Involvement of Bacillus subtilis ClpE in CtsR Degradation and Protein Quality Control

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The heat-inducible CtsR regulon of Bacillus subtilis codes for three Clp proteins with chaperone or protease activity. While the importance of ClpC and ClpP has been elucidated for a wide range of cellular adaptation processes, this study deals with the physiological role of B. subtilis ClpE. Northern experiments and reporter gene analyses revealed that ClpE is essential both for efficient CtsR-dependent gene derepression and for repression under conditions beyond the thermal optimum. This leads to the formation and accumulation of toxic protein aggregates that can be visualized as electron-dense particles by electron microscopy. The comparison of radiolabeled aggregated protein fractions of wild-type and clpE mutant cells during heat stress displayed a significant delay of protein disaggregation in the absence of ClpE. A kinetic Western blotting approach confirmed the long-term residence of ClpE in the insoluble cell fraction rather than in the cytoplasmic fraction. These observations indicate the involvement of ClpE in global protein disaggregation. As a characteristic structural element of ClpE, the N-terminal zinc finger domain was proven to be essential for basal in vitro ATPase activity.

Heat stress adaptation is one of the best-characterized physiological stress responses of mesophilic bacteria. A sudden thermal upshift has various severe consequences for the cellular system. One important issue is the immediate aggregation of cellular protein. Many vegetative proteins are not able to fold properly under conditions beyond the thermal optimum. This leads to the formation and accumulation of toxic protein aggregates that can be visualized as electron-dense particles by electron microscopy (24, 32). For disaggregation and protection of protein folding under heat stress, different groups of heat shock proteins (HSPs) are strongly induced. Functionally, the HSPs are mainly organized in oligomeric complexes that may act as molecular chaperones or proteolytic systems (13). Many of the HSPs are members of the AAA+ superfamily that comprises all ATPases associated with a variety of cellular activities (8, 30). Depending on the sizes of the formed complexes, the HSPs are divided into several families. Members of the HSP100 family represent proteins that form the largest chaperone and protease complexes in bacterial cells. Among them are the Clp (caseinolytic protease) proteins that are widely distributed among pro- and eukaryotes and commonly show a broad activity spectrum.

The Bacillus subtilis ClpP protease was shown to act centrally in global protein quality control (21). A clpP mutant shows a pleiotropic phenotype revealing the involvement of ClpP in development of competence, motility, thermostability, degradative enzyme synthesis, and sporulation (11, 21, 27). The ClpC ATPase is the interacting partner of ClpP in many physiologically important processes such as competence gene expression or adaptation to diverse stress parameters (9, 23). Several ClpCP substrates were described previously (22, 25, 31, 39), and ClpCP-dependent degradation was shown to be mediated by adapter proteins (8, 35). Furthermore, B. subtilis ClpXP controls competence development as well as the degradation of SsrA-tagged polypeptides (29, 42).

So far, a physiological function for the B. subtilis ClpE ATPase has not been described. ClpE was originally visualized on two-dimensional gels in 1987 and was described as unknown heat shock protein Hsp1 (14). The clpE gene was discovered and annotated as a member of a novel Hsp100-type ATPase subfamily and was characterized as part of the heat-inducible CtsR regulon (7). The ClpE ATPase can be assigned to the class I of the AAA+ superfamily (34).

ClpE homologues were found in several gram-positive bacteria. In Listeria monocytogenes, ClpE is required for prolonged survival at elevated temperature, virulence, and regulation of cell septation (28). ClpE of Streptococcus pneumoniae seems to be the major thermostolerance Clp ATPase and is also partly involved in virulence (5). A detailed study of Lactococcus lactis ClpE revealed specific effects on CtsR-dependent clp expression at elevated temperature and demonstrated the essentiality of the N-terminal zinc finger domain for proper ClpE function in vivo (40). In B. subtilis, the genes coding for ClpC, ClpE, and ClpP are members of the CtsR (class three stress gene repressor) regulon (6). Regulation of CtsR activity is controlled by a fine-tuned phosphorylation mechanism including the modulators McsB and McsA and has been studied in detail (20, 25). Among the CtsR-regulated genes, clpE is most tightly repressed (7). ClpE was found to be a very short-lived protein, that is, at least in the cytoplasmic cell fraction, degraded mainly by ClpCP (12). Although the interaction of ClpE and ClpP could be proven by coimmunoprecipitation...
immunogold detection of ClpE by electron microscopy was performed as described previously (24). Strains with clpE-bgaB fusions were created using BHL1 as the donor strain for the clpE-bgaB construct that was integrated at the amyE locus of the wild type (wt) and the clpE mutant. The strains were grown in LB medium and, after setting the heat stress, samples (including two parallels for statistical improvement) were taken from control and heat stress cultures at 0, 15, 30, 60, and 90 min. Preparation of the samples and determination of BgaB activity were done as described earlier (11).

