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ATP-dependent Proteases Differ Substantially in Their Ability to Unfold Globular Proteins*[S]

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Prakash Koodathingal[‡§1], Neil E. Jaffe[‡§1], Daniel A. Kraut[‡§], Sumit Prakash[‡§], Susan Fishbain[‡§], Christophe Herman[¶], and Andreas Matouschek[‡§2]

From the [‡]Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, Evanston, Illinois 60208, the [§]Robert H. Lurie Comprehensive Cancer Center, Northwestern University, Chicago, Illinois 60611, and the [¶]Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas 77030

ATP-dependent proteases control the concentrations of hundreds of regulatory proteins and remove damaged or misfolded proteins from cells. They select their substrates primarily by recognizing sequence motifs or covalent modifications. Once a substrate is bound to the protease, it has to be unfolded and translocated into the proteolytic chamber to be degraded. Some proteases appear to be promiscuous, degrading substrates with poorly defined targeting signals, which suggests that selectivity may be controlled at additional levels. Here we compare the abilities of representatives from all classes of ATP-dependent proteases to unfold a model substrate protein and find that the unfolding abilities range over more than 2 orders of magnitude. We propose that these differences in unfolding abilities contribute to the fates of substrate proteins and may act as a further layer of selectivity during protein destruction.

Energy-dependent proteolysis is responsible for more than 90% of the protein turnover inside the cell (1). This process both removes misfolded and aggregated proteins as part of the response of the cell to stress and controls the concentrations of regulatory proteins (2, 3). In prokaryotes and eukaryotic organelles, energy-dependent proteases fall into five classes as follows: ClpAP, ClpXP, Lon, HslUV (also referred to as ClpYQ), and HflB (also referred to as FtsH). In Archaea, analogous functions are performed by the archaebacterial proteasome, consisting of the proteasome-activating nucleotidase (PAN),[3] working with the 20 S proteasome (4); in the cytoplasm and nucleus of eukaryotes, these same functions are performed by the 26 S proteasome (5). These different proteases show little sequence conservation outside the ATP-binding domains, but they share their overall architecture. They all form oligomeric, barrel-shaped complexes composed of one or more rings with the active sites of proteolysis sequestered inside a central degradation chamber (6). Access channels to these sites are narrow, and proteins have to be unfolded to gain entry (6). Regulatory particles belonging to the AAA family of molecular chaperones assemble on either end of the proteolytic chamber and recognize substrates destined for degradation. After recognition, the regulatory particles translocate the substrate through a central channel to the proteolytic chamber and in doing so unravel folded domains within the substrate. Translocation and unfolding are driven by ATP hydrolysis by the regulatory particles, with conformational changes in the protease transmitted to the substrate by conserved residues in the loops lining the channel (7–10).

Protein degradation by AAA proteases is tightly regulated. Most proteins are targeted to ClpAP, ClpXP, HslUV, Lon, HflB, and PAN by sequence motifs in their primary structure (11–17). Sometimes adaptor proteins recognize and bind sequence elements in substrates and deliver them to the protease, and other times the protease recognizes sequence elements directly (18, 19). In contrast, proteins are typically targeted to the 26 S proteasome through the covalent attachment of polyubiquitin chains (20). Thus, substrates appear to be selected for degradation based on the presence of specific recognition elements in the protein substrates.

However, other mechanisms may also affect the specificity of degradation by prokaryotic proteases. Individual proteases recognize a wide range of targeting signals (11, 16). (For example, *Escherichia coli* ClpXP recognizes sequences belonging to five distinct classes of consensus sequences (11), and ClpAP, Lon, and FtsH can bind to unstructured regions in proteins with a wide range of amino acid sequences (21–23).) One illustration of the loose specificity in targeting signals is the ability of a mitochondrial presequence to target proteins to the proteases ClpAP (24) and HslUV *in vitro* (see below). In addition, substrates are commonly acted upon by several different proteases in *E. coli*. For instance, proteins containing the 11-residue ssrA peptide at their C termini can be recognized by ClpAP, ClpXP, FtsH, Lon, and the archaebacterial proteasome (4, 25–27). Similarly, some substrates of Lon can be degraded by HslUV *in vivo* (28).

It is not clear how degradation remains selective despite the loose specificity of targeting signals. We propose that the intrinsic protein unfolding ability of AAA proteases and the stabilities of substrates against unfolding play a role in deter-

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[1] Both authors contributed equally to this work.

[2] To whom correspondence should be addressed: Dept. of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, 2205 Tech Dr., Evanston, IL 60208-3500. Tel.: 847-467-3570; Fax: 847-467-6489; E-mail: matouschek@northwestern.edu.

[3] The abbreviations used are: PAN, proteasome-activating nucleotidase; DHFR, dihydrofolate reductase; DTT, dithiothreitol; ATPγS, adenosine 5′-*O*-(thiotriphosphate).

mining the fate of cellular proteins. For example, ClpXP releases hard-to-unfold substrates when it encounters them and degrades destabilized titin variants 20-fold faster than wild type titin (29). The membrane-bound AAA protease FtsH has a weak unfolding ability, which allows this protease to act selectively on damaged and unfolded polypeptides (30). Here we find that the relative unfolding abilities of ATP-dependent proteases vary more than 100-fold and that the unfolding abilities of proteases belonging to the same class but originating from different species appear to be conserved. The unfolding abilities also seem to be intrinsic properties of the proteases themselves rather than other cytosolic factors, such as chaperones. Differences in protease unfolding abilities may contribute to substrate selectivity during protein degradation. For example, expression of a protease with a weak unfolding ability during a stress response could allow the selective elimination of unfolded, misfolded, or otherwise aberrant proteins and spare stable proteins from destruction (30).

EXPERIMENTAL PROCEDURES

Substrates—Protease substrates contained domains derived from *Bacillus amyloliquefaciens* barnase (31), *B. amyloliquefaciens* barstar (32), *E. coli* DHFR (33), *E. coli* chemotaxis response regulator CheY (34), or mouse DHFR (35). N-terminal targeting signals for ClpAP or HslUV consisted of 65 amino acid extensions derived from the signal sequence of yeast cytochrome b_2 (36), for PAN/20S of the first 65 residues of barnase, for ClpXP of the first 18 residues of the bacteriophage protein λO (37), and for Lon of the first 40 amino acids of the *E. coli* protein UmuD, a subunit of DNA polymerase V. C-terminal targeting signals for ClpAP, ClpXP, FtsH, and PAN/20S contained the 11-residue ssrA peptide (15) and for Lon and HslUV the *E. coli* cell division inhibitor protein SulA (169 amino acids) (28). The 26 S proteasome substrates were targeted using N- or C-terminal fusions of the first 60 amino acids of the yeast Sic1 protein (39), which was ubiquitinated *in vitro* as described below. In addition, a hexahistidine tag and a Strep-tag II (Qiagen, Valencia, CA) were appended to the termini to aid in purification. The simple sequence repeats are the same as those used previously for 26 S proteasome degradation experiments (40). All constructs were made using standard molecular biology techniques in pGEM-3Zf (+) (Promega) or pET-3a (Novagen) vectors and verified by DNA sequencing.

Radioactive fusion proteins were expressed from a T7 promoter by coupled *in vitro* transcription and translation in a reticulocyte extract (Promega), supplemented with [^{35}S]methionine, and proteins were partially purified by high speed centrifugation and ammonium sulfate precipitation, as described (36). For experiments using fully purified substrates, His-tagged fusion proteins were purified from the translation mix using nickel-nitrilotriacetic acid magnetic beads (Qiagen) following standard protocols as provided by the supplier.

26 S proteasome substrates were expressed in Rosetta (DE3)pLysS *E. coli* (Novagen) in EZ Rich Defined Media lacking methionine (Teknova) and supplemented with [^{35}S]methionine and -cysteine. After cell lysis, protein was purified on nickel-nitrilotriacetic acid and then on *Strep*-tactin resins using standard protocols (Qiagen), leading to single band on a Coomassie-stained gel. The substrates were then ubiquitinated *in vitro* at 25 °C for 2–3 h in a reaction mixture containing 25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 4 mM $MgCl_2$, 1 μM DTT, 83 nM yeast E1 (Boston Biochem), 1 μM recombinant UbcH5a (Boston Biochem), 1.9 μM purified Rsp5 (39), 0.33 mg/ml ubiquitin, an ATP regeneration system consisting of 40 mM creatine phosphate, 0.4 mg/ml creatine kinase and 4 mM ATP, and ~1 μM substrate (supplemental Fig. 4).

