Functional Role of the N-Terminal Region of the Lon Protease from *Mycobacterium smegmatis*

Stanislav G. Roudiak and Thomas E. Shrader*

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

Received April 27, 1998; Revised Manuscript Received June 18, 1998

ABSTRACT: Lon protease homologues contain a poorly conserved N-terminal region of variable length. To better understand the role of the N-terminal region of Lon in the complicated reaction cycle of ATP-dependent protein degradation, we expressed and characterized mutants of the Lon protease from *Mycobacterium smegmatis* (Ms-Lon) lacking 90, 225, and 277 N-terminal residues (N-G91, N-E226, and N-I278, respectively). N-I278 displayed neither peptidase nor ATPase activity despite the fact that it was stable and soluble in vivo, had a near-wild-type CD spectrum, and the deleted residues included neither the catalytic nucleophile for peptide bond hydrolysis (S675) nor the ATP binding regions. N-G91 and N-E226 retained peptidase activities against small unstructured peptides that were stimulated, to near-wild-type levels, by the Ms-Lon substrate protein α-casein. By contrast, N-G91 and N-E226 retained basal ATPase activities, but these activities were only stimulated weakly by α-casein. Ms-Lon, N-E226, and N-G91 all exhibited low-level peptidase activity in assays containing nonhydrolyzed nucleotide analogues. However, these peptidase activities were stimulated strongly by α-casein in the case of Ms-Lon but weakly by α-casein in the cases of N-G91 and N-E226. Strikingly, despite the near-wild-type peptidase activities of N-G91 and N-E226, both were severely impaired in their degradation of the Ms-Lon protein substrates α-casein in vitro and RcsA in vivo. Overall, N-G91 and N-E226 displayed catalytic properties similar to *Escherichia coli* Lon (Ec-Lon) in the presence of the PinA inhibitor, suggesting that PinA inhibits Ec-Lon protease by inhibiting the function of Ec-Lon's N-terminal region. In vivo protease assays further revealed that, in contrast to the inactive Ms-Lon point mutant S675A, N-G91 and N-E226 did not reduce the cellular activity of RcsA. This same defect was observed previously for Ms-Lons with multiple mutations in their peptidase active sites. We conclude that proteolytically inactive mutants of Ms-Lon retain the ability to reduce the cellular activity of RcsA but that both the N-terminal region and the peptidase active site region of Ms-Lon are required for this activity of wild-type Ms-Lon. The inabilities of N-G91 and N-E226 to degrade larger protein substrates and to reduce the cellular activity of RcsA were not the result of drastic alterations in their quaternary structures. Gel filtration profiles of N-G91 and N-E226 revealed that each was primarily tetrameric, with an increased percentage of dimeric species and a decreased percentage of trimeric species relative to Ms-Lon. The observed shifts in the dimer/trimer ratios of the N-terminal truncation mutants suggest that the Ms-Lon tetramer contains two types of subunit−subunit interactions.

ATP-dependent proteases mediate both the turnover of damaged proteins and the constitutive or conditional turnover of naturally short-lived proteins (reviewed in refs *1* and *2*). The ATP-dependent protease Lon from *Escherichia coli* (also called La and differentiated as Ec-Lon here) is a homotetramer or octamer of 87 kDa subunits (*3*). Ec-Lon's large native molecular mass, ∼350000 Da, identified it as a structurally complex protease in comparison with secreted proteases such as trypsin and elastase (*3*). However, Lon homologues are structurally less complex, in terms of both their total size and subunit complexity, than other known ATP-dependent proteases such as the 26S proteasome (*4*), the ClpAP and ClpXP proteases (*5*, *6*), and the HslUV protease (*7*−*9*).

Ec-Lon is responsible for the degradation of the majority of abnormal proteins synthesized in *E. coli* cells (*1*), revealing that Ec-Lon recognizes a very broad range of polypeptide substrates. In addition, several normal cellular polypeptides have been identified as Ec-Lon substrates (*2*). The basis for the extremely broad substrate recognition by Ec-Lon, or any ATP-dependent protease, remains only partially understood (*10*, *11*). Interestingly, Ms-Lon degrades RcsA (*12*), a natural substrate of Ec-Lon (*2*), despite the significant sequence divergence between these two enzymes and the evolutionary distance between the two organisms. This cross-species substrate recognition favors mechanisms of substrate recognition in which Lon proteases recognize global structural properties in their substrate proteins, such as the lack of compactness characteristic of many damaged proteins, rather than specific regions of primary structure.

* To whom correspondence should be addressed. Phone: (718) 430-2892. Fax: (718) 430-8565. E-mail: shrader@aecom.yu.edu.

S0006-2960(98)00945-3 CCC: $15.00
Published on Web 07/21/1998

Lon proteases degrade proteins and small peptides with a cleavage site specificity most closely resembling that of chymotrypsin (*3*). Protein degradation by Lon requires nucleotide hydrolysis, and the ratio of peptide bond hydrolysis to ATP hydrolysis by Ec-Lon is strikingly constant for several protein substrates (~2 ATP/peptide bond) (*3*). However, the obligate coupling of the peptide hydrolysis and ATPase reactions was ruled out by demonstrations that Lon peptidase activity was supported by nonhydrolyzed nucleotide analogues (*13*) and also that removal of the active site Ser nucleophile abolished Lon peptidase activity but not its ATPase activity (*12*, *14*). However, even conservative substitutions in the peptidase active sites of Lon proteases have measurable effects on their ATPase activities, revealing that the peptidase and ATPase regions of Lon proteases interact structurally (*12*, *15*).

Protein substrates of Lon proteases stimulate both their peptidase and ATPase activities (*3*, *12*). By contrast, small peptide substrates of Ec-Lon do not stimulate its ATPase activity (*3*). Goldberg and co-workers have demonstrated that protein substrates stimulate the peptidase activity of Ec-Lon via catalysis of ADP release. ADP release allows Lon to rebind ATP and isomerize to an activated conformation (*16*). ADP release is also the rate-limiting step in the ATPase reaction of Ec-Lon (*16*). Structural interconversions of Ec-Lon, driven by nucleotide binding and hydrolysis, were further demonstrated by experiments revealing that peptide chloromethyl ketones inactivate the ATP−Lon complex but not the ADP−Lon complex (*3*). In addition, unfolded proteins stimulate binding of nonhydrolyzed nucleotide analogues to Ec-Lon, consistent with the "opening" of additional active sites on the enzyme (*3*). Overall, despite being the least complex structurally of the known ATP-dependent proteases, Lon has multiple catalytic activities that are affected differently by interactions with its substrates

EXPERIMENTAL PROCEDURES

Bacterial Strains, Media, Reagents, and DNA Manipulations. *E. coli* strain MC1061 [*hsdR mcrB araD139* Δ*(araABC -leu)7679 galU galK rpsL thi*] was used for plasmid preparation (*17*), and strain MC1061 pREP4 (a kan^R- and $lacI^Q$-containing vector from Qiagen) was used for protein overexpression. Construction of strain Sgr-9 [MC4100, *cpsB10::lacZ(immλ) leu::Tn10(Tet^s) lon146::Tn10 SulB**] was described previously (*12*). Where appropriate, *E. coli* cells were grown in Luria broth containing ampicillin (Sigma) at 100 μg/mL and kanamycin (Sigma) at 25 μg/mL.

