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Regulatory role of cardiolipin in the activity of an ATP-dependent protease, Lon, from *Escherichia coli*

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Lon is an ATP-dependent serine protease that plays a significant role in the quality control of proteins in cells, degrading misfolded proteins and certain short-lived regulatory proteins under stresses as such heat-shock and UV irradiation. It is known that some polymers containing phosphate groups regulate enzymatic activity by binding with Lon. We focused on the phospholipids of biological membrane components such as phosphatidylethanolamine, phosphatidylcholine, phosphatidylglycerol and cardiolipin (CL), and examined whether or not liposomes containing these phospholipids regulate the enzymatic activity of Lon. CL-containing liposomes specifically inhibited both the proteolytic and ATPase activities of Lon in a dose-dependent manner. In addition, on pull-down assay, we found that CL-containing liposomes selectively bound to Lon. The interaction between CL-containing liposomes and Lon changed with the order of addition of Mg^{2+}/ATP. When CL-containing liposomes were added after the addition of Mg^{2+}/ATP to Lon, the binding of CL-containing liposomes to Lon was significantly decreased as compared with the reversed order. In fact, we found that CL-containing liposomes bound to Lon, resulting in inhibition of the enzymatic activity of Lon. These results suggest that Lon interacts with CL in biological membranes, which may regulate the functions of Lon as a protein-degrading centre in accordance with environmental changes inside cells.

Keywords: AAA^+ protein/ATPase/liposome/phospholipid/proteolysis.

Abbreviations: CL, cardiolipin; MBP, maltose-binding protein; PC, 1, 2-dipalmitoyl-*sn*-glycero-3-phosphocholine; PE, 1, 2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; PG, 1, 2-Dipalmitoyl-*sn*-glycero-3-phosphoglycerol.

Lon is an adenosine 5′-triphosphate (ATP)-dependent serine protease that is located in the cytosol of prokaryotes, and in the mitochondria and peroxisomes of eukaryotes (*1*–*7*). Lon plays an important role in the quality control of proteins by degrading abnormal and misfolded proteins (*2*, *8*, *9*). In fact, Lon selectively degrades short-lived regulatory proteins such as bacteriophage λN protein, the SulA cell division regulator and RcsA, which is a positive regulator of capsule synthesis (*6*, *10*–*13*). Lon is a homo-oligomer of six identified subunits, each of which contains an amino-terminal (N) domain involved in substrate binding, an ATPase (A) domain, a Substrate Sensor and Discriminatory (SSD) domain, which may also be involved in substrate binding, and a carboxyl-terminal (P) domain containing the Ser-Lys dyad at the proteolytic active site (*4*, *14*–*20*). These subunits form a ring-shaped structure with a central cavity, in which the active sites for both ATPase and proteolysis reside, depending on Mg^{2+}, but not on ATP (*21*). It is speculated that the energy of ATP hydrolysis helps the entry of a protein substrate into the central cavity, and then the translocation of the substrate to the active site of a protease, where degradation of the substrates occurs.

Escherichia coli Lon has been identified as a DNA binding protein, and binds to double-stranded DNA (dsDNA) with relatively higher affinity than in the case of single-stranded DNA (ssDNA) (*22*, *23*). Several *in vitro* studies have shown that Lon interacts non-specifically with large DNA molecules such as DNA plasmids and a calf thymus DNA, leading to stimulation of both the ATPase and protease activities of Lon (*22*, *24*, *25*). Moreover, Lon also interacts with inorganic polyphosphate (polyP) under amino acid starvation in *E. coli*, and this interaction facilitates Lon-mediated proteolysis of free ribosomal proteins (*26*, *27*). Both *in vivo* and *in vitro* studies have revealed that polyP competitively inhibits the DNA binding ability of Lon. Furthermore, the polyP–Lon complex is released from DNA, which initiates degradation of free ribosomal proteins, and thus results in down-regulation of protein translation (*26*, *27*). These findings have led us to the idea that a polymer including phosphate groups has the ability to modulate the function of Lon. In this study, we focused phospholipids as phosphate-rich molecules in the inner membrane, and investigated the interaction between Lon and liposomes. We found that cardiolipin (CL) selectively binds to Lon, and then inhibits both the ATPase and protease activities of Lon. Finally, it was suggested that Lon is involved in the regulation of protein degradation by interacting with CL in response to various environmental stimuli.

Experimental Procedures

Materials

1, 2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (PE), 1, 2-dipalmitoyl-*sn*-glycero-3-phosphocholine (PC), and CL were obtained from Sigma (St Louis, MO, USA). 1, 2-Dipalmitoyl-*sn*-glycero-3-phosphoglycerol (PG) was obtained from NOF Corporation (Tokyo, Japan). 1, 2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(biotinyl) was purchased from Avanti Polar Lipids (AL, USA).

