Structural and Functional Conversion of Molecular Chaperone ClpB from the Gram-Positive Halophilic Lactic Acid Bacterium *Tetragenococcus halophilus* Mediated by ATP and Stress$^V$

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In this study, we report the purification, initial structural characterization, and functional analysis of the molecular chaperone ClpB from the gram-positive, halophilic lactic acid bacterium *Tetragenococcus halophilus*. A recombinant *T. halophilus* ClpB (ClpB<sub>tha</sub>) was overexpressed in *Escherichia coli* and purified by affinity chromatography, hydroxypapitate chromatography, and gel filtration chromatography. As demonstrated by gel filtration chromatography, chemical cross-linking with glutaraldehyde, and electron microscopy, ClpB<sub>tha</sub> forms a homohexameric single-ring structure in the presence of ATP under nonstress conditions. However, under stress conditions, such as high-temperature (>45°C) and high-salt concentrations (>1 M KCl), it dissociated into dimers and monomers, regardless of the presence of ATP. The hexameric ClpB<sub>tha</sub> reacted with heat-aggregated proteins dependent upon the DnaK system from *T. halophilus* (KJE<sub>tha</sub>) and ATP. Interestingly, the mixture of dimer and monomer ClpB<sub>tha</sub>, which was formed under stress conditions, protected substrate proteins from thermal inactivation and aggregation in a manner similar to those of general molecular chaperones. From these results, we hypothesize that ClpB<sub>tha</sub> forms dimers and monomers to function as a holding chaperone under stress conditions, whereas it forms a hexamer ring to function as a disaggregating chaperone in cooperation with KJE<sub>tha</sub> and ATP under poststress conditions.

The bacterial heat shock protein ClpB and its eukaryotic homolog HSP104 belong to a class of molecular chaperones whose expressions are strongly induced by various types of stress. The structure and function of ClpB have been well characterized in *Escherichia coli* and *Thermus thermophilus*. ClpB contains two nucleotide-binding domains (NBD1 and NBD2) that are separated by a middle domain forming a coiled-coil structure (1, 2, 17, 31, 34). Moreover, ClpB forms a hexameric single-ring structure and cooperates with the DnaK chaperone system (DnaK, DnaJ, and GrpE; termed KJE) in the solubilization and refolding of aggregated proteins; these reactions are dependent on the presence and hydrolysis of ATP (3, 12, 19, 20, 33). In these reactions, ClpB and KJE may act sequentially or simultaneously. Although the protein disaggregation mechanism mediated by the DnaK-ClpB bichaperone system is still under discussion, Bukau and colleagues recently provided direct evidence for the mechanism (26, 32). One study demonstrated that aggregated proteins are solubilized by the continuous extraction of unfolded polypeptides dependent upon the DnaK-ClpB bichaperone system and not by the fragmentation of large aggregates (26). Here, the middle domain of ClpB is thought to play a crucial role in the initial disaggregation reaction. The other study reported that aggregated proteins are translocated through the central pore of the ClpB hexameric ring and then refolded by KJE (32). However, it was reported that, unlike other chaperones, the ClpB homologs were unable to prevent the aggregation of denatured proteins (11).

In gram-negative bacteria and eukaryotes, the disaggregating activities of ClpB and HSP104 have been reported to be important for resistance to high-temperature stress, cold acclimation, and induced thermotolerance to lethal stress (8, 9, 12, 24). In gram-positive bacteria, a few reports have described the in vivo functions of ClpB: (i) the mutation of clpB did not affect the resistance of *Lactococcus lactis* MG1363 to high-temperature, salt, and puromycin stress (13), and (ii) ClpB was required for the induced thermotolerance and virulence of *Listeria monocytogenes* (4). However, the in vitro characterization of ClpB from gram-positive bacteria has yet to be accomplished.

*Thetragenococcus halophilus* is a moderately halophilic gram-positive lactic acid bacterium with a NaCl optimum of approximately 2 M and an upper limit of approximately 4 M; it is currently exploited in the brewing of Japanese soy sauce (5). In a previous study, we cloned a dnaK gene of *T. halophilus* and confirmed that the expression of the dnaK gene was induced by salt stress as well as by heat stress (10). Moreover, the in vitro

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and in vivo characterization of the DnaK proteins was also performed under various salinity conditions (28). Because, as mentioned above, DnaK and ClpB are known to form a bichaperone system that efficiently mediates the ATP-dependent reactivation of aggregated proteins, we have a considerable interest in the biochemical properties of ClpB and the DnaK-ClpB bichaperone system in *T. halophilus*.

