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Thermotolerance Requires Refolding of Aggregated Proteins by Substrate Translocation through the Central Pore of ClpB

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Summary

Cell survival under severe thermal stress requires the activity of the ClpB (Hsp104) AAA+ chaperone that solubilizes and reactivates aggregated proteins in concert with the DnaK (Hsp70) chaperone system. How protein disaggregation is achieved and whether survival is solely dependent on ClpB-mediated elimination of aggregates or also on reactivation of aggregated proteins has been unclear. We engineered a ClpB variant, BAP, which associates with the ClpP peptidase and thereby is converted into a degrading disaggregase. BAP translocates substrates through its central pore directly into ClpP for degradation. ClpB-dependent translocation is demonstrated to be an integral part of the disaggregation mechanism. Protein disaggregation by the BAP/ClpP complex remains dependent on DnaK, defining a role for DnaK at early stages of the disaggregation reaction. The activity switch of BAP to a degrading disaggregase does not support thermotolerance development, demonstrating that cell survival during severe thermal stress requires reactivation of aggregated proteins.

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Introduction

Cells have evolved a powerful network of chaperones and proteases to counteract the adverse effects of protein misfolding. In the cytosol of bacteria, two chaperone systems with folding capacity, the DnaK chaperone with its DnaJ and GrpE cochaperones (KJE) and the GroEL chaperonin with its GroES cochaperone (ESL), bind misfolded proteins and assist their refolding to the native state (Hartl and Hayer-Hartl, 2002). Alternatively, misfolded proteins are degraded by several different ATP-dependent proteolytic systems. These systems are composed of ring-shaped oligomeric assemblies of several subunits of a regulatory AAA+ chaperone with ATPase activity (ClpA, ClpX, HslU) and an associated peptidase that is either covalently attached to the ATPase (Lon, FtsH) or diffusible (ClpP, HslV) (Wickner et al., 1999).

Severe heat stress results in massive protein misfolding that exceeds the buffering capacity of this quality control network and results in the aggregation of proteins, which is linked to cell death. Several factors may contribute to cell death, including toxic effects of intermediate and final forms of the aggregated proteins, and the net loss of active proteins. An intriguing observation is that survival under severe heat stress is increased by orders of magnitude through short preincubation of cells at sublethal heat shock temperatures. This evolutionary conserved phenomenon of acquired thermotolerance depends critically on the stress-inducible AAA+ (*A*TPase *a*ssociated with diverse cellular *a*ctivities) chaperone ClpB in bacteria and its homolog Hsp104 in yeast (Sanchez and Lindquist, 1990; Squires et al., 1991). ClpB as well as its eukaryotic counterparts does not associate with a peptidase but rather acts in collaboration with the DnaK (Hsp70) system to solubilize and refold aggregated proteins (Glover and Lindquist, 1998; Goloubinoff et al., 1999; Mogk et al., 1999; Motohashi et al., 1999; Parsell et al., 1994). It is an unsolved question whether the essential role of ClpB/Hsp104 in thermotolerance relies on its ability to solubilize aggregated proteins and thereby to eliminate potentially toxic effects or, alternatively, on its ability to reactivate the lost proteins.

The mechanism of ClpB/Hsp104-dependent protein disaggregation is still unknown; however, several models have been proposed. One model predicts that ClpB/Hsp104 breaks large protein aggregates into fragments through a "crowbar" mechanism (Glover and Lindquist, 1998; Lee et al., 2003). This crowbar activity of ClpB/Hsp104 may be achieved by its unique M domain that is essential for ClpB-mediated protein disaggregation (Cashikar et al., 2002; Kedzierska et al., 2003; Mogk et al., 2003b). The recently solved crystal structure of *Thermus thermophilus* ClpB revealed that the M domain forms a large coiled-coil structure that is located on the outer surface of a hexameric ClpB model (Lee et al., 2003). Interestingly, the M domain is flexible, and its mobility is crucial for protein disaggregation (Lee et al., 2003). An alternative model suggests that the fragmentation of large aggregates is achieved by deoligomeriza-

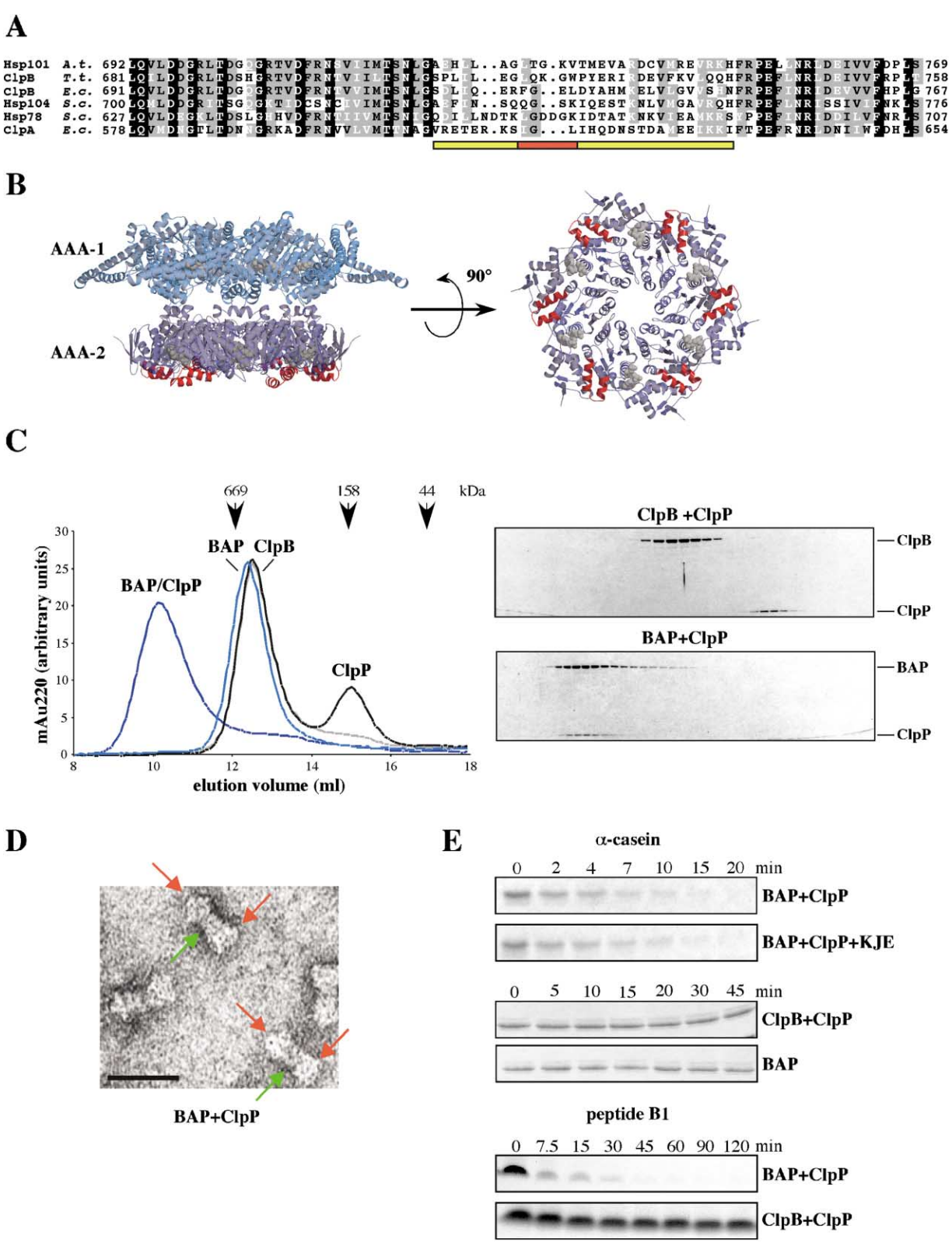

Figure 1. Incorporation of the ClpP Interaction Loop into ClpB Allows Association with ClpP