**Construction of a clpE deletion mutant.** A clpE deletion mutant was constructed following the PCR-synthesis method using marker cassettes with long flanking homology regions (41). Regions upstream and downstream of the B. subtilis clpE gene were amplified with the following primer pairs: CLPE_UP_FOR (5′-AAACCCGACCCGACCCGACCTACAG-3′) and CLPE_UP_REV (5′-GTCTTT GCCGATCACCTATGTTAACGTTAGGCAAATATTACCGCATTACAAACC-3′) and CLPE_DOWN_FOR (5′-GCATATGTTAAGCCGCCGACCTACGCAGAATTGGGT GAGG-3′) and CLPE_DOWN_REV (5′-GGGGCTCTAC TGGAATTAACGACCTGTCG-3′). The resulting PCR fragments contained complementary ends to the spectinomycin cassette of pUS19 (underlined). Using pUS19 as a template and the PCR fragments as megaprimers, a fusion construct was generated containing the spectinomycin cassette of pUS19 flanked by the up- and downstream homologous regions of clpE. The construct was 3′-adenylated using the A-addition kit (QIAGEN) and ligated into the pGEM expression vector; Ap′. The correct orientation of the appropriate treated plasmid pX2 to create pX2clpE vector (Promega) to create pGEM-clpE fragment. The construct was linearized and the chromosomal insertion was verified by PCR. DNA sequencing of the new open reading frame. Xylose inducible promoter. Chloramphenicol-resistant transformants were selected, and the deletion of the clpE gene was verified by PCR and Western analysis using ClpE-specific antibodies.

**Constitution of a cpe conditional mutant.** A 500-bp promoterless cpe fragment starting with the Shine-Dalgarno site was amplified with the following primers: CLPE_FOR_X2 (5′- CGGAGATCCAAAAATTATTTAGGGTTTGCTG-3′) and CLPE_REV_X2 (5′- CGGAGATCCAAAAATTATTTAGGGTTTGCTG-3′) and CLPE_REV_X2 (5′- CGGAGATCCAAAAATTATTTAGGGTTTGCTG-3′). The resulting PCR fragments contained complementary ends to the spectinomycin cassette of pUS19 (underlined). Using pUS19 as a template and the PCR fragments as megaprimers, a fusion construct was generated containing the spectinomycin cassette of pUS19 flanked by the up- and downstream homologous regions of clpE. The construct was 3′-adenylated using the A-addition kit (QIAGEN) and ligated into the pGEM expression vector; Ap′. The correct orientation of the insert was confirmed by PCR and restriction digestion. The vector construct was transformed into B. subtilis and integrated via Campbell-type integration into the chromosomal locus of clpE, leaving the full-length transcript under control of the P_C promoter. Chloramphenicol-resistant transformants were selected, and the chromosomal insertion was verified by PCR. DNA sequencing of the new generated cpe locus confirmed the expected changes in the promoter region and displayed no nucleotide changes in the cpe open reading frame. Xylose induc-

(12), substrates of ClpEP degradation have so far not been described.

This study presents first insights into the physiological role of ClpE in B. subtilis. The presence of ClpE in the cells after heat shock is shown to be important for the destabilization of CtsR and disaggregation of heat-denatured proteins. In this context, a clpE mutant displays a retardation of CtsR-dependent induction as well as a delayed restoration of the repressed stage. In summary, the results indicate a ClpEP-dependent pathway of CtsR degradation and the involvement of ClpEP in overall protein quality control in response to heat stress.

**MATERIALS AND METHODS**

**General culture conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* and *B. subtilis* strains were cultivated routinely under agitation at 37°C in Luria-Bertani (LB) medium or Belitsky cultures were divided during exponential growth (optical density at 540 nm previously described (16, 33). For protein analyses, cells were washed and re-suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and then disrupted by sonication (three times at 55 W for 1 min). Soluble and insoluble protein fractions were separated by centrifugation (22,000 × g at 4°C for 60 min). Protein quantitation was done by Roti-Nanoquant assay (Roti GmbH, Karlsruhe, Germany). For resolubilization of cell pellet protein, cell pellet fractions were resuspended in a buffer containing 50 mM Tris-HCl (pH 8.0), 50 mM dithiothreitol, 8 M urea, and 10% glycerol and incubated for 2 h at 40°C. Protein extracts were separated by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Each lane was loaded with 25 μg soluble protein or equal volumes of resolubilized protein. Western analysis was performed according to the described protocol using existing antibody stocks (12). Preparation of cells for analysis using ClpE-specific antibodies.