Proteases—*E. coli* ClpA and ClpX clones were the gift of Dr. Michael Maurizi (National Institutes of Health). ClpA was purified as described (41). ClpX was expressed in BL21 (DE3) cells. After sonication and centrifugation, the soluble fraction was ultracentrifuged for 1 h at 100,000 × *g* to remove membranes and cell debris, and the resulting soluble fraction was precipitated by adding $(NH_4)_2SO_4$ to 40%. The pellet was resuspended and applied to a phenyl-Sepharose CL-4B column (Sigma) equilibrated with buffer PS-A (50 mM phosphate buffer, pH 7.5, 2 mM DTT, 10% glycerol, 0.5 M $(NH_4)_2SO_4$). ClpX eluted halfway through a 100 to 0% $(NH_4)_2SO_4$ gradient. Fractions containing ClpX were dialyzed, applied to a Q-Sepharose column (GE Healthcare), and eluted with a 0.1–1.0 M KCl gradient in buffer QS (50 mM Tris-HCl, pH 8, 5 mM $MgCl_2$, 5 mM DTT, 10% glycerol). Fractions containing ClpX were loaded onto a Mono Q column (GE Healthcare) and eluted with a 0.1–0.5 M KCl gradient in buffer MQ (50 mM Tris-HCl, pH 8, 5 mM $MgCl_2$, 5 mM DTT, 10% glycerol). Eluates were estimated to be at least 90% pure by SDS-PAGE. ClpP was purified as described (41). *Haemophilus influenzae* HslUV in pRSET-B was a gift of Dr. David McKay (Stanford University). HslU and HslV were co-expressed in BL21 (DE3) pLysS cells for 2.5 h at 37 °C after induction with 1 mM isopropyl 1-thio-β-D-galactopyranoside. Soluble lysate was applied to a Q-Sepharose column (GE Healthcare) in 10 mM Na_2PO_4, pH 7.0, 5 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, 10% glycerol and eluted with a 0.1 to 0.4 M KCl gradient. Eluates containing both HslU and HslV were pooled and precipitated in 70% saturated $(NH_4)_2SO_4$. The protein complex was further purified by gel filtration using an S-200 column (GE Healthcare). Eluates containing HslUV were pooled and applied to a Mono Q column (GE Healthcare) in 10 mM Na_2PO_4, pH 7.0, 5 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, 10% glycerol and eluted with a 0.1 to 0.4 M KCl gradient. HslU and HslV were assessed >95% pure by SDS-PAGE. *E. coli* HslUV was PCR-amplified from *E. coli* strain DH5α, cloned into pRSET-B, and expressed and purified as described for *H. influenzae* HslUV. FtsH was purified as described previously (30). Clones of *Methanococcus jannaschii* PAN and *Thermoplasma acidophilum* 20 S were a gift of Dr. Christopher Hill (University of Utah). PAN and 20 S were purified essentially as described (42). *E. coli* Lon was purified using a hexahistidine tag attached to the C terminus. Lon was PCR-amplified from *E. coli* strain DH5α, cloned into plasmid pQE-60 (Qiagen), and expressed in M15 cells. The expressed protein was purified as described (43). C-terminally hexahistidine-tagged mitochondrial Lon was expressed from plasmids in *Saccharomyces cerevisiae* (43); mitochondria were isolated from *S. cerevisiae* as described previously, and the histidine-tagged Lon was purified using previously published protocols. Yeast proteasome was purified as described previously (39).

Degradation Assays—Experiments were performed at 30 °C unless otherwise noted. Protease was in excess of unfoldase (except for HslUV, where HslU and HslV were co-purified as a 1:1 complex) to prevent free unfoldase and 26 S proteasome, which was purified as an intact complex. ClpAP degradation assays were performed in a total of 50 μl of Clp degradation buffer (50 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.02% Triton X-100, 20 mM $MgCl_2$). 25 μl of substrate protein produced by *in vitro* translation (see above) were ammonium sulfate-precipitated and resuspended in 25 μl of pre-warmed degradation buffer. Degradation reactions were started by the addition of 10 μl of substrate to 40 μl of pre-warmed degradation buffer containing 4 mM ATP, 1 mM DTT, 20 mM creatine phosphate, 0.1 mg/ml creatine kinase, and either 0.1 μM $ClpA_6$, 0.2 μM $ClpP_{14}$ for the N-terminally targeted substrates, or 0.2 μM $ClpA_6$, 0.4 μM $ClpP_{14}$ for the C-terminally targeted substrates. Methotrexate (100 μM) was included in the reaction as indicated. At designated times 4-μl samples were transferred to 35 μl of SDS-PAGE sample buffer to stop proteolysis. Samples were analyzed by SDS-PAGE and electronic autoradiography. ClpXP degradation assays were performed under the same conditions as ClpAP degradation assays, except using 0.3 μM $ClpX_6$ and 0.8 μM $ClpP_{14}$. HslUV degradation assays were performed similarly to ClpXP degradation assays, but in 50 μl of HslUV degradation buffer (25 mM Tris-HCl, pH 7.8, 150 mM KCl, 5 mM $MgCl_2$) with 0.05 μM $HslU_6V_6$. Lon degradation assays were performed in a total of 50 μl of Lon assay buffer (20 mM Tris-HCl, pH 8, 20 mM $MgCl_2$) with 0.1 μM Lon_6 for both N-terminal and C-terminal targeting signal. HflB degradation assays were performed as described (30). PAN/20S degradation assays were performed in 50 μl of degradation buffer (50 mM Tris-HCl, pH 7.5, 100 mM KCl, 20 mM $MgCl_2$) with 0.2 μM PAN_6, 0.4 μM 20 S_{14}. The 26 S proteasome degradation assays were conducted using purified yeast proteasome essentially as described previously (44). Degradation was in 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 5% glycerol, 5 mM ATP, 25 mM creatine phosphate, 0.1 mg/ml creatine kinase, 10 μM α-casein (to inhibit free 20 S proteasome), 2 mM DTT, 10–80 nM proteasome, and 1–10 nM ubiquitinated substrate (with proteasome in excess of substrate).

ATPase Assay—ATPase rates were measured by a coupled pyruvate kinase-lactate dehydrogenase assay (45, 46) in the presence of 100-fold excess of α-casein. The concentration of the complexes carrying the proteolytic sites was set at 15-fold molar excess over the concentration of the ATPase complexes (ranging from 0.05 to 1 μM) for ClpAP, ClpXP, HslUV, and PAN/20S so that measurements reflected the ability of the ATPase-protease complex, not the free ATPase subunits (47). Reactions were performed in 50 mM HEPES, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 100 μM DTT, 200 μM NADH, 1 mM phosphoenolpyruvate, 20 μg/ml lactate dehydrogenase, and 23 μg/ml pyruvate kinase. Reactions (50 μl) were performed in triplicate at 30 °C.

RESULTS

Sequential Degradation by AAA Proteases—The ATP-dependent proteases ClpAP, ClpXP, PAN/20S, and the eukaryotic 26 S proteasome can unfold and degrade many stable proteins (24, 48–53). In contrast, FtsH lacks a robust unfolding ability, and degradation by FtsH depends on spontaneous

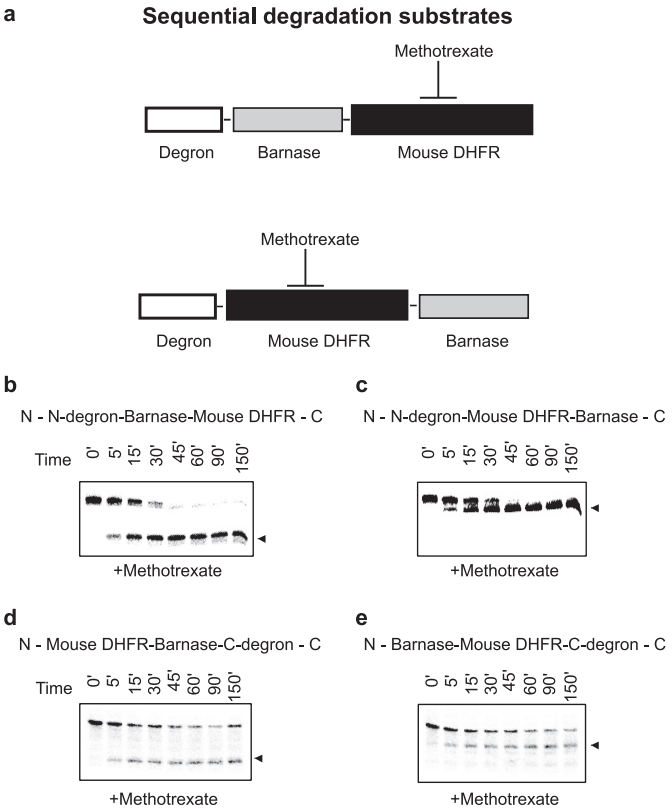


FIGURE 1. **Sequential degradation of barnase-DHFR fusion proteins by ATP-dependent proteases.** *a,* schematic drawing of fusion proteins used for the sequential degradation experiments. *b–e, E. coli* HslUV-mediated degradation of *N*-degron-barnase-mouse DHFR-*C* and *N*-degron-mouse DHFR-barnase-*C* (*b* and *c*) and *N*-mouse DHFR-barnase-degron-*C* and *N*-barnase-mouse DHFR-degron-*C* substrates (*d* and *e*). Stabilizing DHFR by 100 μM methotrexate prevented the degradation of the fusion protein only when the DHFR domain was adjacent to the targeting signal but not when barnase was inserted between the targeting signal and DHFR. Experiments were performed at 30 °C. The *arrow* indicates the position of the methotrexate-stabilized degradation intermediate. Degradation stopped ~40 residues before the DHFR (data not shown) domain, implying that the protease stalled because it was unable to unfold the DHFR domain.

unfolding of its substrate proteins (30). These observations suggest that AAA proteases differ in their intrinsic abilities to unfold proteins. We propose that these differences may affect whether a protein with an appropriate targeting signal can be degraded. To compare the unfolding abilities of the various ATP-dependent proteases, we first tested whether their basic mechanisms of degradation are similar.