In this study, we cloned the *clbP* (*clpB*-*tha*) gene of *T. halophilus* and characterized its product overexpressed in *E. coli*. The purified ClpB*Tha* protein formed a hexameric single-ring structure in the presence of ATP and, in cooperation with the *T. halophilus* DnaK chaperone system (*KJE*-*Tha*), reactivated heat-aggregated proteins. Under stress conditions, it dissociated into dimers and monomers, and the resultant low molecular species protected substrate proteins from thermal denaturation and subsequent aggregation. These results provide a novel insight into the structure-function relationship of ClpB under both stress and poststress conditions.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** The *T. halophilus* JCM8588 and *E. coli* strains used in this study (Table 1) were grown as previously described (10, 28, 29). The *T. halophilus* was grown at 30°C in MRS medium (Oxoid, Hampshire, England) containing 1 M NaCl. *E. coli* JM109 (Promega, Madison, WI) and BL21 (DE3) (Invitrogen, Carlsbad, CA) were grown at 37°C with shaking in LB broth. When the growth was appropriate for clonal selection, *BL21 (DE3) (Invitrogen, Carlsbad, CA)* were grown at 37°C with shaking in Luria-Bertani (LB) broth. When the growth was appropriate for clonal selection, *BL21 (DE3) (Invitrogen, Carlsbad, CA)* were grown at 37°C with shaking in Luria-Bertani (LB) broth.

**TABLE 1. Strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Characteristic(s)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. halophilus</em> JCM8588</td>
<td>Wild type</td>
<td>This work</td>
</tr>
<tr>
<td><em>E. coli</em> JM109</td>
<td>recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 (lac-proAB) [F’ traD36 proAB’ lacIq lacZ M15]</td>
<td>Invitrogen</td>
</tr>
<tr>
<td><em>E. coli</em> BL21(DE3)</td>
<td>F’ompT hsdR34(a-tha-1 the-1 dcm)</td>
<td>Invitrogen</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
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<td></td>
</tr>
<tr>
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<td>Amp’</td>
<td>Promega</td>
</tr>
<tr>
<td>pET100/D-TOPO</td>
<td>Directional TOPO expression vector</td>
<td>Amp’</td>
</tr>
<tr>
<td>pClpB<em>Tha</em>-M</td>
<td>Partial gene encoding the middle region of <em>T. halophilus</em> ClpB cloned in pGEM-T easy</td>
<td>Amp’</td>
</tr>
<tr>
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<td>Partial gene encoding the N-terminal region of <em>T. halophilus</em> ClpB cloned in pGEM-T easy</td>
<td>Amp’</td>
</tr>
<tr>
<td>pClpB<em>Tha</em>-C</td>
<td>Partial gene encoding the C-terminal region of <em>T. halophilus</em> ClpB cloned in pGEM-T easy</td>
<td>Amp’</td>
</tr>
<tr>
<td>pClpB<em>Tha</em></td>
<td><em>T. halophilus clpB</em> gene cloned in pET100/D-TOPO</td>
<td>Amp’</td>
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**Purification of the ClpB*Tha* protein.** The ClpB*Tha* protein containing the His tag was purified by nickel affinity chromatography (10, 28, 29). Since the ClpB*Tha* protein was PCR amplified from *T. halophilus* genomic DNA with two oligonucleotide primers, *clpB*-*Tha*-ds1 and *clpB*-*Tha*-ds2, the amplified fragment was cloned into an *E. coli* expression vector, pET100/D-TOPO (Invitrogen), according to the manufacturer’s instructions. The vector pET100/D-TOPO carried a His tag, which was thus incorporated in the protein at the N terminus of the *clpB*-*Tha* gene product. The constructed plasmid, named pClpB*Tha*, (Table 1), was transformed into *E. coli* BL21(DE3). The overexpression of ClpB*Tha* was performed as previously reported (10, 28, 29). The *E. coli* cells were grown to an optical density at 600 nm in LB medium containing 50 μg/ml ampicillin at 37°C of 0.5, and then IPTG was added to a concentration of 1 mM. After a further 3 h at 37°C, the cells were harvested by centrifugation and stored at −80°C.

