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The DnaK/ClpB chaperone system from *Thermus thermophilus*

S. Schlee and J. Reinstein*

Max-Planck-Institute for Molecular Physiology, Department of Physical Biochemistry, Otto-Hahn-Str. 11, 44227 Dortmund (Germany), Fax: +49 231 133 2699, e-mail: joachim.reinstein@mpi-dortmund.mpg.de

Abstract. Proteins of thermophilic organisms are adapted to remain well structured and functional at elevated temperatures. Nevertheless like their 'cousins' that reside at medium temperatures, they require the assistance of molecular chaperones to fold properly and prevent aggregation. This review compares structural and functional properties of the DnaK/ClpB systems of *Thermus thermophilus* and, mainly, *Escherichia coli* ($DnaK_{Tth}$ and $DnaK_{Eco}$). Many elemental properties of these systems remain conserved. However, in addition to a general increase of the thermal stability of its components, the $DnaK_{Tth}$ system shows profound differences in its regulation, and genetic as well as oligomeric organization. Whether these differences are unique or represent general strategies of adaptation to life at elevated temperatures remains to be clarified.

Key words. Protein folding; chaperone; DnaK; DnaJ; GrpE; ClpB; heat-shock; thermophile.

Introduction

Life for thermophilic organisms is a challenge. Unlike microbes or higher organisms that live at high salt concentrations or exotic pH values, for example, they cannot evade the unfriendly nature of their environment by simply adjusting their interior milieu through accessory proteins such as ion or proton pumps or compatible solutes to achieve more cozy (ambient) conditions [1, 2]. Rather heat directly effects elementary constituents of the cell. High temperature increases the fluidity of membranes and affects the structure and function of proteins and nucleic acids. Thermophilic organisms have evolved sophisticated mechanisms to cope with these problems.

One of the most prominent and interesting enigmas in that respect is how protein folding and maintenance of well-defined structures are achieved. Since this problem is related to one of the most important and challenging questions – 'the protein folding problem' – thermophilic organisms and their proteins have attracted particular attention in the last 2–3 decades. Identifying strategies of how thermophilic proteins fold and gain stability might also lead to a deeper understanding of folding in general. Indeed, investigation of thermostable proteins brought substantial insights; however, the mechanisms by which stability is achieved are much more diverse than initially anticipated [3].

Although the native fold of a protein is encoded by its amino acid sequence, after de novo synthesis, and especially under stress conditions, many proteins need the assistance of molecular chaperones to reach their native state efficiently. Because thermophilic organisms also provide a complex cellular machinery of molecular chaperones, the intriguing question, How do thermophiles adapt? becomes even more complex. In any case the chaperone system must meet the general requirements that affect every enzymatic function at elevated temperatures [4].

This review aims at a description of the organization and functional properties of the DnaK system of *Thermus thermophilus*, an organism that inhabitates environments of 40–85 °C. In comparison with the properties of the best characterized DnaK system from *Escherichia coli*, particular properties that are connected to the adjustment to higher temperatures may be identified.

* Corresponding author.

General roles and mechanisms of Hsc70/DnaK and Hsp104/ClpB

DnaK

In the simplest case the assistance of chaperones in the folding of proteins can be described by a passive 'holding' mechanism, according to which chaperones increase the yield of folded proteins by binding and holding onto unfolded or partially folded polypeptide chains [5–7]. This withdraws aggregation-prone species from free solution and therefore helps to prevent protein aggregation. Subsequent release of substrate proteins then allows for further folding.

Chaperones of the Hsp70 family (appear to) use the chemical energy that becomes available upon ATP hydrolysis also to drive this binding and release cycle and possibly make it more efficient [8–14]. Since the nucleotide bound to these molecular chaperones (ATP vs. ADP) determines the affinities and exchange rates of substrate proteins [6], the intrinsic ATPase activity of Hsp70 chaperone proteins controls the switch between high and low affinity states for substrate binding [15, 16].