<table>
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tion of clpE was confirmed by Western blotting and immunodetection using ClpE-specific antibodies.

**Site-directed mutagenesis.** To mutate the N-terminal zinc finger of ClpE, the cysteine codons 29 (TGT) and 32 (TGC) of clpE were changed into serine codons (CTT and AGC, respectively). This was done by PCR with primer pair **ClpE_ZNF_MUT_FOR** (5′-GGTCAATAAAGGTTCTTTGAGAAC ACTGGTTAAGCAAGAC-3′) (nucleotide changes are underlined) and **ClpE_ZNF_MUT_REV** (5′-GAAAAGCACTTGGTTATGACGGAG ATTTTTGGC-3′). Using the GeneTailor site-directed mutagenesis system (Invitrogen), the DNA template prSetAcclpE was specifically methylated by DNA methylase. After PCR, the DNA of the reaction mixture was transformed into *E. coli* DH5α-Ti, a strain containing endonuclease MerBC that selectively cleaved the methylated DNA template, allowing only the replication of the nonmethylated PCR product pRSETAcclpEclpP, and AcclpE was added to each sample for an additional incubation time of 2 h, ensuring quantitative capture of CtsR. The beads were washed three times in 500 µl of KI buffer and, finally, boiled in 10 µl of SDS sample buffer for 5 min at 95°C. After protein separation by 15% one-dimensional SDS-PAGE with an appropriate marker (BenchMark prestained ladder; Invitrogen), gels were vacuum dried and exposed on a phosphor screen (Molecular Dynamics) overnight. Autoradiographs were detected by scanning with a Storm 840 (Molecular Dynamics), and the marker was transferred by size comparison with the corresponding gel stencils. CtsR-specific signals were then evaluated by size comparison with the transferred marker. The experiments were conducted three times, and a stabilization of CtsR in the clpE background was observed when the radiolabeling time interval was further shortened to 2.5 min.

**RESULTS**

**ClpE affects CtsR-dependent gene expression.** In order to expose the effects of a clpE deletion on CtsR-dependent gene expression in *B. subtilis*, the transcription of clpC and clpP was monitored by Northern analyses of the wt and a clpE mutant during heat stress. The expression of clpE itself was studied using a transcriptional fusion of the clpE promoter and the bgaB gene of *Bacillus steaetherophilus*.

The Northern analysis of clpP expression (Fig. 1A) showed a very low basal level of clpP transcription under control conditions at 37°C in both the wt and the clpE mutant. After a thermal upshift, clpP was induced in both strains; however, it was stronger in the wt than in the clpE mutant. After 25 min of heat shock, clpP expression decreased to the basal level in the wt, revealing a short time window for clpP derepression during heat stress (see references 11 and 15). Contrarily, the clpE mutant showed a retarded clpP induction, indicating a reduced efficiency in CtsR-dependent derepression. Furthermore, the period of derepression was significantly increased. The same kind of Northern analysis displayed a reduced induction and a prolonged derepression of the clpC operon after heat shock in the clpE mutant as well. However, after 45 min of heat shock, the repression of both the clpP gene and the clpC operon was restored to more than 50% compared to the 5-min induction level in the clpE mutant. In addition, the repression of both the clpP gene and the clpC operon was restored to more than 50% compared to the 5-min induction level in the ΔclpE background (data not shown). Because the clp transcripts have half-lives of less than 2 min at 50°C, stabilizing effects at the posttranscriptional level could be neglected (12).

Additionally, cytoplasmic protein fractions of samples from the same cultures were used for Western analyses with ClpC-, ClpE-, and CtsR-specific antibodies. According to the transcriptional data, slight accumulations of the respective gene products were detected in the clpE mutant compared to the wt 25 min after heat induction (data not shown).