ClpAP, ClpXP, FtsH, Lon, and the eukaryotic 26 S proteasome unfold and degrade their substrates sequentially along the polypeptide chains of the substrates, starting from the targeting signal (24, 30, 54–57). Sequential degradation can occur in either the N- to C-terminal or the C- to N-terminal direction (53, 58, 59), depending on the location of the targeting signal. We find that representatives from the remaining families of ATP-dependent proteases, namely the archaebacterial proteasome (PAN/20S) and HslUV, function similarly. We came to this conclusion by following the degradation of two-domain model substrates consisting of *B. amyloliquefaciens* ribonuclease barnase and mouse DHFR as well as either an N-terminal or C-terminal targeting tag (Fig. 1; supplemental Fig. 1). PAN/20S and HslUV degraded these substrates in an ATP-dependent

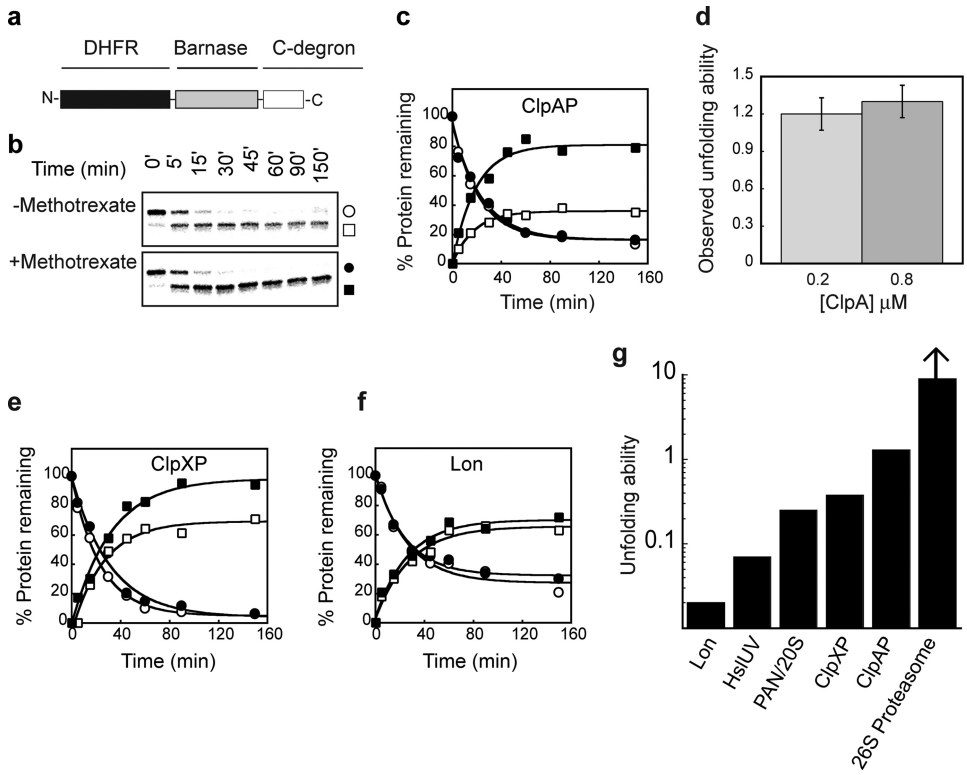
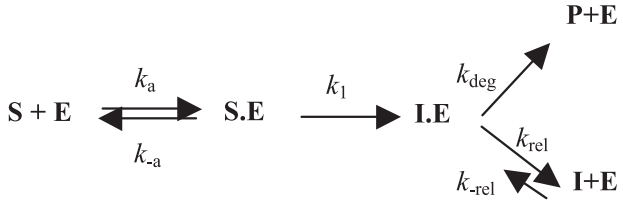


FIGURE 2. **ATP-dependent proteases differ substantially in their unfolding ability.** *a*, linear representation of the multidomain substrates targeted to proteases through the C termini. *b*, degradation of *N*-DHFR-barnase-ssrA-*C* substrate by ClpAP at 30 °C (see under "Experimental Procedures"), with and without 100 μM methotrexate; data shown as autoradiograms of SDS-polyacrylamide gels. *c*, their quantification, amount of substrate proteins remaining (−ligand, ○; +ligand, ●) and the amount of degradation end products (−ligand, □; +ligand, ■). *d*, observed unfolding ability of ClpAP is independent of protease concentration. Unfolding ability values were measured at two different protease concentrations (0.2 μM $ClpA_6$, 0.4 μM Clp_{14} and 0.8 μM $ClpA_6$, 1.6 μM Clp_{14}). Values are means ± S.E. from three experiments. *e* and *f*, degradation of *N*-DHFR-barnase-degron-*C* substrates by ClpXP (*e*) and Lon (*f*) at 30 °C (see under "Experimental Procedures"); plot is shown as quantification of autoradiograms of SDS-polyacrylamide gels. Degradation assay conditions were as shown in *b*. Degradations were performed in the absence (substrates remaining, ○; degradation end products, □) and in the presence of 100 μM methotrexate (substrates remaining, ●; degradation end products, ■). *g*, relative unfolding abilities of ATP-dependent proteases plotted in log scale. Values are means of at least three repeat experiments. 26 S Proteasome degraded its substrates completely in the absence of methotrexate, and no DHFR containing intermediate accumulated to detectable levels, suggesting a U > 9 (represented by the *arrow*).



SCHEME 1

and tag-specific manner (data not shown). Degradation of DHFR is inhibited when protein unfolding is prevented by the tightly binding ligand methotrexate (K_D = 9.5 nM for methotrexate and DHFR (60)) (Fig. 1, *b–e*). Stabilizing DHFR by methotrexate in a substrate in which DHFR is inserted between barnase and the degradation tag protected both the DHFR and barnase from degradation and the fusion protein remained stable (Fig. 1, *c* and *e*). In contrast, when barnase was inserted between the degradation tag and DHFR, stabilizing DHFR no longer protected the barnase domain. Instead, the reaction yielded a partially degraded product (Fig. 1, *b* and *d*). Hence, we conclude that these proteases degrade their substrates sequentially in either an N- to C-terminal or C- to N-terminal direction, starting from the targeting signal.

Relative Unfolding Abilities of AAA Proteases—To compare the unfolding abilities of AAA proteases, we again followed the degradation of model proteins composed of appropriate targeting signals followed by a barnase and then a DHFR domain (Fig. 2*a*). The barnase domain was destabilized by mutagenesis in some experiments so that all proteases were able to unfold and degrade the domain completely. The stability of the DHFR domain was adjusted by changing assay temperature or by replacing mouse DHFR with the more stable *E. coli* DHFR ($\Delta G_{unfolding}$ = 6.1 kcal mol^{-1} for *E. coli* DHFR and $\Delta G_{unfolding}$ = 4.4 kcal mol^{-1} for mouse DHFR (61, 62)). Thus, as the proteases encountered the DHFR domain, some of the time they would succeed in unfolding the DHFR domain and degrade the substrate completely, and some of the time they would fail to unfold the DHFR domain, and the remainder of the substrate would dissociate to yield a partially degraded substrate (Scheme 1).

The simplest mechanism for this reaction is described by Scheme 1, where S represents the two-domain substrate consisting of barnase and DHFR; *E* is the protease; *S.E* is the substrate-protease complex; *I* is the substrate intermediate containing folded DHFR domain; *P* is the product (*i.e.* peptides); k_{deg} is the rate constant by which the intermediate *I* is degraded to peptides; k_{rel} is the rate constant with which the intermediate dissociates from the protease, and k_{-rel} is the rate constant with which the intermediate rebinds the protease. By the time that the proteases encounter the DHFR domain, the targeting signals have already been degraded. Thus, the intermediate, once released, cannot be retargeted to the protease (*i.e.* k_{-rel} is negligible) and instead accumulates as a fragment. Indeed, the amount of fragment formed did not change with protease or substrate concentrations as expected in the absence of rebinding (Fig. 2*d* and supplemental Fig. 2 and data not shown).

The amount of partially degraded intermediate formed relative to the total amount of substrate is a measure of the processivity of a protease and is determined by the ability of the protease to unfold the DHFR domain. A protease with a weak unfolding ability will more often fail in degrading the domain and more intermediate will accumulate. Conversely,

TABLE 1

Unfolding ability measured for barnase-DHFR substrates

Unfolding ability measurements were performed as described, and the values are represented as means ± S.E. from at least three repeat experiments.

ATP-dependent protease	C terminus		N terminus		C terminus at 37 °C
	E. coli DHFR	Mouse DHFR	*E. coli* DHFR	Mouse DHFR	*E. coli* DHFR
ClpAP	1.25 ± 0.1	ND[a]	0.5 ± 0.1	ND[a]	2.2 ± 0.4
ClpXP	0.4 ± 0.1	1.7 ± 0.2	0.2 ± 0.05	ND	0.8 ± 0.1
HslUV (*E. coli*)	0.1 ± 0.03	0.3 ± 0.1	>9	ND	0.2 ± 0.05
PAN/20S	0.3 ± 0.1	1.0 ± 0.2	0.15 ± 0.05	0.6 ± 0.1	0.6 ± 0.2
Lon (*E. coli*)	<0.05	0.1 ± 0.05	~0	<0.1	0.1 ± 0.05
FtsH	ND[b]	ND[b]	ND[b]	ND[b]	~0
26 S proteasome	>9	ND	>9	ND	ND

[a] ClpAP completely degrades DHFR (mouse) in these constructs when targeted through either the N or C terminus.
[b] At 30 °C FtsH failed to degrade the proximal barnase domain when targeted through the C terminus making unfolding ability measurements impossible.

a protease with stronger unfolding ability will more often succeed in degrading the domain and less intermediate will accumulate.

To compare the unfolding abilities of different proteases, it is convenient to define a processivity ratio U as the ratio of complete degradation to partial degradation given by the ratio of the rate constants k_{deg}/k_{rel} (as defined in Equation 1). This ratio U ranges from zero to infinity with increasing processivity of the protease and is related to the difference in activation energy between the degradation and release pathways ($\Delta\Delta G_U$; see Equation 2). None of the proteases investigated here are able to unfold and degrade DHFR bound to methotrexate, and in the presence of the ligand all natively folded substrate that is targeted to the protease is converted into the intermediate. Therefore, the processivity ratio U can be calculated from the amount of intermediate accumulating in the presence and absence of methotrexate as shown in Equations 1 and 2,

$$U = k_{deg}/k_{rel} = (I_{+MTX}/I_{-MTX}) - 1 \quad \text{(Eq. 1)}$$

$$RT\ln(U) = \Delta G^{\ddagger}_{rel} - \Delta G^{\ddagger}_{deg} = \Delta\Delta G_U \quad \text{(Eq. 2)}$$

where I_{+MTX} is the amount of DHFR intermediate accumulated in the presence of methotrexate and I_{-MTX} is the amount of intermediate accumulated in the absence of methotrexate.

