A Membrane-Bound Archaeal Lon Protease Displays ATP-Independent Proteolytic Activity towards Unfolded Proteins and ATP-Dependent Activity for Folded Proteins

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In contrast to the eucaryal 26S proteasome and the bacterial ATP-dependent proteases, little is known about the energy-dependent proteolysis in members of the third domain, *Archaea*. We cloned a gene homologous to ATP-dependent Lon protease from a hyperthermophilic archaeon and observed the unique properties of the archaeal Lon. Lon from *Thermococcus kodakaraensis* KOD1 (Lon_{Tk}) is a 70-kDa protein with an N-terminal ATPase domain belonging to the AAA⁺ superfamily and a C-terminal protease domain including a putative catalytic triad. Interestingly, a secondary structure prediction suggested the presence of two transmembrane helices within the ATPase domain and Western blot analysis using specific antiserum against the recombinant protein clearly indicated that Lon_{Tk} was actually a membrane-bound protein. The recombinant Lon_{Tk} possessed thermostable ATPase activity and peptide cleavage activity toward fluorogenic peptides with optimum temperatures of 95 and 70°C, respectively. Unlike the enzyme from *Escherichia coli*, we found that Lon_{Tk} showed higher peptide cleavage activity in the absence of ATP than it did in the presence of ATP. When three kinds of proteins with different thermostabilities were examined as substrates, it was found that Lon_{Tk} required ATP for degradation of folded proteins, probably due to a chaperone-like function of the ATPase domain, along with ATP hydrolysis. In contrast, Lon_{Tk} degraded unfolded proteins in an ATP-independent manner, suggesting a mode of action in Lon_{Tk} different from that of its bacterial counterpart.

Energy-dependent protein degradation in viable cells plays several important roles in the rapid turnover of short-lived regulatory proteins and in the quality control of intracellular proteins by eliminating damaged or denatured proteins. In eukaryotic cells, target proteins covalently modified with ubiquitin are degraded by the multimeric 26S proteasome. Bacterial cells possess a number of ATP-dependent proteases, for instance, Lon, ClpAP, ClpXP, HslUV (ClpYQ), and FtsH in *Escherichia coli* (13, 14). They processively degrade proteins into short oligopeptides in a highly coupled manner with ATP hydrolysis, and their function allows cells to achieve balanced growth and respond to external stress.

Lon protease was the first ATP-dependent protease to be described (44). Lon from *E. coli* (Lon_{Ec}) is an 87-kDa protein consisting of N-terminal ATPase and C-terminal protease domains on a single polypeptide (5, 11) and forms a homooligomer (a homotetramer or homooctamer) of identical subunits (6, 11). Previous studies demonstrated that a variety of temperature-sensitive mutations in *E. coli* could be suppressed by inactivation of the *lon* gene (16, 17). Lon_{Ec} participates in the rapid degradation of abnormal proteins in cooperation with the heat shock chaperone system DnaK/DnaJ/GrpE (21, 43). In addition, it has been known that Lon proteases are capable of recognizing and degrading specific protein substrates, such

as UmuDC (10), Sula (31), RcsA, (7, 47), CcdA (48), and so on. Lon_{Ec} shows little or no peptide cleavage activity in the absence of nucleotides; however, interestingly, it has been suggested that ATP binding, not hydrolysis, at the high-affinity sites might induce some conformational change of Lon_{Ec} to the open (active) state that allows cleavage of peptide bonds (11, 12, 29).

ClpAP, ClpXP, and HslUV (ClpYQ) represent another type of ATP-dependent proteases with a heteromeric structure. They are comprised of two distinct subunits of ATPase (ClpA, ClpX, and HslU) and protease (ClpP and HslV) (13, 14). In the recently determined crystal structure of HslUV, two hexameric rings of HslU were found to be stacked to both ends of an internal chamber composed of two hexameric rings of the proteolytic core, HslV, forming a structure resembling the eukaryotic 26S proteasome (4, 41, 50). ClpAP is supposed to have a common molecular architecture, except for a tetradecameric proteolytic chamber of ClpP with sevenfold symmetry (49). It has been revealed that the hexameric ring of ClpA is self assembled when ATP is bound (22).

The ATPases in the Lon and Clp proteases share structural and mechanistic features with other chaperone-like ATPases that assist in the assembly, operation, and disassembly of protein complexes, and this sequence set is assigned to the AAA⁺ superfamily (34). ATPases in ATP-dependent proteases also act as molecular chaperones, and recent studies have led to considerable progress in understanding the role of ATPase subunits in the proteolysis by ClpAP and ClpXP (15, 55). That is, ClpA and ClpX ATPases function to unfold protein structures, to bind unfolded proteins, and to translocate unfolded

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proteins to the proteolytic chamber, coupled with ATP hydrolysis (19, 20, 39). Likewise, a chaperone-like activity of the ATPase domain of Lon_{Ec} has been suggested to function during the ATP-dependent unfolding of a specific substrate, CcdA (48). A model for the protein unfolding-coupled translocation mechanism has been proposed from the crystal structure of the HslUV complex (50). In addition, proteolytically inactive Lon of yeast mitochondria could complement a mutation of host cells lacking functional assembly of mitochondrial protein complexes, suggesting a chaperone activity of ATPase domain in yeast Lon (35).

