Polypeptide stimulators of the Ms-Lon protease

STANISLAV G. RUDYAK AND THOMAS E. SHRADER

Department of Biochemistry, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461

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Abstract

Both the peptidase activity against small fluorescent peptide substrates and the ATPase activity of Lon (La) proteases are stimulated by unstructured proteins such as α -casein. This stimulation reveals the simultaneous interaction of Lon with two proteolytic substrates— α -casein and the peptide substrate. To understand the cellular function of this stimulation, it is important to determine the physical properties of Lon stimulators. The abilities of compositionally simple random copolymers of amino acids (rcAAs) to stimulate the peptidase and ATPase activities of the Lon protease from *Mycobacterium smegmatis* (Ms-Lon) and its N-terminal truncation mutant (N-E226) were determined. We report that cationic but not anionic rcAAs stimulated Ms-Lon's peptidase activity but were themselves poor substrates for the enzyme. Peptidase stimulation by rcAAs correlated approximately with the degree of hydrophobicity of these polypeptides and reached levels >10-fold higher than observed previously for Ms-Lon stimulators such as α -casein. In contrast to α -casein, which stimulates Ms-Lon's peptidase activity by 40% and ATPase activity by 150%, rcAAs stimulated peptidase activity without concomitant stimulation of ATPase activity. Active site labeling experiments suggested that both rcAAs and ATP increased peptidase activity by increasing accessibility to the peptidase active site. Peptidase activity assays in the presence of both α -casein and rcAAs revealed that interactions of rcAAs and α -casein with Ms-Lon are extremely complex and not mutually exclusive. Specifically, (1) additions of low concentrations of α -casein (<50 μ g/mL) caused a further stimulation of Ms-Lon's rcAA-stimulated peptidase activity; (2) additions of higher concentrations of α -casein inhibited Ms-Lon's rcAA-stimulated peptidase activity; (3) additions of all concentrations of α -casein inhibited N-E226's rcAA-stimulated peptidase activity. We conclude the Ms-Lon can interact with an rcAA, α -casein, and a substrate peptide simultaneously, and that formation of this quaternary complex requires the N-terminal domain of Ms-Lon. These data support models of Ms-Lon that include two allosteric polypeptide binding sites distinct from the catalytic peptidase site.

Keywords: active site labeling; Lon protease; polypeptide activators; protein degradation

Members of the Lon (originally called La) family of proteases are large multimers of ~85,000 Da subunits whose native molecular weights are >450,000 Da (Goldberg et al., 1994; Roudiak & Shrader, 1998). These enzymes require ATP to catalyze the exergonic process of peptide bond hydrolysis, and the stoichiometries of peptide bond hydrolysis to ATP hydrolysis for the Lon homolog from *Escherichia coli* (Ec-Lon) are remarkably constant for disparate protein substrates (~2 ATP/peptide bond; Goldberg et al., 1994). However, coupling of peptide bond hydrolysis and ATP hydrolysis is not obligatory, as both Ec-Lon and Ms-Lon's peptidase sites are activated by nonhydrolyzed nucleotide analogs (Goldberg & Waxman, 1985; Roudiak et al., 1998), and removal of the active site Ser nucleophiles abolishes the peptidase activities but not the ATPase activities of these enzymes (Fisher & Glockshuber, 1993; Roudiak et al., 1998). Interestingly, even conservative substitutions in the peptidase active site of Lon proteases result in mea-

surable decreases in their ATPase activities, revealing that the peptidase and ATPase regions of Lon proteases interact structurally (Roudiak et al., 1998; Starkova et al., 1998; van Dijk et al., 1998).

An atomic resolution structure is not available for any Lon protease homologue. Recent cryoelectron microscopy data reveal that the Lon homolog from *Saccharomyces cerevisiae* (Sc-Lon) is an approximate toroid containing seven subunits (Stahlberg et al., 1999). Sequence alignments suggest that Lon monomers contain: (1) a poorly conserved 200–500 residue N-terminal region (Wang et al., 1993; Van Dyck et al., 1994; Roudiak & Shrader, 1998); (2) a highly conserved 250 residue central region containing the Walker A- and B-box ATP binding motifs (Chung & Goldberg, 1981; Walker et al., 1982); and (3) a highly conserved 200-residue C-terminal region containing the Ser nucleophile for peptide bond hydrolysis (Amerik et al., 1991; Goldberg et al., 1994). Limited trypsin digestion of Ms-Lon yielded a stable intermediate corresponding to an N-terminal ~24 kDa fragment, suggesting that the ~220 residue N-terminal region of Ms-Lon forms an independently folded domain (Roudiak & Shrader, 1998). Assays with N-terminal truncation mutants of Ms-Lon reveal that the N-terminal

Reprint requests to: Thomas E. Shrader, Department of Biochemistry, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York; e-mail: shrader@aecom.yu.edu.

domain is dispensable without pronounced loss of either peptidase activity against small fluorogenic peptides or ATPase activity (Roudiak & Shrader, 1998). However, this truncation abolishes Ms-Lon's protease activity against larger protein substrates (Roudiak & Shrader, 1998). Limited trypsin digestion of Ms-Lon failed to yield a stable intermediate corresponding to the peptidase domain (Roudiak & Shrader, 1998). However, the isolated peptidase domain of Ec-Lon retains peptidase activity with an altered substrate specificity (Rasulova et al., 1998). Isolated ATPase domains have not been reported for either Ms-Lon or Ec-Lon. However, a 100-residue subdomain was isolated from the Ec-Lon ATPase domain and retains the polypeptide binding activities of native Ec-Lon (Smith et al., 1999).