The observed processivities appear to reflect intrinsic properties of the proteases because degradation experiments performed with purified ClpAP acting on purified substrates yielded the same results as experiments with substrates synthesized by *in vitro* transcription and translation (supplemental Fig. 3). Similarly, the processivity of purified Lon and Lon inside mitochondria coincides (see below). Thus, we conclude that the measured processivity ratios determined for different proteases acting on the same substrate can be used to compare the abilities of these proteases to unfold proteins.

Processivity ratios above 9 and below 0.1, corresponding to 10 and 90% formation of *I*, are prone to experimental error; therefore, we adjusted the stability of the DHFR domain as described above to obtain processivity ratios between 9 and 0.1. The processivities or unfolding abilities of two proteases can then be compared directly only for the same substrates under identical reaction conditions. To compare proteases with very different unfolding abilities, it is necessary to compare them each to a third protease under appropriate conditions. The comparisons can then be made by scaling using pairwise comparisons made under identical conditions.

ATP-dependent Proteases Vary Substantially in Their Ability to Unfold Model Proteins—We first compared protease unfolding abilities for substrates targeted through C-terminal signals (Fig. 2 and Table 1). In these constructs, the DHFR domain is located at the N terminus of the substrate, followed by the barnase domain and the C-terminal targeting signal (Fig. 2*a*). Strikingly, the unfolding abilities of the proteases differed by more than 2 orders of magnitude (Fig. 2*g*). Among the bacterial proteases, ClpAP was the strongest (U = 1.3 ± 0.1 for the more stable *E. coli* DHFR at 30 °C), followed by ClpXP (U = 0.4 ± 0.1) and HslUV (U = 0.1 ± 0.03). Lon exhibited a weak unfolding ability (U ≅ 0), and as expected (30), FtsH failed to degrade wild type DHFR even at elevated temperatures (Table 1). The archaebacterial proteasome PAN/20S showed an unfolding ability comparable with ClpXP (U = 0.3 ± 0.1), but the eukaryotic 26 S proteasome exhibited a greater unfolding ability than any of the prokaryotic proteases (U > 9; see below and supplemental Fig. 4). Thus, ATP-dependent proteases have substantially different unfolding abilities.

An earlier study demonstrated that ClpXP and HslUV have maximum rates of degradation at different temperatures, 37 and 55 °C, respectively (63). We find that the unfolding abilities of all proteases tested are ~2-fold greater at 37 °C than at 30 °C (Table 1), presumably at least in part because the substrate domains are less resistant to unfolding. The hierarchy of unfolding abilities and the relative differences among the proteases are the same at the two temperatures tested (Table 1).

To compare the unfolding abilities of AAA proteases for degradation from the N terminus, we followed proteolysis of substrates where the order of the targeting signal and the two folded domains was reversed. Thus, the proteases again encountered the DHFR domain after degrading the targeting signal and part of the barnase domain (Fig. 3 and Table 1). The observed unfolding abilities of the 26 S proteasome, ClpAP, ClpXP, PAN/20S, and Lon followed the same hierarchy as found for degradation from the C terminus toward the N terminus. However, HslUV represented an exception. For degradation from the N terminus, HslUV has the strongest unfolding ability of all the bacterial proteases, whereas for degradation from the C terminus, HslUV is one of the weaker unfoldases and ranks below the other proteases, apart from FtsH and Lon, in unfolding ability. This observation suggests that HslUV unfolds proteins more effectively when degrading from the N terminus toward the C terminus than in the opposite direction.

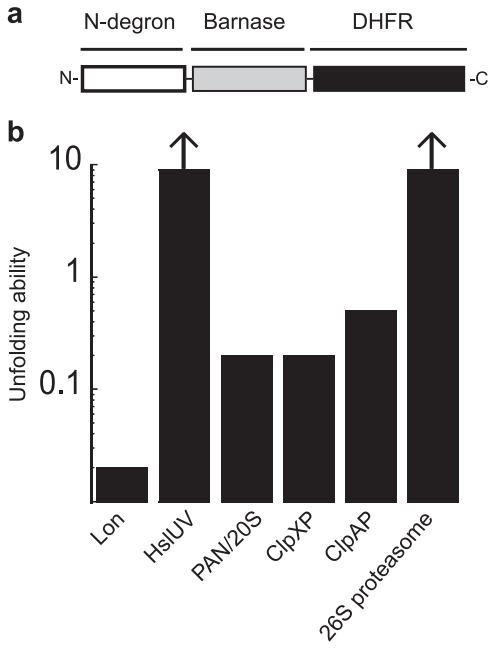


FIGURE 3. **Unfolding ability for substrates targeted through N terminus.** Except for HslUV, the observed unfolding abilities of the 26 S proteasome, ClpAP, ClpXP, PAN/20S, and Lon followed the same hierarchy as found for degradation from the C terminus to the N terminus. *a,* schematic representation of the substrates targeted to proteases by signals at their N termini. *b,* relative unfolding ability of ATP-dependent proteases estimated for substrates targeted through N terminus plotted in log scale. Both 26 S proteasome and HslUV unfold and degrade DHFR completely in the barnase-DHFR fusion constructs when targeted through the N terminus, suggesting a $U > 9$ (represented by the *arrow*). Values are means of at least three repeat experiments.

Conservation of Unfolding Ability between Organisms—Next, we tested whether unfolding ability is conserved between homologous proteases from different organisms. For example, the amino acid sequences of HslUV from *E. coli* and *H. influenzae* are 80% identical, and their unfolding ratios coincided ($U = 0.1 \pm 0.03$ for *E. coli* protein and $U = 0.12 \pm 0.03$ for *H. influenzae* protein). The amino acid sequences of Lon from *S. cerevisiae* and *E. coli* show only 33% overall sequence identity. Yeast mitochondrial Lon is considerably larger than prokaryotic Lon and possesses a 57-amino acid insertion between the ATP-binding sites and proteolytic sites (64, 65). Nevertheless, the unfolding ratios of the two proteases also coincided within experimental error ($U = 0.13 \pm 0.04$ for yeast mitochondrial Lon and $U = 0.1 \pm 0.05$ for *E. coli* Lon using an *E. coli* DHFR-containing substrate at 37 °C). The unfolding ability of mitochondrial Lon protease can also be assessed in an environment that is close to the physiological situation by importing the test substrates into purified yeast mitochondria. We found that the unfolding ability of Lon inside mitochondria also coincided with that of purified mitochondrial Lon acting on substrates purified from the *in vitro* translation reaction ($U = 0.55 \pm 0.08$ inside mitochondria *versus* $U = 0.60 \pm 0.06$ for purified Lon, respectively, measured for mouse DHFR constructs at 37 °C; supplemental Fig. 3). Western blotting for Hsp78 and Hsp70 suggested that the purification procedure removed these chaperones. Therefore, the measured unfolding abilities reflect an intrinsic property of Lon, not assistance in unfolding from mitochondrial chaperones.

In contrast, the unfolding ability of the eukaryotic 26 S proteasome was significantly stronger than that of the archaebacterial proteasome (PAN/20S) for degradation from either direction (Figs. 2 and 3 and Table 1). In the absence of methotrexate, the eukaryotic proteasome degraded its substrates completely, and no DHFR-containing intermediate accumulated to detectable levels (supplemental Fig. 4), suggesting a $U > 9$. Thus, in degradation from the C terminus, the 26 S proteasome had the greatest unfolding ability of all the proteases tested. In degradation from the N terminus, neither the eukaryotic proteasome nor HslUV produced fragments during degradation.

The archaebacterial proteasome is considered to be the evolutionary precursor of the eukaryotic 26 S proteasome (66). The ATPase subunits of the archaebacterial proteasome share 18–20% sequence identity (41–45% similarity) with the ATPases of the 19 S particle of human and yeast proteasome (67). In the 20 S core components, the β-subunits share 20–25% identity (45–50% similarity), and the α-subunits share 28–36% identity (48–54% similarity) between the two proteases (68–70). Nevertheless, the two proteases differed greatly in their ability to unravel and degrade folded proteins, perhaps due to the additional components in the 19 S particle of the yeast proteasome that are absent from the archaebacterial proteasome.

Sequence Dependence of Unfolding Ability—The ability of an AAA protease to unfold proteins depends on both the rate with which the protease moves forward to unravel the domain (k_{deg} in Scheme 1) and the rate with which the protease dissociates from its substrate when it encounters the domain (k_{rel} in Scheme 1). In partially degraded intermediates, the last proteolytic cleavage occurs ~30–90 amino acids before the domain resisting unfolding, depending on the protease. Thus, partial degradation leaves an undegraded tail (24, 29), which presumably connects the folded DHFR domain at the entrance of the translocation channel to the proteolytic sites buried in the core of the protease. The proteases interact with their substrates primarily through this tail because the targeting signals have already been degraded when the proteases reach the folded domain. Thus, changing the tail sequences in the substrates could change the unfolding strengths of the proteases, if the proteases have different affinities for their substrates.

To test this possibility, we replaced the barnase domain in the model substrates with two different proteins, the barnase inhibitor barstar and the *E. coli* chemotaxis protein CheY (Fig. 4*a*). The sequences of these proteins are unrelated, and they differ significantly in their chemical properties, such as hydrophobicity and net charge (data not shown). Nevertheless, the unfolding abilities of the different proteases were unaffected by the amino acid sequences of the substrates (Fig. 4*b*).