Limited Trypsin Digestion of Ms-Lon. Fifty micrograms of Ms-Lon was incubated with 100 ng of trypsin (Sigma) in 50 mM Tris (pH 8.0) and 10 mM $MgCl_2$ in a total reaction volume of 100 μL. At selected time points, aliquots were withdrawn and the digestion was stopped by the addition of 100 μL of 20% trichloroacetic acid. Undigested protein remaining in aliquots was precipitated by incubation on ice for 20 min. Precipitated protein was collected by centrifugation at 14 000 rpm for 10 min, followed by removal of the supernatant. Precipitated proteins were dissolved in SDS-loading buffer and fractionated by 12% SDS−PAGE. Fractionated polypeptides were transferred from the 12% SDS−PAGE to Immobilon-P transfer membrane (Millipore). For the transfer, we employed a Trans-Blott Cell (Bio-Rad) operated at 50 V for 2 h in 10 mM CAPS (pH 11.0) and 10% methanol. The most stable Ms-Lon fragments were visualized by staining the Immobilon-10 membrane with Coomassie Brilliant Blue (Baker) and excised from the membrane. The stable tryptic fragments of Ms-Lon were identified with 10 cycles of automated Edman degradation (performed at the Albert Einstein College of Medicine Laboratory for Macromolecular Analysis).

N-Terminal Truncations of Ms-Lon. All mutated Ms-Lon enzymes were expressed bearing an N-terminal hexa-His affinity domain (*18*) and were purified as described for the wild-type enzyme (*12*). N-Terminal truncations of Ms-Lon were constructed by replacing specific *Eco*RI−*Kpn*I, −*Sac*I, and −*Bam*HI restriction fragments of the wild-type Ms-Lon expression system (pMs-Lon) with double-stranded oligonucleotides (*12*). For mutant N-G91, the *Eco*RI (within the pQE-8 vector)−*Kpn*I (nucleotide 611 of *lon* sequence file AF30688) fragment was replaced with an oligonucleotide with the sequence (sense strand is shown, and the initiation codon of the truncated protein is underlined) 5′-AATTCATTAAAGAGGAGAAATTAACTATGAGAGGATCGCATCACCATCACCATCACGGTAC-3′. For N-E226, the *Eco*RI−*Sac*I(1016) fragment was replaced with an oligonucleotide with the sequence 5′-AATTCATTAAAGAGGAGAAATTAACTATGAGAGGATCGCATCACCATCACCATCACGAGCT-3′. For N-I278, the *Eco*RI−*Bam*HI-(1166) fragment was replaced with an oligonucleotide with the sequence N-I278, 5′-AATTCATTAAAGAGGAGAAATTAACTATGAGAGGATCGCATCACCATCACCATCACAG. Each expression construct contained *Mycobacterium smegmatis* chromosomal DNA through the *Sph*I site (2805) that lies 130 nucleotide downstream of the *lon* termination codon. The final yields were 10−20 mg/L of culture for Ms-Lon and N-E226, 2−5 mg/L for N-G91, and 1−2 mg/L for N-I278. The lower yield of N-G91 was probably the result of its in vivo degradation during expression (breakdown products are clearly visible in Figure 3, right gel). The lower yield of N-I278 was probably the result of its poor binding to the affinity resin as this fragment was expressed at high levels.

Peptidase, Protease, and ATPase Assays. Peptidase assays contained 10 mM $MgCl_2$, 50 mM Tris (pH 7.9), 4.0 mM ATP, 0.3 mM Glt-Ala-Ala-Phe-4Mβ NA (Bachem), and 5−10 μg of Ms-Lon in a total volume of 200 μL. Reaction mixtures were incubated for 30 or 60 min at 37 °C and reactions stopped by the addition of 100 μL 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 (*19*). The amount of 4Mβ NA (4-methoxy-β-naphthylamide) released during peptidase assays was calibrated using the free compound (Sigma). Protease assays contained 10 mM $MgCl_2$, 4.0 mM ATP, 50 mM Tris-HCl (pH 8.0), 1.0 μg of Ms-Lon, and 10 μg of α-casein−fluorescein isothiocyanate type I (Sigma) in a 100 μL total volume (*20*). Reaction mixtures were incubated for 15, 30, 45, 60, or 90 min at 37 °C and reactions terminated by the addition of 10 μL of 10 mg/mL bovine serum albumin (Sigma) and 100 μL of 10% trichloroacetic acid (TCA). Mixtures for terminated reactions were incubated for 10 min on ice and centrifuged for 10 min at 14 000 rpm. Super-

natants were transferred to fresh tubes, and 200 μL 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 in 60 min. ATPase assays were performed as described previously (*12*, *21*).

β-Galactosidase Assays of Sgr-9 Cells. The cellular levels of β-galactosidase in Sgr-9 cells containing plasmids expressing Ms-Lon or its N-terminal truncations were determined using the chromogenic β-galactosidase substrate 2-nitrophenyl β-D-galactopyranoside (ONPG, Boehringer) as described previously (*12*). Briefly, transformants were grown overnight to saturation, without plasmid induction, and 1.0 mL of cells was pelleted in a microcentrifuge and resuspended in 50 μL of 25% sucrose and 0.25 M Tris (pH 8.0). To this mixture was added 10 μL of lysozyme (10 mg/mL) followed by incubation for 15 min on ice. The resulting protoplasts were lysed by the addition of 0.15 mL of 75 mM EDTA and 0.33 M NaCl and incubation on ice for 5 min, followed by the addition of 10 μL of 1% Triton X-100. The supernatant from the lysed cells was prepared by centrifugation at 12000*g* for 15 min at 4 °C. After centrifugation, the insoluble cell debris was removed and ONPG assays were performed by incubating 50 μL of the above lysate in 650 μL of Z buffer [100 mM K_xHPO_4 (pH 7.0), 10 mM KCl, 1.0 mM $MgSO_4$, and 10 mM 2-mercaptoethanol] at room temperature. One hundred microliters of 10 mM ONPG was added to this reaction and the color allowed to develop. The reaction was timed and stopped with 750 μL of 1 M Na_2CO_3 and the optical density of the final reaction determined at 420 nm. These values were normalized to the concentration of total cellular protein as determined by the Bio-Rad protein detection reagent.

Size-Exclusion Chromatography Using a FPLC Superose 6 Column. Ms-Lon and its N-terminal deletions (1−2 mg) were applied to a Superose 6 FPLC column (Pharmacia), equilibrated with 50 mM Tris-HCl (pH 7.9) buffer. Samples were eluted with 50 mM Tris-HCl (pH 7.9), at a flow rate of 15 mL/h, at 4 °C. Molecular masses were estimated via calibration of the column with the following standards: blue dextran (BD) (2 000 000 Da), (a) thyroglobulin (669 000 Da), (b) apoferritin (443 000 Da), (c) catalase (232 000 Da), (d) alcohol dehydrogenase (150 000 Da), and (e) BSA (66 000 Da); all calibration proteins were from Sigma.