In contrast to members of the domains *Bacteria* and *Eucarya*, little is known about the energy-dependent proteolysis system in members of the third domain, *Archaea*. Complete genome sequences have revealed that archaeal genomes also encode putative orthologs of 20S proteasome and proteasome-activating nucleotidases, as well as open reading frames that share significant identity with Lon proteases from bacterial cytosol and eucaryal mitochondria. *clp* and other ATP-dependent protease genes are not found in archaeal genomes, except for the thermophilic methanogen *Methanobacterium thermautotrophicum*, which encodes a Clp-like ATPase (MTH284) but not a Clp-like protease in the genome. Although there have been some reports concerning archaeal proteasomes (27, 38, 56), no investigation has been made for the ATP-dependent protease Lon from archaea.

Thermococcus kodakaraensis (previously called *Pyrococcus kodakaraensis*) strain KOD1 is a hyperthermophilic archaeon isolated from a geothermal spring at a wharf on Kodakara Island, Kagoshima, Japan (32). Various thermostable enzymes from this archaeon, including DNA polymerase (18, 45), DNA ligase (33), ribulose 1,5-bisphosphate carboxylase/oxygenase (8, 24, 26), and so on have been characterized. Many of the proteins show distinct structural and catalytic features from bacterial and eucaryal counterparts. Here, we have characterized an archaeal Lon from this hyperthermophile and report the unique properties of the archaeal Lon protease.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *E. coli* TG-1 and DH5 α and plasmid pUC118 were used for DNA manipulation and sequencing. *E. coli* BL21CodonPlus(DE3)-RIL cells (Stratagene, La Jolla, Calif.) and plasmids derived from pET-21a(+) or pET-16b(+) (Novagen, Madison, Wis.) were used for gene expression. *E. coli* strains were cultivated in Luria-Bertani medium at 37°C, and ampicillin was added to the medium at a final concentration of 50 μ g/ml, when needed.

DNA manipulation and sequencing. DNA manipulations were performed by standard procedures, as described by Sambrook and Russell (36). Restriction enzymes and other modifying enzymes were purchased from Takara Shuzo (Kyoto, Japan) or Toyobo (Osaka, Japan). Small-scale preparation of plasmid DNA from *E. coli* cells was performed with a plasmid mini kit (Qiagen, Hilden, Germany). DNA sequencing was performed with the ABI PRISM kit and model 310 capillary DNA sequencer (Applied Biosystems, Foster City, Calif.). Nucleotide and amino acid sequences were analyzed with GENETYX software (Software Development, Tokyo, Japan).

Cloning and expression of lon_{TK}. The *lon* gene from *T. kodakaraensis* KOD1 (*lon_{TK}*) was identified by random sequencing of a phage library of *T. kodakaraensis* KOD1 genomic DNA, and the nucleotide sequence was determined with the phage DNA as a template. The full-length *lon_{TK}* flanked by the *Nde*I and *Bam*HI sites was amplified by PCR with KOD1 genomic DNA and two primers (sense [5'-ATGCATCATATGGACGAGGAGTCCACCA-3'] and antisense [5'-ATGCATGGATCCAGTTAGGAAGACCCGTAAAGCGGAAG-3']); the italicized sequences indicate the *Nde*I site in the sense primer and the *Bam*HI site in the antisense primer. The amplified DNA fragment was digested with *Nde*I and

*Bam*HI after confirmation of the sequence. The fragment was then ligated with the corresponding sites of plasmid pET-21a(+) to obtain pETlon for the production of recombinant Lon protease from *T. kodakaraensis* KOD1 (*lon_{TK}*) with the native structure. *E. coli* BL21CodonPlus(DE3)-RIL harboring pETlon was induced for overexpression with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) at the mid-exponential growth phase and incubated for 4 h at 37°C. The cells were harvested by centrifugation (7,000 $\times$ g for 10 min at 4°C), washed with 50 mM Tris-HCl buffer (pH 8.0), and then resuspended in the same buffer. The cells were disrupted by sonication and centrifuged (15,000 $\times$ g for 30 min at 4°C). The resulting supernatant was incubated at 70°C for 30 min, followed by centrifugation (15,000 $\times$ g for 15 min at 4°C). The precipitated fraction was treated with 1.5% Triton X-100 in the Tris buffer at 4°C for 2 h and then centrifuged (15,000 $\times$ g for 15 min at 4°C) to obtain solubilized recombinant *lon_{TK}*. The supernatant was applied to a ceramic hydroxyapatite CHT5 column (Bio-Rad, Hercules, Calif.) preequilibrated with 5 mM sodium phosphate buffer (pH 7.0). *lon_{TK}* was eluted by a linear gradient of sodium phosphate buffer (5 to 500 mM) by using a protein purification apparatus (AKTA explorer 10S; Amersham Pharmacia Biotech, Uppsala, Sweden) with a flow rate of 2.0 ml/min. The peak fractions were concentrated by using Centricon-100 (Millipore, Bedford, Mass.), and the sample was applied to a Superdex 200 high resolution 10/30 column (Amersham Pharmacia Biotech) preequilibrated with 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl with a flow rate of 0.2 ml/min.