The structural and mechanistic aspects of the Hsp70 system are best understood for the *Escherichia coli* system, which consists of three components. The principal member of the prokaryotic Hsp70 chaperone system, $DnaK_{Eco}$, binds and hydrolyses ATP. In the ATP-bound state of DnaK, rapid substrate binding occurs. Stable holding of peptides involves a conformational change that is achieved by hydrolysis of bound ATP to ADP. The two co-chaperones or cohort proteins, the nucleotide exchange factor $GrpE_{Eco}$ [17] and the ATPase stimulating protein $DnaJ_{Eco}$ [17, 18], control the switch between the two nucleotide states and thereby regulate the interaction of DnaK with its substrates. $DnaJ_{Eco}$ stimulates the intrinsically low ATPase rate of $DnaK_{Eco}$ by a factor of 500–15,000 [19, 20], and $GrpE_{Eco}$ accelerates nucleotide exchange by a factor of 5000 [21].

ClpB

Members of the Clp/Hsp100 protein familiy exist in the cytosol of eubacteria and some eukarya, as well as within eukaryotic organelles [22]. They are involved in a variety of biological activities such as thermotolerance, proteolysis, DNA transposition and gene regulation. In spite of their diverse functions they seem to employ a common biochemical mechanism: the disassembly of quaternary protein structures and dissolvation of protein aggregates [23, 24].

Based on the number of nucleotide binding sites the Hsp100-proteins are classified in two groups [25]. Prokaryotic ClpB is a member of class 1, typified by two nucleotide binding domains, and is a homologue of yeast Hsp104, which was the first protein to be characterized in the B subfamily [26]. Recently, the functional cooperation between the chaperones Hsp70 and Hsp104 in recovery of aggregated proteins has been described. In eukaryotes this cooperation enables yeast to recover from heat stress in vivo and reactivate proteins that had been chemically denatured and aggregated in vitro [27]. In prokaryotes this functional interaction between the DnaK and ClpB chaperone system was also reported for *E. coli* [28–30] and *T. thermophilus* [31, 32].

ClpB contains two nucleotide-binding domains (NBD1 and NBD2). The two NBDs possess the classical Walker-type consensus sequences and are highly conserved throughout all members of class1 Hsp100 proteins, respectively. However, a comparison of NBD1 and NBD2 shows limited sequence similarity [33, 34]. The NBDs are separated by a middle region whose length varies within the subfamilies and are flanked by amino- and carboxy-terminal regions. This might be an implication for distinct conserved functions of the two binding sites.

Organization of the dnaK/clpB operon

Genomic level

Chaperones are present in the three 'primary kingdoms' of life: bacteria, archaea and eukaryotes. The sequence homology between the proteins from eukaryotes and bacteria illustrates a remarkable conservation of the heat shock genes in evolution. For example, the DnaK protein from *E.coli* is 48% identical to the Hsp70 protein of *Drosophila* [35], the homology beween ClpB from *Thermus thermophilus* and Hsp104 from yeast amounts to 45% (genestream align). While bacteria and archea contain only few *hsp70*-related genes, eukaryotes possess multigene families related to hsp70 and its cochaperones (table 1).

Despite the sequence homology of chaperones from different organisms the organization of the *hsp70* loci differs. Generally, the two co-chaperones GrpE and DnaK are included within the *dnaK* operon (fig. 1, *Bacillus* species and archaea). An exception is *E. coli* where *grpE* is not included. The *dnaK* operon of *T. thermophilus* is different because besides *dnaK* and its co-chaperones, it contains also *dafA* and *clpB*. Interestingly, ClpB has not been identified in any other thermophilic organism so far. Moreover, even Hsp70 has not been found in any of the hyperthermophilic archaea investigated up to this time [36]. The surprising conclusion one has to draw from these data is that thermophilic organisms apparently do not depend on a complete set of the DnaK/ClpB system.

Structure

The structural organization of the DnaK/ClpB system is described with respect to amino acid sequence, secondary, tertiary and quarternary structure.

Table 1. Number of *hsp70* and *hsp104* related genes in different organisms (mainly derived from Prosite). In most species, there are many proteins that belong to the Hsp70 familiy. Some of them are expressed under unstressed conditions and can be found in different cellular compartments. Signatures of DnaJ are the N-terminal 'J' domain and a central domain containing four repeats of a CXXCXGXG motif ('CRR' domain). Some proteins assigned to the DnaJ familiy only contain the 'J'-domain. Whereas all Hsp70 proteins seem to cooperate with J-domain-containing partners, some may be independent of a GrpE analog since ATP hydrolysis and not dissociation of ADP is usually limiting here. Additionally, nucleotide exchange factors like Bag-1, which is structurally unrelated to GrpE, may substitute in that respect [87, 88].

