Mg^{2+}-Linked Oligomerization Modulates the Catalytic Activiy of the Lon (La) Protease from *Mycobacterium smegmatis*†

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Received February 6, 2001

ABSTRACT: Lon (La) proteases are multimeric enzymes that are activated by ATP and Mg^{2+} ions and stimulated by unfolded proteins such as α-casein. The peptidase activity of the Lon protease from *Mycobacterium smegmatis* (Ms-Lon) is dependent upon both its concentration and that of Mg^{2+}. Addition of α-casein partially substitutes for Mg^{2+} in activating the enzyme. In chemical dissociation experiments, higher concentrations of urea were required to inhibit Ms-Lon's catalytic activities after an addition of α-casein. Analytical ultracentrifugation was used to directly probe the effect of activators of peptidase activity on Ms-Lon self-association. Sedimentation velocity experiments reveal that Ms-Lon monomers are in a reversible equilibrium with oligomeric forms of the protein and that the self-association reaction is facilitated by Mg^{2+} ions but not by AMP-PNP or ATPγS. NaCl at 100 mM facilitates oligomerization and stimulates peptidase activity at suboptimal concentrations of $MgCl_2$. Sedimentation equilibrium analysis shows that Ms-Lon associates to a hexamer at 50 mM Tris and 10 mM $MgCl_2$, at pH 8.0 and 20 °C, and that the assembly reaction is Mg^{2+} dependent; the mole fraction of hexamer decreases with decreasing $MgCl_2$ to undetectable levels in 10 mM EDTA. The analysis of experiments conducted at a series of initial protein and $MgCl_2$ concentrations yields two assembly models: dimer ↔ tetramer ↔ hexamer and timer ↔ hexamer, equally consistent with the data. Limited trypsin digestion, CD, and tryptophan fluorescence suggest only minor changes in secondary and tertiary structure upon Mg^{2+}-linked oligomerization. These results show that activation of Ms-Lon peptidase activity requires oligomerization and that Ms-Lon self-association reaction is facilitated by its activator, Mg^{2+}, and stimulator, unfolded protein.

Members of the Lon family proteases are multimers of ~85 000 Da subunits whose native molecular masses by gel filtration are >450 000 Da (*1*, *2*). Cryoelectron microscopy suggests that the Lon protease homologue from *Saccharomyces cerevisae* (Sc-Lon) is a toroid containing seven subunits (*3*). ATP activates both the peptidase and protease activities of Lon proteases. The hydrolysis of ATP is required for degradation of large protein substrates, but only ATP binding is required for the hydrolysis of small peptides (*1*, *4*). Active site labeling experiments suggested that ATP activates peptidase activity, in part by increasing accessibility to the peptidase active site (*5*). ATP is essential for the oligomerization of several ATP-dependent proteases. HslVU, ClpXP, and ClpAP proteases all undergo ATP-dependent oligomerization (*6*−*9*). The ClpA regulatory ATPase, which forms part of ClpAP, itself oligomerizes in the presence of ATP (*8, 10*). It is not known whether ATP or Mg^{2+} regulate the oligomerization of Lon proteases. However, a role for Mg^{2+} in Lon's catalytic activities in addition to its binding to ATP is suggested by the following observations: (1) the concentration of Mg^{2+} required for maximal activity of the Lon protease from *Escherichia coli* (Ec-Lon) is much higher than the saturating concentration of ATP (*1*); (2) the protease activity of IBDVP2 VP4, a viral Lon homologue that lacks an ATPase domain is stimulated by Mg^{2+} (*11*). Finally, Sc-Lon has a less symmetric shape with leglike protrusions in the absence of ATP (*3*). The peptidase and ATPase activities of both Ec-Lon and Ms-Lon are stimulated by unfolded proteins, such as α-casein (*1*, *4*). In contrast, only the peptidase activity of Ms-Lon is stimulated by simple copolymers of amino acids (rcAAs). The existing data support a model of Ms-Lon containing two polypeptide-binding sites distinct from the peptidase active site (*5*). The model is based on the observations that (1) rcAAs stimulated Ms-Lon's peptidase activity without concomitant stimulation of its ATPase activity; (2) α-casein stimulates the peptidase activity of an Ms-Lon mutant lacking its N-terminal domain (N−E226) without concomitant stimulation of its ATPase activity; (3) additions of low concentrations of α-casein cause a further stimulation of the peptidase activity of Ms-Lon already maximally stimulated by rcAAs. In the present study, the model of Ms-Lon structure and function is extended to include the effects of Mg, ATP, and α-casein on protein oligomerization and Ms-Lon's catalytic activities.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Media, Reagents, and DNA Manipulations. Wild-type Ms-Lon and its S675A mutant (S675 is the catalytic serine) were expressed bearing N-terminal His_6 affinity domains in *Escherichia coli* strain SGR9 [MC4100

† The analytical ultracentrifugation studies were supported by NIH Grant GM39929. S.R. was supported by NIH Training Grant 5T32 GM08572.