In order to purify *lon_{TK}* without using Triton X-100, a recombinant Lon flanked with a His₁₀ tag at the N terminus (*lon_{TK}*-NH₁₀) was expressed and purified by Ni²⁺ affinity chromatography. An expression plasmid, pETlonNH₁₀, was constructed by inserting the *Nde*I-*Bam*HI fragment of *lon_{TK}* into pET-16b(+). *E. coli* BL21CodonPlus(DE3)-RIL cells were then transformed with pETlonNH₁₀ and were induced and disrupted as described above. The soluble protein fraction was applied to a Ni²⁺ affinity column (HisBind purification kit; Novagen) and washed with 200 mM imidazole in 160 mM Tris-HCl buffer (pH 7.9) containing 4 M NaCl, and *lon_{TK}*-NH₁₀ was eluted with 1 M imidazole in the buffer.

The protein concentration was determined according to the method of Bradford (44) (Bio-Rad protein assay) with bovine serum albumin as a standard. The protein purity was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis performed according to the standard procedure. The N-terminal amino acid sequences of purified proteins were determined by a protein sequencer (cLC model 491; Applied Biosystems).

Enzyme assays. Peptide cleavage activity was assayed using the fluorogenic peptides glutaryl-Ala-Ala-Phe-4-methoxy- β -naphthylamide (Glt-AAF-MNA) or succinyl-Phe-Leu-Phe-4-methoxy- β -naphthylamide (Bachem, Bubendorf, Switzerland) (52). The reaction mixture was composed of 0.3 mM fluorogenic peptide, 1 or 4 mM ATP, 10 mM MgCl₂, and *lon_{TK}* (5 μ g) in 500 μ l of 50 mM 2-(cyclohexylamino)ethanesulfonic acid (CHES) buffer (pH 9.0). The mixture was incubated at 70°C for 60 min, and the increase in fluorescence (350 nm, excitation; 440 nm, emission) was monitored by spectrofluorometer (model F-2000; Hitachi, Tokyo, Japan).

ATPase activity was assayed by determining P_i liberated from ATP. *lon_{TK}* (2.5 μ g) was incubated with 4 mM ATP and 10 mM MgCl₂ in 100 μ l of 50 mM CHES buffer (pH 9.0) at 70°C for 15 min. Free P_i was determined by the procedure described by Black and Jones (3). The background levels of hydrolysis (no enzyme) were subtracted in each assay.

Proteolytic properties of *lon_{TK}* towards α -casein from bovine milk (Sigma, St. Louis, Mo.), hemoglobin A from human (Sigma), and ribulose-1,5-bisphosphate carboxylase/oxygenase from *T. kodakaraensis* KOD1 (*Rubisco_{TK}*) (8) were analyzed by SDS-PAGE. Substrate protein (3.2 μ g) was incubated with *lon_{TK}* (2 μ g)-1 mM ATP-10 mM MgCl₂ in 100 μ l of 50 mM CHES buffer (pH 9.0) at 37°C for the appropriate amount of time. The mixtures were concentrated to approximately 10 μ l under reduced pressure, applied to SDS-PAGE, and then stained with Coomassie brilliant blue R-250 (Bio-Rad). The experiments were also performed at 70°C with slight modification. In order to avoid the complete consumption of ATP in the mixture, the amounts of protein substrate and *lon_{TK}* were reduced to 0.5 and 0.2 μ g, respectively, and Bio-Rad silver stain was applied for visualization of protein bands. Circular dichroism (CD) spectra of protein substrates were obtained in 10 mM Tris-HCl buffer (pH 8.3) containing 10 mM MgCl₂ by using a spectropolarimeter (model J-820; JASCO, Tokyo, Japan). Matrix-assisted laser desorption/ionization-time of flight mass spectroscopic and electron-spray ionization-time of flight mass spectroscopy/mass spectroscopic analyses of the degradation products from enzymatically dephosphorylated α -casein (Sigma) were carried out with AXIMA-CFR (Shimadzu, Kyoto, Japan) and API QSTAR (Applied Biosystems), respectively, after desalting the mixture by using the ZipTip μ C₁₈ column (Millipore).

Western blot analysis. *T. kodakaraensis* KOD1 was cultivated in artificial seawater (Marine Art SF; Senju Seiyaku, Osaka, Japan) containing 0.5% tryptone and 0.5% yeast extract. The cells grown on 1% pyruvate or 0.1% elemental sulfur in the medium at 85°C for 12 h were harvested, washed with 0.1 M potassium phosphate buffer (pH 7.0) containing 0.4 M NaCl, and then disrupted by French press (96 MPa). The resulting cell extract was ultracentrifuged ($110,000 \times g$ for 2 h at 4°C) to separate the cytosol and membrane fractions. Each fraction (20 μ g of protein) was subjected to SDS-PAGE and followed by Western blot analysis using specific antiserum (rabbit) against the recombinant Lon_{Tk}. A protein A-peroxidase conjugate was used to visualize the specific proteins together with 4-chloro-1-naphthol and hydroxyperoxide.