RESULTS

Expression, Purification, and Characterization of N-Terminal Truncation Mutants of Ms-Lon. An understanding of the domain structure of the Lon protease is an important step toward unraveling the interplay of Lon's peptidase and ATPase activities during the complex process of ATP-dependent protein degradation. Sequence comparisons of the highly divergent Lon proteases from *E. coli* (Ec-Lon) and *M. smegmatis* (Ms-Lon) revealed that the central regions of these enzymes, which contain the ATPase active sites, and the C-terminal regions, which contain the peptidase active sites (*22*), are the most highly conserved (Figure 1).

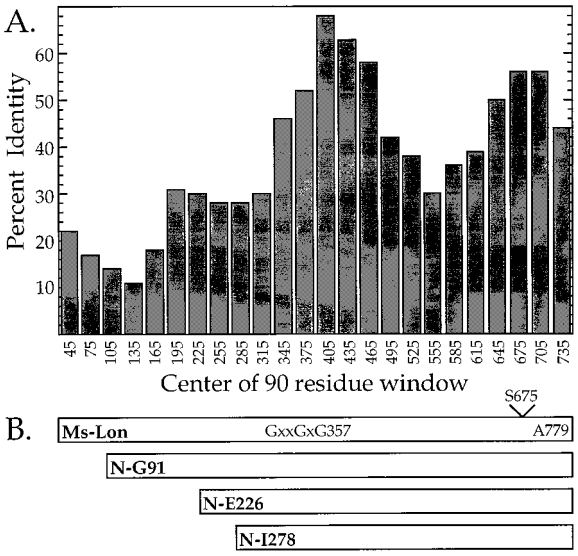

FIGURE 1: Sequence comparison of Ms-Lon and Ec-Lon and schematic structure of Ms-Lon and its N-terminal deletion mutants. (A) Indicated are the percentages of identical residues, between Ms-Lon and Ec-Lon, within a 90-residue window centered on the indicated residue. The numbering is based on the Ms-Lon sequence file AF30688 (*12*). (B) Schematic structure of Ms-Lon and the three N-terminal deletion mutants characterized here. The peptidase active site nucleophile S675 and a portion of the ATP-binding site G-P-P-G-V-G357 are indicated.

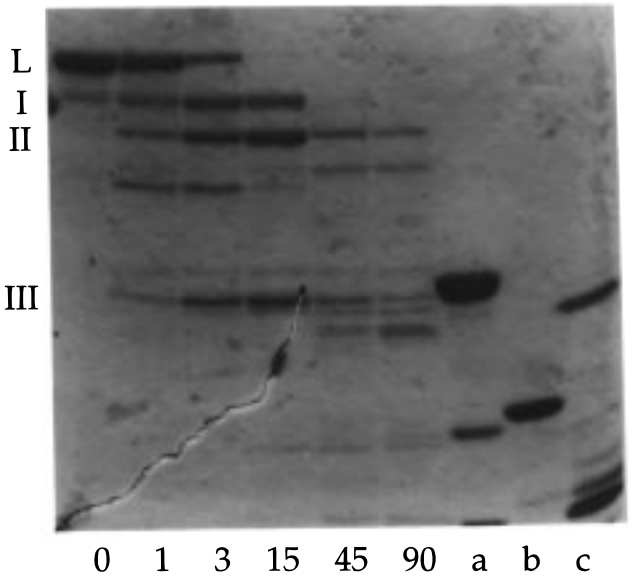

FIGURE 2: Limited trypsin digestion of Ms-Lon revealed a stable N-terminal ∼25 kDa fragment. The indicated fragments of Ms-Lon (L, 85 083 Da; includes affinity tag) had the following N-terminal sequences: I (61 667 Da, assuming mature C terminus) = T205-G-M-E-K-T; II (49 901 Da, assuming mature C terminus) = G335-M-A-V-V-G; III (approximately 24 000 Da) = M (within affinity tag)-R-G-S-H_6. The stability of fragments I and II depended on the addition of $MgCl_2$, but not nucleotide, to the digestion. The numbers represent the minutes of trypsin digestion. a is carbonic anhydrase (M_r = 29 000). b is *Staphylococcus aureus* nuclease (16 000 Da). c is trypsin (24 000 Da).

By contrast, the N-terminal regions of Lon family members are not highly conserved, and these regions contain large extensions in the eukaryotic homologues (*23*−*25*). However, limited trypsin digestion of Ms-Lon revealed that one of the most stable intermediates was an N-terminal ∼25 kDa fragment, suggesting that the 220 N-terminal residues of Ms-

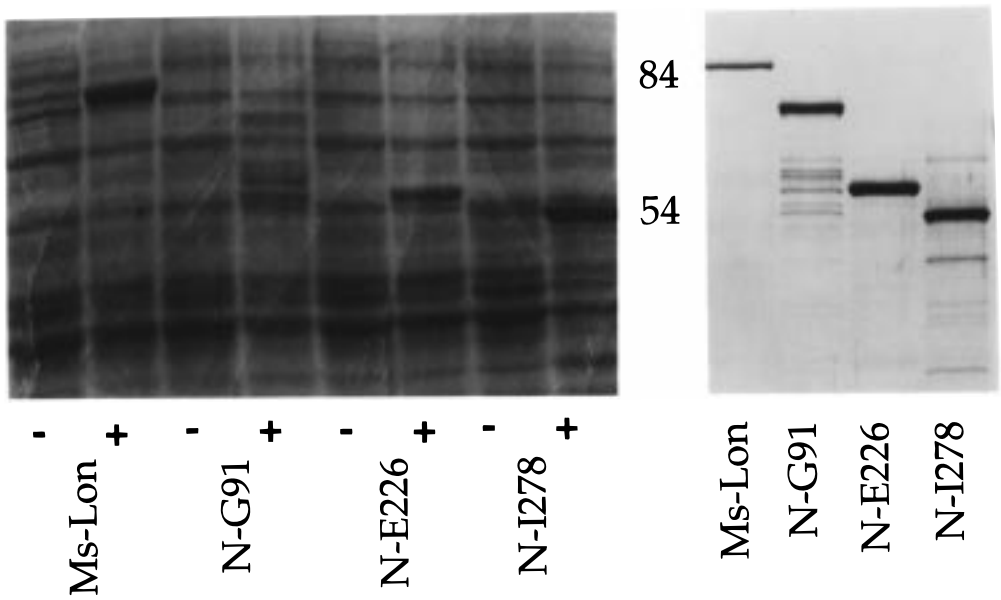

FIGURE 3: Expression and purification of N-terminal truncation mutants of Ms-Lon. (Left) Total cellular protein from cells before and after induction of the indicated expression systems. Proteins were fractionated by 8% SDS−PAGE and stained with Coomassie Brilliant Blue (Baker). Approximate molecular weights, indicated between the two gels, are based on the calculated molecular weights of Ms-Lon and N-I278. (Right) Final affinity-purified proteins from the induced cultures. Proteins were fractionated by 8% SDS−PAGE and stained with silver.