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10.1021/bi0102508 CCC: $20.00
Published on Web 07/12/2001

cpsB10::lacZ(immλ)leu::Tn10 sulB lon146::Tn10*] pREP4 (a *kan*^{R}, *lacI*^{Q} containing vector; Qiagen). Overnight cultures of fresh transformants were diluted 1:100 into fresh LB medium [ampicillin (Sigma) at 100 μg/mL and kanamycin (Sigma) at 25 μg/mL] and grown at 37 °C until the A_{600} value for the culture reached ~1.0, followed by growth in the presence of 1.0 mM IPTG for an additional 3 h. Cells were disrupted by sonication in lysis buffer (50 mM Tris, pH 8.0, 1.0 mM imidazole). Nickel affinity chromatography involved binding cleared lysate from disrupted cells to Ni−NTA−agarose (Qiagen; 5 mL of Ni−NTA−agarose/L of original culture volume), followed by washing with 100−200 volumes of lysis buffer containing 15 mM imidazole and elution with 5 volumes of lysis buffer containing 100 mM imidazole. Affinity-purified His_6-Lon was concentrated using a Centriprep 30 concentrator (Amicon) and further purified on a Hi Load 26/60 Superdex 200 column (Pharmacia) equilibrated in lysis buffer. Additional rounds of gel filtration on a Hi Load 26/60 Superdex 200 column (Pharmacia) were employed to remove minor contaminants. This material was concentrated to 5−10 mg/mL as measured by the A_{280} of the sample (*12*) and analyzed for homogeneity using SDS−PAGE.

Peptidase and ATPase Assays. Peptidase assays contained 50 mM Tris (pH 8.0), either 0.5 or 4.0 mM ATP, 0.3 or 0.5 mM of Glt-Ala-Ala-Phe-4Mβ*NA*, and the indicated concentrations of Ms-Lon and $MgCl_2$ in a total volume of 200 μL. Reactions were incubated for 30 or 60 min at 20 or 37 °C and 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. In continuous peptidase assays, fluorescence increases were directly measured in discrete reactions at 20 °C. The amount of 4Mβ*NA* (4-methoxy-β-naphthylamide) released during peptidase assays was calibrated using the free compound (Sigma). In chemical dissociation experiments Ms-Lon was incubated for 20 h at 20 °C at the indicated concentrations of urea. Reactions were started by addition of ATP and peptide substrate. ATPase assays were performed as described previously (*2*, *13*).

Analytical Ultracentrifugation. Velocity and equilibrium analytical ultracentrifugation studies of Ms-Lon and S675A were performed at 20 °C using a Beckman XL-I analytical ultracentrifuge. Samples were dialyzed overnight against buffer containing 50 mM Tris (pH 8.0) and the indicated concentration of $MgCl_2$ or 10 mM EDTA. Sedimentation velocity experiments were conducted at 20 000 or 40 000 rpm in Al-Epon two-channel centerpieces using interference optics. The concentration of S675A or Ms-Lon was 10−15 μM unless otherwise indicated. The sedimentation boundaries are presented as the change in boundary concentration with time [d*c*/d*t*] vs *s** [$=\ln(r/r_m)/\omega^2 t$] using DCDT+ vl.13 (*14*), where *r* is the radial position, r_m is the radial position of the solution column meniscus, ω is the speed in radians per second, and *t* is time. The observed values were normalized to standard conditions ($s_{20,w}$ and $D_{20,w}$) by correcting for solvent density and viscosity. A value for the partial specific volume, $\bar{v}$, of 0.7361 $cm^3\ g^{-1}$ was calculated from the amino acid composition of the protein using SEDNTERP v1.04 (*15*).

Equilibrium analytical ultracentrifugation experiments were carried out in six-channel centerpieces and monitored by the protein's absorbance at 280 nm. Three initial concentrations of S675A ranging from 1 to 15 μM were centrifuged under each experimental condition analyzed. Absorbance scans were collected after 22 and 24 h at 4000, 6500, and 9000 rpm. Equilibrium was assumed to have been reached if the 22 and 24 h sets of scans were identical. The absorbance offset was determined by "overspeeding" the samples. The equilibrium data were analyzed using the Optima XL-A/XL-I analysis software (Beckman/Coulter) within Origin v4.1 (MicroCal).

Limited Trypsin Digestion of Ms-Lon. Five hundred microliters of the proteolytically inactive Ms-Lon mutant S675A (0.5 μM) was incubated with 0.8 μg of trypsin (Sigma) in 50 mM Tris (pH 8.0), with or without 10 mM $MgCl_2$. At selected time points, aliquots were withdrawn, and the digestion was stopped by the addition of 500 μL of 10% trichloroacetic acid. Aliquots were chilled on ice for 20 min, centrifuged at 14 000 rpm for 10 min, and subjected to 12% SDS−PAGE. The stable S675A fragments were visualized by staining the SDS−PAGE with silver nitrate.

RESULTS

Ms-Lon Peptidase Activity Is Dependent on Mg^{2+} and Protein Concentration. The dependence of Ms-Lon's peptidase activity on Ms-Lon concentration was measured at 1 and 10 mM $MgCl_2$. The slopes of the curves shown in panels A and B of Figure 1 reflect the relative rates of hydrolysis of the Ms-Lon substrate Glt-Ala-Ala-Phe-4Mβ*NA*. In the presence of 10 mM $MgCl_2$, the peptidase activity of Ms-Lon is independent of enzyme concentration from 1.0 to 0.1 μM (Figure 1A). However, further decreasing the Ms-Lon concentration below 0.1 μM resulted in a significant decrease in peptidase activity. In contrast, Ms-Lon peptidase activity depended strongly on Ms-Lon concentrations at 1 mM $MgCl_2$ over the 1.0−0.1 μM range of concentrations (Figure 1B). ATPase activity was also dependent on Ms-Lon concentration at 1 mM but not 10 mM $MgCl_2$ (data not shown). At an Ms-Lon concentration of 0.1 μM, the protein's peptidase activity displays a sigmoidal dependence on $MgCl_2$ concentration (Figure 1C). Together, these results suggest that oligomerization of Ms-Lon is required for its enzymatic activity and that Mg^{2+} mediates the self-association reaction. Therefore, a direct analysis of Ms-Lon self-association was undertaken.