Nucleotide sequence accession number. The nucleotide sequence data reported here will appear in the EMBL/GenBank/DBJ nucleotide sequence databases under accession number AB066562.

RESULTS

Primary structure of Lon_{Tk}. We found a phage clone harboring a gene homologous to known *lon* genes through random sequencing of the *T. kodakaraensis* KOD1 genomic DNA library. The full-length *lon*_{Tk} was subcloned, and the primary structure was determined, as shown in Fig. 1. *lon*_{Tk} (1,905 bp) encoded a protein consisting of 635 amino acids (aa) with a molecular mass of 70 kDa, which was smaller than the 87-kDa Lon_{Ec} (5, 11). Lon_{Tk} possessed a two-domain structure like other Lon proteases. The N-terminal region was an ATPase domain belonging to the AAA⁺ superfamily with Walker A and B, sensor 1 and 2, and several replication factor C box motifs, as previously described by Neuwald et al. (34). Nevertheless, the overall identity of the ATPase domain of Lon_{Tk} was very low compared to the values for Lon_{Ec} (20%) and other bacterial and eucaryal Lon proteases. Interestingly, two transmembrane helices could be identified at positions 128 to 150 and 160 to 182, which were between the Walker A and B motifs in the ATPase domain. The protease domain of Lon_{Tk}, comprising the C-terminal region, showed 37% identity to that of Lon_{Ec}, and Ser679, which functions as a nucleophile during peptide bond cleavage (2, 9), was also conserved in Lon_{Tk} at position 521. In Lon_{Ec}, His665, His667, and Asp676 have been found to be important residues and were supposed to form a catalytic triad together with Ser679, as in the case of classical serine proteases (42). The putative counterparts for His665 and Asp676 were conserved in Lon_{Tk} as His507 and Glu518, respectively, but His667 was replaced by Gln at the corresponding position in Lon_{Tk}.

Recent genome analyses showed the presence of *lon*-homologous genes in other archaea, namely, *Pyrococcus abyssi* (PAB1313), *Pyrococcus horikoshii* (PH0452), *Archaeoglobus fulgidus* (AF0364), *Methanococcus jannaschii* (MJ1417), *M. thermautotrophicum* (MTH785), *Thermoplasma acidophilum* (Ta1081), and *Halobacterium* sp. (VNG0303G). The deduced amino acid sequences of archaeal Lon showed high identities to Lon_{Tk} (50 to 87%) except for that from *M. jannaschii* (36%). In particular, those from Thermococcales were quite similar to Lon_{Tk} (86 to 87%), although putative *lon* genes from *P. abyssi* and *P. horikoshii* were divided by intein sequences. In contrast, similarities were lower between Lon_{Tk} and bacterial orthologs, even from hyperthermophilic *Thermotoga maritima* (TM1633) (24%) and extremely thermophilic *Thermus thermophilus* (51) (23%).

Expression and purification of recombinant Lon_{Tk}. In order to investigate the catalytic properties of Lon_{Tk}, we constructed

an expression plasmid, pETlon, in which *lon*_{Tk} was oriented downstream of the T7 promoter and the designed ribosome binding site of pET-21a(+). *E. coli* BL21CodonPlus(DE3)-RIL was then transformed with pETlon, and crude Lon_{Tk} was obtained as a soluble protein after 0.1 mM IPTG induction. There was no influence of *lon*_{Tk} expression on the growth of the transformant. The crude soluble fraction was then heat treated at 70°C for 30 min to precipitate the proteins from the host cell. Thermostable enzymes derived from thermophiles generally maintained their solubility even at high temperatures so that those expressed in *E. coli* could effectively be separated from host-derived proteins by heat treatment. However, in the case of Lon_{Tk}, the recombinant protein precipitated and was found in the insoluble protein fraction after the heat treatment. This was probably due to the hydrophobic nature derived from the putative transmembrane sequences, since addition of Triton X-100 to the precipitated fraction led to efficient solubilization of an active protein. The solubilized Lon_{Tk} was further purified by hydroxyapatite chromatography, followed by gel filtration chromatography. SDS-PAGE analysis indicated the existence of one major band of 70 kDa together with four faint bands with apparent molecular masses of 64, 50, 38, and 36 kDa (Fig. 2A). The 70-kDa major protein agreed with the predicted molecular mass of Lon_{Tk}, and its N-terminal amino acid sequence was identical to the deduced sequence of Lon_{Tk}. N-terminal sequences of the minor protein bands of 64, 50, and 38 kDa were also identical to that of Lon_{Tk}, and that of the 36-kDa protein corresponded to an internal sequence of Lon_{Tk} from aa 324. These facts indicated that these minor proteins were formed by the partial proteolysis of Lon_{Tk} during the expression and purification steps or by the autolysis of Lon_{Tk}. Therefore, the active fraction after gel filtration was used for further biochemical characterization. We also attempted to obtain recombinant Lon_{Tk} without using any detergent during the purification steps. For this purpose, a plasmid for recombinant Lon_{Tk} with a His₁₀ tag at the N terminus (Lon_{Tk}NHis₁₀) was constructed. Lon_{Tk}NHis₁₀ was expressed in *E. coli* and successfully purified by Ni²⁺ affinity chromatography, although the yield was much lower than that for Lon_{Tk} with the native primary structure described above.