**Similar results were obtained for the clpE promoter activity by clpE′-BgaB measurements (Fig. 1B).** The basal expression at 37°C was low in both strains. During heat stress, however, the wt showed a high initial induction level that subsequently declined as expected. In contrast to that, a different induction pattern was observed in the clpE mutant during heat stress. After 15 min, the induction in the clpE mutant was reduced approximately by half and increased during the first 30 min of heat stress, whereas a slow decrease followed during the next...
hour. The growth of the clpE mutant was slightly diminished in comparison to that of the wt after heat shock.

Further Northern analyses confirmed that the expression of groESL (class I heat shock operon, HrcA regulated [36]) and clpX (class IV heat shock gene, unknown regulation [10, 12]) did not show any significant differences between the wt and the clpE mutant during heat shock (data not shown), suggesting that ClpE is a specific factor for CtsR-dependent gene expression during heat stress.

CtsR is a putative substrate of ClpEP-dependent degradation after heat shock. CtsR degradation was shown to depend on ClpCP after heat-simulating puromycin stress (25). Now, a radiolabeling approach was chosen to determine Clp-dependent effects on CtsR stability. Figure 2 illustrates the stability of radiolabeled CtsR in wt and clpP mutant during heat shock (data not shown), suggesting that ClpE is a specific factor for CtsR-dependent gene expression during heat stress.

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ClpE is localized at heat-generated protein aggregates. For the determination of ClpE distribution in the cells during heat...
shock, soluble (cytoplasmic) and insoluble (“pellet”) protein fractions of wt cells were examined by Western analyses. Figure 3A shows the ClpE amounts in both fractions at different time points. Surprisingly, ClpE was detected for a longer period in the insoluble than in the soluble fraction, suggesting that targets might be heat-generated protein aggregates. For a detailed analysis, the ClpE content was examined in soluble and insoluble fractions of xylose-inducible clpE conditional mutant (BMM11) cells (Fig. 3B). The cytoplasmic fraction showed a ClpE-specific signal already in the control lane due to the presence of xylose, but signals in the corresponding insoluble fractions were detectable only after the cells were exposed to heat stress. Thus, only after heat shock did ClpE in the pellet fraction appear likely to attack heat-generated protein aggregates.

This suggestion was finally proven by localization of ClpE in ultrathin sections of heat-shocked cells using the immunogold labeling technique and electron microscopy (Fig. 4). The concentration of ClpE-specific signals within and around the electron-dense particles that are known to represent the fraction of insoluble protein aggregates (“inclusion bodies”) (see reference 24) confirmed the Western experiments, indicating a functional relationship between ClpE and heat-denatured proteins. At this stage, however, it was not clear whether the majority of ClpE detected in the pellet fraction was active or inactive due to its rapid degradation in the soluble fraction that might also lead to aggregation of truncated ClpE. Therefore, functional ClpE-dependent effects on protein disaggregation were examined subsequently.

ClpE is involved in disaggregation of insoluble heat-denatured proteins. A pulse-chase radiolabeling approach using L-[^35]S]methionine was chosen to detect differences in the stability of heat-generated protein aggregates in the pellet fractions of wt and clpE mutant cells. Figure 5 shows the counts-per-minute values and corresponding protein quantities of the labeled pellet fraction for the wt and the clpE mutant. The prolonged stability of labeled protein in the insoluble fraction of clpE mutant cells after heat shock revealed a delay of protein disaggregation for at least half an hour in comparison to the wt. Twenty minutes after heat shock, a difference of 7 to 33 μg aggregated protein/(OD × ml) was measured in the wt and clpE mutant pellet fractions, respectively, indicating an approx-
imately fivefold reduced protein disaggregation in the clpE mutant at this time point. One hour after heat stress, the quantities of aggregated protein in the wt and the clpE mutant were nearly at the same level.

The counts-per-minute values of the soluble and crude extract fractions were about 1 order of magnitude higher and did not show any significant differences between the wt and the clpE mutant (data not shown).

Thus, the stabilization of heat-generated insoluble protein aggregates in the clpE mutant suggests for the first time a physiological role of the ClpE ATPase in protein quality control in *B. subtilis*.