Ec-Lon and Ms-Lon's peptidase and ATPase activities are stimulated by unfolded proteins such as apomyoglobin, glucagon, and α -casein (for clarity, we designate nucleotide-dependent effects as activations and unfolded polypeptide-dependent effects as stimulations). These properties are conserved in Ms-Lon despite the moderate sequence conservation between the two enzymes (Roudiak et al., 1998). The fact that α -casein stimulated the peptidase activity of Lon, instead of having competed with the peptide for the peptidase active site, showed that Lon contains a polypeptide binding site, distinct from the peptidase active site (Goldberg, 1992). Additional support for this polypeptide binding site was: (1) protein but not peptide substrates promoted the release of ADP from Ec-Lon (Menon & Goldberg, 1987); and (2) the K_m for α -casein hydrolysis by Ec-Lon was higher than the K_a for α -casein-stimulation of Ec-Lon's peptidase activity (Waxman & Goldberg, 1986). α -Casein also stimulated the peptidase activity of N-E226, an Ms-Lon mutant lacking the N-terminal domain. However, stimulation of N-E226's peptidase activity occurred without concomitant stimulation of its ATPase activity (Roudiak & Shrader, 1998). Those data revealed the unusual complexities of Lon–polypeptide interactions and that these interactions may be separately uncoupled from Lon's active sites.

Results

Cationic rcAAs stimulate the peptidase but not the ATPase activity of Ms-Lon

To allow us to understand the role of the stimulation of Ms-Lon by unfolded proteins, we are characterizing the physical properties required for a polypeptide to stimulate Ms-Lon. The abilities of a number of compositionally simple random copolymers of amino acids (rcAAs) to stimulate Ms-Lon's peptidase and ATPase activities were determined. Cationic but not anionic rcAAs stimulated Ms-Lon's peptidase activity (Fig. 1). Peptidase activity was monitored by the increase in fluorescence that occurs after hydrolysis of the Ms-Lon peptide substrate Glt-Ala-Ala-Phe-4MBNA. Stimulation of Ms-Lon's peptidase activity by several cationic rcAAs was much greater than the 40% stimulation caused by α -casein (Roudiak et al., 1998). RcAAs containing the nontranslatable amino acid ornithine (O in Fig. 1) stimulated Ms-Lon to a degree similar to that caused by rcAAs containing the same percentages of Lys. In addition, there was an approximate correlation between the degree of rcAA-stimulation of Ms-Lon's peptidase activity and the hydrophobicity of the rcAA. Finally, the strong stimulation by a strictly alternating copolymer revealed that clusters of hydrophobic residues were not essential to stimulate Ms-Lon's peptidase

Fig. 1. Stimulation of Ms-Lon's peptidase activity by rcAAs. Shown are the percent increases in the peptidase activity of Ms-Lon against Glt-Ala-Ala-Phe-4MBNA resulting from the addition of the indicated rcAA. The rcAA preparations tested range in size between 15,000 and 50,000 Da, and are abbreviated to represent their composition. For example, K1F1 contains 50% Lys and 50% Phe with an approximate length of 250 residues.

activity (polyKF). The maximum stimulatory effects of rcAAs were realized for rcAA additions well below the lowest concentration indicated in Figure 1 (20 µg/mL). For example, titrations of rcAA stimulation of Ms-Lon's peptidase activities for several rcAAs were saturated at concentrations of rcAAs below 5 µg/mL.

In contrast to α -casein, the effects of rcAAs on Ms-Lon's peptidase activity were not accompanied by stimulation of Ms-Lon's ATPase activity (Fig. 2). In fact, most cationic rcAAs slightly inhibited Ms-Lon's ATPase activity while anionic rcAAs did not appreciably affect that activity. Inhibition of Ms-Lon's ATPase activity cannot be explained by a simple interaction between ATP and cationic rcAAs because the nucleotide was present in vast excess (4.0 mM) relative to the concentration of rcAA residues (~0.15 mM) and molecules (~0.5 µM). The separate effects of rcAA additions on Ms-Lon's peptidase activity and ATPase activity are analogous to the separate effects of α -casein on the peptidase activity and ATPase activity of N-E226, an N-terminal truncation mutant of Ms-Lon (Roudiak & Shrader, 1998).