Mg^{2+} Stimulates Self-Association of Ms-Lon. Analytical ultracentrifugation was used to directly determine whether Ms-Lon exists in reversible subunit equilibrium in solution. Initial sedimentation velocity experiments compared Ms-Lon and a proteolytically inactive S675A mutant in the presence and absence of $MgCl_2$. Analyses of these data revealed that Ms-Lon and S675A had essentially superimposable profiles in all tested conditions (data not shown). Thus, subsequent ultracentrifugation experiments employed the S675A mutant in order to avoid possible complications arising from self-proteolysis.

Sedimentation velocity experiments were conducted in order to establish a qualitative picture of Ms-Lon self-association. Figure 2 shows the results obtained upon sedimentation of S675A at concentrations of 13, 6, and 2

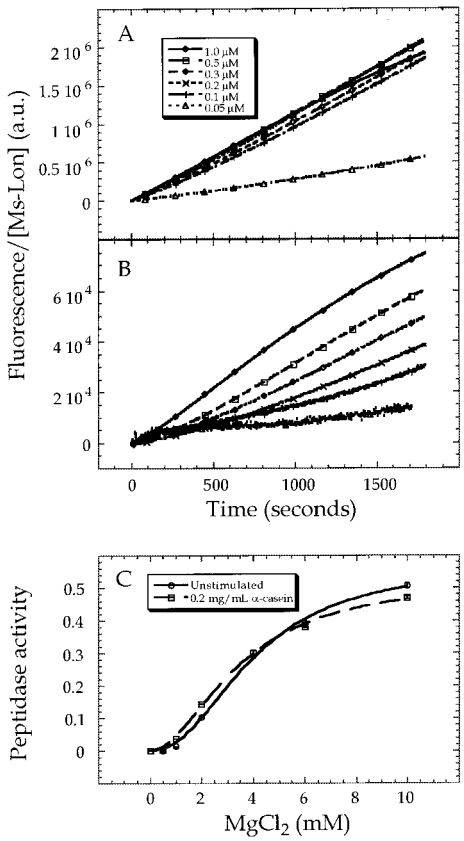

FIGURE 1: The Ms-Lon peptidase activity is dependent on Ms-Lon and Mg^{2+} concentration. The continuous time traces of Ms-Lon peptidase activity were obtained at the following concentrations of $MgCl_2$: (A) 10 mM $MgCl_2$; (B) 1 mM $MgCl_2$. Ms-Lon peptidase activity was measured in a continuous time assay, with Glt-Ala-Ala-Phe-4MβNA as peptide substrate at 20 °C, and the traces were divided by the concentration of Ms-Lon ranging from 0.05 to 1 μM. The fluorophore 4MβNA becomes fluorescent only upon cleavage from peptide. This allows us to measure the Ms-Lon activity in real time. The traces become nonlinear at higher concentrations of Ms-Lon (>2 μM), probably due to inhibition by ADP or substrate exhaustion. The repeats of these experiments produced similar results. (C) Shown are the 30 min peptidase assays containing 0.1 μM Ms-Lon, 0.5 mM Glt-Ala-Ala-Phe-4MβNA and incubated at 20 °C. Reactions contained 0.2 mg/mL α-casein, where indicated. The peptidase activity is in moles of peptide hydrolyzed per mole of Ms-Lon monomer per minute. The solid lines represent the fit of data into the Hill equation (*17*) with values of K_{eq} = 4.0 and 3.5 mM and n_H = 2.2 and 1.9 for samples containing no α-casein or 0.2 mg/mL α-casein, respectively.

μM in buffer containing 10 mM $MgCl_2$ (panel A) and 10 mM EDTA (panel B). The shift of the sedimentation boundaries to higher *s* values with increasing protein concentration is indicative of a reversibly associating system. Other hallmarks of an interacting system exhibited by Ms-Lon include the asymmetry of the boundary shapes and that the shapes change with protein concentration. The shift of the sedimentation boundaries to higher *s* values at 10 mM $MgCl_2$ and the appearance of additional high *s* peaks suggest that Mg^{2+} ion binding facilitates Ms-Lon self-association. Similar studies conducted at 25 and 50 mM $MgCl_2$ did not yield sedimentation profiles appreciably different from those obtained at 10 mM $MgCl_2$ (data not shown).

Sedimentation velocity experiments with samples containing 100 mM NaCl were analyzed in order to ascertain whether the effect of Mg^{2+} ions on Ms-Lon oligomerization was specific. The effect of 100 mM NaCl on Ms-Lon self-association was less dramatic than that of $MgCl_2$, but still measurable. The shift of the Ms-Lon's sedimentation boundaries to higher *s* values reached approximately one-third of that in the presence of 10 mM $MgCl_2$ (Figure 3A). While NaCl at 100 mM was unable to support degradation of Glt-Ala-Ala-Phe-4MβNA in the absence of $MgCl_2$, at low concentrations of $MgCl_2$, 100 mM NaCl stimulated the Ms-Lon's peptidase activity, consistent with its stimulation of oligomerization (Figure 3B).

