other ATP-dependent proteases, forms a hexameric ring structure with its proteolytic active site buried within the central cavity (Krzywda et al., 2002; Niwa et al., 2002). Since this proteolytic chamber is accessible only to unfolded and extended polypeptides, a topological barrier between cytoplasmic proteins and the site of proteolysis is created, thereby helping to avoid aberrant degradation of cellular proteins. In the current model for degradation by ATP-dependent proteases, the protease first selects a protein for degradation, either because it has an accessible tag that specifies protease binding or because an internal degradation signal has become exposed. Following recognition, ATP hydrolysis promotes both unfolding of the substrate and its subsequent translocation into the proteolytic chamber (reviewed in Horwich et al., 1999). Evidence for this dual role of ATP comes primarily from the bipartite ClpAP and ClpXP proteases (Kim et al., 2000; Singh et al., 2000; Weber-Ban et al., 1999). Following translocation, the protein is hydrolysed to small fragments, which are released from the chamber into the cytoplasm. Armed with robust unfoldase activity, these Clp family proteases can degrade proteins whose thermostabilities range from 1 to 15 kcal/mol. Because these proteases can unfold most proteins, recognition of the protein usually serves as the commitment step for degradation.

In the present study, we have investigated the mechanism of degradation by FtsH. We previously showed that FtsH recognized and degraded proteins with nonpolar carboxy-terminal tails in vivo (Herman et al., 1998). Two classes of nonpolar tails were identified: those recognized solely by FtsH in vivo (e.g., 108; Figure 1A) and those recognized by both FtsH- and Clp-dependent proteases (e.g., SsrA, Figure 1A). In the present study, we used this tagging system to investigate the mechanism of degradation by FtsH. Using several soluble proteins and their variants, we find that their rates of degradation by FtsH either in vitro or in vivo were inversely related to their overall thermodynamic stability. This was also true for the degradation of a membrane protein and its variants studied in vivo. Thus, unlike other well-studied ATP-dependent proteases, FtsH appears to lack robust unfoldase activity. Instead, our experiments are consistent with the idea that ATP hydrolysis by FtsH is mainly used to translocate unfolded substrates sequentially from the recognition signal to the active site. We propose that lack of a robust unfoldase enables FtsH to discriminate among proteins based on their thermodynamic sta-

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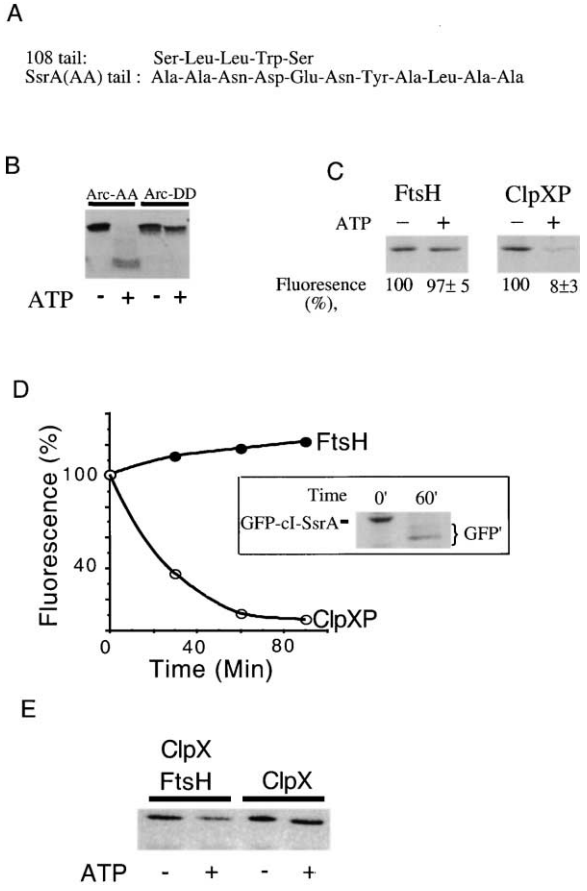

Figure 1. Degradation of Tagged Arc and GFP with Nonpolar Tails

(A) Amino acid sequence of the FtsH specific tail (108) and the universal tail (SsrA) recognized by FtsH and the ClpP-dependent proteases.

(B) Degradation of Arc by FtsH. 30 μM Arc-SsrA (AA) or Arc-SsrA (DD) was incubated with 1 μM FtsH at 37°C for 1 hr. Products were visualized by Coomassie blue staining after separation on a 16.5% Tris-tricine gel. In this and subsequent figures, Arc and FtsH concentrations are calculated in terms of subunit equivalents, and reactions were performed in buffer H with 10 mM ATP, unless otherwise indicated.

(C) Degradation of GFP-SsrA by FtsH or ClpXP. 10 μM of GFP-SsrA was incubated with FtsH (1 μM) or ClpXP (1 μM) at 37°C for 1 hr, separated on a 12% SDS gel and visualized with Coomassie blue staining. Numbers underneath the lanes represent percent of initial GFP fluorescence (509 nm) remaining at the end of the reaction.

(D) Degradation of GFP-CI-SsrA by FtsH or ClpXP. Fluorescence of GFP-CI-SsrA (509 nm) was monitored at different times after addition of 1 μM FtsH or 1 μM ClpXP at 37°C. The inset shows the 0 and 60 min time points of the FtsH reaction, separated on a 12% SDS gel and visualized with Coomassie blue staining. The decrease in size reflects partial or complete degradation of the CI moiety of the fusion protein.

(E) Degradation of GFP-SsrA (10 μM) by FtsH (1 μM) and ClpX (1.5 μM) or ClpX (1.5 μM) alone after a 60 min incubation. Products were visualized by Coomassie blue staining after separation on a 12% SDS gel.

bility, after the initial binding step has occurred. This property of FtsH is likely to be central to its housekeeping and regulatory roles in degrading both membrane-localized and soluble proteins.

Results

FtsH Recognizes Nonpolar Tails

We previously showed that the amino-terminal domain of λcI was degraded by FtsH when tagged with either the universal SsrA tail or the FtsH-specific 108 tail (Herman et al., 1998). We show here that the unrelated protein P22 Arc repressor is also degraded by FtsH in vitro when it is tagged either with SsrA (Figure 1B) or with 108 (Figure 3A). Degradation was tail specific since changing the last two alanines in SsrA to aspartate residues blocked degradation of Arc-SsrA (Figure 1B). These data confirm our previous finding that the SsrA or 108 motif is sufficient to mediate recognition by FtsH.