Purification of Lon

Lon was purified using a maltose binding protein (MBP) fusion system as described previously (*28*). In brief, *E. coli* JM109 strains carrying the pMAL-c2-Lon vector were cultivated in LB medium containing 50 μg/ml ampicillin at 37°C overnight and then diluted with fresh LB (Luria-Bertani) medium to $OD_{550} = 0.1$, followed by incubation at 37°C until $OD_{550} = 0.4$. The production of the MBP-Lon fusion protein was induced by the addition of IPTG (final concentration, 0.3 mM), and the cultivation was continued for a further 3 h. The cells were harvested by centrifugation (2,930 *g* for 20 min) at 4°C, and then suspended in PBS. After centrifugation as described above, the resultant precipitates were resuspended in buffer A comprising of 0.0025% (w/v) lysozyme, 1 mM EDTA and 20% (w/v) sucrose in 20 mM Tris–HCl, pH 8.0. The cells were disrupted with glass beads (Sigma) in a Cell Beater (BIO 101, Fast Prep™ FP120; Thermo Savant, Holbrook, NY, USA), and the crude extracts were centrifuged at 21,130 *g* for 5 min at 4°C. The supernatants were dialysed against buffer B comprising 200 mM NaCl and 20 mM Tris–HCl, pH 8.0, then loaded onto a column of amylose resin (New England Biolabs, Beverly, MA, USA) equilibrated with buffer C comprising 200 mM NaCl, 20% (v/v) glycerol and 20 mM Tris–HCl, pH 8.0. The column was washed with 10-bed volumes of buffer C. The MBP-Lon fusion protein was eluted with 10 mM maltose in buffer C, and then the fractions containing the fusion protein were concentrated with an Amicon Ultra-4 according to the manufacturer's instructions (MILLIPORE, MA, USA). Protein concentrations were determined by the Bradford method using bovine serum albumin as the standard. To separate Lon from MBP, the MBP-Lon fusion protein (400 μg) was incubated with 20 μg of Factor Xa protease (New England Biolabs) at 4°C for 14 h, and then the Lon protein was further purified with a Sephacryl S-200 (GE Healthcare, Buckinghamshire, England) column equilibrated with buffer C. The purified Lon protein was stored at −80°C until use.

Preparation of liposomes

Liposomes with the following compositions were prepared from PE, PC, PG and CL: Liposomes I (Lip I) (PE : PG : CL = 6 : 3 : 1), Liposomes II (Lip II) (PE : PG = 6 : 3) and Liposomes III (Lip III) (PE : PC = 6 : 4). PE and PC in chloroform–methanol (2 : 1, v/v), and PG and CL in chloroform were mixed in the indicated molar ratios, and then the solvent was evaporated off. The resultant mixture was suspended in buffer C, vortexed at 70°C, and then sonicated for 30 s to homogeneity.

Degradation of casein by Lon in the presence of liposomes

Proteolytic activity was measured using α-casein (Sigma) as a substrate (*29*). Under the standard assay conditions, liposomes were pre-incubated with 0.3 μM Lon in 50 mM Tris–HCl, pH 8.0, on ice for 30 min. Then, 25 mM $MgCl_2$ and 1 mM ATP were added successively to the reaction mixtures every 30 min on ice. Finally, 5 μM casein was added, followed by incubation at 37°C for 120 min. For the time-course experiments, 10 μl aliquots of the reaction mixture were collected at intervals during the incubation, which were mixed with 5 μl of 3× SDS sample buffer comprising 0.15% (w/v) bromphenol blue, 25% (v/v) glycerol, 6% (v/v) mercaptoethanol and 3% (w/v) SDS in 150 mM Tris–HCl, pH 7.5. The samples were boiled for 5 min, and then subjected to electrophoresis on 5–20% (v/v) polyacrylamide SDS–slab gels (e-PAGEL; ATTO, Tokyo, Japan). The gels were stained with 0.025% (w/v) Coomassie brilliant blue R-250 in 10% (v/v) acetic acid and 20% (v/v) methanol solution, and then the densities of the protein bands of casein were determined with Multi-Gauge software (Fujifilm, Tokyo, Japan). The results are shown relative to the initial density of the casein band.

ATPase activity of Lon protease in the presence of liposomes

ATPase activity is measured using unlabelled ATP with [γ-^{32}P]ATP (Perkin Elmer, MA, USA) as a substrate (*30*, *31*). Various concentrations of liposomes were mixed with 0.3 μM Lon in 50 mM Tris–HCl, pH 8.0, followed by pre-incubation on ice for 30 min. Then 7.5 mM $MgCl_2$ was added to the reaction mixture, followed by incubation on ice for 30 min. Finally, 1 mM unlabelled ATP with [γ-^{32}P]ATP was added to the reaction mixture, followed by incubation at 37°C for 30 min. The reaction was stopped by the addition of ice-cold PBS(−) containing a mixture of 6% (w/v) Norit A (Nacalai Tesque, Kyoto, Japan) and 0.3% (w/v) Dextran T-500 (GE Healthcare), and the reaction mixture was left on ice for 30 min, and then centrifuged at 11,000 *g* for 15 min. The radioactivity of [^{32}P]orthophosphate in the resultant supernatant was measured by subjecting the mixture to counting with an ACS-II (GE Healthcare) using a Liquid Scintillation Analyzer, Tri-Carb 1600 TR (Packard, USA).

Binding of liposomes to Lon

MagnaBind™ Streptavidin Beads (Thermo Fisher Scientific, Rockford, IL, USA) were washed with buffer D comprising 150 mM NaCl, 0.1 mM EDTA and 0.1% (v/v) Triton X-100 in 10 mM Tris–HCl, pH 7.5 and buffer E comprising 1.15 M NaCl, 0.1 mM EDTA and 0.1% (v/v) Triton X-100 in 10 mM Tris–HCl, pH 7.5, followed by equilibration with buffer B using a Dynal MPC-M (Dynal, Oslo, Norway). For this assay, biotinylated PE was added at 0.5% (w/v) to prepare liposomes, and 0.2 mM of each lot of liposomes was pre-incubated with 0.3 μM Lon in 50 mM Tris–HCl, pH 8.0, on ice for 30 min. Then 25 mM $MgCl_2$ and 1 mM ATP were successively added to the reaction mixtures on ice every 30 min. Finally, the reaction mixtures were incubated with washed magnet-beads, where Lon-biotinylated PE-containing liposome complexes bound to streptavidin-beads by incubation at 4°C for 30 min. The tertiary complexes with the beads were washed three times with buffer B at 4°C for 10 min using the Dymal MPC-M, and then 1× SDS sample buffer was added to the final precipitate. Samples were boiled for 5 min, and the beads were separated by using the Dynal MPC-M. The resultant supernatants were subjected to SDS–PAGE, followed by western blotting, and the recovery of Lon was detected using an anti-Lon polyclonal antibody. The intensities of the protein bands of Lon were analysed using a LAS-1000 imaging analyzer (Fujifilm) and quantitated with Multi-Gauge software as described above.