The N-terminal zinc finger of ClpE is crucial for basal ATPase activity. The ClpE ATPase possesses an N-terminal zinc finger of the C₄ type (Cys₃₋₆, ₂₉₋₃₂) as a characteristic structural element. For a functional characterization of this domain, the *clpE* gene of *B. subtilis* was cloned into a pRSETA expression vector containing an N-terminal His₆ translational fusion tag. The vector construct was subsequently used for a site-directed mutagenesis that altered two cysteine codons of the zinc finger at positions 29 and 32 to serines, leading to the destruction of the zinc finger. Both the native ClpE and the zinc-deficient N-terminal His₆-tag derivative of ClpE were overexpressed and purified. The ATPase activities of both derivatives were determined in vitro by a colorimetric ATPase assay. Measurements were performed without substrates except ATP, to display the basal ATPase activity of the ClpE derivatives. Figure 6 shows the data for three measurements with different amounts of each His₆-ClpE and His₆-ClpE₂₉₋₃₂S. Mean values that were calculated from the linear ranges of the kinetics exhibited a basal ATP turnover of 45.3 molecules of ATP min⁻¹ monomer⁻¹ for His₆-ClpE, but only...
5.3 molecules of ATP min⁻¹ monomer⁻¹ for His₆-ClpEC (C2932S). Thus, the basal ATPase activity of the zinc-deficient ClpE derivative was diminished to about 10% of the native ClpE ATPase activity, indicating that the ClpE zinc finger is crucial for ATPase activity.

DISCUSSION

ClpE was described as a new independent type of the HSP100 ATPases belonging to the CtsR regulon in B. subtilis (7). Whereas no significant physiological function could be allocated to B. subtilis ClpE so far, the involvement of ClpE homologues in important physiological processes has been shown for several other bacteria. These findings prompted us to a closer examination of B. subtilis ClpE ATPase.

A clpE mutant showed a characteristic phenotype concerning the expression patterns of CtsR-dependent genes after heat shock, and absence of ClpE diminished cell growth slightly during heat stress at 50°C (Fig. 1). A lag of growth at 54°C was already described for a B. subtilis clpE mutant (7), while the data for CtsR-dependent gene expression are similar to those of an L. lactis clpE mutant, showing a prolonged derepression of the CtsR-regulated clpP gene after heat shock (40). The observed phenotype might partly be explained by the new data presented in this study.

ClpE was found to be involved in CtsR destabilization in the soluble cell fraction during heat stress. The global repressor CtsR autoinduces its own synthesis by derepression of the clpC operon after heat shock. Former studies showed the involvement of ClpCP in CtsR degradation after heat-simulating puromycin stress (25). However, the partial stability of radioactively labeled CtsR in the clpC mutant observed in this study revealed that this is only one pathway of CtsR degradation after heat shock. Indeed, the data showed a significant stabilization of radioactively labeled CtsR in the clpE mutant in the first 30 min after the thermal upshift, suggesting that ClpEP dominates the CtsR degradation at the early stage of heat stress, thereby ensuring an efficient derepression of class III heat shock genes. This proposal is strongly supported by the data of Fig. 1, showing a significantly reduced heat induction for CtsR-dependent clpE and clpP expression in the clpE mutant. The diminished heat induction might result from a retarded CtsR degradation via the congested ClpCP pathway in the absence of ClpEP and might in addition be one reason for the subsequent delay of CtsR-dependent rerepression. The period of CtsR stabilization in the clpE mutant corresponds to

![FIG. 4. Detection of subcellular localized ClpE in wt cells 10 min after heat shock. Cryosections of the cells were incubated with ClpE-specific antibodies and, afterwards, with primary-antibody-specific gold-conjugated secondary antibodies. Signal detection of the gold particles was carried out by electron microscopy. Accumulation of signals is indicated with the arrows.](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3462456/images/figure4.png)
the time window of ClpE traceability in the cytoplasmic fraction of wt cells (Fig. 3A), indicating that ClpEP mainly acts on CtsR degradation during the short period of derepression immediately after heat shock. These observations indicate an essential role for ClpEP in CtsR-dependent gene derepression by destabilizing the CtsR repressor. After degradation of ClpE in the soluble fraction of wt cells, ClpCP seems to become the main actor of CtsR degradation, because the clpC mutant still showed a stabilized lower level of labeled CtsR after 3 hours of heat stress. In accordance with that, a reduced CtsR-dependent heat induction was also observed in clpC and clpP mutants at the clpE promoter (25).

Thus, CtsR degradation after heat shock turns out to be a fine-tuned mechanism depending on the proteolytic systems ClpCP and ClpEP. The systems might represent two separate CtsR degradation pathways. As the different CtsR destabilization patterns of the corresponding mutants indicate, one pathway leads to rapid CtsR degradation at the early stage of heat induction by ClpEP, the other one to the complete removal of remaining CtsR by ClpCP. The complete stabilization of labeled CtsR in the clpCE double mutant strongly indicates that there are no further Clp ATPases involved in CtsR destabilization after heat shock. Further in vitro experiments concerning the ClpEP-dependent degradation of CtsR are planned to confirm the proposals from the presented in vivo data.