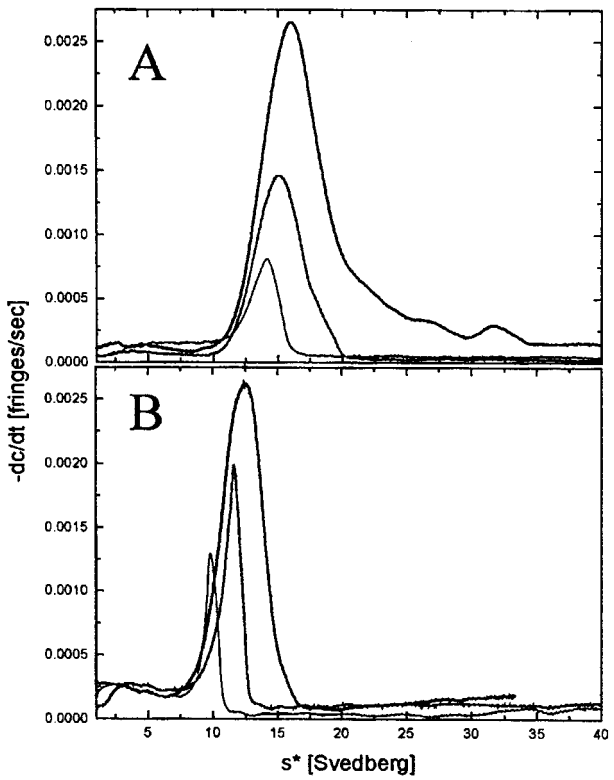

FIGURE 2: Sedimentation velocity profiles in the presence and absence of 10 mM Mg^{2+}. The sedimentation velocity profiles are shown for the samples containing (A) 13, 6, and 2 μM Ms-Lon, 10 mM $MgCl_2$, and 50 mM Tris, pH 8.0, and (B) 13, 6, and 2 μM Ms-Lon, 10 mM EDTA, and 50 mM Tris, pH 8.0. The *s* values corresponding to the maximum of the peaks were 15.8, 14.9, and 14.0 S for samples containing 13, 6, and 2 μM Ms-Lon and 10 mM $MgCl_2$, and 12.4, 11.7, and 9.7 S for samples containing 13, 6, and 2 μM Ms-Lon and 10 mM EDTA, respectively.

Although the results presented above clearly show that nucleotide binding is not required for Ms-Lon self-assembly, the binding of nucleotides has been shown to stimulate self-association of a number of proteases including ClpAP, ClpXP, and HslVU (*6*−*8*). Figure 4 shows that 0.5 mM ATPγS or 0.5 mM AMP-PNP in the presence of 10 mM EDTA has no effect of Ms-Lon self-association. Additional sedimentation velocity studies of Ms-Lon in buffer containing 0.5 mM ATPγS or 0.5 mM AMP-PNP at the conditions where Ms-Lon is partially dissociated (2 mM $MgCl_2$) and fully oligomerized (10 mM $MgCl_2$) were indistinguishable from sample containing no nucleotide. An alternative model, that ADP inhibits Ms-Lon activity by inhibiting its oligomerization, was also tested. However, no change in the sedimentation pattern was observed in the presence of saturating ADP (data not shown). Overall, these results reveal that Mg^{2+} stimulates association of Ms-Lon, while ATP analogues and ADP have little effect on it.

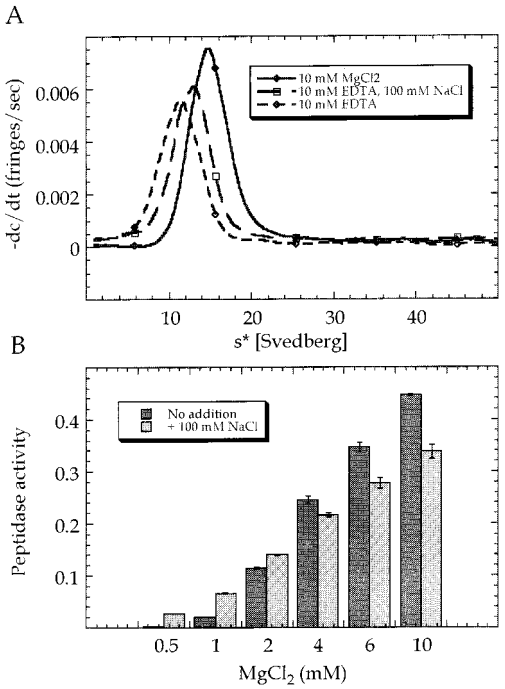

FIGURE 3: Sedimentation velocity profiles and peptidase activity in the presence and absence of 100 mM NaCl and 10 mM Mg^{2+}. (A) The sedimentation velocity profiles are shown for the samples containing ~15 μM Ms-Lon, 50 mM Tris, pH 8.0, and either 10 mM $MgCl_2$ or 100 mM NaCl and 10 mM EDTA or 10 mM EDTA. The *s* values corresponding to the maximum of the peaks were 14.9, 13.1, and 11.4 S, respectively. (B) Shown are the peptidase activities of samples containing 0.2 μM Ms-Lon, 50 mM Tris, pH 8.0, and the indicated concentrations of $MgCl_2$ with or without the addition of 100 mM NaC1.