However, when the amino acid sequences of the substrates were changed more substantially, the unfolding abilities changed. For example, ClpAP degraded substrates containing mouse DHFR completely, but insertion of a glycine-rich region of 95 residues before the DHFR domain led to the accumulation of a partially degraded fragment (Fig. 4, *c* and *e*). The glycine-rich region did not seem to stabilize DHFR against spontaneous global unfolding because resistance of DHFR to degradation by

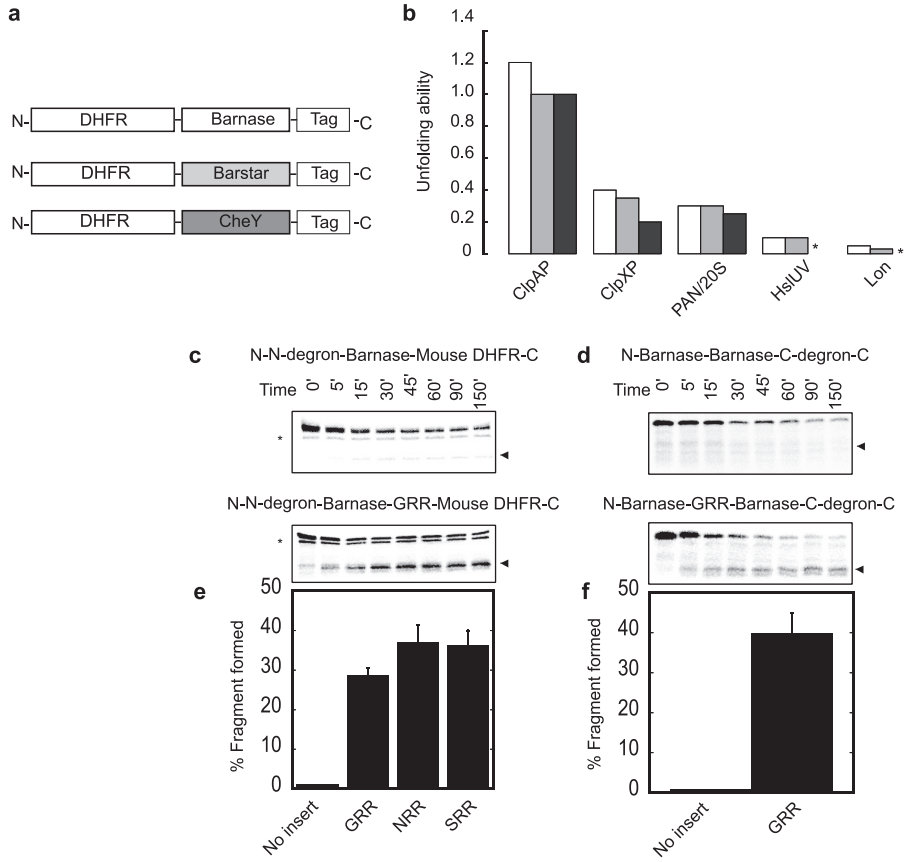


FIGURE 4. **Sequence dependence of unfolding ability.** *a,* linear representation of chimeric proteins used in the degradation assays. The tags used for different proteases are the same as in Fig. 2*a,* and the details are given under "Experimental Procedures." *b,* unfolding ability values estimated for different proteases using barnase (*clear bar graphs*), barstar (*light gray bar graphs*), and CheY (*dark gray bar graphs*). Measurements are means ± S.E. from three repeat experiments. *Asterisk* indicates HslUV and Lon were unable to degrade the proximal CheY domain when targeted from the C terminus, preventing measurement of unfolding ability. *c,* simple sequence insert can prevent progression of degradation by ClpAP. Degradation of mouse DHFR and glycine-rich region (*GRR*)-mouse DHFR at 25 °C is shown. Positions of the partially degraded products are indicated by *arrowheads*. *Asterisk* indicates nonspecific band present in the translation reaction. *d,* degradation of barnase and barnase- glycine-rich region (*GRR*) by ClpXP, targeted through an ssrA tag. Experiments were performed at 30 °C. *e* and *f,* extent of fragment formation by ClpAP (*e*) and ClpXP (*f*) quantified as the percentage of full-length protein degraded.

the ATP-independent protease proteinase K was unaffected (40). Judging from the size of the fragment formed, degradation stopped ~40 residues before the DHFR domain, just as occurs when DHFR is stabilized with methotrexate (Fig. 4*e*), implying that the protease stalled because it was unable to unfold the DHFR domain. Similarly, degradation by ClpXP was stopped by insertion of a glycine-rich region in front of a folded domain (barnase) (Fig. 4, *d* and *f*). This inhibition did not seem to be specific to the identity of amino acids in the simple sequence region, because replacing the glycine-rich region with serine-rich and asparagine-rich regions also inhibited ClpAP-mediated degradation (Fig. 4*e*). However, we cannot rule out a model in which ClpP is unable to digest regions of low complexity amino acids.

Steady-state ATP Hydrolysis Rates for Different Proteases—To gain insight into the biochemical mechanism by which these proteases attain their wide range of unfolding abilities, we compared the rates of ATP hydrolysis for different proteases. Nucleotide hydrolysis by most ATP-dependent proteases has been characterized earlier (14, 30, 55, 71–76). We determined the steady-state kinetic parameters for ATP hydrolysis using a coupled spectrophotometric assay (45, 46). Assays were performed under the degradation assay conditions in the presence of saturating concentrations of a nonspecific protein substrate. The ATPase rates as a function of nucleotide concentration fitted well to the Michaelis-Menten equation (Fig. 5*a*). The maximum rates of ATP hydrolysis (Fig. 5*b* and Table 2) for the proteases did not correlate with their unfolding abilities. For example, Lon and FtsH are weak unfoldases but have robust (Lon, $k_{cat} = 288\ min^{-1}$) and moderate (FtsH, $k_{cat} = 42\ min^{-1}$) ATP hydrolysis rates. In contrast, the 26 S proteasome has a strong unfolding ability but a moderate ATP hydrolysis rate ($k_{cat} = 72\ min^{-1}$). The ATPase rates of the two-component prokaryotic proteases ClpAP, ClpXP, HslUV, and PAN/20S seem to correlate with their unfolding abilities for C-terminally targeted substrates ($R = 0.97$; Fig. 5*c*), but the relationship does not hold for degradation from the N terminus because of the strong unfolding ability of HslUV under these conditions.

DISCUSSION

Conserved Protein Unfolding Mechanism among ATP-dependent Proteases—ATP-dependent proteases unfold and degrade proteins sequentially in N- to C-terminal or C- to N-terminal directions (11, 53, 58, 59). This behavior had been known for the eukaryotic 26 S proteasome, ClpAP, ClpXP, HflB (FtsH), and Lon (24, 30, 49, 54, 56–58, 77), and we show here that the archaebacterial proteasome and HslUV also function similarly. Thus, representatives from all classes of ATP-dependent proteases function by the same overall mechanism. This sequential degradation causes folded domains to be unraveled by the proteases in a process that is different from the global unfolding process induced by heat or denaturant (24, 78–80), and the resistance of the substrate to unraveling depends on the local structure of the protein near the targeting signal but not the overall thermodynamic stability (24, 55, 81). Unfolding by unraveling appears to be particularly effective because this unfolding mechanism can overcome the frequently high cooperativity of global unfolding.

Unfolding Abilities and Substrate Selection—Despite the common degradation mechanism, the unfolding abilities of ATP-dependent proteases differ by more than 2 orders of magnitude. ATP-dependent proteases select proteins for destruc-

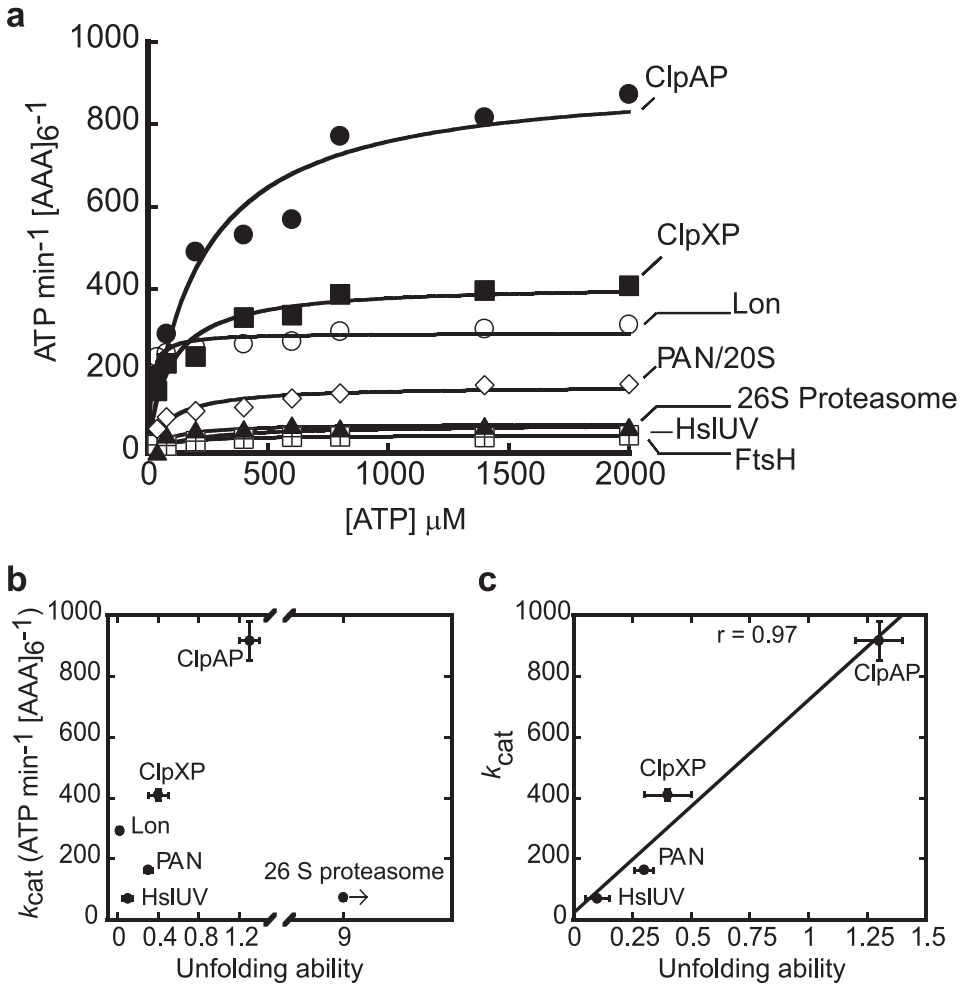


FIGURE 5. **Steady-state kinetic parameters for ATP hydrolysis.** *a,* rate of ATP hydrolysis as a function of nucleotide concentration. *Solid lines* represent fits to the Michaelis-Menten equation. ATP hydrolysis rate of individual ATP-dependent protease in the presence of 20 μM α-casein at 30 °C was measured by a coupled pyruvate kinase-lactate dehydrogenase assay. *b,* relationship between unfolding ability and maximum ATP hydrolysis rates for substrates targeted through the C terminus. The plot shows the ATPase rate at 4 mM ATP. *c,* unfolding abilities of ClpAP, ClpXP, PAN/20S, and HslUV correlate well with the protease maximum ATP hydrolysis rates. The unfolding ability values are for substrates targeted through the C terminus.