(A) Multiple sequence alignment of the second AAA domain of various ClpB proteins. The protein sequence of the second AAA domain of *Escherichia coli* ClpB (P03815) was aligned with the second AAA domain of ClpB proteins from *Thermus thermophilus* (Q9RA63), Hsp104 from *Saccharomyces cerevisiae* (P31539), Hsp78 from *S. cerevisiae* (Q12137), and Hsp101 from *Arabidopsis thaliana* (P42730). *E. coli* ClpA is included as reference. Identical amino acids are boxed in black, conserved residues (black) are boxed in gray, and similar amino acids (white) are boxed in gray. The helix-loop-helix motif of ClpA that mediates ClpP association is indicated in yellow (α helix) and red (loop).

tion of ClpB/Hsp104. ClpB/Hsp104 hexamers would bind to protein aggregates in the ATP bound state, and deoligomerization of ClpB following ATP hydrolysis would generate the mechanical force needed to break apart the aggregates (Schlee et al., 2004). A third model favors ClpB-mediated changes in the aggregate structure, enabling DnaK to solubilize the aggregated proteins in a subsequent reaction (Ben-Zvi and Goloubinoff, 2001). Such changes could be mediated by a "capture-and-release" activity of ClpB, resulting in the partial unwinding of polypeptides that are trapped within the aggregate (Hoskins et al., 2002; Lee et al., 2003). Finally, protein disaggregation might be achieved by translocation of single polypeptides from the aggregate through the central pore of ClpB/Hsp104. Such an activity would be reminiscent of the activity of peptidase-associated AAA+ chaperones for which a translocating activity has been shown (Hoskins et al., 2000; Kim et al., 2000; Ortega et al., 2000; Reid et al., 2001; Singh et al., 2000). The suggested models are not mutually exclusive and may in part also act sequentially or in concert: a possible crowbar activity could facilitate the unwinding of aggregated proteins via a capture-and-release or threading mechanism.

The proposed role of the cooperating DnaK/Hsp70 chaperone system in the disaggregation process differs depending on the models described above. Protein aggregates of reduced size, generated by ClpB either by the crowbar or the deoligomerization mechanism, could serve as substrates for DnaK, which has disaggregation activity toward small protein aggregates (Diamant et al., 2000; Glover and Lindquist, 1998; Mogk et al., 2003a). The capture-and-release mechanism could allow DnaK to work productively on aggregated proteins after ClpB-mediated changes of the aggregate structure. In case of a translocation mechanism, DnaK may be required for efficient substrate threading through the pore, either by facilitating initial unfolding of the substrate or by interacting with the translocating polypeptide at the distal end of the pore, thereby generating a pulling or trapping force. Thus, depending on the model, ClpB and the DnaK system may act sequentially or simultaneously. A physically linked bichaperone complex as the active disaggregation machinery is supported by the observation that cooperation of the two chaperone systems exists only between proteins of the same species (Glover and Lindquist, 1998; Krzewska et al., 2001; Schlee et al., 2004). Evidence for such a complex was recently provided for *T. thermophilus* ClpB/DnaK (Schlee et al., 2004).

We set out to investigate both the working mechanism of ClpB and the precise role of ClpB in thermotolerance development. The central approach was to generate a ClpB variant, BAP, which associates with the ClpP peptidase, thereby converting ClpB from a reactivating to a degrading disaggregase. This ClpB variant enabled us to rigorously test whether protein translocation through the central pore of ClpB is possible and relevant to protein disaggregation. Furthermore, the activity switch of ClpB allowed us to determine in vivo the consequences of changing the fate of solubilized proteins on thermotolerance development.

Results

BAP: A ClpB Variant that Associates with ClpP

To test whether ClpB can be converted into a chaperone that assists in the degradation of aggregated proteins, we constructed a ClpB variant that can associate with the peptidase ClpP. This design was based on the interaction of the peptidase-associated AAA+ proteins ClpA and ClpX with ClpP, which depends on a conserved IGF/L tripeptide located on a helix-loop-helix motif of the AAA domain (Kim et al., 2001; Maurizi and Xia, 2004). This structural motif is also present in ClpB (Lee et al., 2003) and is located at the bottom of AAA-2, similar to its position in ClpA and ClpX (Figures 1A and 1B). However, ClpB lacks the consensus ClpP binding motif and thus does not interact with ClpP.

We replaced the helix-loop-helix motif of ClpB (S722-N748) with the analogous motif of ClpA (V609-I635), resulting in a ClpB variant referred to as BAP (Clp*B*-Clp*A*-*P* loop). Purified BAP did not reveal any oligomerization or structural defects (Figure 1C; data not shown). Using size exclusion chromatography, we showed that BAP, in contrast to ClpB wild-type, formed a complex with ClpP (Figure 1C). BAP/ClpP complexes could also be visualized by electron microscopy, while no complexes were observed between ClpB and ClpP (Figure 1D; data not shown). These results demonstrate that the exchanged helix-loop-helix motif containing the IGF/L tripeptide sequence is sufficient to allow ClpB to associate with ClpP.

BAP/ClpP Forms an Active Proteolytic Complex

To probe for the proteolytic activity of the BAP/ClpP complex, we tested the degradation of α-casein, a naturally unfolded model substrate. α-casein was efficiently degraded by BAP/ClpP but not by BAP alone or ClpB/ClpP (Figure 1E). As proteolysis is strictly dependent on protein translocation into ClpP (Ortega et al., 2000), our results represent the first direct evidence for a translocation activity of ClpB toward substrate. Degradation of α-casein by BAP/ClpP occurred with a comparable rate

(B) Hexameric model of *E. coli* ClpB generated from the structure of *T. thermophilus* ClpB (Lee et al., 2003). The side view shows the position of the AAA-1 (blue) and AAA-2 (purple) domains. The helix-loop-helix motif is located at the bottom of AAA-2 and is colored in red. The bound AMPPNP molecules are shown as CPK models and are colored in gray.

(C) Association of BAP with ClpP. BAP or ClpB was incubated alone (light blue and gray, respectively) or with ClpP (blue and black, respectively), and complex formation was monitored by size exclusion chromatography. Coomassie-stained SDS-PAGE analysis of the indicated runs is shown.

(D) Complex formation between BAP and ClpP was monitored by electron microscopy. BAP and ClpP are indicated by red and green arrows, respectively. Scale bar, 250 Å.

(E) Degradation of substrates by BAP/ClpP. Hydrolysis of α-casein or peptide B1 (AHAWQHQGKTLFISRKTYRIC) was followed in the presence of the indicated proteins, and degradation was monitored at the indicated time points.

as compared to Lon or ClpA/ClpP-dependent hydrolysis (see Supplemental Figure S1A at http://www.cell.com/cgi/content/full/119/5/653/DC1/).

We also followed the degradation of the 21-mer peptide B1, which we recently identified as a ClpB-interacting peptide in a peptide library screen (Schlieker et al., 2004). The peptide was rapidly degraded by BAP/ClpP but remained stable in the presence of ClpB/ClpP (Figure 1E). Finally, we determined the influence of the DnaK chaperone system (KJE), which is essential for ClpB-dependent protein disaggregation, on substrate degradation by BAP/ClpP. α-casein hydrolysis was not affected by KJE (Figure 1E). Together, these results indicate that substrates are threaded through the central channel of BAP to reach the proteolytic chamber of ClpP. This furthermore shows that recognition and translocation of soluble, monomeric substrates by ClpB are independent of KJE.

BAP/ClpP Degrades Aggregated Proteins in a KJE-Dependent Fashion

We next analyzed whether the BAP/ClpP complex can also act on aggregated proteins using malate dehydrogenase (MDH) as model substrate (Figure 2A). Interestingly, BAP/ClpP alone did not have any disaggregation activity and hence did not degrade aggregated MDH. Solubilization of MDH aggregates by BAP remained KJE dependent and then occurred at the same rate as solubilization by ClpB/KJE. The additional presence of ClpP did not significantly influence the disaggregation reaction by BAP/KJE (Figure 2A), even when added in large excess over BAP (data not shown). However, addition of ClpP to BAP/KJE caused a complete switch in activity of the bichaperone system, resulting in degradation instead of reactivation of aggregated MDH (Figure 2A). The kinetics of MDH disaggregation and degradation proceeded with similar rates. A lag phase for MDH hydrolysis was not noticed, giving a first hint that MDH disaggregation and degradation are coupled events (Figure 2B).