	dnaK (hsp70)	dnaJ (hsp40)	grpE	clpB (hsp104)
Thermus thermophilus	1	1	1	1
Escherichia coli	3 (hsp70, hscA, hscC)	3 (dnaJ, cbpA, hscB)	1	1
Saccharomyces cerevisiae	≥ 10 (ssa, ssb, ssc, ssd, sse)	≥ 8 (ydj1, mdj1, xdj1, scj, sis)	1 (mge1)	2 (hsp104, hsp78)
Drosophila melanogaster	≥ 8 (hsp70, hsp68, hsc1–6)	≥ 2 (dnaJ60, csp)	1 (grpE, mitoch.)	?
Arabidopsis thaliana	≥ 3 (hsc1–3)	2 (atj, j10)	≥ 7 (mainly mitochondrial)	1 (clpB)
Mus musculus	≥ 8 (hspA1-6, hsc70, BiP)	≥ 16 (A, B and C subfamily)	2 (grpEL1, grpEL2)	?
Homo sapiens	≥ 8 (hspA1–6, hsc70, BiP)	≥ 15 (hdj1, hdj2, A, B and C subfamily)	1 (Mt-grpE)	?

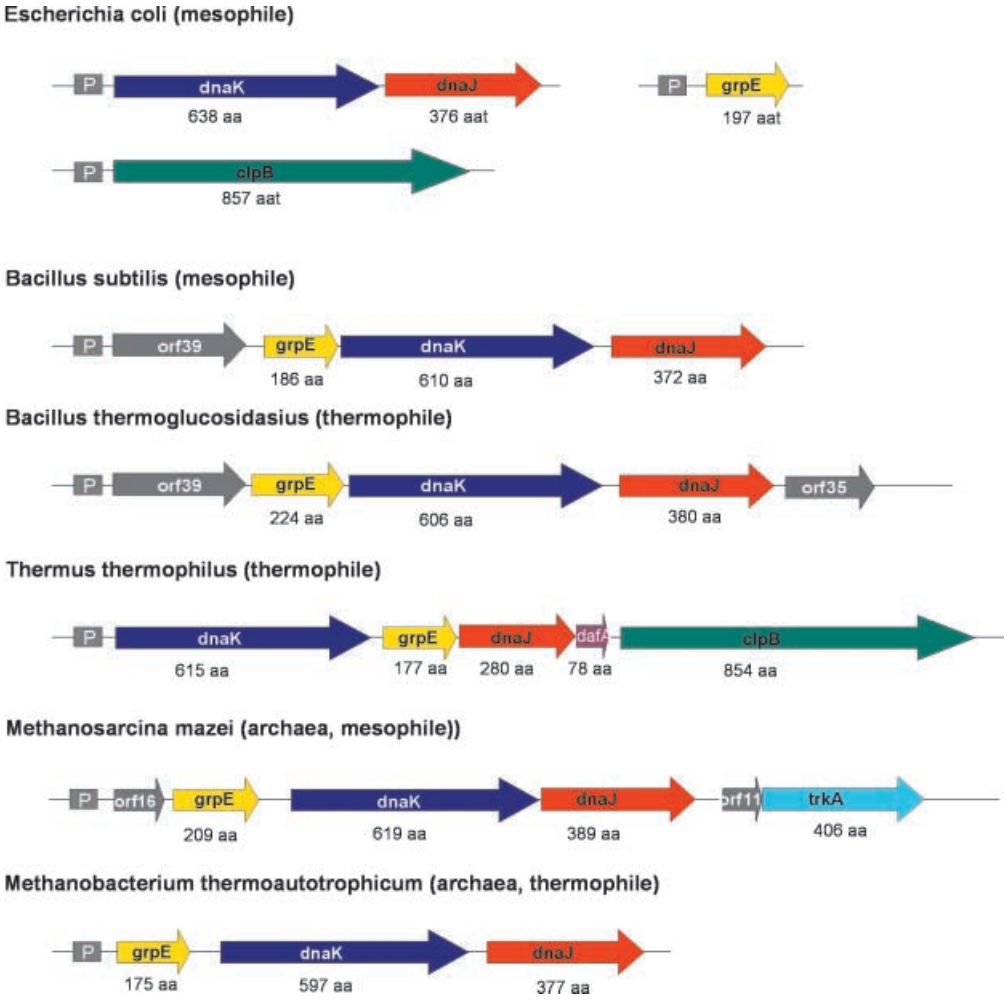

Figure 1. Graphic representation of the *hsp70* (*dnaK*)-locus genes and their organization. The arrows represent the protein-coding regions of the genes; figures below the arrows are numbers of amino acids encoded. References used were the EcoGene Database; *Bac. subtilis* [89], *Bac. thermoglucosidasius* [36], *Thermus thermophilus* [37, 47, 90], archaea [91].

