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LonR9 Carrying a Single Glu^{614} to Lys Mutation Inhibits the ATP-Dependent Protease La (Lon) by Forming Mixed Oligomeric Complexes

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An unusual *lon* mutation (called *lon*R9) is dominant over the wild-type gene, which encodes the ATP-dependent protease La (Lon) in *Escherichia coli,* when present in multicopy plasmids. Here, we cloned and sequenced *lon*R9, and showed that the mutant gene carries a single point mutation in its open reading frame, which leads to replacement of Glu^{614} by Lys. The LonR9 protein and its poly-His-tagged form were purified to apparent homogeneity. Both of the purified proteins were capable of inhibiting the ATP-dependent proteolysis and the protein-activated ATP hydrolysis by protease La. Furthermore, the His-tagged LonR9 protein was found to form mixed oligomeric complexes with protease La, upon analysis by chromatography on a metal-chelating column. These results suggest that the phenotypic dominance of the *lonR9* mutant is due to the formation of mixed oligomeric complexes between LonR9 and protease La, in which the defective components prevent the function of the wild-type subunits.

Protease La, the product of the *lon* gene, is an ATP-dependent protease in *Escherichia coli* (1-4). This enzyme is composed of four or eight identical subunits (87 kDa), each of which contains an ATP-binding site and a Ser active site for proteolysis. It is a heat shock protein and plays an essential role in the degradation of most abnormal proteins and certain short-lived regulatory proteins, such as SulA and RcsA (5-8). Therefore, mutations in the *lon* gene cause a variety of phenotypic alterations including a reduced ability to degrade abnormal proteins, defective cell division, and overproduction of capsular polysaccharides.

Although most *lon* mutants are recessive, an unusual *lon* mutant, called *lon*R9 or *cap*R9, is dominant over the wild type under certain conditions (9). For example, when a wild-type cell is carrying the *lon*R9 allele on multicopy plasmids, it evidently shows the mutant phenotype, such as overproduction of capsular polysaccharides. The product of *lon*R9 has been purified and shown to have nearly identical subunit size with that of protease La (10). However, it is unable to hydrolyze proteins or ATP. Furthermore, it inhibits both the ATP-dependent casein hydrolysis and the protein-activated ATP cleavage by the wild-type enzyme (10,11). Therefore, it has been suggested that the phenotypic dominance may result from interaction of the normal and LonR9 subunits to form mixed oligomers (9,10). However, none is known about the nature of the *lon*R9 mutation at molecular level. In the present studies, therefore, we cloned and sequenced *lon*R9 for determination of the mutation site(s) and for clarification of the biochemical basis for the phenotypic dominance of the mutant allele.

MATERIALS AND METHODS

Materials. Protease La and its mutant forms were purified using conventional chromatographic procedures as described previously (10), and the His-tagged protein was isolated using Ni^{2+}-bound nitrilo-triacetic acid (NTA) column (Qiagen). [^{3}H]Methyl-casein was prepared as described by Jentoft and Dearborn (12) using [^{3}H]-formaldehyde (NEN-Dupont). All other chemicals used were purchased from Sigma, unless otherwise indicated.

Cloning of lonR9. Based on the published sequence of the *lon* gene (13-15), four primers were designed. Two were made complementary to the 5′ and 3′ ends of the adjacent genes and the others were to the internal sequences of the *lon* gene. Their locations are depicted in Fig. 1A. Two separate polymerase chain reactions (PCR) were carried out using pairs of primer-1 and -3 and primer-2 and

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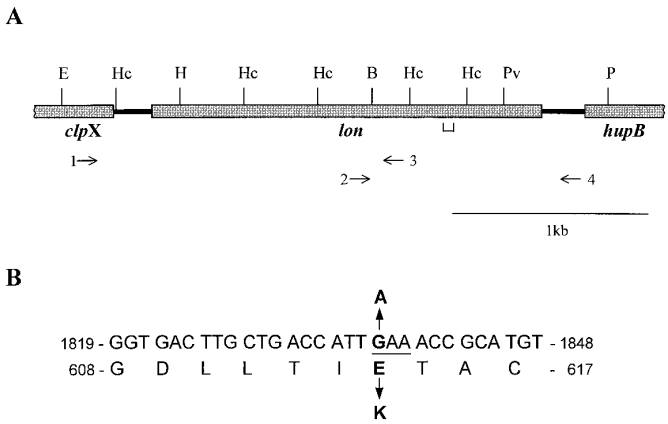