As an alternative substrate, we used urea-denatured luciferase, since its KJE-mediated refolding is strictly dependent on ClpB and thus represents a disaggregation reaction (Krzewska et al., 2001). Addition of ClpP strongly reduced the amount of reactivated luciferase by KJE/BAP, whereas luciferase refolding by KJE/ClpB was not influenced (Figure 2C). Reduced levels of refolded luciferase by KJE/BAP/ClpP could be attributed to luciferase degradation. In agreement with the data obtained for aggregated MDH, the degradation of urea-denatured luciferase by BAP/ClpP was dependent on KJE (Figure 2C). Luciferase degradation and refolding proceeded with similar kinetics, again indicating a direct coupling of luciferase disaggregation and degradation.

As another oligomeric substrate, we used dimeric TrfA, which is monomerized by the joint activity of ClpB and KJE (Konieczny and Liberek, 2002). In accordance with these earlier findings, the degradation of TrfA dimers by BAP/ClpP required the assistance of KJE (see Supplemental Figure S1B on the *Cell* web site).

Disaggregation and Degradation of Aggregated MDH Are Directly Coupled

The degradation of MDH during the disaggregation reaction can be explained in two ways. BAP-mediated disaggregation and degradation of MDH might be physically and kinetically coupled processes. Accordingly, during the disaggregation reaction, aggregated MDH molecules would be directly threaded through the central pore of BAP into the proteolytic chamber of the associated ClpP peptidase. Alternatively, aggregated MDH might first be solubilized by BAP/KJE and, in a second independent step, free molecules of solubilized MDH might be recognized and degraded by BAP/ClpP. In order to distinguish between these possibilities, we determined whether solubilized MDH occurs free in solution prior to its degradation by BAP/ClpP. For this purpose, we took advantage of the GroE chaperonin system (ESL), which does not influence MDH disaggregation by ClpB/KJE but accelerates the MDH refolding reaction, resulting in a direct coupling of the MDH disaggregation and refolding reaction (C. Schlieker et al., submitted). ESL thus only acts on solubilized, unfolded MDH and can be used in order of addition experiments to test for a direct coupling of MDH disaggregation and degradation by KJE/BAP/ClpP.

Fast regaining of MDH activity was observed when ESL was added to either ClpB/KJE or BAP/KJE at the beginning of the disaggregation process. However, upon addition of ClpP to BAP/KJE, only a small fraction (18%) of MDH was refolded by ESL, and no further increase in MDH activity was noticed after 60 min, when the disaggregation reaction was finished (Figure 3A). These data demonstrate that the additional presence of ESL can only partially rescue aggregated MDH from degradation by KJE/BAP/ClpP. To test for a direct coupling of MDH disaggregation and degradation, we added ESL and ClpP either at the start of the BAP/KJE-mediated disaggregation reaction (0 min) or after 45 min, when the disaggregation reaction is almost finished, while the refolding of solubilized MDH species has just begun (Supplemental Figure S2A) (Goloubinoff et al., 1999). Thus, dependent on the time point chosen for the addition of ESL and ClpP (0 or 45 min), different MDH substrate types exist (aggregated MDH or soluble, unfolded MDH, respectively). High MDH refolding yields (68%) were only obtained when ESL and ClpP were added after 45 min to KJE/BAP, indicating that the refolding of soluble, unfolded MDH species by ESL is faster than the degradation by BAP/ClpP (Figure 3B). These data demonstrate that the majority of aggregated MDH could not be handed over to ESL, since it is not released by KJE/BAP/ClpP to the solvent but instead is directly transferred from the aggregate into the proteolytic chamber of the peptidase. Notably, addition of ClpP to KJE/BAP after 45 min blocked further refolding, indicating that soluble, unfolded MDH is also substrate for BAP/ClpP (Figure 3B). The remaining protection of MDH by ESL (added at 0 min) most likely stems from the existence of two different BAP populations in the assay: one BAP population that is associated with ClpP, resulting in MDH degradation; and a noncomplexed population, allowing the subsequent binding to ELS, resulting in MDH refolding. In fact, BAP-ClpP association was lost in the presence of ADP, similar to ClpX-ClpP (Joshi et al., 2004), suggesting that complex dissociation can occur during the functional cycle of BAP/ClpP (Supplemental Figure S6B). In a complementary experiment, we monitored the ClpB/KJE-dependent refolding of aggregated MDH in the additional presence of a substrate-

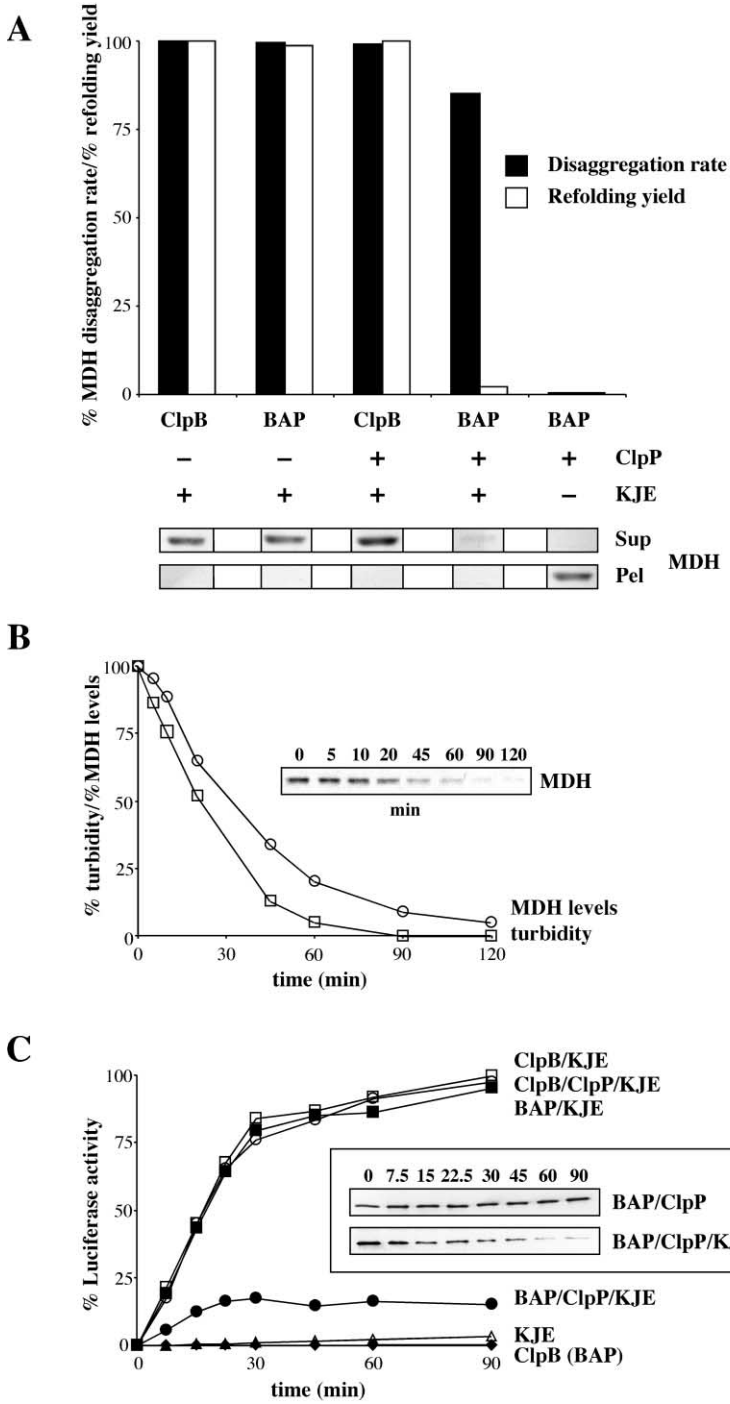

Figure 2. Degradation of Protein Aggregates by BAP/ClpP Is KJE Dependent

(A) Disaggregation and refolding of aggregated MDH was followed in the presence of the indicated components. The MDH disaggregation rates and MDH refolding yields (180 min) were calculated and set as 100% for ClpB/KJE. Soluble and aggregated MDH species were separated by centrifugation after 180 min incubation time and analyzed by SDS-PAGE.