Results

Effects of liposomes on Lon-mediated degradation of casein

We investigated the effect of liposomes on the proteolytic activity of Lon as to degradation of casein. ATP-dependent degradation of casein by Lon was completely inhibited at 30 min after the addition of 0.2 mM Lip I (PE : PG : CL = 6 : 3 : 1), and the inhibition continued to 90 min, but was relieved slightly at 120 min [Fig. 1A(c) and B]. On the other hand, Lip II (PE : PG = 2 : 1) and Lip III (PE : PC = 3 : 2) did not show any effect [Fig. 1A(d, e) and B]. Although CL is composed of the dimeric structure of PG, PG itself did not seem to suppress the activity of Lon. The time-course of casein degradation showed that the inhibitory ability of Lip I was evident at the beginning of the reaction [Fig. 1A(c) and B]. These results suggest that CL-containing liposomes selectively inhibit the proteolytic activity of Lon.

Dose–response analysis with Lip I showed that the inhibition of the protease activity of Lon was dependent on the concentration of Lip I (Fig. 2A and B). The addition of 0.05–0.2 mM Lip I suppressed casein degradation in a concentration-dependent manner at a

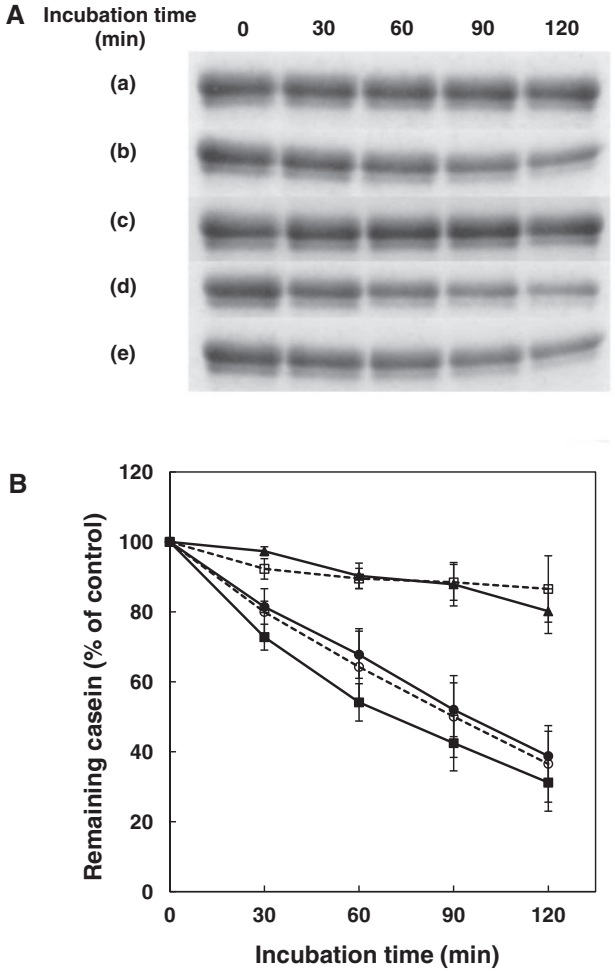

Fig. 1 Effects of liposomes on the degradation of casein by Lon. (A) Time-dependent degradation of casein. The reaction mixture without (a and b) or with 0.2 mM Lip I(c), II(d) or III(e) was incubated with 0.3 μM Lon, 25 mM $MgCl_2$ and 1 mM ATP in 50 mM Tris–HCl, pH 8.0 and then 10 μl aliquots were taken at 0, 30, 60, 90 and 120 min after the addition of α-casein (5 μM) during incubation at 37°C, followed by SDS–PAGE analysis and staining of the gel with Coomassie brilliant blue. In (a), no liposomes or ATP but Mg^{2+} and Lon were added as a negative control, and in (b), no liposomes but Mg^{2+}/ATP and Lon were added as a positive control. (B) Quantitation of casein degradation. The amounts of casein were calculated from the stained gels, corresponding to in A as (a) (open square), (b) (open circle), (c) (filled triangle), (d) (filled square) and (e) (filled circle), by scanning the bands with an image scanner, CanoScan 9900 F (Canon, Tokyo, Japan). The intensities of the bands were quantitatively analysed with Multi-Gauge software (Fujifilm). The means ± SD for four independent experiments are shown.

nearly constant rate throughout the reaction for 120 min. While 0.3 mM Lip I completely inhibited it by 90 min, this inhibition was partially relieved at 120 min, which allowed the degradation of casein slightly. On incubation for a long time and in the presence of highly concentrated Lip I, casein degradation slightly proceeded, because CL-containing liposomes may unfavourably interact with Lon, and/or Lon is unstable. These results suggest that the inhibition of the protease activity of Lon by Lip I is due to the substantial direct interaction of Lip I with Lon.