Table 1: Deconvoloution of the Circular Dichroism Spectra of Ms-Lon, N-E226, and N-I278[a]

name	α-helix	β-sheet	turns	random coil
Ms-Lon	38	32	0	30
N-E226	22	42	5	31
N-I278	25	45	1	29

[a] Circular dichroism spectroscopy was performed on a Jasco J-720 instrument at ambient temperature. Secondary structure estimations were performed using the SSE-338 program that compares the sample's CD spectrum with those of 15 reference proteins (*42*). The program estimates the fraction of the sample's residues in each of four conformations: α-helix, β-sheet, turns, and random coil. Values are in percent.

Lon form an independent domain (Figure 2). To probe the function of Ms-Lon's N-terminal region, N-terminally truncated Ms-Lons were expressed and purified. The inducible overexpression plasmid pMs-Lon (*12*) was modified to express three N-terminal truncations of Ms-Lon lacking 90, 225, and 277 residues (called N-G91, N-E226, and N-I278, respectively). Lysates from *E. coli* cells expressing and overexpressing N-G91, N-E226, and N-I278, and the resultant purified enzymes, are shown in Figure 3. Gel filtration revealed that Ms-Lon, N-G91, and N-E226 were all primarily tetrameric (see below). By contrast, N-I278 was stable and soluble in vivo, but the purified enzyme behaved as a higher multimer with an average molecular weight approximately equal to that of thyroglobulin (M_r = 600000−800000, data not shown). The CD spectra of both N-E226 and N-I278 were similar to that of Ms-Lon (*12*), suggesting that the inactivity of N-I278 (see below) was not the result of denaturation (Table 1).

Lon proteases are remarkable in their abilities to degrade a wide range of damaged and truncated proteins but to discriminate against the vast milieu of correctly folded cellular proteins, even in their nascent states (*1*, *12*). Discrimination against nonsubstrate *E. coli* proteins is broadly conserved between Ec-Lon and Ms-Lon (*12*). Part of our original rationale for generating N-terminal truncations of Ms-Lon was the hypothesis that the N-terminal region is essential for discrimination against nonsubstrate proteins. However, *E. coli* cells tolerated expression of the truncation mutants of Ms-Lon as well as they tolerated expression of indigenous Ec-Lon (*26*) or full-length Ms-Lon (*12*). Uninduced cultures expressed Ms-Lon at levels ∼5-fold higher than the level of wild-type expression of Ec-Lon (0.5% of the total cellular protein for Ms-Lon, N-E226, and N-I278 but lower for N-G91; see the left panel of Figure 3). However, uninduced cultures of cells expressing Ms-Lon or the truncation mutants grew only slightly slower than uninduced cultures containing vector alone, while induction significantly slowed cell growth (data not shown). These results were inconsistent with the rapid degradation of any essential *E. coli* protein by the Ms-Lon truncation mutants. In addition, during peptidase assays, only peptides that were substrates for Ms-Lon were substrates for the truncation mutants (data not shown). We conclude that N-terminal truncation of Ms-Lon did not create a nonspecific protease.

Minimum Size of Ms-Lon Fragments Retaining ATPase and Peptidase Activities. Mutants N-G91 and N-E226 retained near-wild-type peptidase activity with the peptide substrate Glt-Ala-Ala-Phe-4Mβ*NA and also retained basal ATPase activity (Figure 4A,B). By contrast, mutant N-I278 retained less than 1% of the wild-type peptidase activity and less than 2% of the wild-type ATPase activity (data not shown). We conclude that deletion of less than 226 residues from the N terminus of Ms-Lon leaves its basal peptidase and ATPase activities intact. This result might have been expected as the N-terminal regions of Lon homologues are poorly conserved relative to the other regions of the enzyme. However, even small deletions at its N terminus rendered Ms-Lon inactive in the degradation of protein substrates (see below). The complete inactivity of N-I278 was unexpected as this slightly larger deletion still leaves intact both the peptidase active site and the ATPase region (*22*). The ability

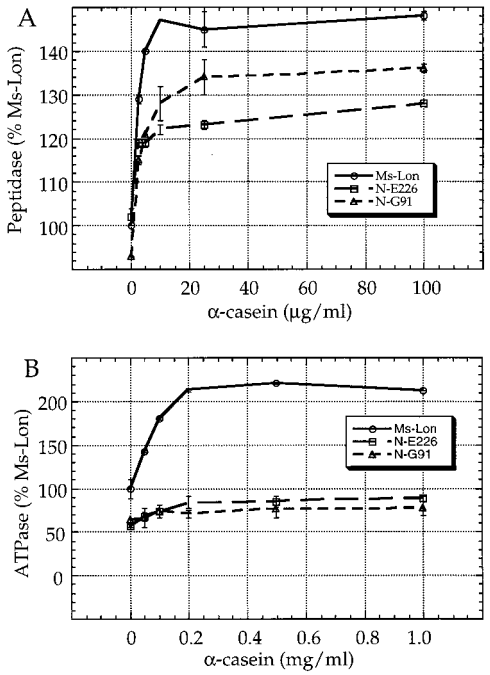

FIGURE 4: Stimulation of peptidase and ATPase activities of Ms-Lon, N-G91, and N-E226 by α-casein. (A) Peptidase activities of Ms-Lon, N-G91, and N-E226 as a function of the concentration of α-casein added to the reaction mixture. The indicated values are the rates of degradation of Glt-Ala-Ala-Phe-4MβNA relative to degradation by Ms-Lon in assays lacking α-casein. (B) ATPase activities of Ms-Lon, N-G91, and N-E226 as a function of the concentration of α-casein added to the reaction mixture. The indicated values are the rates of hydrolysis of ATP relative to hydrolysis by Ms-Lon in assays lacking α-casein.

of N-terminal truncation to abolish the activity of the peptidase site, separated by ~400 residues, reveals that the different regions of Ms-Lon interact structurally. Indeed, it has been demonstrated that the different regions of Ms-Lon interact strongly (*12*, *15*). For example, a substitution of the S675 nucleophile that removes a single hydroxyl group (S675A) resulted in a measurable decrease in Ms-Lon's ATPase activity, while more drastic mutations [exemplified by the mutant P-Ala (G673A/P674A/G677A/V678A/T679A); S675 and A676 are unchanged] have a larger effect (*12*). Finally, the inactivity of N-I278 might have been the trivial ramification of its higher multimeric state which blocked substrate access to the active site. However, attempts to activate N-I278 with low concentrations of urea were not successful.