The native molecular mass of Lon_{Tk} could not be estimated by gel filtration chromatography because the recombinant Lon_{Tk} was eluted from an exclusion volume of the column. This might be due to incorporation of the recombinant protein within micelles of the detergent used for the solubilization. However, this large molecular mass was still observed in the case of Lon_{Tk}NHis₁₀ purified without the use of any detergents, suggesting an intrinsic property of recombinant Lon_{Tk} to form a highly oligomeric structure.

Subcellular localization of Lon_{Tk} in *T. kodakaraensis* KOD1. We examined the localization of Lon_{Tk} carrying two possible transmembrane sequences in the cells of *T. kodakaraensis* KOD1. The cytosol and membrane fractions from *T. kodakaraensis* KOD1 were separated by ultracentrifugation and then subjected to Western blot analysis with specific antiserum against the recombinant Lon_{Tk}, as represented in Fig. 2B. The results clearly indicate that Lon_{Tk} was located in the membrane fractions while no signal was detected in the cytosol fractions from the cells grown on both pyruvate (fermentation) and elemental sulfur (sulfur respiration). Lon_{Tk} is actually a

FIG. 2. (A) SDS-PAGE gel of purified Lon_{Tk} from recombinant *E. coli*. Arrowheads show the four minor bands in the preparation. Lane M, molecular marker. (B) Western blot analysis of Lon_{Tk} in crude extract, cytosol, and membrane fractions from *T. kodakaraensis* KOD1 grown on 1% pyruvate and 0.1% elemental sulfur. Numbers on the left side of the panel are molecular masses in kDa.

membrane-bound protein, which is a significant difference between it and its bacterial and eucaryal counterparts.

Peptide cleavage activity of Lon_{Tk} . Peptide cleavage activity of Lon_{Tk} was determined by using Glt-AAF-MNA (52), which has often been used as a substrate for various Lon proteases. When Lon_{Tk} was incubated with Glt-AAF-MNA in the presence of ATP and Mg^{2+} , a time-dependent increase in fluorescence due to hydrolysis of the fluorogenic peptide at a high temperature (75°C) was observed (Fig. 3A). Surprisingly, we further found that Lon_{Tk} exhibited much higher activity when ATP was omitted from the reaction mixture, as shown in Fig. 3A. The high ATP-independent activity of Lon_{Tk} is an unprecedented feature of Lon proteases. Lon_{Tk} exhibited no cleavage activity without Mg^{2+} , regardless of the presence or the absence of ATP. The optimum pH and temperature for the ATP-independent activity of Lon_{Tk} were determined to be 9.0

and 70°C (Fig. 3B), respectively. Under the optimum condition, Lon_{Tk} showed a specific activity of 44 ± 9.7 pmol of MNA/ μg of protein/h toward Glt-AAF-MNA in the absence of ATP while that in the presence of 1 mM ATP showed an activity of 15 ± 5.1 pmol of MNA/ μg of protein/h. Lon_{Tk} -N-His₁₀ also showed higher peptide cleavage activity without ATP than with ATP (data not shown), which rules out the possibility that the ATP-independent activity was an artifact derived from the influence of detergent used in the purification procedure of Lon_{Tk} .

Succinyl-Phe-Leu-Phe-4-methoxy- β -naphthylamide (52) served as a better substrate for Lon_{Tk} , with a 5.7-fold higher degradation rate than that toward Glt-AAF-MNA, suggesting that Lon_{Tk} preferred hydrophobic substrates. Lon_{Tk} showed the peptide cleavage activity in the presence of various nucleoside triphosphates; however, the highest level of activity was

FIG. 3. (A) Time courses of hydrolysis of fluorogenic peptide by Lon_{Tk} . The reaction was carried out in 50 mM Tris-HCl buffer (pH 8.0) containing 5 μg of Lon_{Tk} and 0.3 mM Glt-AAF-MNA at 75°C. ATP and/or MgCl_2 was added to the mixture as follows: 10 mM MgCl_2 (open circle); 4 mM ATP and 10 mM MgCl_2 (open triangle); no addition (filled circle); or 4 mM ATP (filled triangle). (B) Effects of temperature on peptide cleavage (open circle) and ATPase (filled circle) activities of Lon_{Tk} . The peptide cleavage assay was performed using Glt-AAF-MNA as a substrate. (C) Effects of ATP (open circle) or AMP-PNP (filled circle) concentration on peptide cleavage activity of Lon_{Tk} .