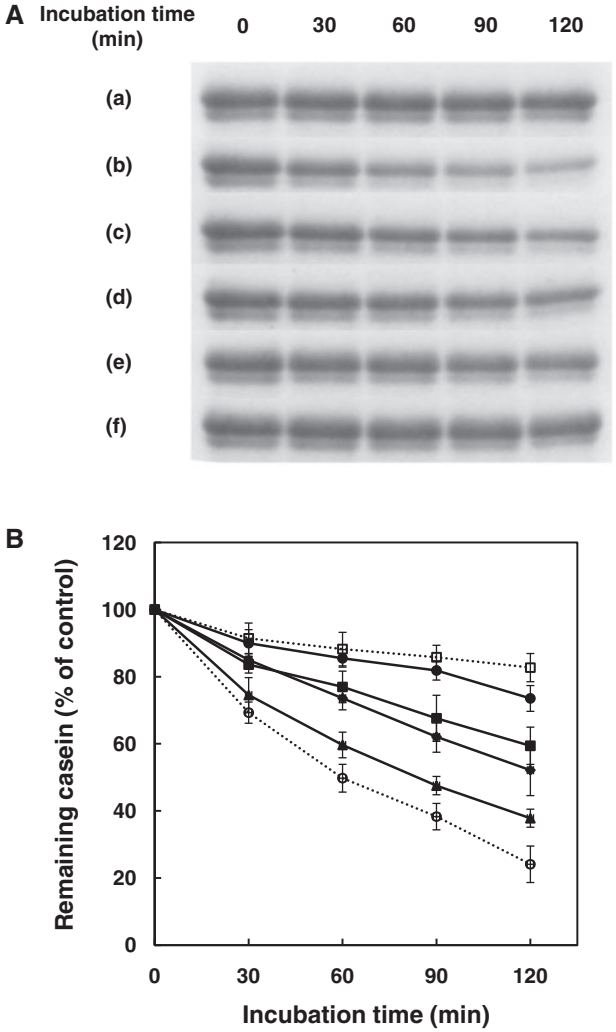

Fig. 2 Dose-dependent effect of Lip I on the degradation of casein by Lon. (A) Time-dependent degradation of casein. The assay was performed as shown in the legend to Fig. 1 (A) except for the amounts of Lip I [0.05 mM (c), 0.1 mM (d), 0.2 mM (e) and 0.3 mM (f)]. (B) Quantitation of casein degradation. The amounts of casein were calculated and analysed quantitatively at 0.05 mM (filled triangle), 0.1 mM (filled diamond), 0.2 mM (filled square) and 0.3 mM (filled circle) Lip I. The results are shown as the means ± SD for four independent experiments.

Inhibition of ATPase activity of Lon by CL-containing liposomes

We next studied the effect of CL-containing liposomes on the ATPase activity of Lon by using unlabelled ATP with [γ-^{32}P]ATP as a substrate. On incubation for 30 min, Lip I completely inhibited the Lon-mediated hydrolysis of ATP, although Lip II or Lip III did not affect it (Fig. 3). These results show that CL in liposomes is important for selective inhibition of the ATPase activity of Lon.

We then examined the dose-dependent effect of Lip I on the ATPase activity of Lon. As shown in Fig. 4, Lip I decreased the ATPase activity of Lon in a concentration-dependent manner, and 0.1 mM Lip I inhibited >90% of the activity of Lon. It is remarkable that the dose of Lip I necessary for the complete inhibition of Lon was 0.3 mM for the proteolytic activity

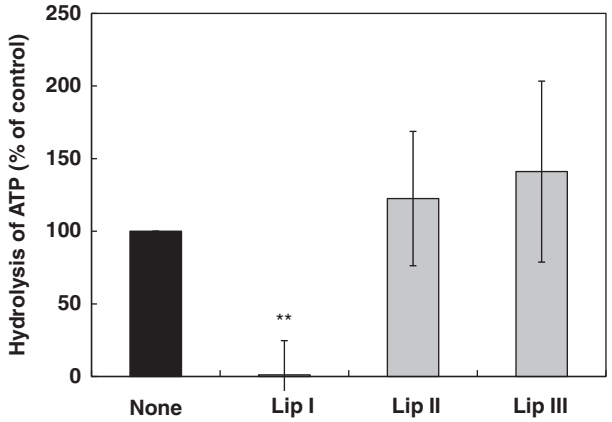

Fig. 3 Effects of liposomes on the ATPase activity of Lon. The reaction mixture, comprising 0.3 μM Lon and 1 mM unlabelled ATP with [γ-^{32}P]ATP in the absence or presence of 0.2 mM Lip I, II or III, was incubated at 37°C for 30 min in 7.5 mM $MgCl_2$, 50 mM Tris–HCl, pH 8.0 and then the radioactivity of [^{32}P]orthophosphate released from [γ-^{32}P]ATP was measured as described under Experimental. The means ± SD for eight independent experiments are shown. The significance of differences was determined by Student's *t*-test, and shown as $^{**}P < 0.001$ versus the control (None).