The Peptidase but Not the ATPase Activities of N-E226 and N-G91 Were Strongly Stimulated by the Unfolded Protein. Goldberg and co-workers have demonstrated that both the peptidase and ATPase activities of Ec-Lon are stimulated by the unfolded protein α-casein but not by folded proteins such as ribonuclease and lysozyme (*27*). We have shown that these properties are conserved in the highly divergent Ms-Lon, suggesting that they are common to Lon proteases (*12*). Figure 4A reveals that the peptidase activities of both N-G91 and N-E226 were stimulated by α-casein to near-wild-type levels. Like that for Ms-Lon, half-maximal stimulation of N-G91 and N-E226's peptidase activities was observed during the course of assays containing <10 μg/mL α-casein (Figure 4A). By contrast, the ATPase activities of N-G91 and N-E226 were only weakly stimulated by α-casein. Half-maximal stimulation of Ms-Lon, N-G91, and

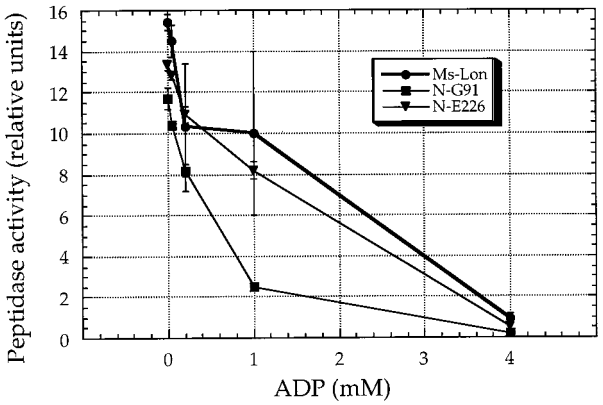

FIGURE 5: N-G91 and N-E226 are inhibited by ADP. Peptidase assays with the substrate Glt-Ala-Ala-Phe-4MβNA (0.3 mM). Each assay contained 5 μg of enzyme, 4.0 mM ATP, and the indicated concentration of ADP.

N-E226's ATPase activities was observed during the course of assays containing <100 μg/mL α-casein (Figure 4B). However, the maximal level of stimulation was much lower for the truncation mutants than it was for Ms-Lon. We conclude that deletion of 226 N-terminal residues of Ms-Lon did not abolish its ability to interact with α-casein. The similar shapes of the curves in panels A and B of Figure 4 for each enzyme suggest that the total overall affinity of Ms-Lon for α-casein was unaltered by N-terminal truncation. However, N-terminal truncation clearly altered either the detailed interactions of α-casein with Ms-Lon or the communication of these interactions to Ms-Lon's active sites.

N-G91 and N-E226 Were Inhibited by ADP. Goldberg and co-workers have demonstrated that bound ADP inhibits Ec-Lon's peptidase activity relative to bound ATP, and that unfolded protein reduces this inhibition by stimulating the release of bound ADP (*16*). ADP release is the rate-limiting step in Ec-Lon's ATPase reaction cycle and allows the enzyme to rebind ATP (*16*). Therefore, a potential explanation for why the peptidase activities of N-E226 and N-G91 were stimulated by unfolded protein in the absence of concomitant stimulation of the ATPase activities would be that the peptidase activities of the truncated enzymes were no longer inhibited by bound ADP relative to bound ATP. The data of Figure 5 contradict this explanation. Increasing concentrations of ADP, in the presence of a constant concentration of ATP, inhibited the peptidase activities of N-G91 and N-E226 at least as strongly as they inhibited the peptidase activity of Ms-Lon.

N-G91 and N-E226 Were Stimulated Weakly, by α-Casein, during the Course of Peptidase Assays with Nonhydrolyzed ATP Analogues. We determined the ability of α-casein to stimulate Ms-Lon, N-G91, and N-E226 in the presence of the nonhydrolyzed nucleotide analogues AMP-PNP and ATPγS. Ms-Lon's peptidase activity was weakly activated by both AMP-PNP and ATPγS (*12*), probably reflecting approximate structural mimicry of ATP by these nucleotide analogues. However, this activation of Ms-Lon's peptidase activity was strongly stimulated by α-casein (Figure 6 and data not shown). Compared to that of Ms-Lon, both N-G91 and N-E226's peptidase activities were weakly stimulated by α-casein (Figure 6). These differences in activation again indicate that N-terminal truncation of Ms-Lon either alters its interactions with unfolded proteins or interferes with the

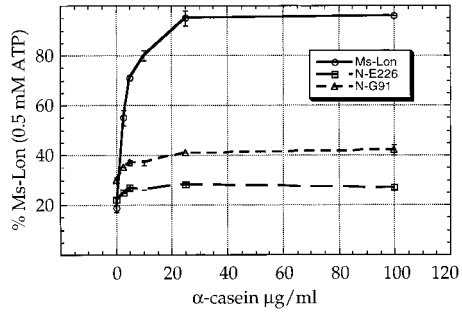

FIGURE 6: α-Casein weakly stimulated N-G91 and N-E226 in peptidase assays with AMP-PNP. Two micrograms of Ms-Lon, N-E226, or N-G91 was incubated with increasing concentrations of α-casein in the presence of 0.5 mM AMP-PNP in 50 mM Tris (pH 8.0), 10 mM $MgCl_2$, and 0.3 mM Glt-Ala-Ala-Phe-4MβNA. Peptidase values are relative to values of Ms-Lon in assays containing 0.5 mM ATP. Values are the average of two independent experiments; standard errors are indicated.

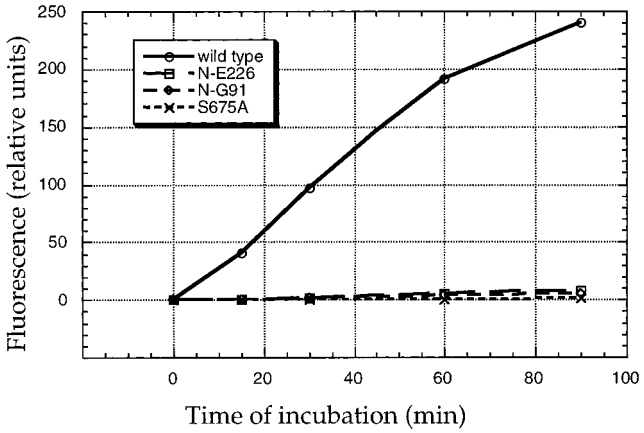

FIGURE 7: N-G91 and N-E226 degraded α-casein poorly in vitro. Shown is the conversion of fluorescein isothiocyanate-labeled α-casein (Sigma) to a TCA soluble form as a function of time. Standard errors for the 15, 30, 45, 60, and 90 min values were as follows(in relative units): Ms-Lon (3.9, 10, 6, 3, and 2), N-E226 (0.2, 0.1, 0.2, 0.1, and 0.1), N-G91 (0.1, 0.2, 0.3, 0.3, and 0), and S675A (0.1, 0.6, 0.6, 0.7, and 1.0). The overall protease activities of the mutants were as follows: N-E226, 3.3% of that of Ms-Lon; N-G91, 2.5%; and S675A, 0.7%. The actual rate of α-casein cleavage by Ms-Lon was 1.5 mg of α-casein cleaved per milligram of Ms-Lon per hour.

communication of these interactions to Ms-Lon's active sites. The concentrations of α-casein required for half-maximal stimulation of the peptidase activities of Ms-Lon, N-G91, and N-E226, in the presence of ATPγS, are all roughly equal (<10 μg/mL; see Figure 6). This equality again suggests that the binding affinities of Ms-Lon, N-G91, and N-E226 for α-casein are similar (see panels A and B of Figure 4).