(B) The kinetics of MDH disaggregation and degradation are coupled. The solubilization of MDH aggregates by BAP/ClpP/KJE was followed by measuring the decrease in MDH turbidity and determining the decrease of MDH levels. Turbidity and amount of aggregated MDH were set as 100%.

(C) Refolding of urea-denatured luciferase by KJE/BAP is sensitive to the presence of ClpP. Refolding of luciferase was monitored in the presence of the indicated chaperones. Degradation of luciferase by either BAP/ClpP or KJE/BAP/ClpP was monitored by Western blotting (insert).

trapping mutant protein of GroEL (EL-D87K), which is deficient in ATP hydrolysis and consequently can trap nonnative substrates (Weber-Ban et al., 1999). In complete agreement with the ESL experiments described above, EL-D87K could only efficiently protect solubilized but not aggregated MDH from degradation by KJE/BAP/ClpP (Supplemental Figures S2B–S2D). These data demonstrate that a direct transfer of aggregated MDH via BAP into the proteolytic chamber of ClpP must have occurred, without MDH becoming accessible to the solvent.

In an alternative approach to demonstrate the crucial importance of substrate translocation for ClpB-dependent protein disaggregation, we investigated the influence of ClpP-S111A, an inactive, propeptide-containing ClpP variant, on protein disaggregation by KJE/BAP. The uncleaved propeptides of ClpP-S111A occupy the digestion chamber and thus limit entry of other molecules (Ortega et al., 2000). Addition of ClpP-S111A to KJE/BAP but not KJE/ClpB inhibited MDH disaggregation (Figure 3C). Consistent with these findings, coexpression of BAP and ClpP-S111A in *E. coli* Δ*clpB* mutant cells also blocked protein disaggregation or the reactivation of heat-aggregated luciferase (data not shown).

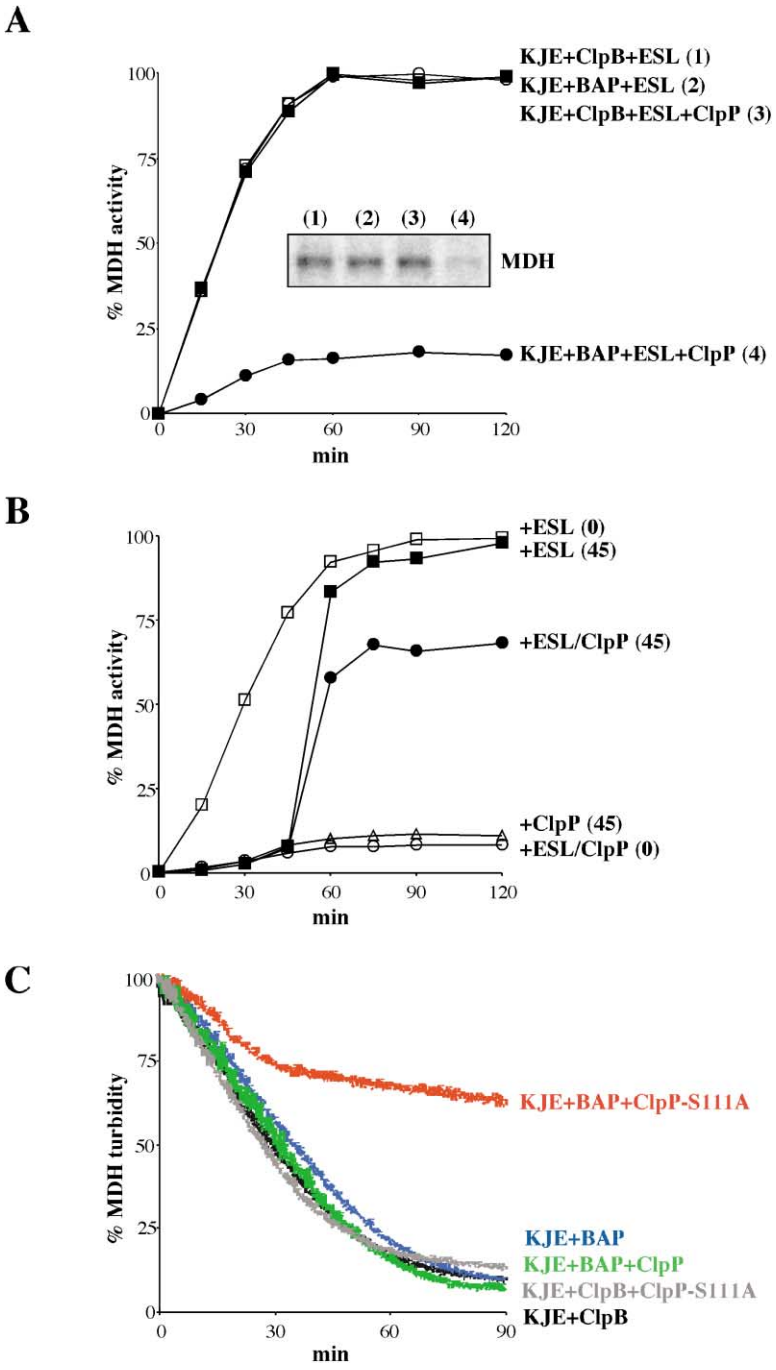

Figure 3. ClpB-Dependent Substrate Translocation Is an Integral Part of the Disaggregation Reaction

(A) Refolding of aggregated MDH by KJE/ClpB (± ClpP) or KJE/BAP (± ClpP) was followed in the additional presence of ESL. MDH levels present after 120 min are given.

(B) Refolding of aggregated MDH by KJE/BAP was analyzed in the additional presence of ESL or ClpP. ESL/ClpP was added either at the beginning of the disaggregation reaction (0) or after 45 min (45).

(C) ClpP-S111A inhibits MDH disaggregation by KJE/BAP. MDH disaggregation was followed in the presence of the indicated components.

The initial decrease of MDH turbidity can most likely be attributed to protein disaggregation by BAP molecules that are not associated with ClpP-S111A. Accordingly, some MDH activity could be regained until the solubilization process was inhibited (data not shown). Together, these findings indicate that ClpB-dependent translocation is an essential part of the disaggregation reaction.

The Conserved Residue Y653 Is Crucial for Substrate Translocation and Protein Disaggregation

The finding that protein disaggregation is linked to substrate translocation through the central pore of ClpB predicts that residues, which are crucial for the translocation activity, should also be important for protein disaggregation. For several peptidase-associated AAA+ chaperones, it has been shown that conserved aromatic residues located at the apical end of the central pore of the AAA+ oligomers are essential for substrate translocation (Siddiqui et al., 2004; Song et al., 2000; Wang et al., 2001; Yamada-Inagawa et al., 2003). Nucleotide-dependent conformational changes of these residues have been suggested to directly couple substrate translocation to substrate unfolding (Wang et al., 2001). The potential translocation channel of ClpB has a diameter of 10 Å at its narrowest points, and, consequently, it is

likely to be too small to allow passage of folded proteins (Supplemental Figure S3). ClpB possesses such conserved aromatic residues (Y251 and Y653) positioned at the central pore of each AAA domain (Schlieker et al., 2004) (Figure 4A). Tyr251 has been implicated in substrate interaction as a ClpB-Y251A mutant is affected in both association with and disaggregation of aggregated proteins (Schlieker et al., 2004). Here, we analyzed the role of Y653 in protein disaggregation by exchanging it to alanine. In addition, we genetically introduced both Y251A and Y653A alterations into BAP to test their roles in substrate translocation and degradation. ClpB-Y653A (and BAP-Y653A) did not exhibit oligomerization defects as determined by size exclusion chromatography and glutaraldehyde crosslinking and displayed a limited proteolysis pattern that is identical to ClpB wild-type (Supplemental Figure S4). The basal ATPase activity of ClpB-Y653A (0.025/s) was comparable to that of ClpB wild-type (0.03/s) and was stimulated by α-casein (2-fold).