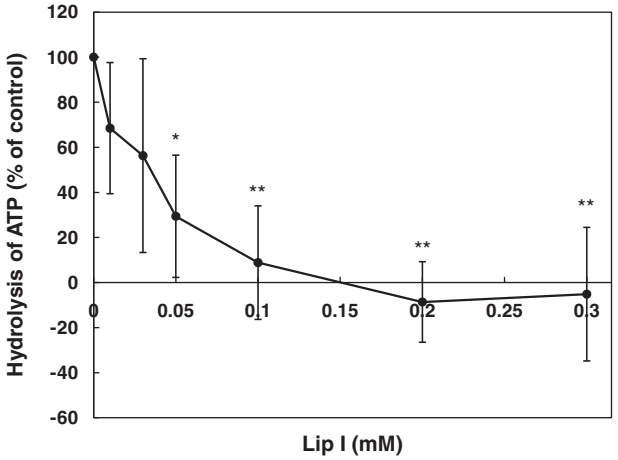

Fig. 4 Dose-dependent effect of Lip I on the ATPase activity of Lon. The reaction mixture, comprising 0.3 μM Lon and 1 mM unlabelled ATP with [γ-^{32}P]ATP in the absence or presence of 0.01–0.3 mM Lip I, was incubated at 37°C for 30 min, and the released radioactivity of [^{32}P]orthophosphate was measured as described under 'Experimental Procedure' section and in the legend to Fig. 3. The results are shown as the means ± SD for four independent experiments with the significance of differences versus the control (None), determined by Student's *t*-test, as $^{*}P < 0.01$ and $^{**}P < 0.001$.

(Fig. 2A and B), but that for the ATPase was 0.1 mM (Fig. 4), suggesting the occurrence of a more susceptible interaction of CL with the ATPase domain than with the protease domain in the Lon protein molecule.

Interaction between CL-containing liposomes and Lon

To examine the interaction between CL-containing liposomes and Lon, the binding of Lon to liposomes was investigated. In this assay, Lips I, II and III were prepared with the slight modification that PE comprised 0.5% (v/v) biotinylated PE, which enabled capture by the streptavidin-coated magnet-beads, leading to separation of unbound from liposome-bound Lon. As shown in Fig. 5A and B, Lon bound specifically to Lip I 4-fold in comparison with none (control), but not to Lip II or III. Additionally, it shows a broad band when liposomes bind to Lon, because the binding of Lon to liposomes may be strong and stable. These results suggest that CL in Lip I exhibits high affinity to Lon, and inhibits both the protease and ATPase activities of Lon.

Factors influencing the inhibitory activity of CL toward Lon

It is known that proteolysis of Lon proceeds in multi-steps, being regulated by such factors as oligomerization, which is dependent on Mg^{2+}, and conformational changes of the catalytic domain, whose proteolytic activity requires both ATP binding and its hydrolysis for maximal efficiency *(21, 32–34)*. To elucidate the mechanisms underlying CL-induced inhibition of Lon, the order of addition of CL-containing liposomes (Lip I) to the reaction mixture was investigated as to whether it is influenced by Mg^{2+} and ATP or not. When Lip I was added in various orders at the same concentration, the extent of the inhibition of casein degradation differed significantly (Fig. 6A–D). When Lip I was added first to the reaction mixture containing only Lon, and Mg^{2+} and ATP were added later, the extent of the inhibition was higher by 20% than when Mg^{2+} and ATP were added to Lon before Lip I addition (Fig. 6B–D). Moreover, the extent when Mg^{2+} was added prior to Lon was almost to the same when ATP was added first (Fig. 6B and C). When the order of addition of Mg^{2+} and ATP was changed before/after Lip I was added to Lon, the extent of the inhibition did not change significantly from the results in Fig. 6B–D. In fact, the inhibitory effect differed between the addition of Mg^{2+}/ATP prior to liposomes and liposomes prior to Mg^{2+}/ATP. These results suggest that Mg^{2+}/ATP is involved in the inhibition of the proteolytic activity by CL-containing liposomes.

To ascertain the importance of the order of Lip I addition to Lon, we studied the interaction between Lip I and Lon using a binding assay with biotinylated PE-containing Lip I and streptavidin-beads, as shown in Fig. 7A and B. When Lip I was added after Mg^{2+} and ATP had been added to Lon, the binding of Lip I to Lon decreased significantly (Fig. 7A), being almost 50% of the control level (Fig. 7B). In fact, Lon interacts with liposomes prior to Mg^{2+}/ATP for maximal binding, as well as the proteolytic activity of Lon. These results suggest that inhibition of Lon by Lip I requires the prior addition of Lip I to Lon before the addition of Mg^{2+}/ATP.

Discussion

Lon has been reported to interact with polymers including phosphate groups such as DNA and