FtsH Cannot Degrade GFP-Tagged Proteins

Because conformational changes in GFP can be monitored in real time by changes in its fluorescence, it has been possible to show that ATP-dependent proteases globally unfold GFP containing appropriate recognition signals prior to degrading them (Benaroudj and Goldberg, 2000; Hoskins et al., 2000; Kim et al., 2000; Singh et al., 2000; Weber-Ban et al., 1999). We investigated whether FtsH can likewise unfold and degrade GFP-SsrA or GFP-108. We found that FtsH does not degrade GFP-SsrA (Figure 1C) or GFP-108 (data not shown) as measured by change in either its fluorescence or amount. ClpXP protease degraded GFP-SsrA (Figure 1C), indicating that our buffer conditions do not interfere with GFP-SsrA degradation. Similar results were obtained in vivo; whereas GFP-SsrA, predominantly recognized by ClpXP, is degraded (Bohn et al., 2002), GFP-108, exclusively recognized by FtsH, is stable (data not shown). Lack of degradation does not result from inaccessibility of the SsrA tag because FtsH was able to degrade the CI-SsrA moiety of a GFP-CI-SsrA fusion protein, but not GFP itself, as demonstrated by both fluorescence measurements (Figure 1D) and the presence of GFP on an SDS gel (Figure 1D, inset). In contrast, ClpXP protease degraded this entire fusion protein, as seen by the rapid decrease of fluorescence (Figure 1D).

GFP is a very stable protein, whose half-life for spontaneous unfolding is estimated to be 20 years at 25°C (Kim et al., 2000). In contrast, the amino-terminal domain of λcI, which is a good FtsH substrate, has a half-life of less than 1 min for spontaneous unfolding at 25°C (cited in Kim et al., 2000). The inability of FtsH to unfold GFP is likely to account for lack of degradation as FtsH degraded unfolded GFP-SsrA. About 60% of the GFP-SsrA was degraded by FtsH when ClpX was simultaneously present to promote unfolding of GFP-SsrA (Figure 1E). Degradation was FtsH dependent since ClpX by itself had no effect on GFP-SsrA degradation, and degradation occurred despite the presence of a serine-protease inhibitor (AEBSF) included in the reaction buffer to inactivate any contaminating ClpP peptidase. Additionally, when GFP-SsrA was unfolded with urea and then rapidly diluted into reaction buffer, FtsH degraded about 40% of the protein. This degradation was initiated by binding to the SsrA tail, since FtsH was unable to degrade unfolded GFP-DD in which the last two residues are replaced with aspartate residues (data not shown). Taken

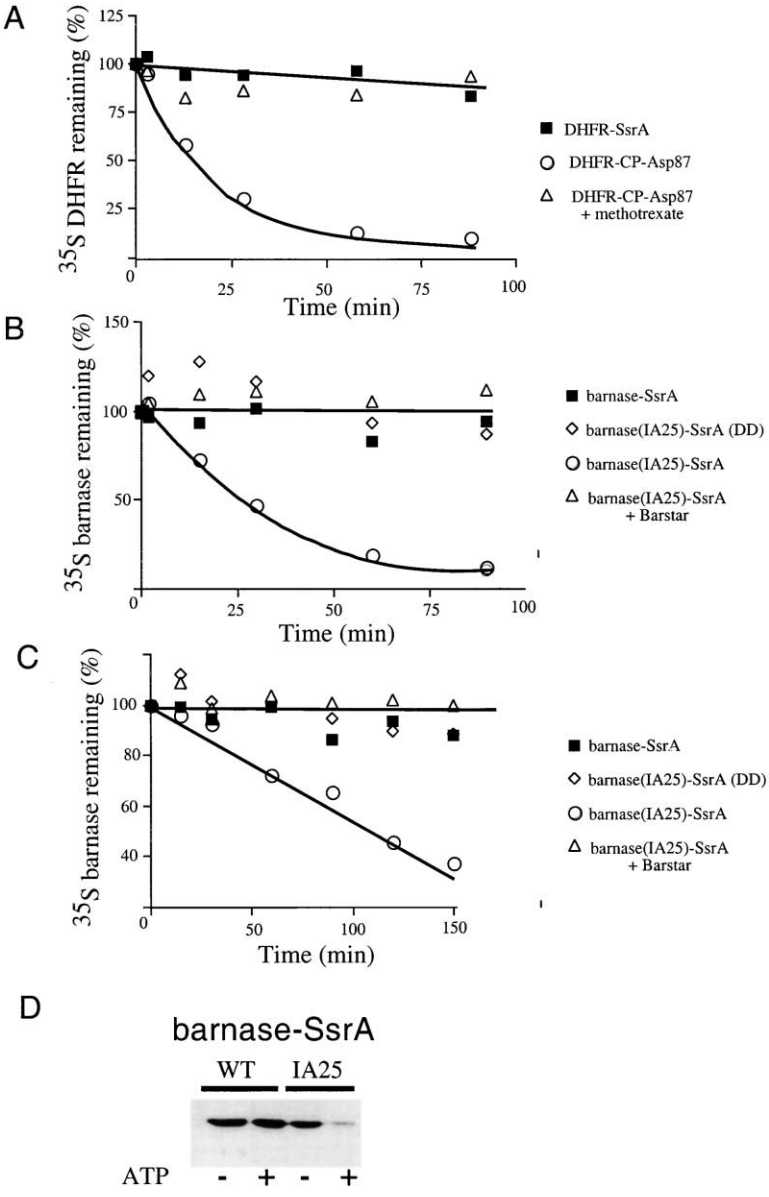

Figure 2. Degradation of DHFR-SsrA or Barnase-SsrA and Its Variants by FtsH

(A and B) Degradation of partially purified (A) [^{35}S]DHFR-SsrA or (B) [^{35}S]barnase-SsrA and its variants by FtsH (0.34 μM) at 37°C. At various times, samples were separated on a 10% SDS gel and visualized by electronic autoradiography. Undegraded [^{35}S] substrate at each time point is plotted as a percentage of the total substrate present at t = 0. Methotrexate (10 μM) and barstar (10 μM) were used when indicated.

(C) Degradation of partially purified barnase-SsrA and its variants by FtsH-enriched IMV. Degradation of [^{35}S]barnase-SsrA variants by FtsH in IMV after incubation at 37°C in buffer H without NP40. At various times after FtsH addition, samples were separated on a 10% SDS gel and visualized by electronic autoradiography. Undegraded [^{35}S] substrate at each time point is plotted as a percentage of the total substrate present at t = 0. Barstar (10 μM) was added when indicated. The slightly lower rate of degradation most likely reflects less FtsH in the IMV than in the solubilized preparation.

(D) Degradation of purified barnase-SsrA and barnase(IA25)-SsrA. Barnase substrates (20 μM) were incubated with FtsH (1 μM) at 37°C for 2 hr and visualized with Coomassie blue staining after separation on a 15% SDS gel.

together, these data suggest that FtsH cannot degrade stable proteins like GFP.