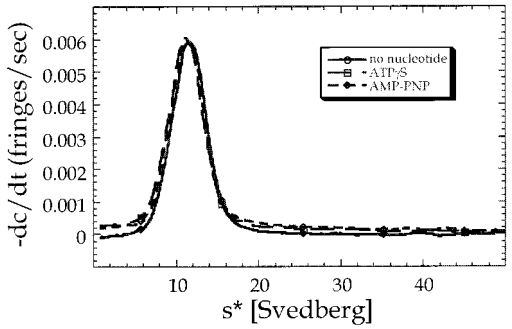

FIGURE 4: Sedimentation velocity profiles in the presence and absence of nucleotides. The sedimentation velocity profiles are shown for the samples containing 0.5 mM ATPγS or 0.5 mM AMP-PNP, 16 μM Ms-Lon, 10 mM EDTA, and 50 mM Tris, pH 8.0. The *s* values corresponding to the maximum of the peaks were 11.6, 11.2, and 11.6 S for samples containing 0.5 mM ATPγS, 0.5 mM AMP-PNP, and no nucleotide, respectively.

Sedimentation equilibrium was used to further explore the Mg^{2+}-linked oligomerization of Ms-Lon. The weight average molecular weight of Ms-Lon increases as a function of $MgCl_2$ concentration (Figure 5) with a dependence comparable to that of peptidase activity. Further increase of $MgCl_2$ concentration up to 50 mM did not detectably increase Ms-Lon self-association (data not shown). Analysis of the equilibrium concentration distributions obtained at each concentration of $MgCl_2$ against the model monomer ↔ *n*-mer with *n* ranging from 4 to 12 yielded the hexamer as the oligomer that overall best describes this ensemble of data (Figure 6). At 10 mM $MgCl_2$ the minimum of the variance shifts to an *n*-mer value greater than six (Figure 6). However, under this condition,

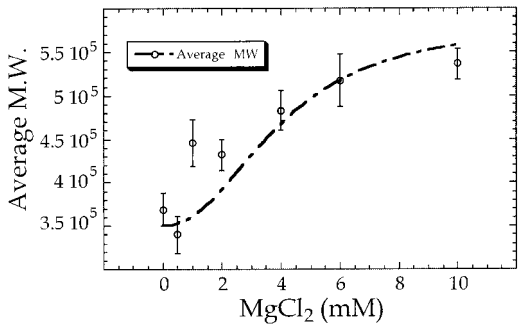

FIGURE 5: Dependence of average molecular weight on Mg^{2+} concentration. The average molecular weights were calculated from equilibrium analytical ultracentrifugation profiles of Ms-Lon at initial concentrations of 1.0, 5.5, and 13 μM for samples containing the indicated concentrations of $MgCl_2$ or 10 mM EDTA and 50 mM Tris, pH 8.0. The error bar represents the 95% confidence interval for the set of two scans. The dotted line depicts the $MgCl_2$ dependence of peptidase activity (Figure 1C).

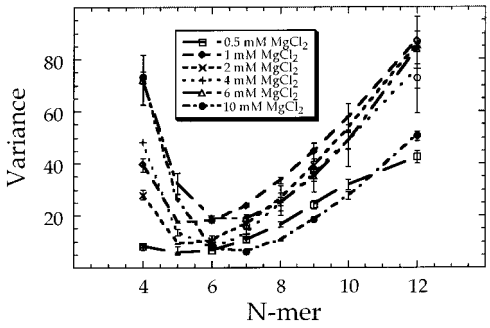

FIGURE 6: Variance for monomer ↔ *n*-mer models. The variance (parameter, which is proportional to the deviation of residual) was calculated for models containing monomer and *n*-mer for samples containing the indicated concentrations of $MgCl_2$. The minimum point for each curve indicates the optimal fitting model.

fast-moving boundaries that comprise 5−10% of the total signal were evident in the sedimentation velocity experiments (Figure 2A), suggesting that higher order Ms-Lon oligomerization is possible. These species could be accounted for in the equilibrium data by extending the fitted model to include dodecamer at this condition (data not shown). Further fitting of alternative oligomerization models indicates the minimal oligomer is larger than a monomer. Equivalently good fits were obtained to the dimer ↔ tetramer ↔ hexamer and trimer ↔ hexamer models (Figure 7) that were superior to the monomer ↔ hexamer and dimer ↔ hexamer models. A dissociation constant of ~4 μM (in Ms-Lon monomer units) was obtained for the trimer ↔ hexamer transition at 10 mM EDTA, decreasing to ~0.5 μM at 1 mM $MgCl_2$ and to undetectable at 10 mM $MgCl_2$, indicating that Ms-Lon is mostly hexamer at the later condition.

The Secondary Structure of Ms-Lon Does Not Change Significantly upon Mg^{2+}-Linked Association. The interpretation of the sedimentation velocity studies assumed that changes in the ultracentrifugation profiles were solely attributable to changes in the oligomerization state of Ms-Lon. However, this assumption would not be appropriate if there were significant Mg^{2+}-dependent rearrangements of the Ms-Lon monomer. To probe Ms-Lon structural change upon Mg^{2+}-linked oligomerization, limited proteolysis by trypsin was employed. A gel showing the trypsin digestion profile of Ms-Lon as a function of $MgCl_2$ concentration is depicted in Figure 8. Previously, we identified the most stable