The possibility that the ClpE ATPase might be directly involved in disaggregation of heat-denatured proteins was supported by localization of ClpE in the insoluble fraction of the cells immediately after heat shock and, more precisely, at electron-dense particles known as heat-generated protein aggregates. A significant difference in protein disaggregation efficiency in the wt and the clpE mutant was confirmed subsequently by radioactive labeling and quantitation of the heat-aggregated protein fractions, showing a delay of protein disaggregation of about 1 hour in the clpE mutant during heat stress. It is likely that ClpE does not act on protein aggregates exclusively as a chaperone but together with ClpP as in the case of CtsR degradation. The accumulation of ClpC and ClpP at heat-generated protein aggregates has been shown in former studies (24). Mutations or deletions of clpC and especially clpP are known to cause severe defects in the removal of aggregated cell protein (21, 23, 27). It is now conceivable that ClpCP and ClpEP might act synergistically on protein disaggregation after heat shock. Both systems might furthermore replace each other in the disaggregation process, so that the loss of the ClpEP protease system caused by clpE deletion might be compensated for by ClpCP. However, the increasing ClpCP amounts after heat shock that have been quantified in the wt (12) are likely to be reduced in the clpE mutant due to the observed stabilization of CtsR that putatively causes the retarded CtsR-dependent heat induction. This leads to a model of stronger and prolonged ClpCP sequestration to heat-denatured proteins in the clpE mutant. That a sufficient level of free ClpC in the cell seems to be critical for the repression of CtsR-dependent genes is supported by a recent study showing that only unsequestered ClpC almost completely inhibits the MesB kinase which negatively regulates CtsR activity (20). Coincidently, in the wt, where the amount of labeled aggregated protein was decreased to the control level after 20 min of heat shock, the CtsR-dependent gene repression was shown to
be completely restored after the first 25 min of heat shock, whereas the rerepression was significantly retarded in the clpE mutant (Fig. 1A). In conclusion, the involvement of ClpE in both CtsR destabilization and protein disaggregation after heat shock might provide the main reason for the observed delay of CtsR-dependent rerepression of the class III heat shock genes in the clpE mutant. As the experimental results indicate, ClpE(P) turns out to be an important backup system of *B. subtilis* for the development of thermotolerance.

One characteristic domain of the ClpE ATPase is the N-terminal C4-type zinc finger. A similar domain is also part of the ClpX ATPase that, in turn, bears only one instead of two domains for ATP binding and hydrolysis and therefore belongs to the AAA⁺ class II proteins (8, 30, 34). A structure-function analysis of the *E. coli* ClpX ATPase revealed that a zinc-deficient derivative was unable to bind ATP, to oligomerize, or to bind to ClpP (2). The focus here was to investigate whether this enzymatic loss of function was also true for a zinc-deficient ClpE ATPase. Indeed, the ClpE derivative with the mutated zinc finger showed an approximately tenfold loss of basal ATPase activity in vitro, showing that this structure element is crucial for the basic ClpE function. A clpE mutant-like phenotype that was observed for an *L. lactis* strain carrying a clpE gene with a mutated zinc finger motif already indicated a relevant function of this domain for ClpE ATPase activity in vivo (40).

Strikingly, the basal ATP turnover of ClpE reached unexpected high values. Measurements at 30°C and 37°C obtained ATP hydrolysis rates of about 300 to 400 molecules of ATP per minute and putative ClpE hexamer. By comparison, the basal ATPase activity of *E. coli* ClpX was recently determined with a turnover rate of 140 molecules of ATP per minute and hexamer at 30°C (4). The combination of this fact with results from further studies showing a direct connection between ATPase activity and the effectiveness of substrate denaturation and degradation by *E. coli* ClpXP (18) supports the suggestion that ClpE might act as an effective protein-disaggregating ATPase under conditions that urgently require such a strong physiological activity. This seems especially important for adaptation to heat and diamide stress (see reference 26) leading to the highest known levels of clpE induction. In terms of genome evolution, it might be speculated that the strong activity of the ClpE ATPase, once established, may have driven its tight epigenetic regulation that is realized at the transcriptional, posttranscriptional, and posttranslational levels (7, 12). Further biochemical characterization of ClpE function and screening for specific substrates should lead to a deeper understanding of its part in cellular protein quality control.

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