Fig. 2. Stimulation of Ms-Lon's ATPase activity by rcAAs. Shown are changes in the ATPase activity of Ms-Lon resulting from the addition of the indicated rcAA. rcAAs were added to a final concentration of 20 µg/mL.

On the mechanism of stimulation of Ms-Lon's peptidase activity by rcAAs

Assays to differentiate between potential k_{cat} and K_m effects of rcAAs on Ms-Lon's peptidase activity were performed. However, plots of peptidase velocity vs. substrate (Glt-Ala-Ala-Phe-4M β NA) concentration showed no plateau region (Fig. 3). Therefore, these data only show that the K_m of Ms-Lon for this peptide is >1 mM. In that region of the velocity vs. substrate concentration plot, the initial slope provides information about the ratio of V_{max} and K_m but not their individual values. The observed reductions in peptidase activity at high Glt-Ala-Ala-Phe-4M β NA concentrations may reflect mild, nonvisible peptide aggregation or increased viscosity of the solution. This statement follows from the facts that (1) solutions of Glt-Ala-Ala-Phe-4M β NA showed an increased anisotropy at concentrations above 1.0 mM; and (2) chymotrypsin yielded a similarly shaped curve of peptidase velocity vs. Glt-Ala-Ala-Phe-4M β NA concentration (data not shown).

The effects of rcAA additions on the accessibility of pyrene maleimide to the Ms-Lon peptidase active site nucleophile were determined. We employed a mutant of Ms-Lon, S675C, that contains a unique Cys residue in place of the catalytic Ser. S675C retains wild-type ATPase activity in vitro and probably some protease activity in vivo (Roudiak et al., 1998). Additions of both ATP γ S and polyKF resulted in measurable increases in the rate of labeling of C675 with pyrene maleimide (measured as the rate of fluorescence increase). Those increases are consistent with active site opening relative to the ADP-bound form of the enzyme. Modest in size, the observed effects (approximately twofold) may underestimate the degree of opening of the peptidase active site as the observed rates ($\sim 4 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$) were lower than expected for a diffusion controlled reaction ($>1 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$). Labeling reactions in the presence of ATP γ S were clearly biphasic because a nonrandom, curved residual (difference between actual and calculated data) resulted when the labeling curve was fit as a single exponential process. Labeling reactions under other conditions were more adequately modeled as a single exponential process (see Fig. 4C). As expected for a weak stimulator of Ms-Lon's peptidase activity (Roudiak et al., 1998), α -casein additions resulted in a measurable but lower increase in the rate of labeling of C675 with pyrene maleimide (not shown).

Fig. 3. Peptidase velocity as a function of peptide concentration. Reactions included 1 μg Ms-Lon and the indicated concentrations of Glt-Ala-Ala-Phe-4M β NA. Where indicated, reactions contained the rcAA at 20 $\mu\text{g}/\text{mL}$.

Fig. 4. Rates of labeling of the active site Cys in the activated and stimulated state. The Ms-Lon mutant S675C, containing a unique Cys in place of the catalytic Ser, was labeled with N-(1-pyrene) maleimide. **A:** The rates of labeling were monitored in the presence of the nucleotides and rcAAs indicated. The rate constants for >7 reactions were averaged to yield a single data point (bar), and this series was repeated to obtain a standard error (error bar). The results of two series of experiments from two independent preparations of S675C are indicated. **B:** Representative labeling progress curves for: Ms-Lon + ATP γ S (a), Ms-Lon + ADP + polyKF (b), Ms-Lon + ADP (c). **C:** Residuals of labeling progress curves (r) after fitting the data of **B** to a single exponential process (ra–rc) or a pair of exponential processes (ra').

Costimulation of Ms-Lon's peptidase activity by rcAAs and α -casein

The strong stimulation of Ms-Lon's peptidase activity by rcAAs such as K4W1 (550%) and K1F1 (270%), relative to α -casein (40%), provided a way to determine whether or not α -casein and rcAAs interacted with the same regions of Ms-Lon. If α -casein and K1F1 interact with the same regions of Ms-Lon, α -casein additions should displace the rcAA and inhibit the peptidase activity of Ms-Lon already stimulated by K1F1. Indeed, the data of Figure 5A reveal that Ms-Lon's ability to degrade the peptide Glt-Ala-Ala-Phe-4M β NA in the presence of saturating K1F1 was inhibited by additions of α -casein to final concentrations above 0.5 mg/mL (assays with 4.0 mM ATP). At the highest concentrations of α -casein (0.5–2 mg/mL), the stimulatory effects of K1F1 were effectively abolished. This effect is seen as the near superposition of the α -casein alone curve (solid) and the α -casein plus rcAA curves (dashed) at α -casein concentrations above 0.5 mg/mL. As expected, if α -casein and rcAAs indeed interact with common sites on Ms-Lon, an increase in the initial concentration of K1F1 from 0.1 to 0.2 mg/mL resulted in a requirement for a higher concentration of α -casein to abolish completely the stimulatory effect of K1F1 (the peptidase activity curves shift to the right for the higher initial rcAA concentration; Fig. 5A,B). By contrast, additions of lower concentrations of α -casein to Ms-Lon peptidase assays in which the peptidase activity had already been stimulated maxi-