TABLE 2

Steady-state kinetic parameters for ATP hydrolysis

Errors are the error of the fit of the data points to the Michaelis-Menten equation.

ATP-dependent protease	k_{cat}[a]	K_M
	min^{-1}	μM
ClpAP	920 ± 70	210 ± 60
ClpXP	410 ± 20	90 ± 20
HslUV (*E. coli*)	69 ± 6	180 ± 60
PAN/20S	163 ± 9	90 ± 30
Lon (*E. coli*)	290 ± 10	12 ± 4
FtsH	44 ± 5	80 ± 20
26 S proteasome	72 ± 8	90 ± 40

[a] k_{cat} is in units of min^{-1}[AAA]$_6^{-1}$.

tion primarily by recognizing amino acid sequences in substrate proteins. However, these targeting preferences are often broad and overlapping. The wide range of unfolding abilities of ATP-dependent proteases may provide an additional mechanism for substrate selectivity. ATP-dependent proteases perform two broad roles in cellular metabolism; they control the concentrations of many specific regulatory proteins, which have to be removed specifically at given times, and they degrade misfolded or damaged proteins as well as subunits that have not been incorporated into their cognate complexes. Proteases with weaker unfolding abilities likely selectively degrade misfolded and loosely folded polypeptide chains while avoiding more stable native proteins and assembled complexes, independent of conventional targeting signals. In contrast, proteases with stronger unfolding abilities are able to degrade regulatory proteins in their native, structured, and thus functional state when they are targeted for destruction. The observation that unfolding abilities seem to be conserved among related proteases agrees with the suggestion that the magnitude of the unfolding strength may play a meaningful biological role. The hierarchy of protease unfolding abilities is conserved for the two domains to be unfolded, either mouse or *E. coli* DHFR and, with the exception of HslUV, for degradation in both directions along the substrate. Degradation from the N or C terminus presents different structures to the protease and is similar to presenting the protease with different domains. Therefore, it appears likely that the relative unfolding abilities found in this model system are conserved among all or most protease substrates. However, we cannot rule out the possibility that certain proteases have evolved to unfold and degrade specific proteins or structural classes of proteins.

Strongly Unfolding Proteases, the Proteasome and Clp Proteases—The unfolding abilities of ClpAP and ClpXP are among the strongest within the prokaryotic proteases. The two proteases are responsible for the majority of degradation of regulatory proteins in prokaryotes (3, 82, 83), and many of their substrates are stably folded. Thus, their unfolding ability appropriately fits their cellular roles in protein destruction. The eukaryotic 26 S proteasome has the strongest unfolding ability of all the proteases we have investigated, and again one of its primary functions is to degrade regulatory proteins.

The relationship between unfolding ability and substrate selection suggests that the proteases with stronger unfolding abilities would require pronounced preferences in target recognition to function selectively. Indeed, the eukaryotic proteasome works together with an exquisitely specific targeting system; the large majority of substrates are targeted to this protease by modification with ubiquitin (20). ClpAP and ClpXP also function with well defined targeting systems. Both pro-

teases can use targeting factors (adaptors) (*e.g.* ClpS for ClpA (84) and SspB for ClpX (18, 85–87)), which deliver substrates to the protease without themselves being degraded. In addition, both ClpAP and ClpXP recognize some well defined primary targeting sequences directly. For example, ClpXP recognizes proteins tagged with the ssrA peptide without the help of an adaptor (11, 15). However, some experiments suggest that ClpAP also degrades proteins containing unfolded regions but lacking specific tag sequences (7, 22) and that ClpXP recognizes a large number of only weakly defined consensus sequences (11). In these examples, the unfolding ability likely has a diminished role in specificity.

Weakly Unfolding Proteases, Lon and FtsH—Lon, from either yeast or *E. coli*, exhibits a substantially weaker unfolding ability than Clp proteases and the eukaryotic 26 S proteasome (>30-fold smaller unfolding ability compared with ClpAP). Lon is conserved from bacteria to humans and is responsible for the rapid degradation of misfolded and oxidized proteins (21, 88–92). Its expression is increased in response to stress when aberrant polypeptides accumulate (93–95). The weak unfolding ability of Lon is consistent with the role of the protease in protein quality control, allowing Lon to be selective toward misfolded and naturally unstable substrates and avoiding degradation of stable native proteins. Lon probably recognizes non-native polypeptides through unfolded regions in the substrates that serve as degradation tags (92). However, disordered regions are relatively common even in native proteins so that recognition of unstructured regions by the protease can lead to the nonspecific elimination of functional proteins (96, 97). Lon may avoid degrading aberrantly targeted native proteins simply because it is unable to unfold them. This strategy may also assist in the selective elimination of unassembled subunits of multiprotein complexes such as the α-, β-, and γ-subunits of the F_1-ATPase, the α-subunit of matrix-processing peptidase α, and free ribosomal subunits (98–100). Thus, increasing the expression of a protease with weak unfolding ability such as Lon may ensure efficient degradation of aberrant and unstable substrates without adversely affecting the concentration of other folded proteins.

FtsH possesses the weakest unfolding ability among all AAA proteases, in agreement with the earlier findings of Herman *et al.* (30). Natural substrates of FtsH include damaged and uncomplexed forms of many membrane proteins, including SecY, YccA, and PhoA (101, 102), but also many short lived transcription factors, including *E. coli* σ^{32}, bacteriophage λ CII, CIII, and Xis (103, 104). If Lon and FtsH both select substrates at least in part based on their thermostability, the two proteases may have common targets, and indeed, they both degrade the λ phage protein Xis (103–105). A similar overlap is observed in yeast mitochondria where the FtsH-like protease Yta10/12 and Lon both degrade a model substrate (90). Interestingly, it has recently been reported that in *Mycoplasma* species, which lack AAA proteases other than Lon and FtsH, Lon has evolved the ability to unfold very stable proteins, suggesting that having a strong protease is indispensable (106).

HslUV protease does not fall neatly into the two groups of proteases discussed above; it is less effective than ClpAP and ClpXP at unfolding proteins from their C termini and more effective than either at unfolding proteins from their N termini. Some earlier findings appear to agree with these observations as HslUV, in contrast to ClpAP and ClpXP (50, 58), cannot degrade C-terminally targeted green fluorescent protein and maltose-binding protein (107, 108) but is able to degrade the stable I27 domain of titin when degrading from the N terminus (63).

Role of Chaperones in Unfolding—We measured the unfolding ability of Lon in an environment that is close to the physiological situation and compared it to purified Lon. Purified Lon and Lon inside mitochondria show the same ability to unfold soluble proteins, suggesting that accessory factors are not general activators of Lon. Hsp70 and Hsp78 chaperones were previously shown to cooperate in Lon-dependent degradation of substrates prone to aggregation in yeast mitochondria (21, 90, 91). Chaperones can prevent substrates from aggregating; thus, their primary function may be to assist in degradation by keeping substrates soluble and accessible to the protease.

Protein Processing—A weak unfolding ability may have a second physiological function in addition to affecting substrate selection. The weaker unfolding ability of some proteases leads to the incomplete degradation of our model substrates, and partial degradation may also occur naturally. One example could be the role of Lon in the production of active maturase enzymes, which are splicing factors that catalyze the removal of introns from mitochondrially encoded genes. Maturase is synthesized from the unspliced pre-mRNA as a larger precursor protein (109), which is subsequently processed by Lon protease (110). Maturase-dependent splicing is impaired specifically when Lon is inactivated (111). Thus, it is possible that Lon functions in this process by degrading the polypeptide tail, whereas the maturase domain itself is protected from proteolysis because it cannot be unfolded by Lon.

Biochemical Basis of the Different Unfolding Abilities—The unfolding strength of a protease is determined by the competition between the forward translocation and unfolding step and the dissociation step (see Equation 1). The dissociation rates are governed by the interaction of protease and substrate. Similarly, if unfolding is caused mechanically by a conformational change in the proteases that is transmitted to the substrate (7, 112), the affinity of the interaction between substrate and protease can be related to the unfolding strength. In both cases tighter substrate binding leads to stronger unfolding.