FIG. 1. The restriction map of the *lon* gene and the nucleotide sequence of the region carrying a single point mutation in *lonR9*. (A) The shaded boxes show the open reading frames of the indicated genes. The restriction sites are also shown: E, *Eco*RI; Hc, *Hin*cII; H, *Hin*dIII; B, *Bam*HI; Pv, *Pvu*I; P, *Pst*I. The nucleotide sequences of the primers used are: primer-1, 5′-GTGGTTATCGACGAGTCGGT-3′; primer-2, CATTGCCAAAGCCACCGGGC; primer-3, GTTCATGGAGTTCGACGTCG; primer-4, CGATCCGCCATCTAACTTAG. (B) The nucleotide sequence of the region containing the point mutation corresponds to the bracket shown in A.

-4, such that to generate the PCR products corresponding to N- and C-terminal fragments of the LonR9 protein, respectively, with a partial overlapping internal sequence. They were then ligated into a pGEM-T-Easy vector (Promega), and the recombinant plasmids were referred to as pGEM/R9-N and pGEM/R9-C. After amplification, pGEM/R9-N was digested with *Bam*HI and *Apa*I and the DNA fragment was ligated into pGEM/R9-C that had been pretreated with the restriction enzymes. The resulting plasmid, pGEM/R9, was propagated in the *E. coli* BL21 strain lacking protease La (Novagen). In order to prepare poly-His-tagged form of the LonR9 protein, the PCR-cloned *lon*R9 in pGEM/R9 was cut out and inserted into pQE31 vector. The resulting plasmid was referred to as pQE/R9.

Assays. Proteolysis was assayed by incubating 10 μg of [^{3}H]-methyl-casein and appropriate amounts of protease La and/or LonR9 in 50 mM Tris-HCl (pH 8), 5 mM $MgCl_2$, and 1 mM ATP (total volume: 0.1 ml). After Incubation, the reaction was terminated by adding 50 μl of 40% (w/v) trichloroacetic acid and 50 μl of 3% (w/v) bovine serum albumin. The radioactivity released into acid-soluble products was then determined (16). ATP hydrolysis was assayed by incubating the same reaction mixtures but in the presence and absence of 10 μg of non-radioactive casein. The inorganic phosphates released during the incubation period were determined as described previously (17,18).

RESULTS AND DISCUSSION

Cloning of lonR9 Carrying a Single Point Mutation

The *lon* gene has been shown to locate in between the *clpX* and *hupB* genes in *E. coli* chromosome (Fig. 1A) (19). Using PCR, *lon*R9 was cloned from the *E. coli* RGC123 strain bearing the mutation in its chromosomal DNA (9). The restriction map of the PCR-cloned *lon*R9 was found to be identical to that of the wild-type gene (data not shown), indicating that the *lon* mutation did not occur at least at the restriction sites. For determining the sequence of *lon*R9, we obtained nine different subclones containing the DNA fragments that are encompassed by the nearest pairs of the restriction sites shown in Fig. 1A. Eight primers were prepared to determine the nucleotide sequences of the subclones. A single G to A point mutation at the 1837th position in the open reading frame of *lon*R9 was identified by aligning its nucleotide sequence with that of the wild-type *lon* gene (Fig. 1B). This mutation was confirmed by direct PCR sequencing of the chromosomal DNA isolated from *E. coli* RGC123 strain bearing the *lon*R9 mutation (data not shown). Thus, the point mutation should lead to replacement of Glu^{614} by Lys and hence to alteration in the isoelectric point of protease La. These results are consistent with the previous report that the subunit of LonR9 has a higher isoelectric point than that of protease La (10).

Although the E614K mutation was not located in any of the putative active site for proteolysis (20) or in ATP-binding region (13), dramatic alteration in the charge of the amino acid might have been sufficient for abolishing both the ATP-dependent proteolysis and the ATP hydrolysis by protease La (10). In addition, the tetrameric LonR9 protein has previously been shown to dissociate more easily into monomers and dimers in the presence of high salt (e.g., 0.2 M NaCl) than the wild-type enzyme (10). Therefore, it appears that Glu^{614} is involved in oligomerization of the subunits of protease La, although it remains unknown how the mutation influences the catalytic function of the enzyme.