TABLE 1. Relative peptide cleavage activity of Lon_{TK} with different nucleotides or divalent cations

Nucleotide	Divalent cation	Peptide hydrolysis (%) ^a
None	None	0
ATP	Mg ²⁺	100
ATP	None	0
None	Mg ²⁺	301
AMP-PNP	Mg ²⁺	94
ADP	Mg ²⁺	57
GTP	Mg ²⁺	66
CTP	Mg ²⁺	193
UTP	Mg ²⁺	77
dTTP	Mg ²⁺	193
None	Ni ²⁺	530
None	Ca ²⁺	316
None	Mn ²⁺	144
None	Co ²⁺	81
None	Zn ²⁺	0

^a Purified Lon_{TK} (5 µg) was incubated with 0.3 mM Glt-AAF-MNA, 4 mM nucleotide, and 10 mM divalent cation at 70°C for 60 min.

obtained when no nucleotide was added to the mixture, as shown in Table 1. These nucleotides seemed to inhibit the activity rather than support it. ADP, a potent inhibitor for Lon_{Ec}, also inhibited the activity of Lon_{TK}, but the enzyme retained 57% activity in comparison to the condition with the addition of ATP. The activity with the nonhydrolyzable analog of ATP, 5'-adenylylimidodiphosphate (AMP-PNP), was similar to that with ATP, as was the case for Lon_{Ec}. Increase in ATP or AMP-PNP concentration resulted in decrease in the activity, and the activity reached half at 1 mM ATP or AMP-PNP (Fig. 3C). These results suggested the inhibition by nucleotide binding and were quite distinct from those for Lon_{Ec}, for which activity elevated with the increase of ATP concentration from 10⁻⁴ to 10⁻² mM (12). Lon_{TK} strictly required divalent cations for the activity, regardless of the presence or absence of ATP (Table 1). Interestingly, Ni²⁺ served as a cofactor better than Mg²⁺ and Ca²⁺ could replace Mg²⁺ to a similar extent. Mn²⁺ and Co²⁺ could also support the hydrolysis, although they were less effective than Mg²⁺, Ni²⁺, or Ca²⁺.

ATPase activity of Lon_{TK}. Even though Lon_{TK} possessed ATP-independent peptide cleavage activity, the enzyme exhibited inherent ATPase activity as in other Lon proteases. We confirmed formation of ADP from [α -³²P]ATP by thin-layer chromatography analysis and did not observe AMP formation (data not shown). The optimum pH and temperature were 9.0 and 95°C (Fig. 3B), respectively, and the specific activity was $7.0 \pm 0.6 \times 10^4$ pmol of P_i/µg of protein/h under the optimum condition, which was 3 orders of magnitude higher than that for peptide cleavage activity. The ATPase activity of Lon_{TK} was efficient and highly thermostable. The activity was strictly dependent on divalent cations such as Mg²⁺, and other divalent cations (Ni²⁺, Ca²⁺, Mn²⁺, and Co²⁺) could support 43 to 59% activity of that with Mg²⁺ (Table 2). UTP could be accepted as a substrate with a hydrolysis rate that was 21% of that for ATP. It is interesting that CTP and dTTP, quite poor substrates for Lon_{TK}, seemed to inhibit the peptide cleavage activity less than other nucleotides.

Previous studies have indicated a regulatory feature of Lon proteases through stimulation of both ATPase and peptide

TABLE 2. Relative hydrolysis rates of different nucleotides by Lon_{TK} and effects of divalent cations

Nucleotide	Divalent cation	Nucleotide hydrolysis (%) ^a
ATP	Mg ²⁺	100
ATP	None	0
AMP-PNP	Mg ²⁺	0
ADP	Mg ²⁺	0
GTP	Mg ²⁺	0
CTP	Mg ²⁺	1
UTP	Mg ²⁺	21
dTTP	Mg ²⁺	2
ATP	Ni ²⁺	46
ATP	Ca ²⁺	43
ATP	Mn ²⁺	56
ATP	Co ²⁺	59
ATP	Zn ²⁺	1

^a Purified Lon_{TK} (2.5 µg) was incubated with 4 mM nucleotide and 10 mM divalent cation at 70°C for 15 min.

cleavage activities by interaction with protein substrates at an allosteric site distinct from the proteolytic site. α -Casein stimulated both the peptide cleavage and ATPase activities of Lon_{Ec} as much as 10-fold and 2.8-fold, respectively (30, 53). In order to examine the allosteric effects of Lon_{TK}, α -casein was added to the reaction mixture because α -casein is a good substrate not only for Lon_{Ec} but also for Lon_{TK} (see below). Without ATP, peptide cleavage activity against Glt-AAF-MNA in the presence of α -casein was 103% of that in the absence of α -casein. In the presence of ATP, the allosteric effects were slightly larger in the peptide cleavage and ATPase activities, which were increased to 113 and 133%, respectively, by the addition of α -casein. Lon_{TK} showed weak allosteric activation when ATP was present, but the effects were not so striking as in those of Lon_{Ec}.