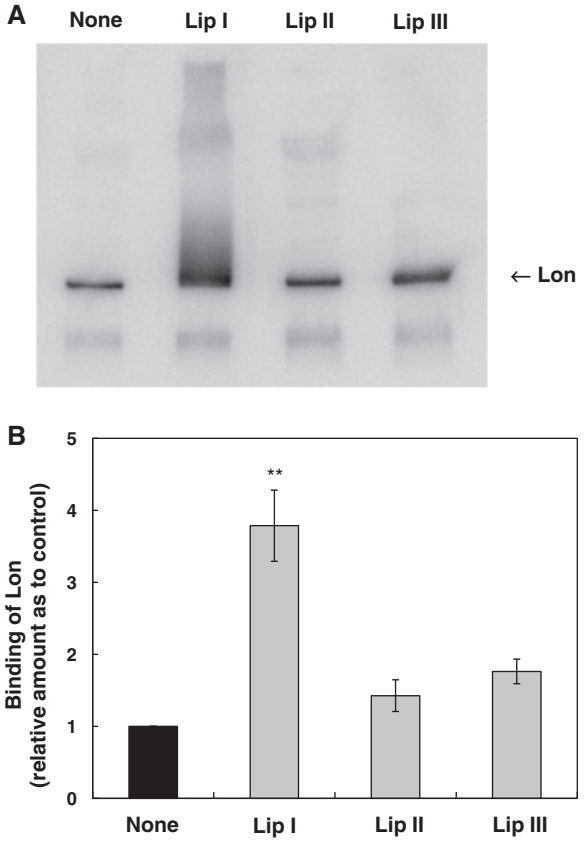

Fig. 5 Binding of Lon to liposomes. (A) Profiles of binding of Lon to Lip I, II and III. Lip I, II or III, containing biotin-PE, was preincubated at 4°C for 30 min with 0.3 μM Lon in 50 mM Tris–HCl, pH 8.0. Then 25 mM $MgCl_2$ and then 1 mM ATP were added sequentially to the reaction mixture on ice at 30 min intervals, followed by pre-incubation at 4°C for 30 min. Finally, streptavidin-beads were added to each reaction mixture, followed by incubation at 4°C for 30 min and washing three times, and then the binding of liposomes to Lon was determined by SDS–PAGE, followed by western blotting analysis. (B) Quantitation of the binding of Lon to liposomes. The amount of Lon bound to Lip I, II or III was determined quantitatively by image analysis with Multi-gauge, as described under 'Experimental Procedure' section. The means ± SD for four independent experiments are shown with the significance of differences, determined by Student's *t*-test, as $^{**}P<0.01$ versus none.

inorganic polyP, leading to significant effects on its enzymatic activity (*22*, *24–27*). Additionally, our studies have shown that lipopolysaccharides, a major component of the outer membrane of *E. coli*, inhibit the enzymatic activity of Lon (Sugiyama, N. and Amano, F., unpublished data). In this study, we investigated whether or not Lon interacts with phospholipids, which are major structural components of biological membranes, by using liposomes with different organizations. The addition of CL-containing liposomes (Lip I) selectively inhibited both the protease and ATPase activities of Lon. On the contrary, the other liposomes devoid of CL did not cause any inhibition (Figs 1 and 3). Besides, this inhibitory effect of CL-containing liposomes was dependent on the dose of the liposomes (Figs 2 and 4), although the minimal concentration of Lip I necessary for complete inhibition of the ATPase activity of Lon was 0.1 mM (Fig. 4), whereas that of the protease activity was about 0.3 mM (Fig. 2), suggesting that the ATPase activity is more sensitive to CL-containing liposomes than the protease activity is. The protease activity of Lon is known to be expressed maximally with binding and hydrolysis of ATP (*32–34*), although ATP hydrolysis is not strictly required for degradation of casein as demonstrated by Goldberg and Waxman (*5*). Therefore it seems feasible that CL-containing liposomes inhibit the ATPase activity first, followed by inhibition of the protease activity of Lon as a result, or that CL-containing liposomes may inhibit the ATPase activity primarily, whereas the protease activity requires ATP binding but may loosely depend on ATP hydrolysis.

The mode of regulation by CL-containing liposomes of the activities of Lon was also supported by the results of an experiment in which the timing of addition of CL-containing liposomes to Lon was changed before or after that of Mg^{2+} and/or ATP (Fig. 6). The highest inhibitory effect of CL-containing liposomes was achieved with prior addition of CL-containing liposomes to Lon (Fig. 6D), and the extent of the inhibition became significantly lower if CL-containing liposomes were added to Lon after the addition of Mg^{2+} and then ATP (Fig. 6B and C). Similar low inhibition was obtained when ATP and then Mg^{2+} were added before CL-containing liposome addition (Fig. 6B and C). These results suggest that the possible site(s) of Lon which CL interacts with might be masked or diminished by Mg^{2+} and/or ATP. In order to explore this possibility, the binding of Lon to CL-containing liposomes was examined. As shown in Fig. 5, Lon bound selectively to CL-containing liposomes (Lip I), and thus this assay method enabled us to examine the mode of interaction between CL-containing liposomes and Lon. When Lon was added to CL-containing liposomes before Mg^{2+}/ATP were added to the reaction mixture, the amount of Lon bound to CL-containing liposomes was significantly higher than otherwise (Fig. 7B). These results seem to support the idea that the interaction of CL with Lon is influenced by Mg^{2+}/ATP, probably through conformational changes of Lon caused by Mg^{2+} (*17*).

It should also be noted that the protease activity of Lon was not completely inhibited when CL-containing liposomes were added after Mg^{2+}/ATP addition to Lon, the extent of inhibition remaining being ≤20% of the original activity (Fig. 6B–D). Further experiments seem to be necessary to determine whether such feasible conformational changes of Lon induced by the addition of Mg^{2+}/ATP prohibit Lon from binding to CL, leading to decreased proteolytic activity of this enzyme. For the details of the mechanisms underlying the conformational changes of Lon, the oligomerization of Lon seems to be a key to understand how CL-containing liposomes inhibit ATPase and proteolytic activities of Lon. As demonstrated by Park *et al.* (*21*), the dimer to hexamer conversion is induced by Mg^{2+} but not by ATP. Defective oligomerization would inhibit both the ATPase and protease activity of Lon. Therefore the most plausible and simplest possibility deduced from the facts that the inhibition of Lon by CL-containing liposomes was decreased by the