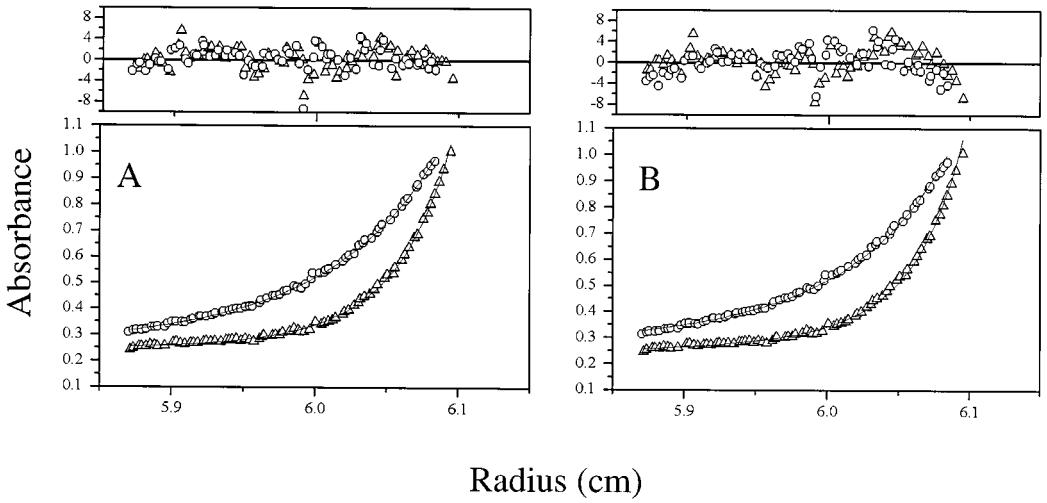

FIGURE 7: Sedimentation equilibrium profiles of Ms-Lon. The fits are shown as solid lines for the following equilibrium models: (A) dimer ↔ tetramer ↔ hexamer; (B) trimer ↔ hexamer. The sample contained Ms-Lon (loading concentration 13.5 μM) in the buffer containing 10 mM EDTA and 50 mM Tris, pH 8.0. The absorbance of the cell was measured at 280 nm at speeds of 6500 and 9300 rpm. The weighted fits for corresponding equilibrium models were calculated as described in Experimental Procedures.

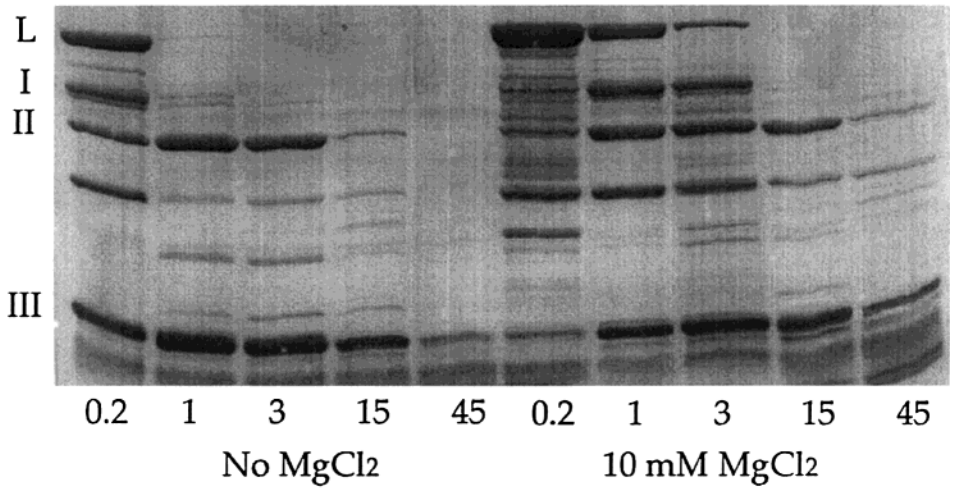

FIGURE 8: Limited trypsin digestion of Ms-Lon. The indicated fragments of Ms-Lon (L) were identified previously as containing the following sequences: I = T205-G-M-E-K-T; II = G335-M-A-V-V-G; III = M (within affinity tag)-R-G-H6. Proteins were fractionated by 12% SDS−PAGE and stained with silver nitrate. Numbers correspond to time of digestion in minutes.

fragments as consisting of highly conserved C-terminal domains and parts of ATPase domains (fragments I and II, corresponding to fragments containing the Ms-Lon sequence starting from residues 205 and 335, respectively) and the 25 kDa N-terminal domain (fragment III) (*2*). The number of stable fragments remained essentially the same upon addition of 10 mM $MgCl_2$; however, the half-life time of the Ms-Lon fragments increased. To control the effect of $MgCl_2$ on trypsin activity, we measured the cleavage of the fluorescent peptide Glt-Glt-Arg-AMC by trypsin in the presence and absence of 10 mM $MgCl_2$. We observed no inhibition of trypsin activity by $MgCl_2$, indicating that stabilization of trypsin digestion fragments is not due to modulation of trypsin activity by Mg^{2+} ions. These results indicate that Ms-Lon trypsin cleavage sites are protected at high $MgCl_2$ concentrations; however, the profile of stable fragments remains unchanged. To probe further the secondary structure changes of Ms-Lon, the CD spectra at 0, 0.5, 1, 2, 4, 6, and 10 mM $MgCl_2$ were determined (data not shown). The differences between CD spectra were minor; only a small 4−6% increase of α-helical structure was observed when the $MgCl_2$ concentration was varied from 0 to 10 mM and Ms-Lon was at 20 μM. Furthermore, tryptophan fluorescence of Ms-Lon showed only a minor change upon $MgCl_2$ addition. The tryptophan fluorescence of Ms-Lon (5 μM) decreased by less than 3−4% upon addition of 10 mM $MgCl_2$, and the emission maximum remained the same at 336 nm. We conclude that Mg^{2+}-linked association does not involve the significant change of the tertiary structure of the monomer.