Proteolytic property of Lon_{TK}. We further examined the proteolytic properties of Lon_{TK} towards three protein substrates with different thermostabilities. α -Casein possessed no specific structure, and the CD spectra of α -casein indeed demonstrated that this protein was unfolded without any secondary structure at different temperatures (37 and 70°C), as shown in Fig. 4A. In SDS-PAGE analyses, the band corresponding to α -casein readily disappeared regardless of the presence or absence of ATP, both at 37 and 70°C (Fig. 4B). These results coincided with the ATP-independent activity of Lon_{TK} toward the fluorogenic peptides. Hemoglobin A from human is an $\alpha_2\beta_2$ heterotetramer with a helix-rich structure at 37°C, but the secondary structure was disrupted at 70°C due to thermal denaturation (Fig. 4C). When hemoglobin A was used as a substrate at 70°C, Lon_{TK} could degrade the unfolded hemoglobin A in either the presence or the absence of ATP (Fig. 4D). In contrast, this protein substrate was degraded only in the presence of ATP at 37°C, the temperature under which hemoglobin A maintains its original folded structure, and the degradation at the low temperature did not occur in the absence of ATP. The third substrate was ribulose-1,5-bisphosphate carboxylase/oxygenase from *T. kodakaraensis* KOD1 (Rubisco_{TK}), composed of 10 subunits with a unique pentagonal structure (8, 24, 26). It was previously demonstrated that the optimum temperature of Rubisco_{TK} is extremely high (90°C) (8) and that its structure is stable even at high temperatures (24). Figure 4E

FIG. 4. Proteolytic properties of Lon_{Tk} with different protein substrates. (A, C, and E) CD spectra of α -casein, hemoglobin A, and Rubisco_{Tk} at 37°C (gray line) and 70°C (black line), respectively. (B, D, and F) Degradation of α -casein, hemoglobin A, and Rubisco_{Tk}, respectively, by Lon_{Tk}. The reaction was carried out at 37 or 70°C with or without the addition of ATP, as indicated in each panel. The mixtures at each reaction time were analyzed by SDS-PAGE as described in Materials and Methods.

shows that Rubisco_{Tk} maintains its secondary structure between 37 and 70°C. Despite the large decameric structure of Rubisco_{Tk}, Lon_{Tk} could degrade the protein in the presence of ATP at 70°C whereas no degradation could be observed when ATP was absent (Fig. 4 F). In these experiments, 15% (0.15 mM) of ATP was confirmed to have remained in the mixture even after the reaction at 70°C for 8 h, since we observed a gradual decrease in the rate of ATP hydrolysis during the incubation.

In contrast to the higher peptide cleavage activity of Lon_{Tk} at a lower ATP concentration (Fig. 3C), the degradation of Rubisco_{Tk} by Lon_{Tk} was maximal at 0.5 mM ATP and the lower ATP concentrations resulted in less degradation efficiency (Fig. 5A). ATP concentrations higher than 0.5 mM also decreased the degradation, probably due to the inhibition by ADP accumulated in the reaction mixture. These results are similar to those for Lon_{Ec}, in which the optimum ATP concentration for protein degradation was 0.6 mM (25). In addi-

FIG. 5. Degradation of Rubisco_{Tk} by Lon_{Tk} with different ATP concentrations (A) and with a nonhydrolyzable analog of ATP, AMP-PNP (B), respectively. The reaction was carried out at 70°C with various concentrations of ATP (0 to 16 mM) for 10 h (A) or with 1 mM AMP-PNP for different reaction periods (B), as indicated in each panel. The mixtures were analyzed by SDS-PAGE as described in Materials and Methods.

tion, Fig. 5B shows that the degradation was not observed when AMP-PNP was added instead of ATP. The results obtained here indicated that coupled hydrolysis of ATP was necessary for degradation of folded proteins by Lon_{Tk}, whereas peptide and unfolded protein substrates could be cleaved by Lon_{Tk} in an ATP-independent manner.

To clarify the peptide bond specificity of Lon_{Tk}, identification of the degradation products from α -casein (enzymatically dephosphorylated) was examined. After the degradation by Lon_{Tk} in the presence of ATP at 70°C for 30 min, the reaction mixture was applied to matrix-assisted laser desorption/ionization–time of flight mass spectroscopic analysis. The molecular masses of the products ranged from 771.5 to 2,461.4 Da (data not shown), indicating that Lon_{Tk} degrades the protein into short peptides (6 to 20 aa), like other known ATP-dependent proteases. We further applied electron-spray ionization–time of flight mass spectroscopy/mass spectroscopic analysis and could determine the sequence of one product [m/z 614.4 (2^+)] as NPWDQVKR_{NA}, an α -S2-casein peptide encompassing aa 107 through 116. This result indicated that Lon_{Tk} cleaved the substrate at the bonds after 106Leu and 116Ala, and these preferences were the same as those reported for Lon_{Ec} (28).

DISCUSSION

As seen in other Lon proteases from various sources, Lon_{Tk} possessed a two-domain structure consisting of N-terminal ATPase and C-terminal protease domains. Although the ATPase domain of Lon_{Tk} showed low overall similarity to those of its bacterial and eucaryal counterparts, it still belonged to the AAA⁺ superfamily, possessing several conserved motifs such as the nucleotide-binding Walker A and B (34). A region including the sensor 2 motif in *E. coli* Lon and Clp proteases has recently been proposed to be a sensor and substrate discrimination domain for recognition and binding to their specific substrates (40, 54). This region was also conserved in Lon_{Tk} and might be involved in the discrimination of unknown specific substrates in *T. kodakaraensis* KOD1. Another candidate for the discrimination region in Lon_{Ec} is a predicted