The Stability of Substrates of Diverse Structure Influences the Rate of FtsH Degradation

To investigate the role of protein stability on degradation by FtsH, we used two model proteins with well-characterized folding properties, barnase and dihydrofolate reductase (DHFR), which differ greatly from each other in structure (Fersht and Daggett, 2002). Both proteins are stable, with an unfolding half-life of hours, and become substrates for degradation by ClpXP or ClpAP when tagged with SsrA (Lee et al., 2001). We synthesized and labeled these tagged substrates in a transcription and translation rabbit reticulocyte lysate, partially purified them, and asked whether they were degraded by FtsH.

FtsH was unable to degrade DHFR-SsrA (Figure 2A), but could degrade DHFR-CP Asp87, a circularly permutated mutant of DHFR, which has a faster rate of unfolding but no gross alterations in structure or enzymatic activity (Iwakura et al., 2000). Addition of methotrexate, a cofactor that stabilizes DHFR-CP Asp87 against unfolding, blocked FtsH degradation. Other mutant proteins with reduced stability (DHFR-CP Ala145, DHFR-CP Pro25) were also degraded by FtsH (data not shown).

FtsH was also unable to degrade barnase-SsrA (Figure 2B), but could degrade barnase(IA25)-SsrA, a variant of barnase with a faster rate of unfolding but no gross alterations in structure or enzymatic activity (Serrano et al., 1992). Degradation was tail specific since changing the last two alanine residues of barnase(IA25)-SsrA to aspartates blocked degradation by FtsH. Addition of barstar, a cofactor that stabilizes barnase(IA25)-SsrA against unfolding, blocked degradation by FtsH. Poor degradation of barnase-SsrA was not due to use of

purified solubilized-FtsH, as similar results were obtained with FtsH-enriched inverted membrane vesicles (IMV) in which FtsH is in its proper membrane environment (Figure 2C). In this and all other experiments performed with IMV, no detergent was present, a serine protease inhibitor, AEBSF, prevented degradation by serine proteases in the membrane, and IMV degradation was shown to be FtsH dependent since it required both ATP and overexpression of FtsH (data not shown). Thus, substrate stability alters the rate of degradation, whether the FtsH is soluble or present in its normal membrane environment. Finally, poor degradation was not due to inhibitory components present in the partially purified substrates—when barnase(IA25)-SsrA and barnase-SsrA were purified from *E. coli*, FtsH degraded only the unstable barnase(IA25)-SsrA variant (Figure 2D). Taken together, these results indicate that for two substrates that differ in structure, the efficiency of FtsH degradation depends on the thermodynamic stability of the substrate.

The Thermostability of Arc Variants Influences the Rate of FtsH Degradation Both In Vitro and In Vivo

To compare degradation by the ATP-dependent protease ClpXP and the ATP-independent protease ArgC to degrade substrates, Burton et al. used a matched series of Arc protein substrates, which differed in their thermostability (Burton et al., 2001). Wild-type P22 Arc repressor is a homodimer having two intertwined subunits, each composed of a β strand and two α helices. The extremely stable NC11 variant, when oxidized, forms a native disulfide bond between the two subunits of the Arc dimer. The somewhat stable PL8 variant has extra hydrogen bonds at the ends of the β strand, and the less stable FA10 variant has alterations in the hydrophobic core. These four substrates were degraded at similar rates by the ATP-dependent protease, ClpXP. In contrast, the intrinsic stability of the substrate altered the rate of degradation by endoproteinase Arg-C, an ATP-independent protease that cannot actively promote unfolding (Burton et al., 2001).

We used these same substrates, tagged with the FtsH-specific 108 tail, to test the effect of substrate stability on FtsH-mediated degradation in vitro. Except for the extremely stable variant $NC11_{OX}$Arc-108, all were degraded by FtsH after a long incubation (Figure 3A). We measured the rates of degradation of purified ^{35}S-labeled wild-type Arc-108, PL8 Arc-108, and FA10 Arc-108 by the appearance of acid-soluble counts at a series of substrate concentrations and took data from the linear portion of the degradation kinetic curves (data not shown). For each variant, we fit the dependence of degradation rates on substrate concentration to a simple Michaelis-Menten model (r value > 0.97) (Figure 3B) and obtained the k_{cat} (units per min per FtsH monomer) and K_M values. Whereas wild-type Arc had a k_{cat} of 0.37 $\pm$ 0.01/min, the hyperstable variant PL8 had a k_{cat} of 0.16 $\pm$ 0.01/min. This difference is not due to altered binding, as the K_M values for the two proteins are quite similar (Figure 3B, legend). The 2.3-fold difference in the degradation rates of these two substrates is very close to the 2.8-fold difference in the degradation rates exhibited by endoproteinase Arg-C, which cannot promote active unfolding. Likewise, the less stable FA10 Arc-108 was degraded more rapidly than wild-type Arc-108. However, FtsH degraded FA10 Arc-108 only 2-fold faster than Arc-108 ($k_{cat} = 0.81 \pm 0.02$ /min), whereas Arg-C degraded the less stable substrate at a 5-fold greater rate. As described in the discussion, this difference may reflect the requirement for FtsH to translocate the substrate to the active site. Together, these experiments indicate that there is a correlation between the thermodynamic stability of the substrate and the kinetics of degradation by FtsH.

We verified that FtsH shows this same dependence on substrate stability when present in its normal location

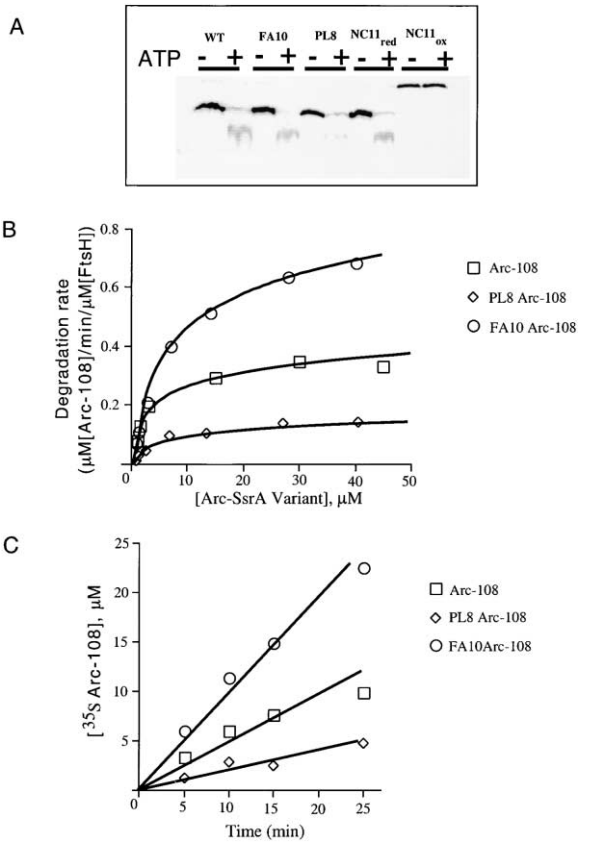

Figure 3. In Vitro Degradation of Arc-108 Variants with Different Thermostabilities

(A) Degradation of Arc-SsrA and its variants. 30 μM substrates (FA10, PL8, NC11 reduced, NC11 oxidized) were incubated with FtsH (1 μM) at 37°C for 8 hr in buffer H containing 0.5 mM β-mercaptoethanol. Products were visualized by Coomassie blue staining after separation on a 16.5% Tris-tricine gel.