The proteases investigated here display similar unfolding strengths for several substrates (Fig. 4*b*) suggesting either that the proteases interact with different amino acid sequences with similar affinities or that dissociation rates do not limit unfolding. However, there are also striking and surprising sequence effects. The apparent unfolding abilities of ClpAP and ClpXP were decreased when the proteases were interacting with their substrates through several stretches of simple amino acid compositions (Fig. 4, *c–f*). Such simple sequences are common in both eukaryotic and prokaryotic genomes (113), and it will be interesting to determine how they affect protein homeostasis. It has been shown that simple sequences play an important role in the regulation of two eukaryotic transcription factors by leading to their partial degradation by the proteasome (40). Similar mechanisms may exist in prokaryotes.

The forward rate of degradation (Scheme 1) depends on the ATP hydrolysis reaction (14, 30, 38, 55, 73). For example, ClpXP can hydrolyze the ATP analogue ATPγS but does so much more slowly. ClpXP can also degrade unfolded substrates in the presence of ATPγS, and the degradation rate is reduced proportionally with the nucleotide hydrolysis rate (38). ATPγS does not support the degradation of folded proteins by ClpXP (38). The correlation between nucleotide and protein hydrolysis does not seem to extend to comparisons of more distantly related proteases and those of different architectures. For example, Lon is a poor unfoldase but hydrolyzes ATP relatively rapidly ($k_{cat} = 288\ min^{-1}$), whereas the eukaryotic proteasome has the strongest unfolding ability but hydrolyzes ATP relatively slowly ($k_{cat} = 72\ min^{-1}$). For degradation from the C terminus, the unfolding abilities of ClpAP, ClpXP, PAN/20S, and HslUV, all of which have a similar simple two-component architecture, correlate with their maximum ATP hydrolysis rates (Fig. 5*c*). This relationship breaks down when degradation from the N terminus is included because of the strong unfolding ability of HslUV in this direction. Thus, it appears that differences in the details of substrate-protease interaction or the mechanism by which ATP hydrolysis is coupled to translocation and unfolding lead to the observed differences in unfolding ability. For example, components of the eukaryotic 19 S assembly might allow the ATPase subunits to be more highly coupled to unfolding than in simpler bacterial proteases, allowing greater force to be applied per ATP hydrolyzed. Furthermore, the single subunit architecture of Lon and FtsH, in which each polypeptide contains both the ATPase and protease domain, might change the way force is transmitted to the substrate.

In summary, we find that the unfolding abilities of ATP-dependent proteases range over several orders of magnitude, and we propose that the ability of a protease to unfold protein domains contributes to its specificity of substrate selection. Thus, the lifetime of a protein in the cell is determined both by the presence and strength of a targeting sequence and by its susceptibility to unfolding by a protease.

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REFERENCES

1. Gottesman, S. (2003) *Annu. Rev. Cell Dev. Biol.* **19,** 565–587
2. Gottesman, S., Wickner, S., and Maurizi, M. R. (1997) *Genes Dev.* **11,** 815–823
3. Wickner, S., Maurizi, M. R., and Gottesman, S. (1999) *Science* **286,** 1888–1893
4. Benaroudj, N., and Goldberg, A. L. (2000) *Nat. Cell Biol.* **2,** 833–839
5. Glickman, M. H., and Ciechanover, A. (2002) *Physiol. Rev.* **82,** 373–428
6. Baumeister, W., Walz, J., Zühl, F., and Seemüller, E. (1998) *Cell* **92,** 367–380
7. Hinnerwisch, J., Fenton, W. A., Furtak, K. J., Farr, G. W., and Horwich, A. L. (2005) *Cell* **121,** 1029–1041
8. Martin, A., Baker, T. A., and Sauer, R. T. (2008) *Nat. Struct. Mol. Biol.* **15,** 1147–1151
9. Martin, A., Baker, T. A., and Sauer, R. T. (2008) *Mol. Cell* **29,** 441–450
10. Martin, A., Baker, T. A., and Sauer, R. T. (2008) *Nat. Struct. Mol. Biol.* **15,** 139–145
11. Flynn, J. M., Neher, S. B., Kim, Y. I., Sauer, R. T., and Baker, T. A. (2003) *Mol. Cell* **11,** 671–683
12. Gottesman, S. (1996) *Annu. Rev. Genet.* **30,** 465–506
13. Ishii, Y., Sonezaki, S., Iwasaki, Y., Miyata, Y., Akita, K., Kato, Y., and Amano, F. (2000) *J. Biochem.* **127,** 837–844
14. Benaroudj, N., Zwickl, P., Seemüller, E., Baumeister, W., and Goldberg, A. L. (2003) *Mol. Cell* **11,** 69–78
15. Keiler, K. C., Waller, P. R., and Sauer, R. T. (1996) *Science* **271,** 990–993
16. Smith, C. K., Baker, T. A., and Sauer, R. T. (1999) *Proc. Natl. Acad. Sci. U.S.A.* **96,** 6678–6682
17. Chiba, S., Akiyama, Y., Mori, H., Matsuo, E., and Ito, K. (2000) *EMBO Rep.* **1,** 47–52
18. Levchenko, I., Seidel, M., Sauer, R. T., and Baker, T. A. (2000) *Science* **289,** 2354–2356
19. Dougan, D. A., Reid, B. G., Horwich, A. L., and Bukau, B. (2002) *Mol. Cell* **9,** 673–683
20. Thrower, J. S., Hoffman, L., Rechsteiner, M., and Pickart, C. M. (2000) *EMBO J.* **19,** 94–102
21. Jubete, Y., Maurizi, M. R., and Gottesman, S. (1996) *J. Biol. Chem.* **271,** 30798–30803
22. Hoskins, J. R., Kim, S. Y., and Wickner, S. H. (2000) *J. Biol. Chem.* **275,** 35361–35367
23. Asahara, Y., Atsuta, K., Motohashi, K., Taguchi, H., Yohda, M., and Yoshida, M. (2000) *J. Biochem.* **127,** 931–937
24. Lee, C., Schwartz, M. P., Prakash, S., Iwakura, M., and Matouschek, A. (2001) *Mol. Cell* **7,** 627–637
25. Gottesman, S., Roche, E., Zhou, Y., and Sauer, R. T. (1998) *Genes Dev.* **12,** 1338–1347
26. Herman, C., Thévenet, D., Bouloc, P., Walker, G. C., and D'Ari, R. (1998) *Genes Dev.* **12,** 1348–1355
27. Choy, J. S., Aung, L. L., and Karzai, A. W. (2007) *J. Bacteriol.* **189,** 6564–6571
28. Wu, W. F., Zhou, Y., and Gottesman, S. (1999) *J. Bacteriol.* **181,** 3681–3687
29. Kenniston, J. A., Baker, T. A., and Sauer, R. T. (2005) *Proc. Natl. Acad. Sci. U.S.A.* **102,** 1390–1395
30. Herman, C., Prakash, S., Lu, C. Z., Matouschek, A., and Gross, C. A. (2003) *Mol. Cell* **11,** 659–669
31. Hartley, R. W. (1975) *Biochemistry* **14,** 2367–2370
32. Hartley, R. W. (1989) *Trends Biochem. Sci.* **14,** 450–454
33. Rood, J. I., Laird, A. J., and Williams, J. W. (1980) *Gene* **8,** 255–265
34. Lopez-Hernandez, E., and Serrano, L. (1996) *Fold. Des.* **1,** 43–55
35. Chang, A. C., and Cohen, S. N. (1978) *J. Bacteriol.* **134,** 1141–1156
36. Matouschek, A., Azem, A., Ratliff, K., Glick, B. S., Schmid, K., and Schatz, G. (1997) *EMBO J.* **16,** 6727–6736
37. Gonciarz-Swiatek, M., Wawrzynow, A., Um, S. J., Learn, B. A., McMacken, R., Kelley, W. L., Georgopoulos, C., Sliekers, O., and Zylicz, M. (1999) *J. Biol. Chem.* **274,** 13999–14005
38. Burton, R. E., Baker, T. A., and Sauer, R. T. (2003) *Protein Sci.* **12,** 893–902
39. Saeki, Y., Isono, E., and Toh-E, A. (2005) *Methods Enzymol.* **399,** 215–227
40. Tian, L., Holmgren, R. A., and Matouschek, A. (2005) *Nat. Struct. Mol. Biol.* **12,** 1045–1053
41. Maurizi, M. R., Thompson, M. W., Singh, S. K., and Kim, S. H. (1994) *Methods Enzymol.* **244,** 314–331
42. Förster, A., Whitby, F. G., and Hill, C. P. (2003) *EMBO J.* **22,** 4356–4364
43. van Dijl, J. M., Kutejová, E., Suda, K., Perecko, D., Schatz, G., and Suzuki, C. K. (1998) *Proc. Natl. Acad. Sci. U.S.A.* **95,** 10584–10589
44. Prakash, S., Inobe, T., Hatch, A. J., and Matouschek, A. (2009) *Nat. Chem. Biol.* **5,** 29–36
45. Bessman, M. (1963) *Methods Enzymol.* **6,** 166–176
46. Seifried, S. E., Wang, Y., and von Hippel, P. H. (1988) *J. Biol. Chem.* **263,** 13511–13514
47. Joshi, S. A., Hersch, G. L., Baker, T. A., and Sauer, R. T. (2004) *Nat. Struct. Mol. Biol.* **11,** 404–411