Fig. 5. Additive stimulation of Ms-Lon's peptidase activity by rcAAs and α -casein. **A:** Peptidase activity of Ms-Lon against Glt-Ala-Ala-Phe-4M β NA in the presence or absence of K1F1 and the indicated concentrations of α -casein. **B:** Peptidase activity of Ms-Lon against Suc-Phe-Leu-Phe-4M β NA in the presence or absence of K4W1 and the indicated concentrations of α -casein. Values are relative to Ms-Lon degradation of Glt-Ala-Ala-Phe-4M β NA. The level of K4W1 stimulation of Ms-Lon's activity against this peptide is lower than that for Glt-Ala-Ala-Phe-4M β NA. **C:** Peptidase activity of Ms-Lon and its N-terminal truncation mutant against Glt-Ala-Ala-Phe-4M β NA in the presence or absence of K4Y1 and the indicated concentration of α -casein. N-E226 is an N-terminal deletion mutant of Ms-Lon lacking the 225-residue N-terminal domain. All assays contained 4.0 mM ATP.

mally by K1F1, resulted in a further stimulation (ascending regions of the dashed curves of Fig. 5A). This further stimulation of Ms-Lon's peptidase activity was independent of both the rcAA and the substrate peptide used: the same curve was qualitatively reproduced for K4W1 and the poor Ms-Lon substrate peptide Suc-Phe-Leu-Phe-4M β NA (Fig. 5B). Finally, at all concentrations, α -casein inhibited the peptidase activity of N-E226 stimulated by K4Y1. In other words, α -casein additions did not result in a further stimulation of N-E226's rcAA-stimulated peptidase activity (there is no ascending region of the curve of Fig. 5C). These data reveal that α -casein, certain rcAAs, and the substrate peptides can interact together with Ms-Lon to form a quaternary complex, but that there is no evidence for this quaternary complex with N-E226.

The effects of rcAA additions on the degradation of α -casein by Ms-Lon

High concentrations of α -casein inhibited Ms-Lon's rcAA-stimulated peptidase activity, suggesting that the two polypeptides occupied the same sites on the enzyme (see Fig. 5A–C). We therefore expected that rcAAs would inhibit the degradation of α -casein by Ms-Lon. The data in Figure 6 reveal that the rcAA inhibition profile of α -casein degradation was complex. First, K1F1 and R4Y1 inhibited strongly the degradation of α -casein ($\sim 100\%$). Second, O4Y1, K4Y1, O1L1, and polyKF inhibited much less completely the degradation of α -casein (20–50%). Incomplete inhibition persisted at saturating levels of those rcAAs (plateau regions in Fig. 7). Third, the least hydrophobic of the cationic rcAAs stimulated the degradation of α -casein. Stimulation of α -casein degradation by those rcAAs was similar in degree to their stimulation of peptidase activity ($\sim 100\%$; see Fig. 1). Finally, clusters of hydrophobic residues were not required to inhibit α -casein degradation, as shown by the fact that additions of polyKF were inhibitory (PolyKF is a strictly alternating rcAA). Again, the incomplete inhibition of α -casein degradation by certain rcAAs reflect the ability of Ms-Lon to interact simultaneously with both the rcAA and α -casein.

The degree of inhibition of α -casein degradation by an rcAA did not correlate with the rcAA's ability to be degraded by Ms-Lon. Although all rcAAs were degraded very slowly relative to α -casein, the strongly stimulating K1A1 was eventually degraded to 70% completion. By contrast, the weakly stimulating K4W1 was degraded to very low levels (0.01–0.1% completion), while the strongly inhibiting K4Y1 and O4Y1 were degraded to intermediate levels (10% completion). Those results reveal that a polypeptide (rcAA) need not be a proteolytic substrate of Ms-Lon to stimulate its peptidase activity.

Relative rcAA stimulation of Ms-Lon and N-E226's peptidase activities

We previously showed that (1) α -casein stimulated N-E226's peptidase activity to the same extent that it stimulated Ms-Lon's peptidase activity in assays with ATP; (2) ATP activated Ms-Lon's

Fig. 6. rcAA inhibition of the degradation of FITC-labeled α -casein (FITC- α -casein) by Ms-Lon. The concentration of each rcAA was 0.25 mg/mL, while the concentration of FITC- α -casein was 0.025 mg/mL. Values are in percent conversion of FITC- α -casein to a TCA-soluble form, relative to assays without rcAA additions.