Peptidase Activity Stimulation by α-Casein Is Mg^{2+} Concentration Dependent. Previous studies demonstrated that both the peptidase and ATPase activities of Lon proteases are stimulated by unfolded proteins, such as α-casein (*1*, *4*). We measured the stimulation of Ms-Lon's peptidase activity by α-casein at 0.5, 1, 2, 4, 6, and 10 mM $MgCl_2$ (Figure 9). The stimulation reached several hundred percent when the $MgCl_2$ concentration was below 1 mM (Figure 9). By contrast, the peptidase activity was stimulated only by few percent when $MgCl_2$ concentration was >4 mM. The stimulation was also temperature dependent and was practically nonexistent at room temperature. However, α-casein

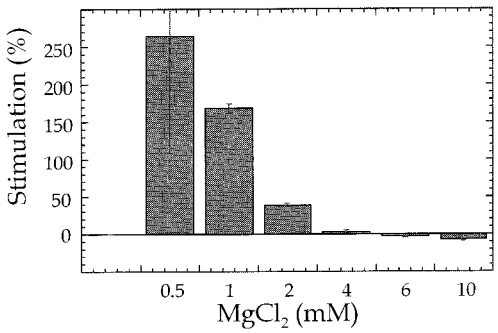

FIGURE 9: Stimulation of peptidase activity by α-casein. Shown are the peptidase assays containing 0.1 μM Ms-Lon, 0.5 mM Glt-Ala-Ala-Phe-4Mβ NA, 0.2 mg/mL of α-casein, and varying $MgCl_2$ concentrations. The reactions were incubated for 30 min at 20 °C. The stimulation of peptidase activity is in percent to the corresponding value of peptidase activity at each tested $MgCl_2$ concentration.

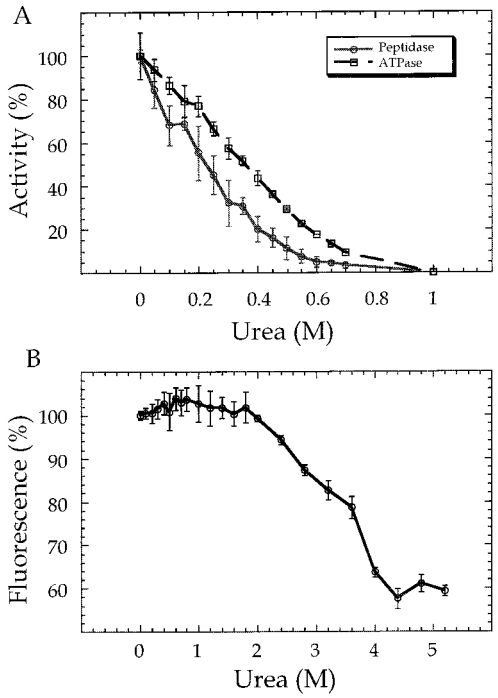

FIGURE 10: The peptidase and ATPase activities of Ms-Lon are inhibited by low concentrations of urea. (A) Peptidase and ATPase activities of Ms-Lon (0.2 μM). The reactions were incubated for 60 min at 37 °C with or without 0.2 mg/mL α-casein after denaturation with urea for 20 h. (B) Ms-Lon tryptophan fluorescence. The samples containing 0.2 μM Ms-Lon were incubated for 20 h in the presence of the indicated concentration of urea. The fluorescence spectra were recorded at an emission wavelength of 345 nm (excitation at 295 nm) in duplicate.

is stimulatory even at room temperature when Ms-Lon is incubated at low $MgCl_2$ concentration (Figure 9). Ms-Lon peptidase and ATPase activity is inhibited at concentrations of urea (0.5−0.8 M, Figure 10A) significantly lower than the 2−3 M urea required for the denaturation-induced decrease in Ms-Lon tryptophan fluorescence (Figure 10B) and shifting of the maximum of tryptophan fluorescence to longer wavelengths (data not shown). These results suggest that the observed inhibition of Ms-Lon's catalytic activities by urea is not due to protein denaturation. An alternative explanation suggested by the data presented above is that urea destabilizes the Ms-Lon oligomer and hence its peptidase activity. To test this hypothesis, sedimentation velocity

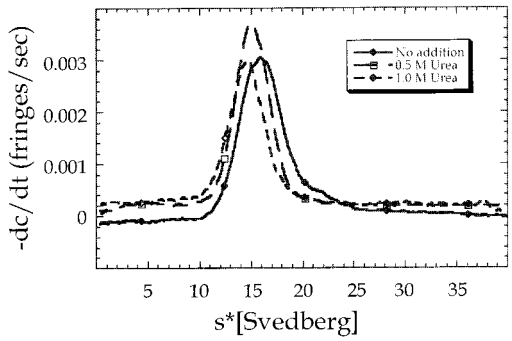

FIGURE 11: Sedimentation velocity profiles in the presence and absence of urea. Sedimentation velocity profiles of samples containing ∼17 μM Ms-Lon and the indicated concentration of urea in 10 mM $MgCl_2$ and 50 mM Tris, pH 8.0. The profiles were corrected for the viscosity and density of the solutions. The *s* values corresponding to the maximum of the peaks were 14.8, 14.9, and 15.9 S for the samples containing 1 M, 0.5 M, and no urea, respectively.