48. Weber-Ban, E. U., Reid, B. G., Miranker, A. D., and Horwich, A. L. (1999) *Nature* **401,** 90–93
49. Singh, S. K., Grimaud, R., Hoskins, J. R., Wickner, S., and Maurizi, M. R. (2000) *Proc. Natl. Acad. Sci. U.S.A.* **97,** 8898–8903
50. Kim, Y. I., Burton, R. E., Burton, B. M., Sauer, R. T., and Baker, T. A. (2000) *Mol. Cell* **5,** 639–648
51. Navon, A., and Goldberg, A. L. (2001) *Mol. Cell* **8,** 1339–1349
52. Johnston, J. A., Johnson, E. S., Waller, P. R., and Varshavsky, A. (1995) *J. Biol. Chem.* **270,** 8172–8178
53. Prakash, S., Tian, L., Ratliff, K. S., Lehotzky, R. E., and Matouschek, A. (2004) *Nat. Struct. Mol. Biol.* **11,** 830–837
54. Reid, B. G., Fenton, W. A., Horwich, A. L., and Weber-Ban, E. U. (2001) *Proc. Natl. Acad. Sci. U.S.A.* **98,** 3768–3772
55. Kenniston, J. A., Baker, T. A., Fernandez, J. M., and Sauer, R. T. (2003) *Cell* **114,** 511–520
56. Ondrovicová, G., Liu, T., Singh, K., Tian, B., Li, H., Gakh, O., Perecko, D., Janata, J., Granot, Z., Orly, J., Kutejová, E., and Suzuki, C. K. (2005) *J. Biol. Chem.* **280,** 25103–25110
57. von Janowsky, B., Knapp, K., Major, T., Krayl, M., Guiard, B., and Voos, W. (2005) *Biol. Chem.* **386,** 1307–1317
58. Hoskins, J. R., Yanagihara, K., Mizuuchi, K., and Wickner, S. (2002) *Proc. Natl. Acad. Sci. U.S.A.* **99,** 11037–11042
59. Okuno, T., Yamanaka, K., and Ogura, T. (2006) *Genes Cells* **11,** 261–268
60. Rajagopalan, P. T., Zhang, Z., McCourt, L., Dwyer, M., Benkovic, S. J., and Hammes, G. G. (2002) *Proc. Natl. Acad. Sci. U.S.A.* **99,** 13481–13486
61. Endo, T., and Schatz, G. (1988) *EMBO J.* **7,** 1153–1158
62. Iwakura, M., Nakamura, T., Yamane, C., and Maki, K. (2000) *Nat. Struct. Biol.* **7,** 580–585
63. Burton, R. E., Baker, T. A., and Sauer, R. T. (2005) *Nat. Struct. Mol. Biol.* **12,** 245–251
64. Van Dyck, L., Pearce, D. A., and Sherman, F. (1994) *J. Biol. Chem.* **269,** 238–242
65. Suzuki, C. K., Suda, K., Wang, N., and Schatz, G. (1994) *Science* **264,** 273–276
66. Zwickl, P., Ng, D., Woo, K. M., Klenk, H. P., and Goldberg, A. L. (1999) *J. Biol. Chem.* **274,** 26008–26014
67. Zwickl, P., Baumeister, W., and Steven, A. (2000) *Curr. Opin. Struct. Biol.* **10,** 242–250
68. Zwickl, P., Grziwa, A., Pühler, G., Dahlmann, B., Lottspeich, F., and Baumeister, W. (1992) *Biochemistry* **31,** 964–972
69. Löwe, J., Stock, D., Jap, B., Zwickl, P., Baumeister, W., and Huber, R. (1995) *Science* **268,** 533–539
70. Groll, M., Ditzel, L., Löwe, J., Stock, D., Bochtler, M., Bartunik, H. D., and Huber, R. (1997) *Nature* **386,** 463–471
71. Singh, S. K., Guo, F., and Maurizi, M. R. (1999) *Biochemistry* **38,** 14906–14915
72. Yoo, S. J., Seol, J. H., Kang, M. S., and Chung, C. H. (1996) *Biochem. Biophys. Res. Commun.* **229,** 531–535
73. Vineyard, D., Patterson-Ward, J., Berdis, A. J., and Lee, I. (2005) *Biochemistry* **44,** 1671–1682
74. Vineyard, D., Patterson-Ward, J., and Lee, I. (2006) *Biochemistry* **45,** 4602–4610
75. Tomoyasu, T., Gamer, J., Bukau, B., Kanemori, M., Mori, H., Rutman, A. J., Oppenheim, A. B., Yura, T., Yamanaka, K., Niki, H., *et al*. (1995) *EMBO J.* **14,** 2551–2560
76. Hoffman, L., and Rechsteiner, M. (1996) *J. Biol. Chem.* **271,** 32538–32545
77. Ishikawa, T., Beuron, F., Kessel, M., Wickner, S., Maurizi, M. R., and Steven, A. C. (2001) *Proc. Natl. Acad. Sci. U.S.A.* **98,** 4328–4333
78. Wilcox, A. J., Choy, J., Bustamante, C., and Matouschek, A. (2005) *Proc. Natl. Acad. Sci. U.S.A.* **102,** 15435–15440
79. Huang, S., Ratliff, K. S., Schwartz, M. P., Spenner, J. M., and Matouschek, A. (1999) *Nat. Struct. Biol.* **6,** 1132–1138
80. Sato, T., Esaki, M., Fernandez, J. M., and Endo, T. (2005) *Proc. Natl. Acad. Sci. U.S.A.* **102,** 17999–18004
81. Kenniston, J. A., Burton, R. E., Siddiqui, S. M., Baker, T. A., and Sauer, R. T. (2004) *J. Struct. Biol.* **146,** 130–140
82. Mogk, A., Dougan, D., Weibezahn, J., Schlieker, C., Turgay, K., and Bukau, B. (2004) *J. Struct. Biol.* **146,** 90–98
83. Jenal, U., and Hengge-Aronis, R. (2003) *Curr. Opin. Microbiol.* **6,** 163–172
84. Erbse, A., Schmidt, R., Bornemann, T., Schneider-Mergener, J., Mogk, A., Zahn, R., Dougan, D. A., and Bukau, B. (2006) *Nature* **439,** 753–756
85. Wah, D. A., Levchenko, I., Baker, T. A., and Sauer, R. T. (2002) *Chem. Biol.* **9,** 1237–1245
86. Bolon, D. N., Wah, D. A., Hersch, G. L., Baker, T. A., and Sauer, R. T. (2004) *Mol. Cell* **13,** 443–449
87. Dougan, D. A., Weber-Ban, E., and Bukau, B. (2003) *Mol. Cell* **12,** 373–380
88. Rosen, R., Biran, D., Gur, E., Becher, D., Hecker, M., and Ron, E. Z. (2002) *FEMS Microbiol. Lett.* **207,** 9–12
89. Tomoyasu, T., Mogk, A., Langen, H., Goloubinoff, P., and Bukau, B. (2001) *Mol. Microbiol.* **40,** 397–413
90. Savel'ev, A. S., Novikova, L. A., Kovaleva, I. E., Luzikov, V. N., Neupert, W., and Langer, T. (1998) *J. Biol. Chem.* **273,** 20596–20602
91. Wagner, I., Arlt, H., van Dyck, L., Langer, T., and Neupert, W. (1994) *EMBO J.* **13,** 5135–5145
92. Bota, D. A., and Davies, K. J. (2002) *Nat. Cell Biol.* **4,** 674–680
93. Phillips, T. A., VanBogelen, R. A., and Neidhardt, F. C. (1984) *J. Bacteriol.* **159,** 283–287
94. Jelinsky, S. A., Estep, P., Church, G. M., and Samson, L. D. (2000) *Mol. Cell. Biol.* **20,** 8157–8167
95. Hori, O., Ichinoda, F., Tamatani, T., Yamaguchi, A., Sato, N., Ozawa, K., Kitao, Y., Miyazaki, M., Harding, H. P., Ron, D., Tohyama, M. M., Stern, D., and Ogawa, S. (2002) *J. Cell Biol.* **157,** 1151–1160
96. Dunker, A. K., Brown, C. J., Lawson, J. D., Iakoucheva, L. M., and Obradović, Z. (2002) *Biochemistry* **41,** 6573–6582
97. Tompa, P. (2005) *FEBS Lett.* **579,** 3346–3354
98. Van Dyck, L., and Langer, T. (1999) *Cell. Mol. Life Sci.* **56,** 825–842
99. Rep, M., van Dijl, J. M., Suda, K., Schatz, G., Grivell, L. A., and Suzuki, C. K. (1996) *Science* **274,** 103–106
100. Kuroda, A., Nomura, K., Ohtomo, R., Kato, J., Ikeda, T., Takiguchi, N., Ohtake, H., and Kornberg, A. (2001) *Science* **293,** 705–708
101. Kihara, A., Akiyama, Y., and Ito, K. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92,** 4532–4536
102. Kihara, A., Akiyama, Y., and Ito, K. (1999) *EMBO J.* **18,** 2970–2981
103. Leffers, G. G., Jr., and Gottesman, S. (1998) *J. Bacteriol.* **180,** 1573–1577
104. Kobiler, O., Oppenheim, A. B., and Herman, C. (2004) *J. Struct. Biol.* **146,** 72–78
105. Herman, C., Thévenet, D., D'Ari, R., and Bouloc, P. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92,** 3516–3520
106. Gur, E., and Sauer, R. T. (2008) *Proc. Natl. Acad. Sci. U.S.A.* **105,** 16113–16118
107. Seong, I. S., Oh, J. Y., Yoo, S. J., Seol, J. H., and Chung, C. H. (1999) *FEBS Lett.* **456,** 211–214
108. Kwon, A. R., Trame, C. B., and McKay, D. B. (2004) *J. Struct. Biol.* **146,** 141–147
109. Weiss-Brummer, B., Rödel, G., Schweyen, R. J., and Kaudewitz, F. (1982) *Cell* **29,** 527–536
110. Muroff, I., and Tzagoloff, A. (1990) *EMBO J.* **9,** 2765–2773
111. van Dyck, L., Neupert, W., and Langer, T. (1998) *Genes Dev.* **12,** 1515–1524
112. Siddiqui, S. M., Sauer, R. T., and Baker, T. A. (2004) *Genes Dev.* **18,** 369–374
113. Sim, K. L., and Creamer, T. P. (2002) *Mol. Cell. Proteomics* **1,** 983–995