Effects of LonR9 and Its His-Tagged Form on the Proteolytic Activity of Protease La

In order to determine whether the recombinant LonR9 is capable of inhibiting protease La, the mutant protein was purified from the *E. coli* BL21 cells transformed with pGEM/R9 as described previously (10). To determine whether the subunits of poly-His-tagged LonR9 (His-tagged LonR9) and protease La interact with each other, we also purified the His-tagged protein from the cells carrying pQE/R9 using a NTA-column. The purified proteins were then electrophoresed on polyacrylamide slab gels containing SDS and 2-mercaptoethanol. Fig. 2 shows that both proteins are purified to apparent homogeneity. Due to the fusion of poly-His to N-terminus of LonR9, the His-tagged protein migrated slightly slower than LonR9. Note that protease La itself runs as a 94-kDa protein in the gel (10, 21), despite the fact that its calculated molecular mass from its nucleotide sequence is 87 kDa (13-15).

We then examined whether the recombinant LonR9 and its His-tagged form are capable of inhibiting the proteolytic activity of protease La. As shown Fig. 3A, both of the purified proteins inhibited the hydrolysis of

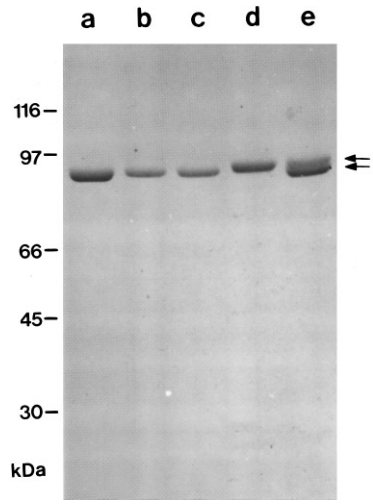

FIG. 2. SDS-polyacrylamide gel electrophoresis of protease La and the LonR9 proteins. The purified proteins (3 μg each) were subjected to electrophoresis on 8.5% (w/v) polyacrylamide slab gels containing SDS and 2-mercaptoethanol (22). Lane a, protease La; lane b, LonR9 derived from the chromosome of the *E. coli* RGC123 strain; lane c, PCR-derived, recombinant LonR9; lane d, His-tagged, recombinant LonR9; lane e, protease La plus His-tagged LonR9. The arrows from top to bottom indicate His-tagged LonR9 and protease La, respectively.

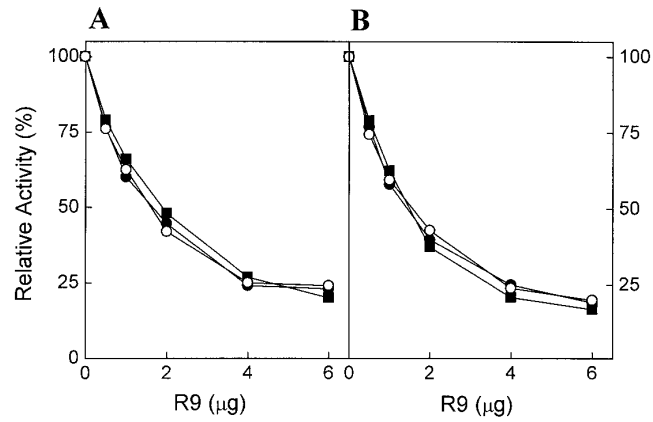

FIG. 3. Effects of the LonR9 proteins on the proteolytic activity (A) and the protein-activated ATPase activity (B) of protease La. Proteolysis was assayed by incubating 10 μg of [^{3}H]methyl-casein and protease La (2 μg) in the absence and presence of increasing amounts of LonR9 derived from the chromosome of the *E. coli* RGC123 strain (○), PCR-derived, recombinant LonR9 (●), or His-tagged, recombinant LonR9 (△). After Incubation for 1 h at 37°C, the radioactivity released into the acid-soluble products was determined as described under Materials and Methods. ATP hydrolysis was assayed by incubating the same reaction mixtures but in the presence of 10 μg of non-radioactive casein. The activity seen with protease La alone was expressed as 100% and the others were as its relative values.

casein by protease La nearly as well as the LonR9 protein derived from the chromosome of *E. coli* RGC123 strain did. They also inhibited the protein-activated ATPase activity of protease La to the same extents seen with the chromosome-derived LonR9 protein (Fig. 3B). In addition, both of the proteins showed a higher tendency to dissociate into monomers and dimers in the presence of salts (*e.g.*, 0.2 M NaCl) than protease La, similar to the chromosome-derived LonR9 protein (data not shown). Thus, it seems clear that the E614K mutation found in the recombinant LonR9 proteins is not due to any PCR error.