N-G91 and N-E226 Lacked in Vitro Protease Activity. Mutants N-G91 and N-E226 were highly active in peptidase assays employing small unstructured peptides, suggesting that the poorly conserved Lon N-terminus can be dispensed for its cellular functions (see Figure 4A). However, the inability of α-casein to stimulate maximally the ATPase activities of N-G91 and N-E226 (see Figure 4B) raised the possibility that larger protein substrates might be more diagnostic for subtle functional defects in these enzymes. Therefore, we tested the ability of N-G91 and N-E226 to degrade fluorescently labeled α-casein. As seen in Figure 7, both mutants had near-baseline activity in this assay (<3.5% of that of Ms-Lon). We conclude that despite their retention of

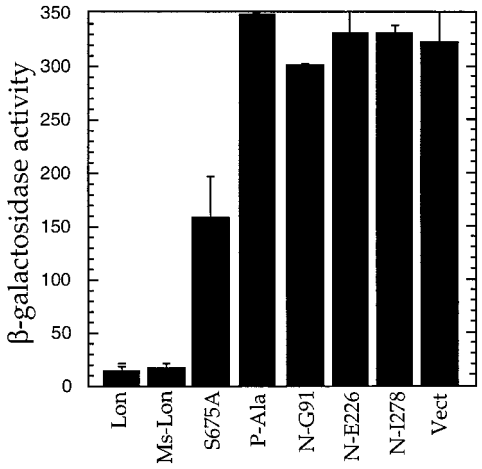

FIGURE 8: N-G91 and N-E226 did not sequester RcsA from its cellular targets or present RcsA to other cellular proteases in vivo. Shown are the β-galactosidase activities of cultures of Sgr-9 pREP4 cells containing the indicated expression plasmids. Sgr-9 is an *E. coli lon* mutant expressing a *cpsB*−*lacZ* fusion protein in response to the cellular RcsA activity (see Experimental Procedures). Results are the average of two independent experiments; standard errors are indicated.

peptidase activity, N-G91 and N-E226 were unable to degrade larger, yet relatively unstructured, protein substrates (α-casein is a near-random coil that is degraded by many proteases).

N-G91 and N-E226 Did Not Degrade RcsA in Vivo. To extend the results of the in vitro protease assays, we determined the ability of N-G91 and N-E226 to degrade RcsA, a natural substrate of Ec-Lon that is also degraded by Ms-Lon (*12*). Degradation of RcsA was monitored with an in vivo assay that measures the cellular activity of RcsA (*12*, *28*, *29*). This assay is based on the previous observation that capsular polysaccharide synthesis is positively regulated by RcsA in *E. coli* cells (*28*). Therefore, *E. coli lon* mutants, which degrade RcsA poorly, have increased steady-state cellular activities of RcsA and, correspondingly, synthesize excess polysaccharide. To monitor the cellular activity of RcsA, we utilized strain Sgr-9, an *E. coli lon* mutant that contains a lysogenized λ-bacteriophage containing a *cpsB*−*lacZ* gene fusion (see Experimental Procedures) (*29*). Lysogens of the *cpsB*−*lacZ*-containing phage express β-galactosidase in response to cellular RcsA activity because RcsA is required for expression from the *cpsB* promoter and the *cpsB* promoter is responsible for expression of *lacZ* in this phage. The RcsA assay is extremely sensitive and was used to demonstrate protease activity of a S675C mutant of Ms-Lon that lacked measurable in vitro peptidase or protease activity (*12*).

As described previously, the cellular levels of β-galactosidase for cultures of Sgr-9 pREP4 cells containing either Ec-Lon or Mc-Lon expression plasmids are reduced ~20-fold relative to that in Sgr-9 pREP4 cells containing vector alone (Figure 8). By contrast, expression of mutants N-G91, N-E226, or N-I278 all failed to reduce the cellular level of RcsA below that of cells containing vector alone (Figure 8). We conclude that neither N-G91 nor N-E226 degrades RcsA in vivo. Overall, N-terminal truncation of Ms-Lon creates enzymes that retain in vitro peptidase activity but lack either in vitro or in vivo protease activity.

N-G91 and N-E226 Did Not Reduce RcsA's Interaction with the cpsB Promoter in Vivo. In vivo expression of a proteolytically inactive Ms-Lon mutant, lacking its Ser nucleophile (S675A), nonetheless reduced the cellular activity of RcsA (as revealed by 2-fold reduction in the expression of the *cpsB*−*lacZ* fusion) (ref *12*, reproduced in Figure 7). On the basis of this result, we concluded that the S675A mutant either binds, unfolds, and renders RcsA a substrate for other cellular proteases (termed RcsA presentation) or binds RcsA and inhibits its binding to the *cpsB* promoter (termed RcsA sequestration). Both interpretations are stoichiometrically plausible because the cellular level of Ms-Lon (∼0.5% of total cellular protein) was much greater than that of RcsA (expressed at extremely low levels) (*30*). Both interpretations are also supported by the demonstrated ability of Ec-Lon and other ATP-dependent proteases to form stable complexes with their substrates and display chaperone-like unfolding activity (*31*−*35*).

Figure 8 reveals that expression of either N-G91 or N-E226 in Sgr-9 pREP4 cells did not reduce the cellular level of RcsA below that of vector alone. Regardless of the exact molecular mechanism of the reduction of RcsA activity by S675A, both N-E226 and N-G91 have lost this ability. In other words, while S675A has lost the ability to hydrolyze RcsA, N-G91 and N-E226 have lost both the abilities to hydrolyze RcsA and either to sequester or present RcsA. We conclude that N-G91 and N-G226 have altered interactions with their substrates. We have previously shown that the Ms-Lon mutant P-Ala, in which six peptidase active site residues have been changed to Ala, failed to inhibit RcsA's interaction with its cellular targets (ref *12* and Figure 7). Overall, we conclude that both the active site region and the N-terminal region of Ms-Lon are required for its wild-type interaction with RcsA.

N-G91 and N-E226 Displayed Altered Intersubunit Interactions but Remained Primarily Tetrameric. A possible explanation for the inability of N-G91 and N-E226 to degrade larger proteins and to sequester or present RcsA would be a drastic alteration in their quaternary structures. However, gel filtration profiles of the N-G91 and N-E226 truncation mutants revealed that this was not the case (Figure 9). Both truncation mutants were primarily tetrameric with molecular masses of ∼400000 Da. As observed for Ec-Lon (*3*), Ms-Lon tetramers behave during gel filtration as if they are slightly larger than their calculated molecular masses indicate. Interestingly, the profiles of both N-G91 and N-E226 revealed increased percentages of dimeric species and decreased percentages of trimeric species relative to wild-type Ms-Lon. These changes in the dimer/trimer ratios must be indicative of changes in the subunit−subunit interactions of Ms-Lon brought about by N-terminal truncation.

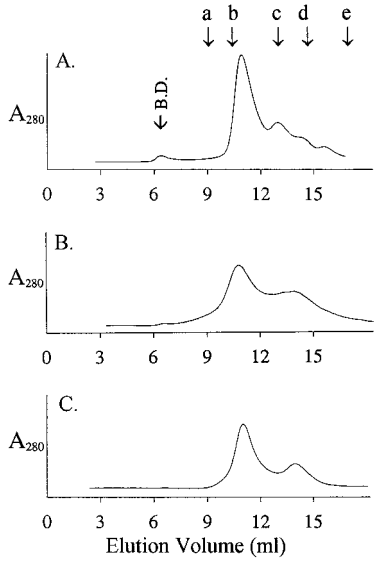

FIGURE 9: N-G91 and N-E226 were primarily tetrameric. Gel filtration profiles of (A) Ms-Lon, (B) N-G91, and (C) N-E226. Molecular mass estimates were as follows: (A) Ms-Lon, first peak, 420 000 Da, second peak, 230 000 Da, third peak, 140 000 Da, and fourth peak, 95 000 Da; (B) N-G91, 470 000 and 160 000 Da; and (C) N-E226, 440 000 and 170 000 Da. Molecular masses were estimated via calibration of the Superose 6 column with (B.D.) blue dextran (2 000 000 Da), (a) thyroglobulin (669 000 Da), (b) apoferritin (443 000 Da), (c) catalase (232 000 Da), (d) alcohol dehydrogenase (150 000 Da), and (e) BSA (66 000 Da); all calibration proteins were from Sigma.