To demonstrate an involvement of these residues in substrate translocation, we tested for degradation of two different soluble substrates (peptide B1, α-casein) by BAP-Y251A and BAP-Y653A in the presence of ClpP (Figure 4B). Degradation of peptide B1 by BAP-Y251A/ClpP was only slightly affected, whereas hydrolysis of α-casein was 5-fold reduced, comparable to the defects determined for MDH disaggregation (Figure 4C). We suggest that the different dependencies on substrate unfolding are the basis for these findings, as the peptide was still degraded in presence of ATPγS, whereas degradation of α-casein strictly depended on ATP hydrolysis (Supplemental Figure S5). ClpB-Y653A was affected more severely and showed an almost complete loss of activity. Complex formation of both BAP pore mutants with ClpP was unchanged, excluding a defect in ClpP association as the reason for the hydrolysis defects (Supplemental Figure S6A). Similar to the observed degradation defects, BAP-Y251A displayed only partial activities in MDH and luciferase disaggregation in vitro and reactivation of heat-aggregated luciferase in vivo, whereas BAP-Y653A exhibited a complete activity loss and did not restore protein disaggregation and thermotolerance in Δ*clpB* mutant cells (Figures 4C and 4D; Supplemental Figure S7). Consistent with this notion, BAP-Y653A/ClpP/KJE could no longer degrade dimeric TrfA (data not shown). Together, these data demonstrate that Tyr653 is essential for substrate translocation and protein disaggregation.

In order to provide direct evidence for a role of Tyr653 in substrate translocation and protein disaggregation, we replaced Tyr653 in vivo by the photocrosslinking amino acid p-benzoyl-L-phenylalanine (Phe*) (Chin et al., 2002). We monitored the interaction of this residue with peptide B1*, which was labeled with a biotin moiety to allow detection of crosslink products, and urea-denatured luciferase. Efficient crosslinking between ClpB-Phe*653 and peptide B1* was obtained in the presence of ATP and especially ATPγS, probably because the interaction with the substrate is less transient in this nucleotide state (Figure 4E). Crosslink products between ClpB-Phe653* and urea-denatured luciferase were obtained only in the additional presence of KJE (Figure 4F). These findings demonstrate that substrates enter the translocation channel of ClpB and directly contact the pore-located Tyr653 residue in the interior of ClpB. Notably, the ability of Tyr653 to contact substrates reflects the dependency of substrate degradation by BAP/ClpP on KJE: peptide B1 is degraded without KJE, whereas the degradation of urea-denatured luciferase requires the additional presence of KJE. Thus, these data provide further evidence for a function of KJE upstream of the translocation activity of ClpB.

The ClpP-Dependent Activity Switch of BAP/KJE Causes Loss of Thermotolerance

At present, it is unclear whether cell death during severe heat stress is caused by the formation of toxic protein aggregates or by the massive loss of active proteins. Consequently, it is also unknown whether the crucial role of ClpB in ensuring cell survival under such conditions relies on its ability to solubilize aggregates or on its ability to reactivate aggregated proteins. The ClpP-dependent activity switch of BAP/KJE is an ideal tool to distinguish between these two possibilities.

We first assessed the fate of the thermolabile model protein luciferase after heat-induced aggregation in Δ*clpB* mutant cells carrying plasmid-encoded *clpB*, *BAP*, and *clpP* under the control of IPTG-regulatable promotors. The plasmids differed in copy number, allowing the production of ClpB or BAP to wild-type levels (at 30°C) and ClpP to higher levels allowing saturating formation of BAP-ClpP complexes (Supplemental Figure S8). Heat shock to 45°C caused quantitative aggregation of luciferase, which was largely solubilized and refolded during a recovery phase at 30°C upon expression of ClpB or BAP (Figure 5A). Coproduction of ClpB and ClpP did not affect the reactivation of heat-aggregated luciferase during the recovery phase at 30°C. In contrast, the refolding of aggregated luciferase, which also occurred in cells producing BAP only, was almost completely abolished upon coproduction of BAP and ClpP, and, consistently, no luciferase could be regained in the soluble fraction (Figure 5A). Thus, in agreement with the data obtained in vitro, ClpP also switches BAP/KJE in vivo from a refolding to a degrading disaggregation machinery.

These results set the basis to determine whether this activity switch affects the development of thermotolerance.

We first determined the consequences of BAP and ClpP coproduction on cell viability upon heat shock to different temperatures (Figure 5B). BAP/ClpP coexpression did not significantly affect viability up to 48°C. However, dramatic cell death was observed at 50°C, similar to the phenotype of Δ*clpB* mutant cells (Figure 5B). In contrast, cells expressing either BAP alone or ClpB/ClpP remained viable. Notably, the reduced viability at 50°C correlated with maximal protein aggregation at this temperature, suggesting that the inability of cells that either lack ClpB or express BAP/ClpP to refold aggregated proteins causes cell death (Figure 5B).

Next, we followed the influence of BAP/ClpP coproduction on the development of induced thermotolerance. The production of plasmid-encoded BAP alone or ClpB with and without ClpP allowed the induction of thermotolerance in Δ*clpB* cells. In striking contrast, cells