(B) Dependence of FtsH degradation rate on the concentration of Arc-108 and its variants. Rates of degradation of [^{35}S] Arc-108 and its variants by FtsH (0.6 μM) were measured at a series of concentrations of each substrate by the release of TCA soluble counts. Each line is a nonlinear least squares fit of the combined data of each substrate to the Michaelis-Menten equation. The K_M values for the data shown in Figure 4B are: Arc-108 = 4 $\pm$ 0.4 μM, PL8 Arc-108 = 6 $\pm$ 1.2 μM, and FA10 Arc-108 = 7 $\pm$ 0.5 μM.

(C) Degradation of Arc-108 variants by FtsH-enriched IMV. Degradation of [^{35}S] Arc-108 variants (40 μM) by FtsH in IMV after incubation at 37°C in buffer H without NP40 was assayed by release of TCA soluble counts. This substrate concentration ensures at least 90% saturation of FtsH.

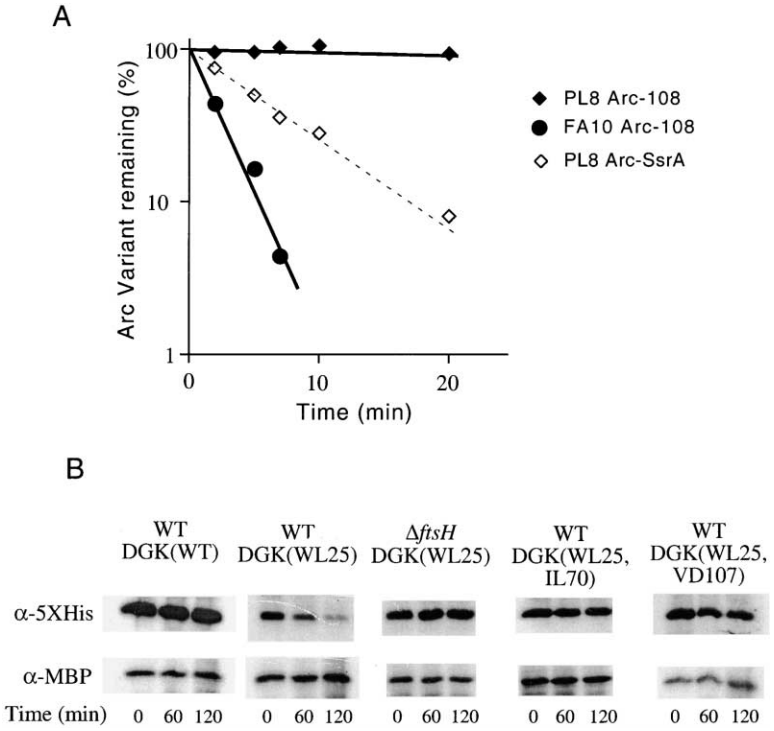

Figure 4. In Vivo Degradation of Arc-108 Variants with Different Thermostabilities

(A) Degradation of FA10 Arc-108, PL8 Arc-108, and PL8 Arc-SsrA in BL21 cells. Cultures growing exponentially at 37°C in M9 glucose (supplemented with kanamycin and all amino acids except methionine and cysteine) were induced, pulse labeled with [^{35}S] methionine, and chased. Samples were removed at indicated times into cold TCA (5% final). Precipitated pellets were resuspended and electrophoresed in 16.5% Tris-tricine SDS gel, and the Arc bands were quantified by electronic autoradiography (Herman et al., 1998).

(B) Degradation of the membrane protein, diacyl-glycerol-kinase (DGK). Western blot analysis of His_6-DGK variants in wild-type or Δ*ftsH* cells (JM105) after blocking protein synthesis with spectinomycin. Cultures growing exponentially at 37°C in M9 glucose (supplemented with ampicillin and all amino acids) were induced for 50 min before addition of spectinomycin. Samples were removed at the times shown into cold TCA (5% final). Precipitated pellets were resuspended and electrophoresed in 15% SDS-polyacrylamide gels. Gels were blotted and probed with anti-His antibodies to visualize DGK and with anti-MBP antibodies to visualize MBP as a blotting control.

in the inner membrane. FtsH in IMV degraded the hyperstable PL8 Arc-108 protein 4.5-fold more slowly than the unstable FA10 Arc-108 protein (Figure 3C), as was observed with detergent-solubilized FtsH. Thus, substrate stability alters the rate of degradation, whether the FtsH is soluble or present in its normal membrane environment.

To determine whether FtsH degradation also depends on thermostability in vivo, we compared the in vivo stability of the hyperstable and less stable Arc variants. Whereas the less stable FA10 variant is rapidly degraded, the hyperstable PL8 variant is stable in vivo (Figure 4A). Although we previously showed that the108 tail is FtsH specific in vivo (Herman et al., 1998), we directly verified that FA10 variant degradation is solely dependent on the presence of the FtsH protease and also showed that the half-life of this variant was unchanged in a double mutant strain lacking both Lon and ClpP peptidase (data not shown). As expected, when variant tagged with SsrA, which is recognized mainly by the ClpXP protease in vivo (Levchenko et al., 2000), the hyperstable PL8 is degraded (Figure 4A). Taken together, these experiments demonstrate that the rate of degradation by FtsH is dependent on protein thermodynamic stability, both in vitro and in vivo. These experiments imply that FtsH, like the ATP-independent endoproteinase Arg-C, lacks a robust unfoldase activity.

Stability of a Membrane Protein Influences FtsH Degradation

One of the major functions of FtsH is to degrade membrane proteins. In this context, FtsH recognizes an N-terminal cytosolic tail which must be >20 amino acid residues in length, without regard to its exact sequence (Chiba et al., 2000). Motivated by our finding that the thermostability of cytoplasmic substrates influences the rate of FtsH degradation, we asked whether that was also true for membrane proteins. Diacyl-glycerol-kinase (DGK) is a small membrane protein containing three transmembrane helices and an amino-terminal cytoplasmic helix of about 34 amino acids (Lau and Bowie, 1997). Mutations that increase and decrease the thermostability of this protein have been identified (Lau and Bowie, 1997; Zhou and Bowie, 2000). Whereas wild-type DGK is stable in vivo, the WL25 variant with decreased thermostability is degraded (Figure 4B). Degradation is FtsH dependent since no degradation was observed in the Δ*ftsH* strain, which carries an extragenic suppressor permitting growth. When mutations that increase the thermostability of wild-type DGK (IL70, VD107) were introduced into the unstable WL25 variant, each of these stabilizating mutations block degradation by FtsH. These results suggest that the thermostability of membrane proteins influences their degradation by FtsH.