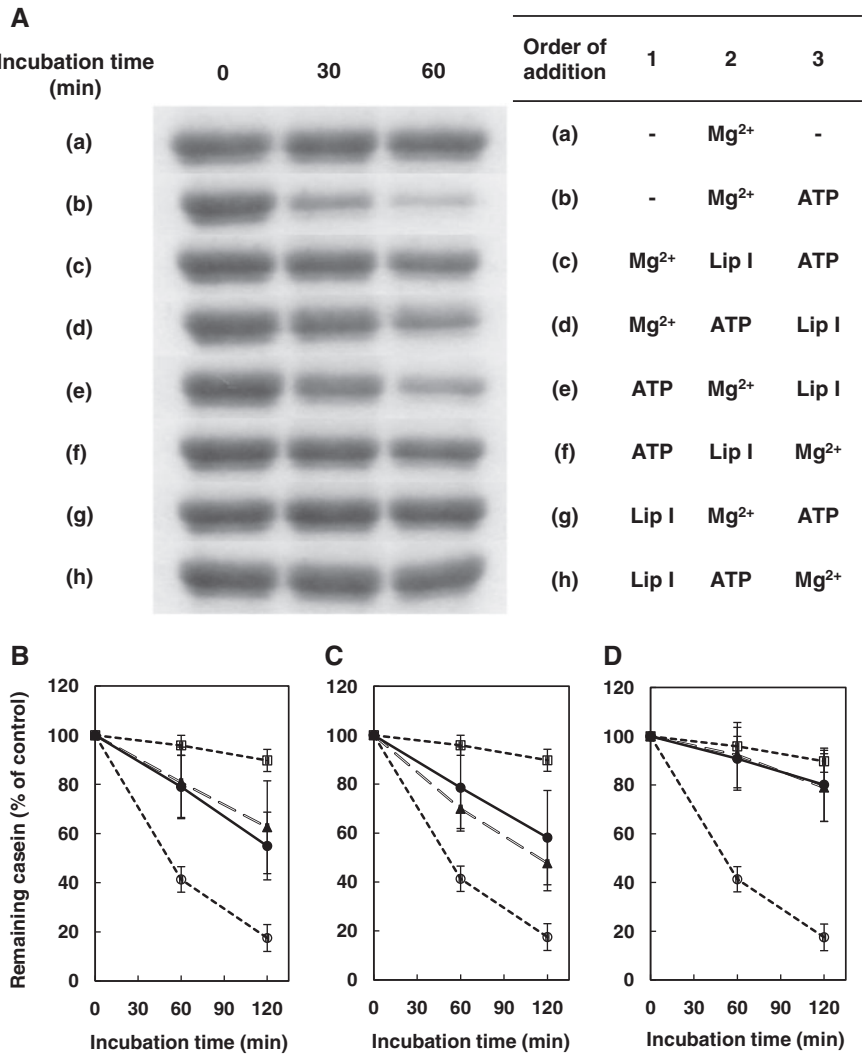

Fig. 6 Effect of the order of Lip I addition to the reaction mixture on the protease activity of Lon. (A) ATP-dependent degradation of casein. Either 25 mM Mg^{2+} (c and d), 1 mM ATP (e and f) or 0.2 mM Lip I (g and h) was added first to each reaction mixture containing 0.3 μM Lon in 50 mM Tris–HCl, pH 8.0, followed by mixing and storage on ice for 30 min. Next, either 25 mM Mg^{2+} (e and g), 1 mM ATP (d and h) or 0.2 mM Lip I (c and f) was added to the corresponding reaction mixture, followed by mixing and pre-incubation at 4°C for 30 min. Then, either 25 mM Mg^{2+} (f and h), 1 mM ATP (c and g) or 0.2 mM Lip I (d and e) was added to the corresponding reaction mixture, followed by mixing and pre-incubation at 4°C for 30 min. Finally, α-casein (5 μM) was added to every reaction mixture, followed by mixing and incubation at 37°C, and then degradation of casein was analysed as described under 'Experimental Procedure' section and in the legend to Fig. 1. In (a), no liposomes or ATP but Mg^{2+} and Lon were added as a negative control, and in (b), no liposomes but Mg^{2+}/ATP and Lon were added as a positive control. (B–D) Quantitation analysis of casein degradation. In (B), the amounts of casein were calculated from the stained gels, corresponding to in (A) as (c) (filled triangle) and (d) (filled circle) as well as negative (open square) and positive (open circle) controls. In (C), (e) (filled triangle) and (f) (filled circle), and in (D), (g) (filled triangle) and (h) (filled circle) correspond to in (A), as shown in the legend to Fig. 1B. The means ± SD for four independent experiments are shown.

addition of Mg^{2+} [Fig. 6A(c)], but that this inhibition was not observed when Mg^{2+} was added after the addition of CL-containing liposomes to the reaction mixture [Fig. 6A (g)], seems to be the specific prevention of the oligomerization of Lon by CL. However, addition of ATP to the reaction mixture prior to CL-containing liposomes [Fig. 6A (f)] also resulted in decreased inhibition of Lon protease by CL-containing liposomes as compared with the control [Fig. 6A (h)]. Taken together, the results in Fig. 6 seem to suggest that either Mg^{2+} or ATP, or the both are involved in the changes of Lon that might avoid the inhibitory activity of CL-containing liposomes. At present, it is not certain whether oligomerization of Lon alone is responsible for the conformational changes that are most susceptible to CL-containing liposomes. We are now trying to identify the binding domain(s) of Lon to CL-containing liposomes.