**Protease sensitivity assay.** Prior to the addition of trypsin, 10 μl of purified ClpB*Tha* protein (1 μM) was preincubated in Tris-HCl buffer (pH 7.4) containing 100 mM KCl, 20 mM MgCl2, and 1 mM dithiothreitol (DTT) for 10 min on ice with and without 50 mM ATP or ADP. Trypsin (Sigma) was then added to the reaction mixtures to a final concentration of 10 μg/ml, and the mixtures were incubated at 37°C. At the indicated incubation time (from 0 to 60 min), the reactions were quenched by the addition of 5 μl of SDS-Polyacrylamide gel
electrophoresis (PAGE) sample buffer containing Tris-HCl (pH 8.0), glycerol, 1% (wt/vol) 2-mercaptoethanol, 1% (wt/vol) SDS, and 0.1% (wt/vol) bromophenol blue. Samples were separated by electrophoresis using 12% SDS-polyacrylamide gels. The gels were stained with Coo massie brilliant blue (CBB) R-250 (Nacalai Tesque).

**Gel filtration chromatography analysis.** Twenty-five micromolar, purified ClpB_{Tha} protein was incubated for 10 min on ice with or without 2 mM ATP, pH 7.4. The samples (50 μl) were centrifuged at 4°C for 15 min before loading onto a TSK G4000SW XL gel filtration column (Tosoh, Tokyo, Japan). The column was developed with 50 mM Tris-HCl buffer (pH 7.4) containing 100 mM KCl, 20 mM MgCl₂, 1 mM DTT, and 2 mM ATP (if required), with a flow rate of 0.5 ml/min, and monitored by absorbance at 290 nm.

The apparent molecular masses of ClpB_{Tha} proteins, including thyroglobulin (669,000 Da), apoferritin (440,000 Da), alcohol dehydrogenase (150,000 Da), and BSA (60,000 Da), were calculated using a molecular weight standard kit (Sigma).

**Chemical cross-linking.** The purified ClpB_{Tha} protein was dialyzed against 50 mM Tris-HCl (pH 7.4) containing 100 mM KCl, 20 mM MgCl₂, and 1 mM DTT. Forty-microliter samples (2.5 μM) were incubated on ice for 1 h after the addition of 5 mM ATP, ADP, AMP, or adenosine 5'-O-(thiotriphosphate) (ATP~S), a nonhydrolyzable ATP analog. Cross-linking was initiated by the addition of 0.1% glutaraldehyde and terminated by the addition of 10 μl of SDS-PAGE sample buffer after incubation at 30°C for 20 min. The samples were boiled for 5 min and resolved by electrophoresis on a 3 to 10% SDS-polyacrylamide gradient gel. The gels were stained with CBB R-250.

**Electron microscopy.** The molecular shape of the ClpB_{Tha} protein was examined by negative staining using 0.5% uranyl acetate in a 1% gluteraldehyde electron microcope (JEOL, Tokyo, Japan) at 100 kV (29). Since the ATP-induced oligomer was unstable and readily dissociated during the preparation of samples for electron microscopy, the oligomer was cross-linked with 0.1% glutaraldehyde.

**Reactivation of heat-inactivated protein.** One micromolar lactate dehydrogenase (LDH) from Leuconostoc mesenteroides (Oriental Yeast, Osaka, Japan) was thermally denatured at 50°C for 15 min. The denatured LDH protein was divided into soluble and insoluble fractions by centrifugation at 20,000 × g for 30 min, and each fraction was analyzed by SDS-PAGE. Since LDH was detected in only the insoluble fraction, the heat-treated LDH was used as an aggregated protein substrate. Aggregated LDH was diluted 10-fold with 50 mM Tris-HCl buffer (pH 7.4) containing 100 mM KCl, 20 mM MgCl₂, and 1 mM DTT in the presence or absence of KJE_{Tha} (Dnak, 2 μM/Dnaf, 0.5 μM/Grpe, 0.25 μM), ClpB_{Tha} (2 μM), and ATP (5 mM) and was incubated at 30°C. Aliquots were taken up at different time points and tested for LDH activity.

**Prevention of the denaturation and aggregation of substrate proteins.** LDH (100 nM) was incubated at 45°C in 50 mM Tris-HCl buffer (pH 7.4) containing 100 mM KCl, 20 mM MgCl₂, and 1 mM DTT in the presence or absence of ClpB_{Tha} (0.5 μM). Aliquots were withdrawn at different time points and tested for residual LDH activity at 30°C as previously described (28). The activity measured just before heating was defined as 100%. BSA (1 μM) was used instead of ClpB_{Tha} as a control.