Fig. 7. Concentration dependence of rcAA-inhibition of Ms-Lon's degradation of FITC- α -casein. Assays contained 0.025 mg/mL FITC- α -casein and the indicated concentration of the rcAA. The curves may overestimate the inhibition of FITC- α -casein degradation by rcAAs because at high levels, these polymers begin to precipitate in the presence of ATP (>0.2 mg/mL). The data points corresponding to 50 μ g/mL rcAA are probably the most informative, as rcAA-stimulation of Ms-Lon's peptidase activities saturates at this concentration (see Fig. 8). Values are in percent conversion of FITC- α -casein to a TCA-soluble form, relative to assays without rcAA additions.

peptidase activity ~ 10 -fold more strongly than did the nonhydrolyzed nucleotide analogs AMP-PNP or ATP γ S; (3) α -casein stimulated Ms-Lon's peptidase activity much more strongly than it stimulated N-E226's peptidase activity in assays with AMP-PNP or ATP γ S (Roudiak & Shrader, 1998). We next determined the effects of rcAA additions on Ms-Lon and N-E226's peptidase activities in assays with AMP-PNP. These assays removed the possibilities of indirect effects of unfolded polypeptides on the exchange of ADP and ATP (Menon & Goldberg, 1987) from more direct effects of rcAA additions on peptidase activity.

In contrast with α -casein, O1L1 stimulated N-E226 much more strongly than it stimulated Ms-Lon in peptidase assays with AMP-PNP (Fig. 8A; see point 3 above). These effects were reproduced qualitatively for several rcAAs. In no assay with AMP-PNP was an rcAA found to stimulate Ms-Lon to a higher final peptidase activity than it did N-E226. In addition, in peptidase assays with ATP (strongly activated Ms-Lon) plus K1Y1 (weakly stimulating rcAA), N-E226 was stimulated to a higher final peptidase activity than was Ms-Lon (Fig. 8B). Finally, in assays with ATP (strongly activated Ms-Lon) plus O1L1 (strongly stimulating rcAA), Ms-Lon and N-E226's peptidase activities converged to similar high levels (Fig. 8C). This level of $\sim 700\%$ stimulation probably represents the maximal peptidase rate for this peptide substrate (Glt-Ala-Phe-4M β NA).

Discussion

The two-site model of Ms-Lon

The existing data characterizing the interaction of Ms-Lon and its mutants with α -casein and rcAAs support a model of Ms-Lon containing two polypeptide binding sites distinct from the peptidase active site (Fig. 9, model II). The schematics of Figure 9 depict: the ATPase active site (A); the peptidase active site (P); a polypeptide binding site the occupancy of which stimulates peptidase activity without concomitant stimulated ATPase activity (PB-P);

Fig. 8. The N-terminal domain inhibits rcAA-stimulation of Ms-Lon's peptidase activity. Stimulation of the peptidase activities of Ms-Lon and N-E226 against Glt-Ala-Ala-Phe-4M β NA: (A) assays contained 0.5 mM AMP-PNP and the indicated concentrations of O1L1, (B) assays contained 4.0 mM ATP and the indicated concentrations of K4Y1, and (C) assays contained 4.0 mM ATP and the indicated concentrations of O1L1. All values are relative to the rate of degradation of Glt-Ala-Ala-Phe-4M β NA in assays with 4.0 mM ATP without rcAA additions.

and a polypeptide binding site the occupancy of which stimulates both ATPase and peptidase activities (PB-AP). Also depicted is a single polypeptide binding site the occupancy of which can stimulate either peptidase activity, ATPase activity, or both (PB; Fig. 9, model I). The PB-AP site is depicted as residing within Ms-Lon's N-terminal domain, but current data demonstrate only that it requires an intact N-terminal domain to function (Roudiak & Shrader, 1998). We depict the PB-P site as residing within the central ATPase domain and expect that this site corresponds to the fragment of Ec-Lon shown recently to bind polypeptides with a specificity mirroring that of intact Ec-Lon (Smith et al., 1999). Model II for Ms-Lon is analogous to two PB site models proposed for the

Fig. 9. Schematic models of Ms-Lon. **Model I:** Schematic of Ms-Lon containing one polypeptide binding site (PB) distinct from the peptidase active site. Also indicated are the ATPase site (A) and the peptidase site (P). The arrow indicates the requirement for an intact N-terminal domain for polypeptide interactions at the PB site to stimulate the A site. **Model II:** Schematic of Ms-Lon containing two polypeptide binding sites (PB-P and PB-AP) distinct from the peptidase active site. See main text for additional details of the model.

ClpXP protease, but differs from ClpXP models in that no sequence homology exists between the two PB sites in Ms-Lon (Levchenko et al., 1997).