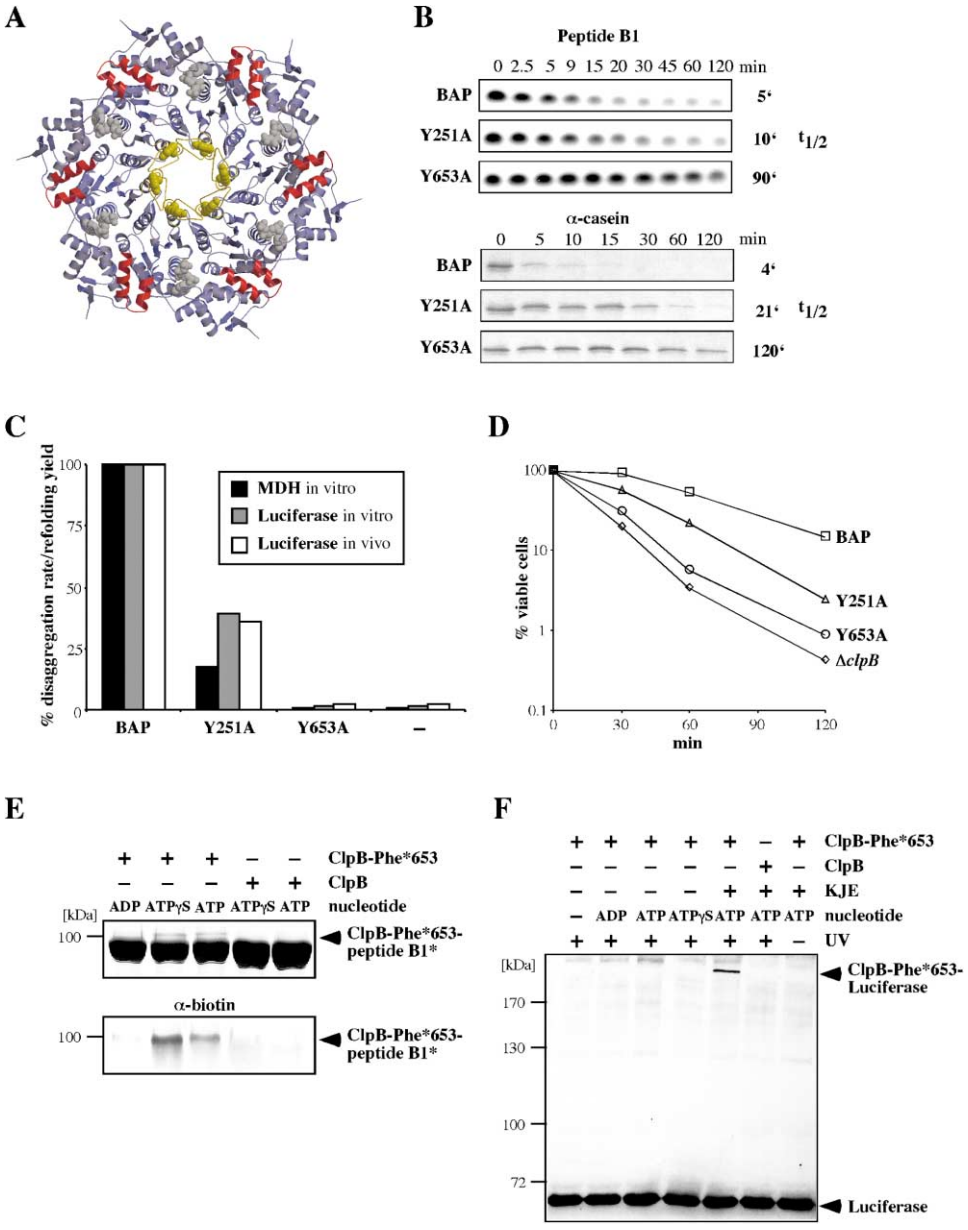

Figure 4. The Pore-Located Tyr653 Residue Is Crucial for Substrate Degradation and Protein Disaggregation

(A) Tyr653 is located close to the pore of the second AAA domain. Bottom-down view of a hexameric model of AAA-2 of *E. coli* ClpB. Tyr653 and the bound AMPPNP molecules are shown as CPK models and are colored in gold and gray, respectively. The helix-loop-helix motif is colored in red. Residues that are part of the Tyr653-containing loop are colored in gold.

(B) Degradation defects of BAP pore mutants. Degradation of the indicated substrates by BAP wild-type, BAP-Y251A, and BAP-Y653A was monitored in presence of ClpP. Calculated half-life times of substrates are given.

(C) Disaggregation defects of ClpB pore mutants in vitro and in vivo. The disaggregation activity of ClpB pore variants was determined in vitro by following the decrease of MDH turbidity (black bars) or by following the refolding of urea-denatured luciferase (gray bars) and by determining the yield of reactivated luciferase (180 min) after heat aggregation in vivo. The disaggregation activity of ClpB wild-type was set as 100%.

(D) ClpB pore mutants are affected in thermotolerance development. The survival rate at 50°C of *E. coli* Δ*clpB* mutant cells carrying plasmid-encoded *clpB* wild-type and mutant alleles was determined. The number of viable cells before heat shock to 50°C was set as 100%.

(E) Tyr653 directly interacts with substrate. ClpB-Phe*653 or ClpB was incubated with peptide B1*, bearing a biotin moiety, in the presence of indicated nucleotides. Crosslink products were analyzed by SDS-PAGE and silver staining (upper panel) or Western blotting (lower panel).

(F) Crosslinking of ClpB-Phe653* to urea-denatured luciferase is KJE dependent. ClpB-Phe*653 or ClpB was incubated with urea-denatured luciferase in the absence or presence of nucleotides and the DnaK chaperone system (KJE). Crosslinked mixtures were analyzed by Western blotting. A protein standard is given.

coproducing BAP and ClpP failed to develop thermotolerance and died at 50°C with a rate identical to Δ*clpB* mutant cells (Figure 5C). It is important to note that this loss of thermotolerance development was not caused by inefficient protein disaggregation, since aggregate solubilization was restored in Δ*clpB* mutant cells by co-production of BAP and ClpP (Figure 5D).

We also investigated whether uncontrolled proteoly-

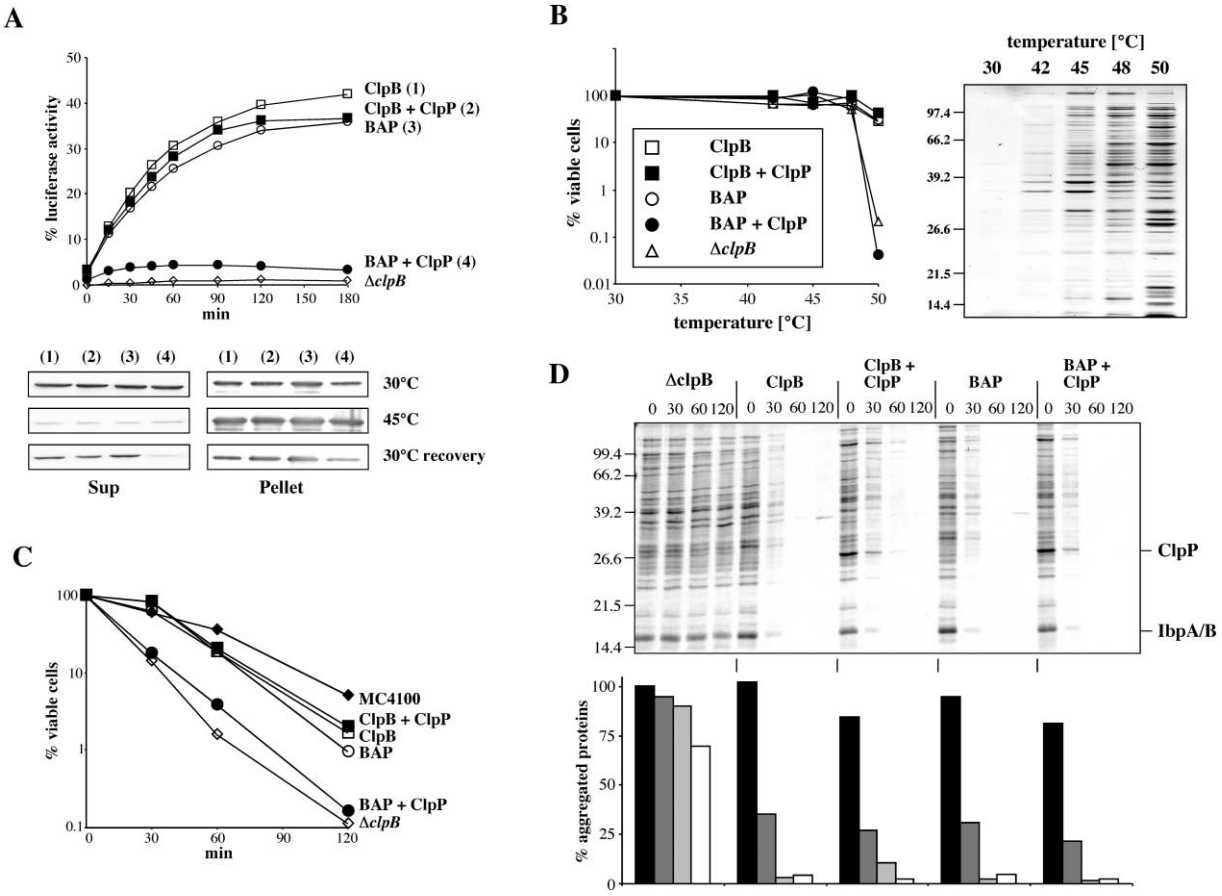

Figure 5. Reactivation of Aggregated Proteins Is Essential for Thermotolerance Development

(A) In vivo reactivation of heat-aggregated luciferase. The recovery of luciferase activity after heat shock was determined in the presence of the indicated plasmid-encoded components. The luciferase activity before heat shock was set as 100%. Immunoblot analysis of soluble and insoluble luciferase species before and after heat shock and after a 180 min recovery phase at 30°C are given.

(B) Temperature-dependent correlation between protein aggregation and cell viability. *E. coli* Δ*clpB* mutant cells expressing the indicated components were grown at 30°C to midexponential growth phase and heat shocked to the indicated temperatures for 30 min. Subsequently, the levels of aggregated proteins and cell viability were determined.