**Yeast enolase (1 M, Sigma) was incubated at 50°C for 1 h in 50 mM Tris-HCl buffer (pH 7.4) containing 20 mM KCl, 10 mM Mg acetate, and 2 mM DTT, in the presence or absence of ClpB_{Tha} (0.5 μM). After incubation, samples were centrifuged at 20,000 × g for 30 min and divided into soluble and insoluble fractions. Each fraction was analyzed by 12% SDS-PAGE.**

**E. coli proteins (1.5 mg/ml) were incubated at 50°C in 50 mM Tris-HCl buffer (pH 7.4) containing 100 mM KCl, 20 mM MgCl₂, and 1 mM DTT in the presence or absence of ClpB_{Tha} (0.5 μM). Aggregation was monitored for 10 min as the increase in absorbance at 320 nm.**

**RESULTS**

Cloning and nucleotide sequence analysis of the clpB locus in *T. halophilus* JCM5888. The highly conserved amino acid sequences of the ClpB proteins from *L. lactis* subsp. *lactis* IL-1-1403, *E. faecalis* V583, and *S. mutans* UA159 were used to construct the degenerative primers for the identification of a putative clpB gene in the *T. halophilus* JCM5888 genome. In this way, a single open reading frame encoding an 872-amino-acid protein with a predicted molecular mass of 98,536 Da was identified. The protein exhibits high similarities to the ClpB proteins from a wide variety of bacteria, and the highest similarity (84.4%) is to that from *E. faecalis*. Upstream of clpB, a putative promoter region was found with a −35 sequence, 5'-TTAACA-3', and a −10 sequence, 5'-TATAC-3'. On the other hand, a deduced rho-independent transcriptional terminator region, which had a free energy of −34.5 kcal · mol⁻¹, was identified immediately downstream of clpB. By comparing putative regulatory elements for clpB genes from various gram-positive bacteria, we identified a putative CtsR binding site in the *clpB* locus from *T. halophilus*. CtsR is a negative regulator of class III heat shock genes and is proposed to bind to a consensus sequence containing a direct repeat hepta-nucleotide sequence separated by three nucleotides: A/GGTCAAA/ANA/GGTCAAA (6), where the italic type indicates a direct repeat. The CtsR binding site located on the *clpB* gene of *T. halophilus* overlapped with the −35 promoter region and had an orientation inverse to that of the consensus sequence. TTT GACCAATTTTACCC (where the italic type indicates a direct repeat), similar to that of *L. lactis* clpB and clpP (30). These results suggest that the expression of the *clpB* gene is regulated by CtsR in a manner similar to those of other gram-positive bacteria.

The amino acid alignment with various bacterial ClpB homologs demonstrated that ClpB_{Tha} possesses two NBD, NBD1 (amino acids 183 to 409) and NDB2 (amino acids 548 to 729), each harboring both Walker A and B motifs characterized by Walker-type ATPase (data not shown). The two NBDs are separated by a spacer-middle domain of 139 amino acids (410 to 547) that has the potential to form a coiled-coil structure.

**Nucleotide-induced oligomerization of ClpB_{Tha}.** The ClpB homologs are known to undergo conformational changes following the addition of ATP (1, 2, 17, 22, 31, 34). In order to obtain similar information for ClpB_{Tha}, we examined the effect of ATP on the conformational changes of ClpB_{Tha} by means of protease digestion. ClpB_{Tha} was incubated in the presence and absence of ATP or ADP for several minutes at 37°C and then analyzed by SDS-PAGE. In the absence of nucleotides, ClpB_{Tha} was digested into several 30- to 70-kDa fragments and the amount of intact ClpB_{Tha} was significantly decreased, as shown in Fig. 1A. In the presence of ATP or ADP, ClpB_{Tha} was relatively protected and a large proportion of the ClpB_{Tha} remained intact. These results indicate that ClpB_{Tha} undergoes conformational changes in the presence of ATP or ADP.

In order to determine whether the changes in the proteolytic sensitivity of ClpB_{Tha} are due to changes in the oligomeric states, the purified ClpB_{Tha} was subjected to analytical gel filtration chromatography by using a TSK G4000SW XL column in the presence or absence of ATP. In the absence of ATP, ClpB_{Tha} was eluted at the position of a 200-kDa standard (Fig. 1B) and the peak was tailed. Thus, in the absence of ATP, ClpB_{Tha} forms both dimers and monomers. In the presence of ATP in the running buffer, the elution position of ClpB_{Tha} was shifted dramatically and slightly behind a 669-kDa standard, indicating that ClpB_{Tha} apparently forms a hexamer in the presence of ATP (Fig. 1B). In the absence of ATP in the running buffer, a preincubated ClpB_{Tha} with ATP was also analyzed by analytical gel filtration chromatography. ClpB_{Tha} was eluted at the position of ca. 200 kDa with a long tail, and the bound ATP was released from ClpB_{Tha} inside the column (data not shown); this suggests that the ClpB_{Tha} hexamer dis-
associates into dimers and monomers under conditions of ATP depletion.