Temperature Dependence of FtsH Degradation

We previously showed that degradation of the FtsH-specific substrate λcI-108 increases with temperature in vivo (Herman et al., 1998). To determine whether this resulted solely from the intrinsic temperature dependence of FtsH, as it has recently been suggested (Kobiler et al., 2002), we compared the temperature dependence of FtsH-mediated degradation of β-casein (an unfolded substrate) with that of λcI-SsrA (a folded substrate). The rate of degradation of β-casein increases about 3-fold between 25°C and 42°C (Figure 5A), which is similar to the increase in the intrinsic ATPase activity of FtsH in this temperature range (Figure 5B). In sharp contrast, the k_{cat} values for λcI-SsrA degradation by FtsH varied from 0.04 ± 0.005/min at 25°C to 0.69 ± 0.02/min at 42°C,

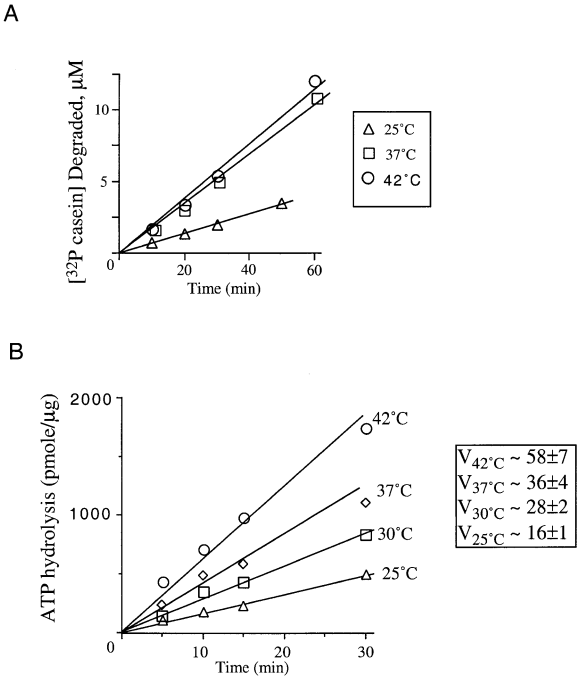

Figure 5. Effect of Temperature on FtsH Activity

(A) Rate of β-casein degradation by FtsH at different temperatures. [32P] β-casein (70 μM) was incubated with FtsH (0.6 μM) in the presence of 1 mM ATP. The vertical axis represents the amount of [32P] TCA soluble products released by FtsH. The best fit straight line through the data points is shown.

(B) ATPase activity of FtsH at different temperatures. The rate of ATPase activity was determined at 1 mM ATP with 0.6 μM of FtsH. Unit of ATPase activity (pmole/min) are indicated in the inset. The best fit straight line through the data points is shown.

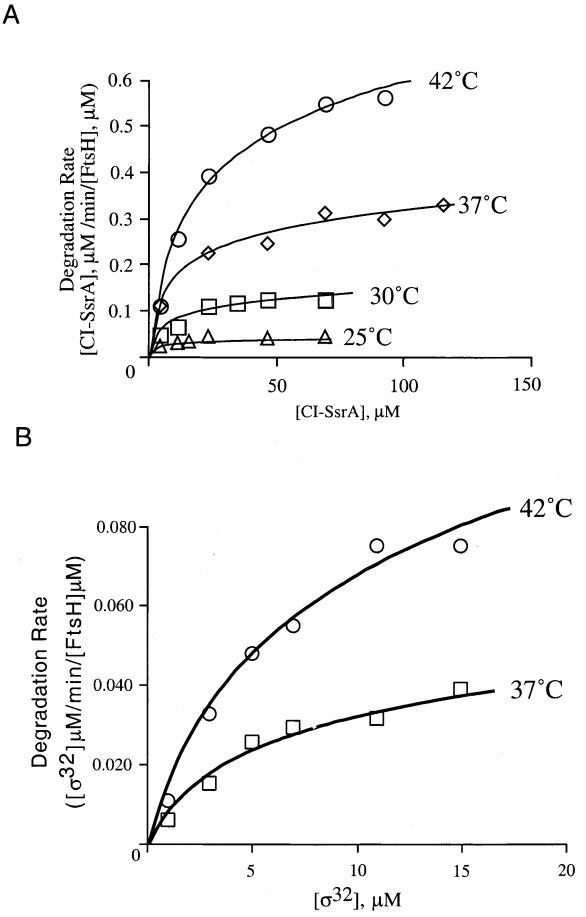

Figure 6. Temperature Dependence of FtsH Degradation of λcI-SsrA and σ^{32}

(A and B) Rates of degradation of (A) [35S] λcI-SsrA and (B) [35S] σ^{32} by FtsH (0.6 μM) were measured at a series of concentrations of each substrate by the release of TCA-soluble counts. Each line is a nonlinear least squares fit of the combined data of each substrate to the Michaelis-Menten equation (r value > 0.97). Substrate and FtsH concentrations are calculated in terms of subunit equivalents. The K_M values for λcI-SsrA are: 5 ± 2 μM (25°C), 11 ± 3 μM (30°C), 12 ± 3 μM (37°C), and 20 ± 2 μM (42°C). The K_M values for σ^{32} are: 4 ± 2 μM (37°C) and 10 ± 2 μM (42°C). The k_{cat} values for λcI-SsrA are: 0.04 ± 0.005 (25°C), 0.15 ± 0.01 (30°C), 0.35 ± 0.02 (37°C), and 0.69 ± 0.02 (42°C). The k_{cat} values for σ^{32} are: 0.042 ± 0.007 (37°C) and 0.112 ± 0.01 (42°C).

indicating a 17-fold increase at the higher temperature (Figure 6A), far greater than the 4-fold increase in ATPase activity between these two temperatures (Figure 5B). This increase is not due to increased binding, as the K_M was actually higher at 42°C (20 ± 2 μM) than at 25°C (5 ± 2 μM) (Figure 6A, legend). These experiments indicate that a temperature-dependent change in the substrate, most likely substrate unfolding, in addition to an increase in FtsH activity is necessary to account for the temperature dependence of degradation for folded substrates.

Degradation of the Heat Shock Factor σ^{32}

FtsH degrades the heat shock factor σ^{32} rapidly in vivo (t1/2 = 1 min at 30°C and 20 s at 42°C) (Straus et al., 1987), but very poorly in vitro (Tomoyasu et al., 1995). Poor degradation is not caused by poor binding as we find that the apparent K_M of σ^{32} for FtsH at 37°C is 4 ± 2 μM (Figure 6B, legend), similar to that exhibited by the good FtsH substrate λCI-SsrA (Figure 6A). σ^{32} is degraded slightly better at 42°C (k_{cat} 42°C/37°C = 2.7), even though the apparent K_M of σ^{32} for FtsH at 42°C is 10 ± 2 μM. As this increase is greater than the increase in intrinsic enzyme activity in this temperature range (Figures 5A and 5B), substrate-dependent changes, most likely unfolding, must partially account for increased degradation at higher temperatures. As σ^{32} is intrinsically a rather stable protein (Blaszczak et al., 1995), we suggest that σ^{32} is a poor substrate for FtsH in vitro because it has a low rate of spontaneous unfolding.