(C) The ClpP-induced activity switch of BAP/KJE leads to a loss of induced thermotolerance. Survival rates of *E. coli* wild-type (MC4100) or *E. coli* Δ*clpB* mutant cells expressing the indicated components were determined at 50°C.

(D) Coexpression of ClpP does not affect general protein disaggregation in vivo. Soluble and aggregated proteins of ^{35}S-L-methionine-labeled *E. coli* Δ*clpB* mutant cells expressing the indicated components were isolated directly after heat shock to 45°C (0 min) and at the indicated time points during a recovery phase at 30°C and analyzed by SDS-PAGE. The positioning of ClpP and the small heat shock proteins IbpA/B that coaggregate with thermolabile proteins during heat shock is indicated. A protein standard (kDa) is given. The amount of aggregated proteins was determined by scintillation counting and set as 100% for Δ*clpB* mutant cells (0 min).

sis by BAP/ClpP might be responsible for the loss of thermotolerance. Total protein degradation of short-lived polypeptides was increased at 30°C and 42°C upon coproduction of BAP and ClpP. However, the level of total protein turnover remained low (Supplemental Figure S9), and, accordingly, no differences in the levels of *E. coli* bulk proteins were detectable (Supplemental Figures S9 and S10). In agreement with these findings, coproduction of BAP and ClpP did not affect cell growth at 30°C or 42°C and decreased cell viability only slightly upon prolonged incubation at 45°C, comparable to the defects of Δ*clpB* mutant cells (Supplemental Figures S11 and S12). These data indicate that coproduction of BAP and ClpP does not lead to reckless protein degradation but only affects cell viability under conditions in which ClpB function becomes essential. We therefore conclude that reactivation but not pure solubilization of aggregated proteins is essential for the survival of cells during severe heat stress.

Discussion

We developed the ClpB variant BAP, which associates with the peptidase ClpP, to investigate the mechanism of ClpB-dependent protein disaggregation and the biological consequences of switching ClpB from a refolding to a degrading disaggregase.

Through introduction of a single structural element from ClpA, the P element containing helix-loop-helix motif that mediates ClpA interaction with ClpP, it was possible to convert ClpB into a chaperone that physically interacts and cooperates with ClpP in the degradation of substrates. The easiness by which this functional switch could be achieved is a manifestation of the close

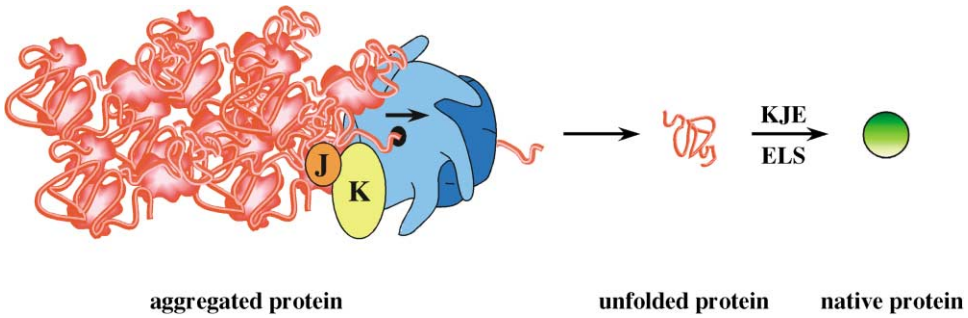

Figure 6. Model of Protein Disaggregation by ClpB/KJE

Protein disaggregation depends on the extraction of single, unfolded proteins from the aggregate surface via a translocation activity of ClpB. The DnaK chaperone system (KJE) is required for initial unfolding processes, potentially helping ClpB to extract polypeptides from the aggregate. After translocation, the extracted polypeptide is released to the solvent and refolded by a chaperone network, involving the GroEL (ESL) and DnaK chaperone systems.

structural and functional relationship between reactivating and degrading AAA+ chaperones.

Several conclusions regarding the working mechanism of ClpB can be drawn from our analysis of BAP. First, the demonstration that the BAP-ClpP complex is capable of degrading substrates provides, for the first time, direct evidence for substrate translocation through the central pore of ClpB. This conclusion is based on the solid evidence that, for all proteolytic systems tested, substrate translocation through the central pore of the AAA+ chaperone is a prerequisite for degradation by the associated peptidase (Hoskins et al., 2000; Kim et al., 2000; Ortega et al., 2000; Reid et al., 2001; Singh et al., 2000).

Second, we could show that the substrate translocation activity of ClpB is dependent on the conserved aromatic residues Y251 and Y653 that are located near the pores of the AAA domains. Roles for aromatic residues corresponding to Y251 of ClpB, located at the apical pore entrance of the AAA domain, have been described to be crucial for substrate degradation by several different peptidase-associated AAA+ chaperones (Siddiqui et al., 2004; Song et al., 2000; Yamada-Inagawa et al., 2003). Perhaps even more intriguing is the essential role of Y653, located inside the ClpB hexamer at the pore of the second AAA domain. A substitution of Y653 by alanine results in a complete loss of chaperone activity of ClpB in vivo and in vitro, in agreement with recently published data for a corresponding mutant in yeast Hsp104 (Lum et al., 2004). We furthermore show that introduction of the Y653A alteration into BAP results in the complete loss of substrate degradation activity of this variant. This finding provides independent strong evidence for the existence of a substrate translocation mechanism that critically depends on Y653. Consistently, we could demonstrate that substrates enter the translocation channel of ClpB during the disaggregation process and directly contact Y653. The exact role of the aromatic residues in the translocation process requires further investigations.

Third, several findings demonstrate that the substrate translocation activity of ClpB is an integral part of the disaggregation reaction. The BAP-mediated disaggregation and degradation processes are kinetically coupled. Furthermore, a tight physical connection between both processes exists, which is manifested by the direct transfer of substrate from the aggregate onto BAP and then into the proteolytic chamber of the associated peptidase without substrate becoming accessible in bulk solution. Finally, complexes between BAP and a ClpP-S111A variant, which is compromised in the uptake of translocated substrates, are affected in protein disaggregation. In case of wild-type ClpB, the extracted polypeptide is released to the solvent and refolded by a chaperone network, involving the GroEL (ESL) and DnaK (KJE) chaperone systems (Figure 6). According to this proposed mechanism, unfolded single polypeptides would be continuously extracted from the aggregates. We could indeed show that such an extraction process is operative in the solubilization of aggregated MDH by ClpB/KJE (C. Schlieker et al., submitted).

A fourth conclusion concerns the role of the DnaK chaperone system in the disaggregation process. Substrate translocation by ClpB is necessary but not sufficient for protein disaggregation, since solubilization of aggregated proteins remains KJE dependent. We find here that KJE is required neither for the basal translocation activity of ClpB nor for ClpB-substrate interaction. Furthermore, the "hand over" of translocated substrates from BAP to ClpP cannot replace KJE function, indicating that KJE does not have an essential activity downstream of ClpB in the disaggregation process. These observations strongly suggest that KJE is required for initial substrate unfolding processes, potentially helping ClpB to extract polypeptides from aggregates (Figure 6), and, consistently, the interaction of Y653 with urea-denatured luciferase was dependent on KJE. We do not exclude a second potential function of KJE downstream of the disaggregation process, which might be to take over the translocated polypeptide chain from ClpB, thereby preventing reassociation of solubilized proteins with aggregates and ensuring substrate refolding.

The role of the M domain of ClpB in protein disaggregation remains unclear. However, since ClpA, the closest ClpB homolog of *E. coli*, is missing the M domain, it is rather unlikely that this extra domain is needed for the basal translocation activity of ClpB. The M domains might be involved in the initial disentanglement of protein aggregates, providing an extra (crowbar) activity to facilitate substrate solubilization and translocation.

Alternatively, M domains might be needed to coordinate the cooperation with the DnaK chaperone system.