Since mobility in gel filtration chromatography is often influenced by the shape of the protein, cross-linking procedures were used as an alternative method to examine the oligomeric state of ClpB$_{Tha}$ on an SDS-polyacrylamide gel (Fig. 1C). The cross-linked monomer band migrated rapidly compared to the non-cross-linked one; this is probably a consequence of the intramolecular cross-linking described previously (22). The ClpB$_{Tha}$ cross-linked in the absence of ATP revealed two bands corresponding to dimer (ca. 200 kDa) and monomer (ca. 100 kDa) subunits. On the other hand, the subunits of ClpB$_{Tha}$ are cross-linked to form a hexamer (ca. 600 kDa) in the presence of ATP. Under these conditions, other oligomers, such as heptamers and pentamers, were not detected. We also examined the effects of ADP, AMP, and ATP$_{γ}$S (a nonhydrolyzable ATP analog) on hexamer formation. ADP and ATP$_{γ}$S induced hexamerization, whereas AMP did not.

In order to further analyze the oligomeric structure of ClpB$_{Tha}$, we performed electron microscopy analysis using a purified protein after cross-linking reactions in the presence or absence of ATP. The electron micrograph of the negatively

FIG. 1. Effect of nucleotides on the conformational change of ClpB$_{Tha}$. (A) Proteolytic sensitivity of ClpB$_{Tha}$ in the presence and absence of ATP or ADP. Ten microliters of purified ClpB$_{Tha}$ (1 μM) was incubated at 37°C for the indicated periods with 100 ng of trypsin either in the presence or absence of 5 mM ATP or ADP. The resultant fragments were separated by electrophoresis with a 12% SDS-polyacrylamide gel and stained by CBB R-250. The numbered bars to the left of the figure indicate the migration positions of molecular mass markers in kilodaltons. (B) The oligomerization of ClpB$_{Tha}$ was analyzed by gel filtration chromatography. Samples (25 μM) were preincubated without (i) or with (ii) 2 mM ATP and subjected to gel filtration chromatography using the running buffer without ATP. Sample preincubated without ATP was also subjected to gel filtration chromatography using the running buffer with 2 mM ATP (iii). In all cases, elution was monitored by the absorbance at 290 nm (ABS$_{290}$). (C) The oligomeric structure of ClpB$_{Tha}$ was analyzed by cross-linking. ClpB$_{Tha}$ (2.5 μM) was cross-linked at 30°C with 0.1% glutaraldehyde for 20 min in the presence and the absence of 5 mM indicated nucleotides. Cross-linking reaction was terminated by the addition of SDS-PAGE sample buffer. Samples were resolved by electrophoresis on a 3 to 10% SDS-polyacrylamide gradient gel. The gels were stained with CBB R-250. Cross-linked phosphorylase B proteins (Sigma) were also resolved as a high-molecular-weight marker. (D) ClpB$_{Tha}$ (2.5 μM) was incubated in the absence of ATP on ice for 1 h and cross-linked with 0.1% glutaraldehyde for 20 min. The cross-linking reaction was terminated by the addition of 1 M glycine. Oligomeric structure was visualized by electron microscopy. (E) Oligomeric structure of ClpB$_{Tha}$ in the presence of 5 mM ATP was also analyzed by electron microscopy as described in Fig. 1D.
stained ClpB<sub>T</sub> in the absence of ATP exhibited small amorphous particles as shown in Fig. 1D, while that in the presence of ATP revealed ring-shaped structures (Fig. 1E); this is consistent with the observations of other bacterial and eukaryotic ClpB homologs (1, 2, 14, 17, 31, 34). Although the resolution of the electron micrograph was not sufficiently high to determine the number of subunits, taking into consideration the sequence similarity of the protein to those characterized in other bacterial systems and the abovementioned results of gel filtration chromatography and the cross-linking experiment (Fig. 1B and C), it is highly likely that the ClpB<sub>T</sub> was assembled into a hexamer in the presence of ATP or ADP.