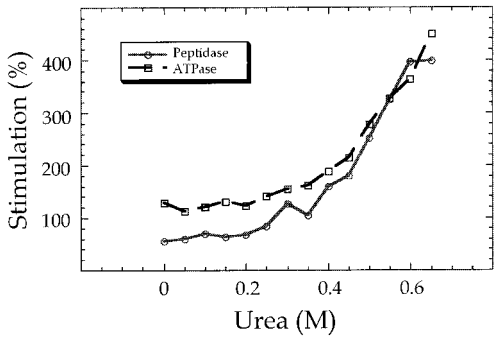

FIGURE 12: The peptidase and ATPase activities of Ms-Lon are stimulated by α-casein. Stimulation of peptidase and ATPase activities by α-casein. Ms-Lon (0.2 μM) was incubated for 60 min at 37 °C with or without 0.2 mg/mL α-casein after denaturation with urea for 20 h.

experiments were performed in the presence of up to 1 M urea. Although the concentration of Ms-Lon in the sedimentation velocity experiments is appreciably higher than that present in the peptidase assays, protein dissociation is observed as a function of urea. Values of *s* (peak) corrected for viscosity and density are 15.9, 14.9, and 14.8 S for 0, 0.5, and 1.0 M urea (Figure 11), respectively. The stimulation of the peptidase and ATPase activities of Ms-Lon was measured in the presence of 0.2 mg/mL α-casein (Figure 12). The peptidase activity of Ms-Lon at 37 °C and 10 mM $MgCl_2$ in the absence of urea is stimulated ∼50% by α-casein, increasing to several hundred percent at higher concentrations of urea (Figure 12). In addition, the concentration of urea required to half-maximally inhibit Ms-Lon peptidase activity in the presence of α-casein increased by ∼0.2 M. The ATPase activity of Ms-Lon in the absence of urea is stimulated 150% by α-casein, also increasing to several hundred percent in the presence of subdenaturing concentrations of urea (Figure 12). The stimulatory effect of α-casein on Ms-Lon activity as a function of urea is comparable to that described above for Mg^{2+} ions and is most plausibly linked to urea-induced dissociation of the catalytically active Ms-Lon oligomer. Initial attempts to demonstrate α-casein stimulation of Ms-Lon oligomerization by analytical ultracentrifugation were not successful due to self-association of α-casein.

DISCUSSION

All major ATP-dependent bacterial proteases are either homo- or heterooligomers. The results presented in this paper show that oligomerization is also required for Ms-Lon activity. By analogy with other proteases, we expected that the regulation of oligomerization might occur through an ATP-dependent mechanism. However, Ms-Lon oligomerization requires only ions, not nucleotide binding. At the low Mg^{2+} concentrations found in cellular milieu, the equilibrium favors low oligomeric forms of Ms-Lon. At high Mg^{2+}, Ms-Lon existed almost exclusively as higher order oligomers. While the present data cannot distinguish between the trimer ↔ hexamer and dimer ↔ tetramer ↔ hexamer assembly models, the data do suggest that the Ms-Lon monomers are minimally present, if at all, even in the absence of Mg^{2+}.

Ms-Lon peptidase activity was nonlinearly dependent on $MgCl_2$ concentration. In addition, peptidase activity was self-stimulated at low concentrations of $MgCl_2$, when Ms-Lon was in the dissociated form. These results are consistent with the linkage of peptidase activity to a higher order assembly reaction and suggest that Mg^{2+} modulates both Ms-Lon activity and oligomerization. Monovalent as well as divalent ions stimulate Ms-Lon oligomerization although not as effectively, a result paralleled by the stimulation of Ms-Lon oligomerization by NaCl at the conditions of partial dissociation. At l mM $MgCl_2$ the dissociation constant of ~0.5 μM describing the trimer ↔ hexamer assembly obtained by ultracentrifugation is comparable to the concentration of Ms-Lon, required for the half-maximal stimulation of peptidase activity by Ms-Lon (~0.3 μM; Figure 1B). Together, these results show that Ms-Lon is active only when it is hexameric and that oligomerization is not modulated by ATP binding. While the concentration of Mg^{2+} is regulated within the cell and hence relatively constant, the concentrations of other factors that affect Ms-Lon self-assembly, such as unfolded protein substrates (i.e., α-casein), are likely to vary more widely. Both peptidase and ATPase activities of Ms-Lon and Ec-Lon are stimulated by α-casein. Although the mechanism of such stimulation is partially understood on a molecular level (*5*), an intriguing additional effect is that α-casein promotes self-association of Ms-Lon. However, both the effect of α-casein on activities of chemically dissociated Ms-Lon and effect of α-casein on peptidase activity in Mg^{2+}-linked association assays are only consistent with α-casein promoting the association of Ms-Lon. At low Mg^{2+} or at high urea α-casein stimulated Ms-Lon peptidase and ATPase activities by several hundred percent, much more than it does at conditions where Ms-Lon is fully oligomeric. At biological concentrations of free Mg^{2+} [1−2 mM (*16*)] such stimulation would be important, because Ms-Lon is almost inactive at those conditions (Figure 1C). Hence, at the biological concentration of Mg^{2+}, Ms-Lon is postulated to be inactive until it binds an unfolded protein substrate, thus protecting normal cellular proteins from degradation. This effect would be in addition to the stimulation of peptidase activity through change in accessibility of catalytic nucleophile. We have previously demonstrated that unstructured polypeptides increase the rate of labeling of the cysteine substituted for the catalytic nucleophile by pyrene maleimide, indicating opening of the active site (*5*). Hence, activation of Ms-Lon by unfolded protein through increased oligomerization is not the only mechanism of activation.

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BI0102508