Evidence for the two-site model of Ms-Lon

Stimulation of the peptidase activities of Ec-Lon and Ms-Lon, rather than the expected competition for the P site, revealed one or more polypeptide binding sites distinct from the P site (depicted as the PB site in Fig. 9, model I; Goldberg, 1992). The lack of competition between protein and peptide substrates also requires that the chemical steps of peptide bond hydrolysis and product release are fast relative to the correct orientation of protein substrates in the P site. We further demonstrated that α -casein stimulated the peptidase activity of N-E226 without concomitant stimulation of its ATPase activity (Roudiak & Shrader, 1998). Those data are consistent with model I if N-terminal truncation uncouples the polypeptide binding event from the A site but not from the P site. However, those data are also consistent with model II containing two independent sites, PB-P and PB-AP, if these sites interact differently with Ms-Lon's A and P sites. In model II, α -casein interacts with both the PB-P and PB-AP sites resulting in stimulation of both the A and P sites. N-terminal truncation to yield N-E226 destroys or inactivates the PB-AP site so that α -casein interacts solely with the PB-P site resulting in stimulation of only the P site.

RcAAs stimulated Ms-Lon's peptidase activity without concomitant stimulation of its ATPase activity (see Figs. 1, 2). These data are more difficult to reconcile with model I. Model I requires that rcAAs and α -casein both interact with the single PB site but elicit very different effects on the enzyme's catalytic activities. These data are more consistent with the model II containing two allosteric sites that interact differently with Ms-Lon's A and P sites. In model II, rcAAs interact only with the PB-P site, resulting in stimulation of the P site without concomitant stimulation of the A site (see Figs. 1, 2). The PB-P site does not require an intact N-terminal domain, so the peptidase activity of N-E226 is stimulated by rcAAs (see Fig. 8).

Finally, additions of low concentrations of α -casein caused a further stimulation of the peptidase activity of Ms-Lon already maximally stimulated by rcAAs (see Fig. 5). This result reveals

that α -casein, rcAAs, and the substrate peptide can simultaneously interact with Ms-Lon in a quaternary complex. This quaternary complex provides strong evidence for model II: Ms-Lon contains two polypeptide binding sites distinct from the P site. In model II, α -casein at low concentrations interacts with the PB-AP site while the rcAA interacts with the PB-P site. Both interactions stimulate peptidase activity. At increasing concentrations, α -casein displaces the rcAA from the PB-P site, resulting in a reduced peptidase activity that eventually converges to the peptidase activity of Ms-Lon in the presence of α -casein alone. Importantly, higher initial concentrations of the rcAA require additions of greater amounts of α -casein to compete out their stimulatory effect (a shifting of the peptidase activity curve to the right in Figs. 5A and 5B).

Additional features of model II revealed by interactions of α -casein with Ms-Lon and N-E226

Additions of α -casein stimulated both the peptidase and ATPase activities of Ec-Lon and Ms-Lon. In model II, these effects result from interactions of α -casein with both the PB-P and PB-AP sites. Based on results with Ec-Lon, ATPase stimulation occurs via either stimulation of ADP/ATP exchange or increased affinity for ATP relative to (Menon & Goldberg, 1987). However, even in assays with AMP-PNP, α -casein stimulated Ms-Lon more strongly than it stimulated N-E226 (Roudiak & Shrader, 1998). Those data show that α -casein interacts with the PB-AP site (absent from N-E226) to stimulate peptidase activity via mechanisms partially independent of ADP/ATP exchange.

Additional features of model II revealed by interactions of rcAAs with Ms-Lon and N-E226

rcAAs stimulated Ms-Lon's peptidase activity much more strongly than does α -casein (100–700% vs. 40%, see Fig. 1) without concomitant stimulation of ATPase activity. In model II, these effects result from rcAAs interaction with only the PB-P site (present in both N-E226 and Ms-Lon). These effects do not require ADP/ATP exchange as they occur in assays with nonhydrolyzed nucleotide analogs (see Fig. 8). Those data indicate that rcAA interactions at the PB-P site stimulated peptidase activity by mechanisms partially independent of ADP/ATP exchange.

RcAAs interact with only the PB-P site and stimulate N-E226's peptidase activity more strongly than they stimulate Ms-Lon's peptidase activity in assays with AMP-PNP (see Fig. 8). Those data show that the N-terminal domain of Ms-Lon inhibits stimulation of Ms-Lon's peptidase activity by rcAA interactions at the PB-P site. α -Casein interacts with both the PB-P and PB-AP sites and stimulate Ms-Lon's peptidase activity more strongly (>300%) than it stimulates N-E226's peptidase activity in assays with AMP-PNP (<50%; Roudiak & Shrader, 1998). Taken together, those two sets of data show that the N-terminal domain's inhibition of stimulation of peptidase activity by polypeptide interactions at the PB-P site is removed, and the N-terminal domain becomes stimulatory, when the polypeptide also interacts with the PB-AP site. Importantly, the N-terminal domain's inhibitory effects are only apparent during peptidase assays employing rcAAs, or other polypeptides, that interact with the PB-P site but not the PB-AP site (Figs. 8A–C). For example, the N-terminal domain's inhibitory effects of α -casein stimulation at the PB-P site are obscured by the independent α -casein stimulation at the PB-AP site (Roudiak & Shrader, 1998).