Taken together, these observations suggest that the binding of ATP or ADP is involved in the hexamerization of ClpB<sub>T</sub> and the removal of these molecules results in the dissociation of the complex into dimeric and monomeric structures.

**Effects of temperature and salt concentration on the ATP-induced hexamerization of ClpB<sub>T</sub>**

The oligomeric structures of ClpB homologs have often been analyzed under nonstress conditions (1, 2, 14, 17, 31, 34); however, to date, little is known about these structures under stress conditions. To address this issue, we examined the effects of temperature and salinity on the stability of the ATP-induced ClpB<sub>T</sub> hexamer by a cross-linking procedure.

Figure 2A shows the effects of temperature on the hexamerization of ClpB<sub>T</sub>. After 30 min of cooling at 4°C or heat treatment at 30°C or 40°C in the presence of ATP, a single band corresponding to the hexamer was detected. On the other hand, the intensity of the hexamer band decreased and low molecular bands, indicating dimer and monomer subunits, appeared after an incubation at 45°C, even in the presence of ATP. At temperatures of 50°C or more, the ATP-induced hexamer was completely dissociated into dimers and monomers; similar dissociation was also observed in the case of the ADP-induced hexamer (data not shown). ClpB<sub>T</sub> remained folded at 55°C, which was confirmed by circular dichroism spectra measurements (data not shown), indicating that heat stress at 45°C or more did not change the secondary structure of ClpB<sub>T</sub>; however, heat stress does dissociate an ATP-induced hexamer into dimers and monomers.

Figure 2B shows the effects of salt concentration on the ATP-induced hexamerization of ClpB<sub>T</sub>. Because it has been reported (23) that *T. halophilus* accumulates high concentrations of potassium ions, more than 1 M in the cell, we examined the effects of 1 to 2 M KCl on the oligomeric state of ClpB<sub>T</sub>. In the presence of KCl at concentrations of 1 M or more, regardless of the presence of ATP, a hexameric structure could not be detected, while dimeric and monomeric structures became predominant (Fig. 2B). Similar dissociations were observed in the presence of 1 M NaCl instead of KCl and in the case of the ADP-induced hexamer (data not shown). The results of the circular dichroism spectra analysis demonstrated that ClpB<sub>T</sub> was not unfolded even at high concentrations of KCl beyond 1 M (data not shown).

Since *T. halophilus* accumulates large amounts of compatible solutes as well as potassium ions when grown in medium containing such organic compounds (23), we analyzed the effects of glycine betaine, a known compatible solute, on the oligomerization of ClpB<sub>T</sub> (Fig. 2C). Although, as mentioned above, high concentrations of KCl dissociated the ATP-induced hexamer, glycine betaine counterbalanced the dissociation effect. Glycine betaine at a concentration of 1 M completely stabilized the ATP-induced hexamer, even in the presence of 1 M KCl. These results indicate that charge screening is responsible for the change in quaternary structure and that such changes can be prevented by the presence of glycine betaine.

Gel filtration analysis was also conducted to verify the results
of the cross-linking (Fig. 2) and showed that the ClpB$_{Tha}$ oligomer was dissociated at 50°C or in the presence of 0.5 M KCl; its status was not affected at various protein concentrations ranging from 2.5 to 20 μM (data not shown). Therefore, we conclude that the ATP-induced hexamer dissociates into dimers and monomers under heat stress or salt stress (without glycine betaine) conditions.

**Renaturation of heat-aggregated proteins by the DnaK-ClpB bichaperone system.** The hexameric ClpB homologs have been well characterized and have been demonstrated to cooperate with the DnaK system (KJE) in the solubilization and reactivation of aggregated proteins (3, 12, 19, 20, 33). In order to confirm whether ClpB$_{Tha}$ possesses the ClpB-specific disaggregation activity, we performed a reactivation assay using heat-aggregated LDH as a substrate protein (Fig. 3). The LDH activity was not recovered spontaneously. KJE$_{Tha}$ or ClpB$_{Tha}$ alone exhibited very little recovery of activity. Only when incubated in the presence of both KJE$_{Tha}$ and ClpB$_{Tha}$ was the aggregated LDH significantly renatured; its activity reached 23% of the initial value after a 3-h incubation at 30°C. However, the recovery of LDH activity by the DnaK$_{Tha}$-ClpB bichaperone system was not observed in the absence of ATP. These results indicate that ClpB$_{Tha}$ forms a hexamer in the presence of ATP and, in cooperation with KJE$_{Tha}$, reactivates aggregated proteins. Here, we demonstrate that ATP is required for both the hexamerization of ClpB$_{Tha}$ and the disaggregation activity of the bichaperone system.