FtsH Degrades Its Substrates Sequentially from the SsrA Tag

We utilized multidomain proteins to probe the direction of FtsH degradation. If degradation proceeds sequentially from the SsrA tag, then stabilizing the domain adjacent to the tag should stabilize the entire protein, whereas stabilizing the distal domain should permit degradation of the domain adjacent to SsrA. Fusion proteins containing destabilized mutants of DHFR and barnase in both permutations, DHFR Lys38CP-barnase(IA25)-SsrA and barnase(IA25)-DHFR Lys38CP-SsrA, were synthesized and labeled by in vitro translation in a rabbit reticu-

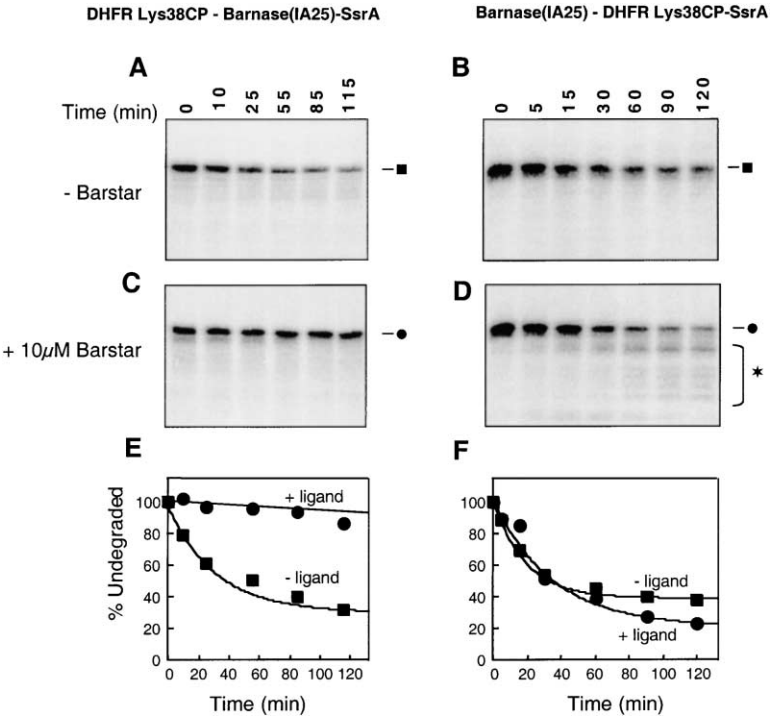

Figure 7. Sequential Degradation of a Two-Domain Protein

(A–D) Autoradiograms of SDS-PAGE gels showing degradation by FtsH of the partially purified [^{35}S] DHFR Lys38CP-barnase(IA25)-SsrA fusion protein (A and C) or the [^{35}S] barnase(IA25)-DHFR Lys38 CP-SsrA fusion protein (B and D) in the absence (A and B) or presence (C and D) of 10 μM barstar as described in Figure 3 except that 1 mM PMSF was present. The full-length protein bands are indicated as closed squares (A and B) and closed circles (C and D). Degradation end products in (D) are indicated (star).

(E–F) Quantification of the amount of full-length [^{35}S] DHFRLys38 CP-Barnase(IA25)-SsrA (E) or [^{35}S] barnase(IA25)-DHFR Lys38 CP-SsrA (F) remaining in the absence (closed squares) or presence (closed circles) of 10 μM barstar at the indicated time points. Curves through the data points are the best fits to a single exponential or a straight line.

locyte lysate. FtsH efficiently degraded both proteins (Figures 7A, 7B, 7E, and 7F). We then stabilized the barnase(IA25) domain by barnstar so that it cannot be degraded by FtsH (Figure 2B). Consistent with the expectations of the sequential degradation model, degradation of DHFR Lys38CP-barnase(IA25)-SsrA was prevented (Figures 7C and 7E), whereas Barnase(IA25)-DHFR Lys38CP-SsrA disappeared at the normal rate and accumulated a lower molecular weight protein, presumably barnase (Figures 7D and 7F). We conclude that FtsH degrades these substrates sequentially from the SsrA tag.

Discussion

ATP-dependent proteases play crucial housekeeping and regulatory roles in the cell. A great deal was known about the mechanism of degradation by the ATP-dependent ClpXP and ClpAP proteases, but almost nothing was known about this process for FtsH, the only ATP-dependent protease that is universally present in eubacterial cells. The ClpXP and ClpAP proteases can unfold and degrade most cellular proteins once they are recognized. Hence their main commitment step is recognition. The principal finding of this work is that the rate of degradation by FtsH is influenced by the thermostability of its protein substrate both in vitro and in vivo, indicating that FtsH lacks a robust unfoldase. The absence of a strong unfoldase allows FtsH to use the thermostability or folding state of proteins in addition to substrate recognition as components of the degradation decision. This property may account for the ubiquitous distribution of FtsH in the bacterial world.

FtsH Lacks Robust Unfoldase Activity

Several ATP-dependent proteases, including ClpXP, ClpAP, the proteasome, and the archaeal proteasome-regulatory ATPase complex PAN possess a robust unfoldase activity that allows degradation of thermostable proteins (Benaroudj and Goldberg, 2000; Kim et al., 2000; Lee et al., 2001; Singh et al., 2000; Weber-Ban et al., 1999). Surprisingly, multiple lines of evidence suggest that FtsH lacks a robust unfoldase activity both in vitro and in vivo. First, FtsH degrades structurally intact, destabilized mutant variants of DHFR and barnase, but not the wild-type proteins. Second, substrate unfolding, in addition to changes in the intrinsic activity of FtsH are required to explain the temperature dependence of λCI-SsrA degradation. Third, although susceptibility of proteins to degradation by ClpAP, ClpXP, and the proteasome is predominately determined by the local structure of the folded domain adjacent to the degradation tag (Lee et al., 2001), FtsH was unable to degrade even those substrates that were highly sensitive to degradation by the ATP-dependent proteases studied previously. Most importantly, the fact that the rate at which both FtsH and the ATP-independent endoproteinase Arg-C degraded the same set of Arc substrates depended on substrate thermostability, suggested that FtsH, like Arg-C, relies on local spontaneous substrate unfolding for proteolytic activity. Moreover, the in vivo rate of degradation of these same Arc substrates by FtsH was also dependent on their thermostability. Thus, our data strongly support the idea that FtsH, unlike other ATP-dependent proteases, cannot unfold thermostable substrates either in vitro or in vivo but relies instead on the local spontaneous unfolding of the substrates for degradation. Interestingly, the in vivo difference between the degradation rates of the stable PL8 Arc-108 variant and the unstable FA10 Arc-108 variant was greater than the difference in vitro. Natural substrates of FtsH may compete with PL8 Arc-108 for binding to FtsH in vivo, thereby displacing PL8 Arc-108 before it

has a chance to spontaneously unfold and become degraded, or additional factors may directly influence the degradation by FtsH. Thus, the cellular milieu might amplify the effect of thermostability on the degradation rate of FtsH.