Simultaneous binding of α -casein and rcAAs to Ms-Lon

At the higher levels tested, additions of α -casein caused a displacement of rcAAs from the Ms-Lon PB-P site. Displacement of the rcAA (strongly stimulating) by α -casein (weakly stimulating) resulted in the lower peptidase activity characteristic of α -casein-stimulated Ms-Lon (see Figs. 5A–C). However, the expected reciprocal ability of rcAAs to inhibit α -casein degradation by Ms-Lon was not always observed. Certain rcAAs strongly inhibited α -casein degradation, but others stimulated it. The ability of an rcAA to stimulate maximally Ms-Lon's peptidase activity without inhibiting completely the degradation of α -casein reveals the mechanism of Ms-Lon. The PB-P site that interacts with α -casein and rcAAs must be large enough to allow the simultaneous binding of multiple polypeptides (at least of α -casein and certain rcAAs). Strong inhibition of α -casein degradation probably results only for the rcAAs that occupy the same subregions of the PB-P site.

Nucleotide binding opens the P site

When either ATP or dATP are bound to Ms-Lon, the P site is activated (increased peptidase activity). Nonhydrolyzed analogs cause a weak activation of the P site, consistent with their approximate molecular mimicry of ATP. Data directly demonstrating that both ATP and rcAAs open the P site are presented in Figure 4. However, the requirement of ATP binding for peptidase activity is absolute for Ms-Lon. No addition of polypeptide stimulators resulted in detectable peptidase activity in the absence of ATP or its nonhydrolyzed analogs (data not shown). Therefore, ATP binding must activate Ms-Lon by mechanisms in addition to opening of the peptidase active site. For example, a reorientation of catalytic groups essential for the protease activity may occur. Interestingly, the opening of the P site by ATP binding suggests that effectors that independently open the P site should inhibit Lon's ATPase activity. These effects have been observed for both small peptides, that must occupy the P site (Goldberg et al., 1994), and certain rcAAs, that open the P site indirectly by interacting with the PB-P site (see Fig. 2).

Optimal stimulation of Ms-Lon's peptidase activity by rcAAs parallels optimal substrate binding by several molecular chaperones

The requirement for the presence of basic residues in the polypeptides that stimulate Ms-Lon's peptidase activity is intriguing as a similar requirement for positively charged residues have been identified for optimal peptide binding to the molecular chaperones: DnaK (Rudiger et al., 1997; Mayer & Bukau, 1998), GroE (Brazil et al., 1998), and ClpX (Levchenko et al., 1997). Interestingly, optimal binding to DnaK requires clusters of four to five hydrophobic residues while Ms-Lon is strongly stimulated by the strictly alternating rcAA, polyKF (see Fig. 1). Those similar but distinct sequence rules for chaperone binding vs. protease activation may indicate the mechanisms cells use to differentiate refoldable vs. nonrefoldable damaged proteins during cellular quality control (Gottesman et al., 1997). The rcAAs tested include cationic polypeptides, anionic polypeptides, cationic hydrophobic polypeptides, and anionic hydrophobic polypeptides. The failure of any of those sequences to stimulate Ms-Lon's ATPase activity suggests that the requirements for a polypeptide to interact with the PB-AP site include greater sequence complexity than can be achieved by a

random copolymer of two amino acids. An alternative proposal is that the low level occurrence of defined secondary structures in the rcAAs was the cause of their lack of interaction with the PB-AP site.

Materials and methods

Bacterial strains, media, reagents, and DNA manipulations

E. coli strain MC1061 [*hsdR mcrB araD139 Δ (araABC -leu)7679 galU galK rpsL thi*] pREP4 (a *kan^R*, *lacI^Q* containing vector from Qiagen, Valencia, California) was used for protein overexpression (Casadaban & Cohen, 1980). Ms-Lon and N-E226 were expressed bearing N-terminal His₆-affinity domains and purified as described previously (Roudiak et al., 1998). Where appropriate, *E. coli* cells were grown in Luria broth containing ampicillin (Sigma, St. Louis, Missouri) at 100 μ g/mL and kanamycin (Sigma) at 25 μ g/mL.