**Effect of ClpB$_{Tha}$ on the denaturation and aggregation of substrate proteins.** In order to assess the intrinsic chaperone activities of the dimeric and monomeric ClpB$_{Tha}$, which to date have not been reported in ClpB homologs, we tested the protection of LDH from thermal inactivation in vitro. BSA, known as a protective agent for enzymes, did not significantly protect LDH from thermal inactivation at 45°C (Fig. 4A). On the contrary, ClpB$_{Tha}$ remarkably suppressed the inactivation in the absence of ATP, suggesting that the dimers and monomers of ClpB$_{Tha}$ protect the proteins from irreversible denaturation leading to aggregation.

We then tested the suppression of substrate thermal aggregation using enolase as a substrate. Enolase was incubated at 50°C for 1 h and then separated into supernatant and precipitate fractions by centrifugation; each fraction was analyzed by SDS-PAGE (Fig. 4B). When enolase was incubated alone or with BSA, it formed large aggregates and was precipitated. Incubation of enolase with ClpB$_{Tha}$ in the absence of ATP resulted in the decrease of aggregated enolase and, consequently, large amounts of enolase remained in the soluble fraction. Since we speculated that the effect of ClpB$_{Tha}$ on the
thermal aggregation of enolase might be specific for this enzyme, we used proteins extracted from E. coli as an alternative substrate (Fig. 4C). The extracted proteins were incubated at 50°C, and the aggregation was monitored by measuring the absorbance at 320 nm, the wavelength usually used for monitoring the aggregation of proteins (16, 27). When the E. coli-extracted proteins were incubated alone or with BSA, the absorbance increased gradually. On the other hand, the rate of aggregation and the maximum absorbance value at 320 nm within 10 min in the presence of ClpB$_{Th}$ were lower than those in the absence of it. These results indicate that dimer- and monomer-forming ClpB$_{Th}$ in the absence of ATP suppresses the thermal aggregation of the substrate proteins and that it possesses general chaperone activities.

**DISCUSSION**

Until now, the biochemical characterization of ClpB homologs has been conducted to determine their structures and functions (1–3, 17, 19, 20, 31, 33, 34). Despite the fact that these molecules are required for resistance to high-temperature stress, cold acclimation, and induced thermotolerance to lethal stress (8, 9, 18, 24), these in vitro studies were performed mostly under nonstress conditions. Moreover, unlike gram-negative bacteria and eukaryotes, little is known about the structure and function of gram-positive bacterial ClpBs; this is despite the fact that, with the exception of Bacillus subtilis, these molecules are carried on the genomes of all gram-positive bacteria (21). In this report, we described a novel aspect of the ClpB from the gram-positive halophilic lactic acid bacterium *T. halophilus*, that is, a structural and functional conversion mediated by ATP and stress.

As demonstrated by gel filtration chromatography, chemical cross-linking with glutaraldehyde, and electron microscopy, ClpB$_{Th}$ formed a hexameric single-ring structure in the presence of ATP, ADP, and ATPγS, while it existed as dimeric and monomeric structures in the absence of them (Fig. 1). Considering that ClpB$_{Th}$ did not hydrolyze ADP (data not shown) and that ATPγS induced hexamerization, nucleotide binding, but not its hydrolysis, was required for hexamer formation, as in the case of other characterized bacterial ClpBs (1, 14, 31, 34). Although the overall ring structure of the oligomer in the presence of ATP appears to be conserved among bacterial ClpB homologs, the effects of ADP on their oligomerization appears to differ among species. Saccharomyces cerevisiae HSP104 (22, 25) and S. cerevisiae mitochondrial HSP78 (15), the most closely related to ClpB and HSP104, formed hexamers in the presence of ADP similar to ClpB$_{Th}$ (Fig. 1). On the other hand, *E. coli* ClpB and *T. thermophilus* ClpB formed small oligomers (2- to ~5-mer) but not a hexamer in the presence of ADP (31, 34). Moreover, the oligomeric states of ClpB homologs in the absence of nucleotides also differ among species. *T. thermophilus* ClpB formed small oligomers (~5-mer) in the absence of nucleotides, while *S. cerevisiae* HSP104 and mitochondrial HSP78 formed monomers (15, 22). Interestingly, *E. coli* ClpB formed a heptamer in the absence of nucleotides and a hexamer in the presence of ATP; therefore, the binding of ATP induces a change in the self-association state of *E. coli* ClpB from that of a heptamer to that of a hexamer (1, 14). ClpB$_{Th}$ differs from those aforementioned in that it exists as a mixture of dimers and monomers (Fig. 1). Nonetheless, the hexameric ring structure of the ClpB homologs, which is believed to be the functional structure, is widely conserved from prokaryotes to eukaryotes.