CL, having the structure of two PGs connected through a glycerol backbone in the centre, has four alkyl chains and two phosphate groups per molecule. Different from PG, in CL, the hydroxyl groups of phosphate and those of the glycerol backbone in the centre form stable intramolecular hydrogen bonds, forming a bicyclic resonance structure. This structure has the negative surface charge suitable for the trapping of one proton from other molecules under physiological conditions (*35*, *36*). Such a structural difference

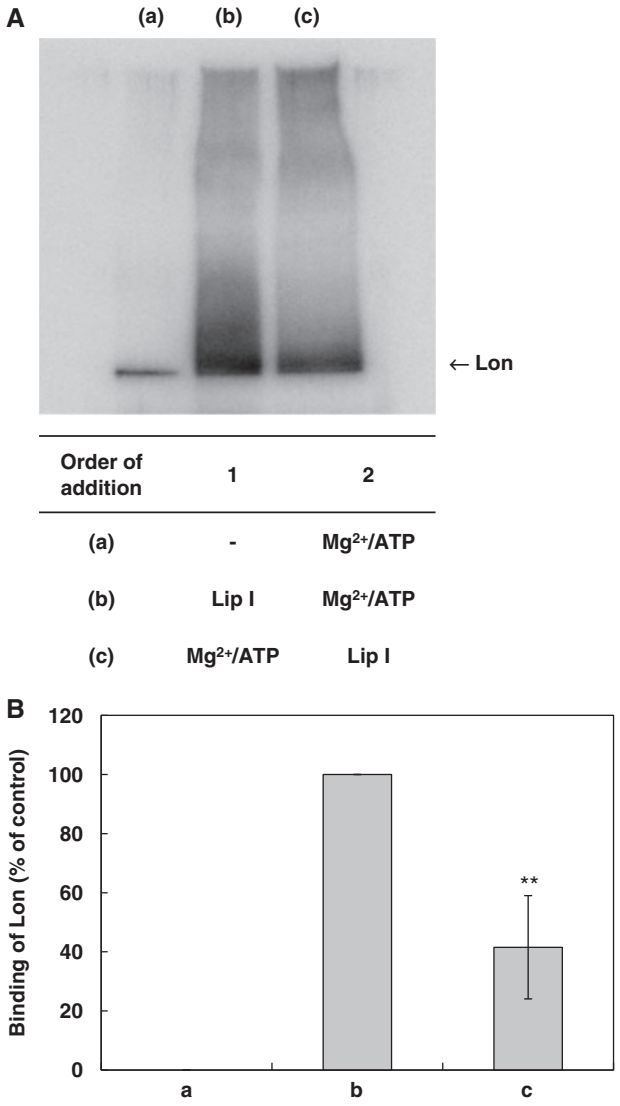

Fig. 7 Effect of the order of Lip I addition to the reaction mixture on binding of Lon to liposomes. (A) Profile of binding of Lon to Lip I. Either 0.2 mM Lip I (b) or 25 mM $MgCl_2$ and 1 mM ATP (c) were added to each reaction mixture comprising 0.3 μM Lon in 50 mM Tris–HCl, pH 8.0, followed by mixing and storage on ice for 30 min. Then either Mg^{2+}/ATP (b) or Lip I (c) was added to the corresponding reaction mixture, followed by mixing and pre-incubation at 4°C for 30 min. Finally, streptavidin-beads were added to each reaction mixture, followed by mixing and incubation at 4°C for 30 min, and then the binding of liposomes to Lon protease was analysed as described under 'Experimental' and in the legend to Fig. 5. In (a), Mg^{2+}/ATP and Lon without liposomes were utilized as a negative control. (B) Quantitation of the binding of Lon to Lip I. The amounts of Lon bound to Lip I were determined as described in the legend to Fig. 5B. The results are shown relative (%) to the values where the amount in (b) was set at 100%, and that in (a) at 0% in each experiment. The means ± SD for five independent experiments are shown with the significance of differences between (c) and (b), determined by Student's *t*-test, as $^{**}P < 0.01$.

between PG and CL might be one of the reasons for the different inhibition of the enzymatic activities of Lon.

In this study, it was suggested that CL-containing liposomes bind directly to Lon, and inhibit the ATPase activity and then the protease activity of Lon. Lip I, comprising PE : PG : CL = 6 : 3 : 1, strongly inhibited both the ATPase and protease activities of Lon. The composition of the phospholipids in Lip I resembles that of the inner membrane of *E. coli*. Lon is known to be a soluble and cytoplasmic enzyme. However, we made a preliminary observation that Lon was not uniformly distributed in the cytoplasm but rather concentrated at the periphery of the inner membrane and to a nucleoid-like structure on transmission electron microscopic analysis (data not shown). CL is an important component of the inner membrane of *E. coli*, comprising ~20% of the total phospholipids, and playing essential roles in the regulation of F_1/F_0 ATPase, that of DNA replication (*37*), and in the structure and function of the bacterial translocation machinery (*38*). In addition, the results of this study suggest that CL in the inner membrane plays a novel regulatory role as to the proteolytic activity of Lon: If CL really binds to Lon and decreases its catalytic activity in cells, CL seems to have of special regulatory function(s) as to Lon. Lon is a cytoplasmic soluble enzyme and thus has been assumed to act mostly in the cytosol. However, if Lon is translocated to the periphery of the inner membrane where CL is abundant, the activity might be down-regulated. Besides, if Lon is translocated to a DNA-rich region (nucleoid) in the cytosol, the activity of Lon might be up-regulated. These ideas seem to resemble the case of protein kinase C (PKC) in mammalian cells. It is well-known that PKC is translocated from the cytosol to the membrane, and its enzymatic activity becomes up-regulated if the membrane is rich in diacylglycerol, while it becomes down-regulated if the membrane is rich in sphingomyelin (*39*). A recent study by Gold *et al.* (*38*) showed that CL tightly associates with the SecYEG translocon complex, stabilizes the dimer, and creates a high-affinity binding surface for the SecA ATPase. In addition, they suggested the involvement of CL in the modification of the sub-cellular distribution of the translocon complex. At present, we have not clarified the mechanism underlying the CL-Lon interaction in *E. coli* cells in detail, but this study seems to provide important and interesting evidence supporting the idea that the translocation of Lon in *E. coli* is another example of regulation of enzymatic activity through binding to a membrane component, CL.

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Conflict of interest
None declared.

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