coiled-coil structure in the N-terminal region (approximately 310 aa) flanked to the AAA⁺ module (7), but such an additional sequence was not found in Lon_{Tk}. One significant difference in Lon_{Tk} from other known Lon proteases is the presence of two possible transmembrane regions within the ATPase domain, and indeed, Lon_{Tk} was revealed to be located in the membrane of *T. kodakaraensis* KOD1 (Fig. 2 B). This protein shared considerably high homology to putative Lon proteins identified in archaea, and the presumable transmembrane sequences were seen in all archaeal Lon. HslU, an ATPase component of another bacterial ATP-dependent protease, also has a large intermediate domain (134 aa) between the Walker A and B motifs. These intermediate domains, supposed to participate in the binding to protein substrates, are extended outward from the complex (41, 50). The common position of these insertion sequences suggests that the transmembrane regions of archaeal Lon proteases may also project outward from the enzyme core, forming structures for anchoring to the membrane. Bacteria also possess a different type of ATP-dependent protease, FtsH, that is a zinc metalloprotease and a membrane-bound protein anchored at the N-terminal region (37, 46). Although the positions of transmembrane segments and catalytic mechanisms were different between archaeal Lon and bacterial FtsH, Lon might play important roles in the quality control of the membranes of archaeal cells, like FtsH in bacterial cells (1, 23). In contrast to the ATPase domain, the protease domain of Lon_{Tk} showed relatively high similarity to other Lon from bacteria and eucarya.

Beyond our expectation, Lon_{Tk} exhibited activity to cleave fluorogenic peptides in the absence of any nucleotides higher than that in the presence of nucleotides, a remarkable catalytic difference between Lon_{Tk} and other known Lon proteases. The peptide cleavage activity was maximal when ATP was absent but diminished with increases in ATP or AMP-PNP concentration. These results demonstrate that Lon_{Tk} already had an open conformation without nucleotide binding. Lon_{Tk} also possessed thermostable ATPase activity, and this activity was much higher than the peptide cleavage activity of this enzyme.

FIG. 6. Schematic model of the function of Lon_{Tk} in an archaeal membrane in the absence of ATP (A) and in the presence of ATP (B).

Not only did the ATPase activity of Lon_{Tk} require divalent cations but so did the ATP-independent peptide cleavage activity of Lon_{Tk}, indicating two independent roles for the cations. As previously proposed (11, 29), these metal cations must serve as a factor that contributes to proteolytic activity, in addition to their function as a cofactor for ATP binding.

We further examined the proteolytic activity of Lon_{Tk} with three protein substrates. Schematic models of the action of Lon_{Tk} in the archaeal membrane are presented in Fig. 6. Lon_{Tk} could degrade proteins that lost their secondary structures without consumption of ATP (Fig. 6A), while it required ATP for the degradation of proteins maintaining their original secondary structures (Fig. 6B). The degradation of folded proteins occurred in an energy-dependent manner, since nonhydrolyzable AMP-PNP could not support the degradation. It has been recently clarified that ATPase components in ATP-dependent proteases are molecular chaperones functioning in the unfolding of protein structures (15, 19, 20, 39, 48, 55). As for Lon_{Tk}, ATP hydrolysis was required for the degradation of protein substrates with folded structures, such as hemoglobin at 37°C or Rubisco_{Tk}. It should be noted that the *in vitro* degradation required a long incubation period. Although this low degradation efficiency is probably due to the fact that they are not native substrates for Lon_{Tk}, our results suggested a specific role for ATP hydrolysis in the disruption of the structures of folded protein substrates, a role shared by the ATPase components of other ATP-dependent proteases. The nucleotide binding might induce some conformational change in Lon_{Tk} for the degradation of substrate proteins with folded structures, when intracellular ATP concentration is sufficient.

On the other hand, Lon_{Ec} requires ATP hydrolysis for efficient degradation of α -casein, a protein without a specific secondary structure (12), indicating that ATP hydrolysis is still needed for the translocation of large unfolded proteins to the proteolytic sites. Unlike Lon_{Ec}, Lon_{Tk} showed ATP-independent proteolytic activity against not only peptide substrates but also large protein substrates lacking secondary structure (Fig. 4B and D). These facts point out a further unique property of Lon_{Tk}; that is, the translocation of unfolded polypeptide chains to proteolytic sites did not require ATP hydrolysis. In the

crystal structures of bacterial HslUV (4, 41, 50) and ClpP (49), the entrance pore from the ATPase ring to the proteolytic chamber is very small (<10 Å), possibly leading to the ATP requirement for translocation of already unfolded substrates through the small pore. In contrast, the ATP-independent translocation in Lon_{Tk} suggested easier access of unfolded proteins to the proteolytic core without energy consumption. One of the possibilities accounting for the higher accessibility might be a larger entrance pore in Lon_{Tk} than that in Lon_{Ec}.

We here reported the first characterization of an archaeal Lon protease. Lon_{Tk} was a novel membrane-bound protease, and the presence of putative transmembrane helices is a common feature among archaeal Lon orthologs. It can be presumed that archaeal Lon degrades specific short-lived proteins in addition to abnormal proteins, as well as Lon (14) and FtsH (37) from *E. coli*. Identification of the specific substrates will help to better understand the important roles of Lon in archaeal cells.

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