DISCUSSION

N-Terminal Truncation Has Different Effects on Ms-Lon's Catalytic Activities. N-Terminal truncation affected differently the ability of α-casein to stimulate Ms-Lon's peptidase activity (which is inhibited by ADP) and ATPase activity (for which ADP release is rate-limiting). This difference revealed that α-casein's well-documented ability to stimulate ADP release from Ec-Lon (*16*) cannot be its only mode of activation of Ms-Lon's peptidase activity. However, the differential effects of N-terminal truncation on the catalytic activities of Ms-Lon support existing models of Ec-Lon in which α-casein interacts with sites on the enzyme distinct from the peptidase active site (*1*, *36*). Our working model proposes that the first α-casein interaction site on Ms-Lon resides in, or requires, the N-terminal region for its activity. The existence of this site has been previously proposed, and it is termed the allosteric site (*1*, *36*). We propose that interaction of α-casein with Ms-Lon's allosteric site results in stimulation of Ms-Lon's ATPase activity, at least partially, via stimulation of ADP exchange. This follows from the weak stimulation, by α-casein, of the ATPase activities of N-G91 and N-E226 (see Figure 4B). The second site of Ms-Lon that interacts with α-casein remains poorly defined. We propose that this site is very near the peptidase active site, in analogy to the extended substrate binding sites observed for other proteases. Localization of the second α-casein interaction site to an extended substrate binding site is suggested by the inability of the P-Ala mutant, which has a drastically altered peptidase active site, to inhibit RcsA from interacting with its cellular targets (see Figure 8). We propose that interaction of Ms-Lon with α-casein at the extended substrate binding site results in stimulation of Ms-Lon's peptidase site via mechanisms, at least partially, independent of ADP exchange. This follows from the differential stimulation, by α-casein, of the ATPase and peptidase activities of N-G91 and N-E226 (see Figure 4B). The proposal that there are multiple mechanisms by which unfolded protein stimulates Ms-Lon is also supported by data demonstrating that certain poly(amino acids) strongly stimulate the peptidase activity of Ms-Lon and its truncation mutants while weakly inhibiting their ATPase activities (S. G. Roudiak and T. E. Shrader, unpublished).

Stimulation of Ms-Lon by α-casein, during ATP-PNP supported peptidase assays, further supports the conclusion that stimulation of ADP exchange is not the sole mechanism of stimulation of Ms-Lon's peptidase activity by unfolded protein. In addition, the >4-fold level of this stimulation suggests that the observed stimulatory effect was not the sole result of opening additional peptidase active sites on the Ms-Lon tetramer (*37*). Overall, our data suggest that α-casein binding (at both the allosteric and extended substrate binding sites of Ms-Lon; see above) and ATP binding cooperate to stimulate maximally the individual peptidase active sites of Ms-Lon. In the presence of ATP, Ms-Lon's peptidase activity is strongly activated, allowing only a 30−50% further stimulation by α-casein (*12*). This level of stimulation can be achieved by α-casein binding to the extended substrate binding site alone (see Figure 4A). In the presence of the nonhydrolyzed analogue ATPγS or AMP-PNP, which are approximate structural mimics of ATP, Ms-Lon's peptidase activity is weakly activated. allowing a higher level of additional stimulation by α-casein. This level of stimulation requires α-casein binding at both the extended active site and the allosteric site (see Figure 6). Overall, our working model proposes that these effects are in addition to the aforementioned effect of α-casein binding at Ms-Lon's allosteric site, stimulation of Ms-Lon's peptidase activity via stimulation of ADP−ATP exchange (*16*). Stimulation of Ms-Lon's ATPase activity requires the N-terminal region (see Figure 4B).

The Catalytic Properties of the N-G91 and N-E226 Truncation Mutants of Ms-Lon Are Similar to Those of Ec-Lon in the Presence of the Inhibitor PinA. The PinA protein from bacteriophage T4 inhibits the *E. coli* Lon protease, making cells that express PinA phenotypically *lon*$^-$ (*38*). Maurizi, Simon, and co-workers have demonstrated that PinA forms a tight complex with Ec-Lon ($K_d \sim 10$ nM) (*39*). However, PinA inhibits neither Ec-Lon's peptidase activity against small unstructured polypeptides nor the ability of α-casein to stimulate this activity (*40*). By contrast, PinA strongly inhibits both Ec-Lon's hydrolysis of α-casein and the stimulation of Ec-Lon's ATPase activity by α-casein (*40*). Overall, both PinA and N-terminal truncation abolish the protease activity but not the peptidase activity of Lon proteases and inhibit the abilities of unfolded proteins to stimulate their ATPase activities. The similarities in the effects of PinA inhibition and N-terminal truncation suggest that the PinA inhibitor functions by inhibiting the activity of the N-terminal region of Ec-Lon. This inhibition could result from either a direct interaction of PinA with Ec-Lon's N-terminal region or an indirect effect that functionally isolates the N-terminal region of Ec-Lon from communicating with its ATPase site.

Altered Subunit−Subunit Interactions in N-G91 and N-E226. In addition to the subtle effects of N-terminal truncation on Ms-Lon's interaction with α-casein, these modifications also alter the subunit−subunit interaction within the Ms-Lon tetramer (see Figure 9). The importance of subtle subunit−subunit communication in Ec-Lon was previously indicated during studies of the CapR9 mutant of Ec-Lon. CapR9 dissociates into monomers and dimers more readily than wild-type Ec-Lon, and the enzyme lacked in vivo protease activity (*41*). Indeed, subtle subunit−subunit interactions are likely to be essential to Lon function if the mechanism of protein degradation by Lon involves either the cooperative unfolding of substrate proteins by multiple Ms-Lon subunits or the reciprocal binding and release of substrates between Ms-Lon subunits (*3*, *36*). In the first case, Lon would function via cooperative ATP hydrolysis-driven conformational changes that were coupled to substrate unfolding. Models of this type are supported by the demonstration that ATP hydrolysis is required for Ec-Lon to degrade α-helical peptides but only nucleotide binding is essential for Ec-Lon to degrade random coil peptides (*10*). In a second case, ATP hydrolysis would drive the reciprocal binding and release of substrates to alternating Lon subunits. This intriguing model has been proposed to explain the processivity of Lon during the degradation of larger substrates (*36*). Overall, it remains to be determined if changes in Ms-Lon's intersubunit interactions and communications, such as those that result from N-terminal truncation, result in enzymes lacking protease activity despite their retention of peptidase activity.