Previously, the effect of temperature on the oligomerization of ClpB had been demonstrated only in *T. thermophilus* (31). *T. thermophilus* ClpB formed small oligomers (~5-mer) in the presence of ATP at 20°C, a much lower temperature than the physiological temperature for the host, while it formed a hexamer at 35°C, a temperature at which *T. thermophilus* ClpB is active as a molecular chaperone. This implies that an increase in temperature stabilizes the hexamer of *T. thermophilus* ClpB (31). In contrast, an increase in temperature in excess of 45°C, which is considered as a sublethal temperature for *T. halophilus* (10), destabilized the hexameric structure of ClpB$_{Th}$ (Fig. 2A). To our knowledge, this is the first report that describes the oligomeric structure of ClpB homologs under heat stress conditions.

The effects of high concentrations of salts on the oligomerization of ClpB homologs were also previously reported (7, 14, 25, 31). Their oligomers were destabilized when the KCl and NaCl concentrations were increased. However, the effects of these salts were counterbalanced by the presence of ATP. Surprisingly, as demonstrated by cross-linking under high-salinity conditions, ClpB$_{Th}$ formed both dimers and monomers, even in the presence of ATP (Fig. 2B). It is notable that the intracellular concentrations of potassium ions in *T. halophilus* can accumulate to levels in excess of 1 M (23). Can ClpB$_{Th}$ form hexamers in vivo under such high-salinity conditions? To address this question, we then analyzed the effect of glycine betaine on the oligomerization of ClpB$_{Th}$ in the presence of ATP and 1 M KCl. The results demonstrated that glycine betaine counterbalanced the dissociation effect of salt (Fig. 2C), suggesting that in vivo ClpB$_{Th}$ can form a hexamer in the presence of ATP. However, an excess amount of KCl com-
pared to glycine betaine induced the dissociation of the hexamer. Moreover, there was no effect of glycine betaine on the structural change of ClpB<sub>Tha</sub> under heat stress conditions (Fig. 5). Taken together, our observations indicate that the ATP-induced oligomerization of ClpB<sub>Tha</sub> was affected by the surrounding environmental conditions, such as a high temperature and the salt concentration.

Disaggregation activities are widely conserved among the ClpB homologs. In fact, ClpB<sub>Tha</sub> also reactivated heat-aggregated LDH in an ATP-dependent cooperation with the DnaK<sub>Tha</sub> system (Fig. 3). Interestingly, ClpB<sub>Tha</sub> exhibited general chaperone activities, such as the protection of substrates from both thermal inactivation and aggregation, in the absence of ATP (Fig. 4). Under these conditions, as demonstrated by cross-linking (Fig. 2A), ClpB<sub>Tha</sub> formed dimers and monomers. After the dissociation of the hexamer into dimers and monomers, these species appear to expose the hydrophobic regions and substrate binding sites hidden inside the ring; this might enable the molecules to interact with unfolded proteins. This behavior may be similar to that of other chaperones, including the DnaK and GroEL systems. Why, nevertheless, does ClpB<sub>Tha</sub> possess intrinsic chaperone activities? One possible explanation is that ClpB<sub>Tha</sub> compensates for the impaired function of the DnaK<sub>Tha</sub> system whose in vitro chaperone activity is lower than that of the E. coli system (S. Sugimoto et al., unpublished data). The independent and/or cooperative functions of the ClpB<sub>Tha</sub> and DnaK<sub>Tha</sub> systems are now under investigation in vivo.

Based on the results presented in this study, we propose the following model for the structural and functional conversion of ClpB<sub>Tha</sub> (data not shown). Under stress conditions, particularly at high temperatures, intracellular labile proteins might be easily denatured and form aggregates. Accumulations of aggregates formed in response to stress would be repaired by the ClpB<sub>Tha</sub>-chaperone system. This facilitates a rapid first defense against stress, including heat shock and stress recovery. The ClpB<sub>Tha</sub> system provides a unique insight into the role of ClpB chaperone-mediated protein-disaggregation under stress.

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