Peptidase, protease, and ATPase assays

Peptidase assays contained 10 mM MgCl₂, 50 mM Tris (pH 8.0), either 4.0 mM ATP or 0.5 mM AMP-PNP, 0.3 mM of either Glu-Ala-Ala-Phe-4MBNA or Suc-Phe-Leu-Phe-4MBNA (Bachem, King of Prussia, Pennsylvania), and 1–5 μ g of Ms-Lon in a total volume of 200 μ L. Reactions were incubated for 60 min at 37 °C, and stopped by the addition of 100 μ L of 1% SDS and 1.2 mL of 0.1 M sodium borate (pH 9.2). Fluorescence was measured in a Photon Technologies QM-1 spectrofluorometer with excitation at 335 nm and emissions were monitored at 410 nm (Waxman & Goldberg, 1985). The amount of 4MBNA (4-methoxy- β -naphthylamide) released during peptidase assays was calibrated using the free compound (Sigma). Peptidase rates were constant for greater than 3 h in the absence of stimulators. Protease assays contained 10 mM MgCl₂, 4.0 mM ATP, 50 mM Tris-Cl (pH 8.0), 0.5–1.0 μ g of Ms-Lon and 5 μ g of α -casein-fluorescein isothiocyanate type I (Sigma) in 200 μ L total volume (Twining, 1984). Reactions were incubated for 60 min at 37 °C and terminated by the addition of 10 μ L of 10 mg/mL bovine serum albumin (Sigma) and 200 μ L of 10% trichloroacetic acid (TCA). Terminated reactions were incubated for 10 min on ice and centrifuged for 10 min at 14,000 rpm. Supernatants, containing TCA-soluble peptides were transferred to fresh tubes, and 400 μ L of 0.5 M CHES-Na (pH 12.0, Sigma) was added. Fluorescence values were measured in a Photon Technology International QM-1 Spectrometer with excitation at 490 nm and emission at 525 nm. The rate of degradation was calculated by normalizing the fluorescence of the α -casein cleaved into TCA soluble peptides by Ms-Lon with the fluorescence of the α -casein exhaustively cleaved into TCA soluble peptides by chymotrypsin. ATPase assays were performed as described previously (Black & Jones, 1983; Roudiak et al., 1998).

Random copolymers of amino acids (rcAAs)

The synthetic copolymers of α L-amino acids (rcAAs) with molecular weights between 15 and 50 kDa were purchased from Sigma. The rcAAs included the following: K1A1 = poly (Lys, Ala) with the molar ratios 1:1, K3S1 = poly (Lys, Ser) 3:1, K1F1 = poly (Lys, Phe) 1:1, K4W1 = poly (Lys, Trp) 4:1, K4Y1 = poly (Lys, Tyr) 4:1, O4Y1 = poly (Orn, Tyr) 4:1, O1L1 = poly (Orn, Leu) 1:1, R4Y1 = poly (Arg, Tyr) 4:1, E6A4 = poly (Glu, Ala) 6:4,

E4Y1 = poly (Glu, Tyr) 4:1. The rAAAs polyK and polyE were homopolymers of Lys and Glu with molecular weights between 15 and 50 kDa. The rAA polyKF was a sequential copolymer of alternating Lys and Phe with molecular weights between 5 and 15 kDa. Gel filtration over NAP-5 columns revealed that the stimulatory effects of rAAAs on Ms-Lon's peptidase activity were not due to small molecule contaminants (not shown). Aliphatic rAAAs each showed baseline absorbances above 250 nm revealing that these polymers retained negligibly few protecting groups from the original polymerization reactions (not shown). To monitor rAA degradation by Ms-Lon, rAAAs were labeled with fluorescein succinimidyl ester (Fluorescein Amine Labeling Kit, PanVera). Labeling reaction included ~0.5 mg of each rAA in 0.5 mL of coupling buffer supplemented with 0.4 M NaCl to which 1 μ L of 20 mM stock of fluorescein succinimidyl ester in DMSO was added, on ice. After 1 h incubation at 37 °C, reactions were quenched with 10 μ L of 1 M Tris, pH 8.0, and passed through a NAP-10 gel filtration column (Pharmacia, Uppsala, Sweden) to remove unincorporated fluorescein. rAA-degradation assays were similar to the FITC- α -casein degradation assays (Roudiak & Shrader, 1998) and included 0.2–2 μ g of rAA and up to 10 μ g of Ms-Lon or 0.2 mg of trypsin per 200 μ L reaction (10 mM MgCl₂, 4 mM ATP, 50 mM Tris, pH 8.0). Trypsin digestions were used as an approximation of complete rAA digestion for normalization of Ms-Lon digestion results.

Stopped-flow fluorescence measurement of labeling of S675C with N-(1-pyrene)maleimide

The S675C mutant of Ms-Lon was specifically labeled with environment-sensitive fluorescent label N-(1-pyrene)maleimide (Molecular Probes, Eugene, Oregon) at the C675 residue in the active site. C675 is the only Cys residue in this mutant. Fluorescent yield of this label increases by at least two orders of magnitude upon adduct formation, allowing measurement of labeling rates directly by fluorescence spectroscopy. Measurements were made on an Applied Photophysics instrument (Model SX.17MV) using an excitation wavelength of 345 nm, and collecting emission of the light not blocked by a 375 nm high-pass filter. All reactions were performed at 37 °C. The typical reaction contained twofold molar ratio of S675C (0.5 μ M) to pyrene, 4 mM ATP (0.4 mM in case of ATP γ S), and/or 20 μ g/mL of polyKF in labeling buffer (10 mM MgCl₂, 50 mM Tris pH 8.0). The average of seven scans were fitted to the equation describing a single exponential process using the Applied Photophysics data analysis software, which employs a Marquardt algorithm. All sets of experiments were repeated twice with independent preparations of the S675C.

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