Finally, the observed shifts in the dimer/trimer ratios of the N-terminal truncation mutants (see Figure 9) are most easily explained if the Ms-Lon tetramer contains two classes of subunit−subunit interactions. Two classes of subunit−subunit interactions within a tetramer are characteristic of a tetramer composed from two copies of a dimer (for example, two dumbbells stacked with collinear 2-fold axes). The gel filtration data suggested that one class of interaction, resulting in a dimer, was unaffected by N-terminal truncation while the second class of interaction, which results in tetramers, was reduced to the point where a particle containing a single interaction of this type (a trimer) is not stable. Two types of interactions, within the Ms-Lon tetramer, are less consistent with either toroidal or tetrahedral models of the enzyme, as in these structures subunit−subunit interactions are all identical.

ACKNOWLEDGMENT

We thank Dr. Nancianne Knipfer, Dr. Thomas Leyh, and Dr. Michael Toney for helpful discussions and comments on the manuscript. We thank Yuan Shi for N-terminal sequence analysis. We thank Dr. George Orr and Dr. Joseph Geavy for help with the gel filtration experiments.

REFERENCES

1. Goldberg, A. L. (1992) *Eur. J. Biochem. 203*, 9−23.
2. Gottesman, S., and Maurizi, M. R. (1992) *Microbiol. Rev. 56*, 592−621.
3. Goldberg, A. L., Moerschell, R. P., Chung, C. H., and Maurizi, M. R. (1994) *Methods Enzymol. 244*, 350−375.
4. Coux, O., Tanaka, K., and Goldberg, A. L. (1996) *Annu. Rev. Biochem. 65*, 801−847.
5. Maurizi, M. R., Clark, W. P., Katayama, Y., Rudikoff, S., Pumphrey, J., Bowers, B., and Gottesman, S. (1990) *J. Biol. Chem. 265*, 12536−12545.
6. Wang, J., Hartling, J. A., and Flanagan, J. M. (1997) *Cell 91*, 447−456.
7. Chuang, S.-E., Burland, V., Plunkett, G., Daniels, D. L., and Blattner, F. R. (1993) *Gene 134*, 1−6.
8. Rohrwild, M., Pfeifer, G., Santarius, U., Muller, S. A., Huang, H. C., Engel, A., Baumeister, W., and Goldberg, A. L. (1997) *Nat. Struct. Biol. 4*, 133−139.
9. Kessel, M., Wu, W.-f., Gottesman, S., Kocsis, E., Steven, A. C., and Maurizi, M. R. (1997) *FEBS Lett. 398*, 274−278.

10. Van Melderen, L., Thi, M. H. D., Lecchi, P., Gottesman, S., Couturier, M., and Maurizi, M. R. (1996) *J. Biol. Chem. 271*, 27730−27738.
11. Levchenko, I., Smith, C. K., Walsh, N. P., Sauer, R. T., and Baker, T. A. (1997) *Cell 91*, 939−947.
12. Roudiak, S. G., Seth, A., Knipfer, N., and Shrader, T. E. (1998) *Biochemistry 37*, 377−386.
13. Goldberg, A. L., and Waxman, L. (1985) *J. Biol. Chem. 260*, 12029−12034.
14. Fisher, H., and Glockshuber, R. (1993) *J. Biol. Chem. 268*, 22502−22507.
15. Starkova, N. N., Koroleva, E. P., Rumsh, L. D., Ginodman, L. M., and Rotanova, T. V. (1998) *FEBS Lett. 422*, 218−220.
16. Menon, A. S., and Goldberg, A. L. (1987) *J. Biol. Chem. 262*, 14929−14934.
17. Casadaban, M. J., and Cohen, S. N. (1980) *J. Mol. Biol. 138*, 179−207.
18. Smith, M. C., Furman, T. C., Ingnolia, T. D., and Pidgeon, C. (1988) *J. Biol. Chem. 263*, 7211−7215.
19. Waxman, L., and Goldberg, A. L. (1985) *J. Biol. Chem. 260*, 12022−12028.
20. Twining, S. S. (1984) *Anal. Biochem. 143*, 30−34.
21. Black, M. J., and Jones, M. E. (1983) *Anal. Biochem. 135*, 233−238.
22. Amerik, A. Y., Antonov, V. K., Gorbalenya, A. E., Kotova, S. A., Rotanova, T. V., and Shimbarevich, E. V. (1991) *FEBS Lett. 287*, 211−241.
23. Wang, N., Gottesman, S., Willingham, M. C., Gottesman, M. M., and Maurizi, M. R. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 11247−11251.
24. Van Dyck, L., Pearce, D. A., and Sherman, F. (1994) *J. Biol. Chem. 269*, 238−242.
25. Suzuki, C. K., Suda, K., Wang, N., and Schatz, G. (1994) *Science 264*, 273−276.
26. Goff, S. A., and Goldberg, A. L. (1987) *J. Biol. Chem. 262*, 4508−4515.
27. Waxman, L., and Goldberg, A. L. (1986) *Science 232*, 500−503.
28. Stout, V., and Gottesman, S. (1991) *Mol. Microbiol. 5*, 1599−1606.
29. Dervyn, E., Canceill, D., and Huisman, O. (1990) *J. Bacteriol. 172*, 7098−7103.
30. Stout, V., Torres-Cabassa, A., Maurizi, M. R., Gutnick, D., and Gottesman, S. (1991) *J. Bacteriol. 173*, 1738−1747.
31. Wickner, S., Gottesman, S., Skowyra, D., Hoskins, D., McKenney, J., and Maurizi, M. R. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 12218−12222.
32. Levchenko, I., Luo, L., and Baker, T. A. (1995) *Genes Dev. 9*, 2399−2408.
33. Wawrzynow, A., Wojtkowiak, D., Marszalek, J., Banecki, B., Jonsen, M., Graves, B., Georgopoulos, C., and Zylicz, M. (1995) *EMBO J. 14*, 1867−1877.
34. Suzuki, C. K., Rep, M., Maarten van Dijl, J., Suda, K., Grivell, L. A., and Schatz, G. (1997) *Trends Biochem. Sci. 22*, 118−123.
35. Higashitani, A., Ishii, Y., Kato, Y., and Koriuchi, K. (1997) *Mol. Gen. Genet. 254*, 351−357.
36. Maurizi, M. R. (1992) *Experientia 48*, 178−201.
37. Menon, A. S., and Goldberg, A. L. (1987) *J. Biol. Chem. 262*, 14921−14928.
38. Skorupski, K., Tomaschewski, J., Ruger, W., and Simon, L. D. (1988) *J. Bacteriol. 170*, 3016−3024.
39. Hilliard, J. J., Maurizi, M. R., and Simon, L. D. (1998) *J. Biol. Chem. 273*, 518−523.
40. Hilliard, J. J., Simon, L. D., Van Melderen, L., and Maurizi, M. R. (1998) *J. Biol. Chem. 273*, 524−527.
41. Chung, C. H., Waxman, L., and Goldberg, A. L. (1983) *J. Biol. Chem. 258*, 215−221.
42. Yang, J. T., Wu, C. S., and Martinez, H. M. (1986) *Methods Enzymol. 130*, 208−269.

BI980945H