FtsH shows a lower relative increase in degradation of the less stable Arc protein compared to the wild-type protein (2-fold) than does the Arg-C endoproteinase (5-fold) in vitro. If FtsH had an additional rate-limiting step in degradation, not present in the Arg-C endoproteinase, this could explain why FtsH exhibits a more modest increase. Translocation into the active site could be such a step, as several lines of evidence suggest that translocation in FtsH is ATP dependent and rate limiting for its degradation of unstable proteins. Small peptides, which can easily diffuse into the FtsH proteolytic chamber, are degraded in the presence of ATPγS, whereas denatured proteins like β-casein, which must be translocated into the chamber prior to degradation, require ATP hydrolysis (Karata et al., 2001), indicating that FtsH utilizes ATP in the translocation process. That the degradation rate of an unfolded substrate, β-casein, is proportional to the rate of ATP hydrolysis by FtsH (Figures 5A and 5B) suggests that translocation is rate limiting for degradation. Moreover, preliminary evidence confirms a striking prediction of this idea: that the degradation rate of stable proteins should be relatively insensitive to partial inhibition of FtsH ATPase (and therefore translocation) because they should be limited by their rate of unfolding, whereas the degradation rate of unstable proteins should be more sensitive to partial inhibition of FtsH ATPase because they should be limited by translocation. In agreement with this model, inhibiting the FtsH ATPase with 0.5 mM vanadate (Tomoyasu et al., 1995) or with an excess of ATPγS decreases the rate of degradation of an unstable Arc variant 3-fold but decreases degradation of wt Arc only 1.3-fold (data not shown). Likewise, degradation of another relatively thermostable substrate, σ^{32}, was only slightly inhibited by 0.5 mM vanadate (Tomoyasu et al., 1995). Together, these results support the idea that the ATPase activity of FtsH is utilized primarily for translocation and that this step is rate limiting in the degradation of unstable substrates. This may also be true of the *m-* and *i-AAA* proteases, mitochondrial homologs of FtsH. These proteases, which mediate degradation of membrane proteins in an ATP-dependent manner, stop degradation when they encounter a stably folded domain (Leonhard et al., 2000). We note that these results do not imply that FtsH completely lacks an unfoldase activity. Because of the cooperative nature of the folding process, some unfolding of substrates is likely to occur during the translocation process itself.

The Unique Cellular Roles of FtsH Require a Weak Unfoldase Activity

FtsH is the only protease completely conserved among all eubacteria examined to date and is essential for viability in *E.coli*. Homologs of FtsH are present in both mitochondria and chloroplasts, where they play important roles (Langer, 2000). Why does this protease, which lacks robust unfoldase activity, play such a key role in the bacterial cell? We suggest that lack of a robust unfoldase activity is a prerequisite for certain of its cellular functions.

The major housekeeping function of FtsH is to sequentially degrade membrane proteins that are either abnormal or present in excessive amounts starting from the cytoplasmic face of the protein (Kihara et al., 1999). Importantly, the primary criterion for membrane proteins recognition is that they possess a cytoplasmic tail of sufficient length ($\geq$20 amino acids) (Chiba et al., 2000, 2002). If the recognition signal is so generic, how then does FtsH know when to degrade its substrate? We suggest that the missing information is provided by the requirement for proteins to unfold readily in order to be degraded. The fact that SecY is degraded by FtsH only when it is improperly folded because it is not associated with its partner protein (Kihara et al., 1995) supports this idea. Our finding that FtsH can degrade a destabilized membrane protein, DGK, but not wild-type DGK and that this destabilized DGK is refractory to degradation when it contains a hyperstabilizing mutant is also consistent with this notion. Thus, a generic recognition signal allows FtsH to recognize many membrane proteins and then to determine their fate with respect to degradation by assessing their folding state in the membrane. This strategy may also allow FtsH to sense and respond to protein damage in the periplasmic portion of membrane proteins. Interestingly, cytoplasmic substrates may also have a rather generic recognition signal, as FtsH recognized all of the randomly selected nonpolar tails that we tested (Herman et al., 1998). In contrast, only a subset of these tails were recognized by ClpP-dependent proteases. By utilizing a rather generic recognition signal and then scanning for proteins of low thermodynamic stability, FtsH can accomplish its housekeeping functions.

There is circumstantial evidence that FtsH has chaperone activities in the cell. FtsH mutants are defective in anchoring integral membrane proteins, export of secretory proteins and colicin tolerance (Akiyama et al., 1994a, 1994b; Qu et al., 1996). Since these activities are rescued by overexpression of cytoplasmic chaperones, it suggests a chaperone function for FtsH (Shirai et al., 1996). In the ClpAP and ClpXP proteases, the regulatory subunits can carry out a chaperone function when they are separate from the peptidase (Mhammedi-Alaoui et al., 1994). The fact that FtsH can do so in association with its peptidase is likely to result from the fact that stable substrate domains can be bound without being degraded.

One important function of ATP-dependent proteases is to carry out regulated degradation of specific proteins. FtsH is known to degrade two key cytoplasmic regulatory proteins: (1) the alternative σ factor, σ^{32}, which is responsible for transcription of the heat shock genes (Herman et al., 1995; Tomoyasu et al., 1995), and (2) LpxC, which carries out the committed step in lipid A biosynthesis and is an important point of regulation in setting the balance between lipid A and phospholipid biosynthesis (Ogura et al., 1999). Their degradation has several features in common: (1) both are rapidly degraded in vivo, (2) degradation of both is responsive to physiological conditions, and (3) both are poorly degraded in vitro. Our experiments indicate that slow degradation of σ^{32} in vitro results from its intrinsic stability

and that FtsH lacks a robust unfoldase in vivo just as it does in vitro. Why then can FtsH degrade σ^{32} rapidly in vivo? We suggest that the cell has specific unfoldases that permit LpxC and σ^{32} to be rapidly degraded by the FtsH protease. The DnaK chaperone machine (DnaK, DnaJ, and GrpE), which is required for the rapid degradation of σ^{32} in vivo (Straus et al., 1990; Tilly et al., 1989) does not fulfill this role, as it cannot enhance degradation in vitro (Blaszczak et al., 1999) (data not shown). By making the amount or activity of these unfoldases responsive to the particular physiological condition to be sensed, the cell can modulate the rate at which FtsH degrades these key regulatory molecules. This mode of regulated proteolysis capitalizes on the inability of FtsH to unfold its substrates efficiently. This regulatory mode has an interesting parallel to the one documented for Clp-dependent proteases. Here, substrates are usually selected at the level of substrate recognition; regulated degradation is accomplished with additional factors that modulate substrate recognition (Dougan et al., 2002).