Mixed Oligomer Formation between Protease La and LonR9

To determine the biochemical mechanism for the inhibitory effect of the LonR9 protein, a fixed amount of the His-tagged LonR9 protein was mixed with increasing amounts of protease La (i.e., to vary their molar ratio) in the presence of 0.4 M NaCl followed by incubation at 37°C for 10 min. Under this condition, all of the His-tagged LonR9 protein completely dissociated into monomers, while protease La became a mixture of monomers and dimers (data not shown). After incubation, the mixtures were diluted 10-fold with 20 mM Tris/HCl (pH 7.8) buffer containing 5 mM $MgCl_2$ but not NaCl for re-association of the subunits into oligomeric forms. Each of the samples was then loaded on a NTA-column, washed with the Tris buffer containing 50 mM imidazole, and eluted with 250 mM imidazole. When protease La alone was chromatographed as above, all of the proteins were recovered in the unbound fraction (Fig. 4A). On the other hand, the His-tagged LonR9

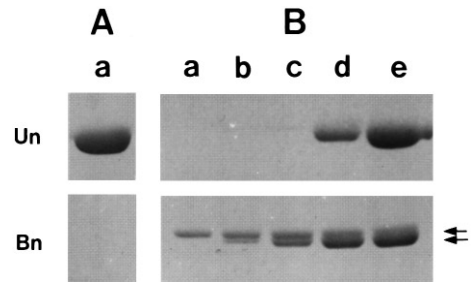

FIG. 4. Formation of mixed oligomeric complexes between protease La and His-tagged LonR9. (A) Protease La alone (150 μg) was incubated for 10 min at 37°C in 50 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, and 0.4 M NaCl. The enzyme sample was diluted 10-fold with the same buffer but lacking NaCl. The resulting solution was chromatographed on a NTA-column (0.2 ml) equilibrated with the dilution buffer as described in the text. (B) Incubations were performed as above but in the absence (lane a) or presence of 10 μg (lane b), 30 μg (lane c), 90 μg (lane d), and 180 μg (lane e) of protease La and 30 μg of the His-tagged LonR9 protein, so that to make molar ratios of 1:3, 1:1, 3:1, and 6:1, respectively. The same aliquots of the unbound (Un) and bound (Bn) fractions were then subjected to gel electrophoresis as in Fig. 2. The arrows from top to bottom indicate His-tagged LonR9 and protease La, respectively.

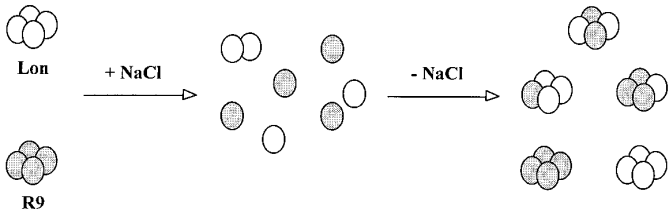

FIG. 5. A schematic model for the formation of mixed tetrameric complexes in the presence of equal amounts of protease La and the LonR9 protein. This model is based on the assumption that both proteins are tetramers. Under this condition, the probability for formation of homo-oligomers of LonR9 and protease La should be much lower than that of their mixed oligomers, although one each of the oligomers that can possibly be formed were depicted.

protein alone was solely recovered in the bound fraction (Fig. 4B, lane a).

When their mixtures were chromatographed at 1:3 or 1:1 molar ratio of protease La to the His-tagged LonR9, most of the wild-type proteins were retained in the column (Fig. 4B, lanes b and c, respectively). In the presence of excess amounts of protease La over the His-tagged LonR9 protein (i.e., at the ratios of 3:1 and 6:1), the amounts of the wild-type protein retained in the column further increased (lanes d and e, respectively). However, unlike in the presence of low concentrations of protease La (i.e., at the ratios of 1:1 and 1:3), the increase in the amounts of the bound protease La was not proportional to the increase in the amount added to the incubation mixtures, since the probability for the formation of homo-oligomeric protease La must also increase. These results indicate that the LonR9 protein interacts with protease La and forms mixed oligomeric complexes, in which the ratio of the subunits of LonR9 to those of protease La varies depending on the concentrations of the two proteins. Thus, the phenotypic dominance of *lonR9* over the wild type appears to result from interaction of the normal and LonR9 subunits to form mixed oligomers, in which the defective components prevent the function of the wild-type subunits. Fig. 5 represents a schematic model for the formation of mixed oligomeric complexes in the presence of equal amounts of the two proteins.

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