The ClpP-dependent conversion of BAP from a refolding to a degrading disaggregase allowed us also to clarify the precise role of the protein disaggregation reaction in thermotolerance development. We could demonstrate that the removal of protein aggregates by degradation is not sufficient to ensure cell survival during severe heat stress. Survival instead depends essentially on the solubilization and subsequent reactivation of aggregated proteins. Previous work has identified more than 300 different protein species to be prone to aggregation in heat-shocked cells of *E. coli*, among them many essential proteins such as EF-Tu and subunits of RNA polymerase (Mogk et al., 1999). Our data indicate that the refolding of such essential proteins in vivo is favored over proteolysis. This competitive advantage of refolding systems over proteolytic systems in the interaction with polypeptides extracted from aggregates documents the efficiency of the protein reactivation machinery, with ClpB as the central component, which ensures cell survival under severe stress conditions.

Interestingly, Riezman and coworkers demonstrated that increased proteolysis can restore growth at increased temperatures in yeast mutant cells lacking induction of major heat shock proteins (Friant et al., 2003). However, cell viability was only regained within a temperature range that does not require Hsp104 function and therefore does not depend on protein disaggregation. We suggest that the severity of the growth conditions (e.g., heat shock) and thereby the degree of lost proteins by aggregation are decisive for the dependence of cell viability on protein reactivation by ClpB/Hsp104.

Experimental Procedures

Strains, Plasmids, Proteins, and Peptides

E. coli strains used were derivatives of MC4100. Δ*clpB* mutant cells were plated on M9 plates containing arabinose and casamino acids, and arabinose-resistant colonies were selected. Cells were grown in Luria broth (LB) or M9 minimal medium supplemented with glucose (0.2%), and all L-amino acids (30 μg mg^{-1}) except L-methionine. Mutant derivatives of *clpB* were generated by PCR mutagenesis and standard cloning techniques in pUHE21-2fdΔ12 and were verified by sequencing. *clpB*/*BAP* and *clpP* were introduced into pHSG575 and pBB529 derivatives, respectively, allowing IPTG-dependent gene expression (Tomoyasu et al., 2001). Luciferase was expressed from pBB532 (Tomoyasu et al., 2001). ClpP-S111A was constructed by PCR mutagenesis and standard cloning techniques in pDS56 (C-His).

Wild-type and mutant ClpB was purified as described after overproduction in Δ*clpB::kan* cells (Mogk et al., 2003b). Purifications of DnaK, DnaJ, GrpE, ClpA, Lon, GroEL, and GroES were performed according to published protocols (Goloubinoff et al., 1999; Reid et al., 2001; Zehnbauer et al., 1981). N-terminally His_6-tagged TrfA and C-terminally His_6-tagged ClpP or ClpP-S111A were purified by Ni-NTA affinity chromatography following standard protocols. Pyruvate kinase and α-casein were purchased from Sigma; malate dehydrogenase (MDH) of pig heart muscle and firefly luciferase were from Roche. Peptides were chemically synthesized and purified by HPLC. MDH was labeled by use of N-succinimidyl [2,3-^{3}H] propionate (Amersham) as described (Weibezahn et al., 2003). Protein concentrations were determined with the Bio-Rad Bradford assay.

Biochemical Assays

ATP hydrolysis rates under steady-state conditions were determined as described (Mogk et al., 2003b). ClpB disaggregation activities were determined by following the solubilization and refolding of aggregated MDH according to published protocols (Mogk et al., 2003b). Refolding of urea-denatured luciferase followed published protocols (Krzewska et al., 2001). Chaperones were used at the following concentrations: 1 μM ClpB (wild-type or derivatives); 1 μM DnaK, 0.2 μM DnaJ, 0.1 μM GrpE; 14 μM GroEL-D87K; 2 μM ClpP; and 3 μM GroEL, 3 μM GroES.

Oligomerization and structural integrity of ClpB variants were tested as described (Mogk et al., 2003b). Complex formation between ClpB and ClpP was followed by size exclusion chromatography (Superose 6, Amersham) in buffer Z (25 mM Tris [pH 7.5], 10 mM $MgCl_2$, 75 mM KCl, 2 mM DTT) at room temperature. ClpB (6 μM) (wild-type and derivatives) was incubated with 7 μM ClpP in the presence of 2 mM ATPγS for 5 min at 30°C. Complex formation was monitored by UV signals (AU_{220}) and SDS-PAGE.

The incorporation of the photocrosslinking amino acid p-benzoyl-L-phenylalanyl (pBpa) at the ClpB pore (Y653) followed published protocols (Chin et al., 2002). Crosslinking of ClpB Phe*653 (1 μM) to substrates (5 μM peptide B1*, 50 nM urea-denatured luciferase) was induced by UV irradiation and visualized by immunoblotting using either an avidin-alkaline phosphatase fusion protein or luciferase-specific antisera.

Degradation Assays

All degradation assays were carried out using 1 μM BAP and 1.5 μM ClpP in the presence or absence of the DnaK chaperone system (KJE, 1 μM DnaK, 0.2 μM DnaJ, 0.1 μM GrpE). Substrates were used at the following concentrations: 2 μM α-casein, 50 μM peptide B1, 2 μM TrfA, and 1 μM MDH (aggregated). Substrate degradation was followed by gel electrophoresis according to published protocols. Coomassie-stained SDS-PAGEs were quantified using MacBas software.

Protein Sequence Alignment, Modeling of ClpB, and Electron Microscopy

Protein sequence alignments were carried out using ClustalW program at the Pole Bio-Informatique Lyonnais, Network Protein Sequence Analysis (http://npsa-pbil.ibcp.fr). A hexameric model of *E. coli* ClpB was developed based on the crystal structure and single particle reconstitution by cryo electron microscopy of *T. thermophilus* ClpB (Lee et al., 2003). Since the loop comprising Tyr653 is disordered in the crystal structure of *T. thermophilus* ClpB, residues 637–660 (*E. coli* ClpB numbering) were replaced by the corresponding residues of *E. coli* ClpA after superimposing the crystal structures of ClpA (Guo et al., 2002) and *T. thermophilus* ClpB via the corresponding AAA-2. For electronmicroscopy, ClpB E279A/E678A derivatives (wt or BAP) and ClpP (each protein 120 nM) were incubated in buffer Z with ATP for 5 min at 30°C. EM was performed essentially as described (Ortega et al., 2000).

In Vivo Activity Assays

Protein disaggregation in vivo was analyzed in cultures of *E. coli* Δ*clpB* mutant strains, bearing plasmid-encoded *clpB* alleles under the control of an IPTG-regulatable promotor. Cells were grown in LB medium or M9 minimal medium in the presence of 250 μM IPTG at 30°C to logarithmic phase, then shifted to 45°C for 30 min followed by a recovery phase at 30°C for 120 min. Soluble and aggregated proteins were isolated as described (Tomoyasu et al., 2001). Gel electrophoresis and immunoblotting were carried out according to published protocols.

Reactivation of heat-aggregated luciferase was analyzed in Δ*clpB* mutant strains, bearing plasmid-encoded luciferase, various *clpB* alleles, and *clpP*. Cells were grown in LB medium containing 250 μM IPTG for 3 hr. Arabinose (0.1% w/v) was added, and cells were grown for another hour. Synthesis of luciferase was blocked by addition of tetracycline (25 μg/ml) and 1% (w/v) glucose, and cells were subsequently shifted to 45°C for 20 min. Reactivation of luciferase was followed during a recovery phase at 30°C by determining the in vivo luciferase activity as described (Tomoyasu et al., 2001).

Survival of *E. coli* cells after exposure to lethal temperature was determined by calculating the plating efficiency. Cells were grown in LB medium to midexponential growth phase at 30°C, followed by a preheat shock to 42°C for 15 min. Subsequently, the cells were incubated at 50°C for the indicated time. Serial dilutions (10^{-3}–10^{-6})

of cells were prepared in LB medium and spotted onto LB plates. Plates were incubated for 24 hr at 30°C, and colony numbers were determined.

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