The universal conservation of the heat shock response among all organisms attests to the necessity of protein-folding homeostasis for cellular survival. We suggest that a "subtle protease" such as FtsH plays a crucial role in this scheme. For most ATP-dependent proteases, substrates are automatically degraded once they are bound. This paradigm is ideal for ridding the cell of proteins where binding is an excellent indicator of defect; for example, SsrA-tagged proteins and proteins whose (normally hidden) degradation tags become exposed. However, there are other cases where additional criteria must be utilized to determine whether to degrade. In such cases, the FtsH protease, which can recognize its substrates without degrading them, is a key player. This feature allows FtsH to selectively degrade those proteins that have low to moderate thermostability or an altered protein folding state, and to function as a chaperone. It may also allow FtsH to regulate the degradation of key proteins by utilizing specific unfoldases whose amount or activity is responsive to cellular conditions. Together, these activities provide FtsH with a unique ability to monitor protein-folding homeostasis and are likely to account for the universality of this protease in the bacterial cell.

Experimental Procedures

Buffers

Buffer A contains 20 mM monoethanolamine (pH 9.0), 20% glycerol, 100 mM KCl, 5 mM $MgCl_2$, 1 mM AEBSF (4-[2-aminoethyl]-benzenesulfonyl fluoride hydrochloride), 1 mM DTT, and 0.1 mM ATP. Buffer B contains 20 mM monoethanolamine (pH 9.0), 10% glycerol, 10 mM KCl, 5 mM $MgCl_2$, 1 mM AEBSF, 1 mM DTT, 2.5% NP40, and 0.2% DHPC (1,2-Diheptanoyl-sn-glycero-3-phosphocholine; Avanti Polar-Lipids, Inc.). Buffer C contains 20 mM monoethanolamine (pH 9.0), 10% glycerol, 10 mM KCl, 5 mM $MgCl_2$, 1 mM AEBSF, 1 mM DTT, and 0.5% NP40. Buffer H contains 50 mM Tris-HCl (pH 7.6), 100 mM KCl, 25 μM zinc acetate, 0.1% NP40, 10 mM $MgCl_2$, and 1 mM DTT.

Bacteria and Plasmids

The strains used to purify proteins (JM105 *ftsH1*[ts] Δ*clpP-lon* and BL21 Δ*clpP-lon*) were constructed by P1Vir transduction. Pulse-chase experiments were performed in BL21 (Novagen) and BL21 Δ*clpP-lon*. Expression vectors were generated by inserting PCR fragments containing the gene of interest into the PQE8 (Qiagen) or PET24 (Novagen) vectors. Inserts were sequenced to check for mutations. Primer sequences and cloning strategies are available upon request. The DGK plasmids are described in Zhou and Bowie (2000).

Proteins and Peptides

Wild-type FtsH protein was expressed in *E.coli* strain JM105 pBHB1. pBHB1 overexpresses FtsH under Arabinose control (Herman et al., 1998). Cultures were grown at 34°C to an OD600 of 0.6 in TB-Mg^{2+} media, and L-arabinose was add to 0.4%. Cells were harvested by centrifugation 2–3 hr later and resuspended in buffer A. The cells were lysed by French press and centrifuged for 10 min at 4000 rpm. The supernatant was collected and centrifuged again at 40,000 rpm. The pellet, containing the membrane fraction, was solubilized in buffer B followed by another centrifugation at 40,000 rpm. The supernatant was load on an FFQ column and eluted with a linear gradient from 10 mM to 1 M NaCl in buffer C. The fractions containing FtsH were then loaded on a Superose 12 column and eluted with buffer C. FtsH-containing fractions were purified further on a Mono P column. The sample was eluted with a linear gradient from 10 mM to 1 M NaCl in buffer C. Purified FtsH was dialyzed in buffer C for 4 hr and stored at −80°C.

Barnase, a ribonuclease, is derived from *Bacillus amyloliquefaciens*; dihydrofolate reductase (DHFR) is derived from *E. coli*. The circular permutants were all derived from *E. coli* DHFR (Iwakura et al., 2000). Radioactive proteins were expressed from a T7 promoter by in vitro transcription and translation in rabbit reticulocyte lysate supplemented with [^{35}S]methionine. Proteins were then partially purified by high-speed centrifugation and ammonium sulfate precipitation as described (Matouschek et al., 1997).

His_6-λcI-L1-SsrA, His_6-barnase-SsrA, and His_6-barnase(IA25)-SsrA were purified by denaturing, and His-GFP-SsrA by nondenaturing nickel NTA chromatography according to the Qiagen protocol. Arc-SsrA, Arc-108 variants, and radioactive proteins were purified as described (Burton et al., 2001). β-casein was purchased from Sigma and labeled with casein kinase II according to the manufacturer's directions (Roche). The purification of His-N-σ^{32} has been described previously (Gamer et al., 1992). ClpX and ClpP were a gift from Tania Baker. Inverted membrane vesicle was prepared as described (Yoshihisa and Ito, 1996); *E.coli* strain JM105 pBHB1 was used to overexpress FtsH.

Degradation of Purified Proteins by FtsH

Unless noted, all degradation assays were performed in buffer H in presence of an ATP regeneration system (100 μg/ml creatine kinase, 10 mM creatine phosphate) and 10 mM or 1 mM ATP. No difference in the rate of β-casein degradation at these two concentrations of ATP was observed. Degradation assays contained 0.6 μM of FtsH and different concentrations of [^{32}P] or [^{35}S] substrates in a volume of 40 μl. At designated times, samples were transferred to ice-cold 10% TCA and centrifuged for 10 min at 14,000 rpm. The radioactivity in the supernatant measured by scintillation counter was used to calculate the rate of degradation. The nonlinear least square fittings of the Michaelis-Menten equation were determined using the Kaleidagraph program (Synergy). All experiments were repeated at least two times with less than 10% fluctuation between experiments.

Degradation of DHFR-SsrA or Barnase-SsrA Variants by FtsH

FtsH proteolytic assays were performed as described above, except that the TCA insoluble pellet was resuspended in SDS-PAGE sample buffer and analyzed by SDS-PAGE; the amount of remaining protein was quantified by electronic autoradiography.

Pulse-Chase Experiment of Arc Variants and Degradation Assay for DGK

The protocol for pulse-chase has been described in (Herman et al., 1998). The protocol followed for DGK degradation is adapted from Gottesman et al. (1998).

ATPase Assay

The ATPase assay has been described (Kamath-Loeb et al., 1995).

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