Not too unexpected, the amino acid sequences of the corresponding chaperones from *E. coli* and *T. thermophilus* share a rather high level of identity with DnaK (55%), GrpE (27%), DnaJ (34%) and ClpB (56%) [31, 37, 38]. Interestingly DnaJ from *T. thermophilus* ($DnaJ_{Tth}$) lacks the Zn^{2+} binding motif that most mesophilic DnaJ proteins have [37]. $DafA_{Tth}$ on the other hand apparently has no counterpart in *E. coli*, nor has any other sequence been found with appreciable homology to $DafA_{Tth}$ in the SwissProtein database.

This high level of sequence homology is also translated to secondary structure. According to secondary structure prediction programs and measurements of circular dichroism spectra (CD), secondary structures of at least DnaK and GrpE from *E. coli* and *T. thermophilus* are comparable [38, 39]. Information about the three-dimensional structures of the thermophile chaperones is not available, and comparisons with known structures of DnaK, GrpE and DnaJ [40–42] determined for the *E. coli* system cannot be made.

DnaK/DnaJ/GrpE quarternary structure

The oligomeric organization of the DnaK system from *E. coli* was summarized in an excellent and critical review [43]. Based on this overview and studies with analytical ultracentrifugation, the oligomeric states of the DnaK system from *E. coli* may be outlined as follows.

$DnaK_{Eco}$ appears to be present as monomer and dimer but also higher-ordered oligomer, depending on the particular experimental conditions and methods used. In the presence of $GrpE_{Eco}$ or $DnaJ_{Eco}$, $DnaK_{Eco}$ oligomers are 'solubilized' and mainly form $DnaK_1GrpE_2$, $DnaK_1DnaJ_1$ or $DnaK_1DnaJ_2$ complexes. The oligomeric state of $DnaJ_{Eco}$ alone is not well-defined; it is reported to form 2- to 8-mers. In contrast, according to most studies, GrpE forms a well-defined dimer in solution. It was reported that Hsc70 forms large oligomers in the presence of ATP and catalytic amounts of DnaJ [44].

In the case of the $DnaK_{Tth}$ system, comprehensive analytical studies about oligomeric states are mostly lacking. $GrpET_{th}$ again has the most well-defined oligomeric state and is dimeric [45]. DnaK and DafA form oligomers of various sizes; according to dynamic light scatter experiments they are highly polydisperse [unpublished results]. One notable exception, however, is the well-defined complex of three molecules of $DnaK_{Tth}$ with three molecules of $DnaJ_{Tth}$ to give a $(DnaK_{Tth} \cdot DnaJ_{Tth})_3$ complex that can be directly isolated from *T. thermophilus* HB8 cells [46]. It was recognized later that this complex also contained three molecules of a 78-amino acid protein named $DafA_{Tth}$ (DnaK-DnaJ association factor) [37]. Analytical studies with the isolated components showed later on that formation of the ternary heterotrimeric $(DnaK_{Tth} \cdot DafA_{Tth} \cdot DnaJ_{Tth})_3$ complex is highly synergistic. Binary complexes between $DnaK_{Tth}$ and $DnaJ_{Tth}$ or $DafA_{Tth}$ with $DnaK_{Tth}$ or $DnaJ_{Tth}$, respectively, could not be observed [47].

Oligomerization of ClpB is highly dynamic and depends on several determinants: ClpB-concentration (obvious), bound nucleotide, environmental factors such as temperature and salt concentration. Furthermore it also seems plausible that binding of substrate proteins and interactions with cooperating chaperones could influence its oligomeric state.

As demonstrated by gel-filtration chromatography, cross-linking studies and electron microscopy for ClpB proteins from *Saccharomyces cerevisiae* [48, 49], *E. coli* [50] and *T. thermophilus* [32, 51] the active ATP-bound form is a ring shaped homo-hexamer with an axial pore. Exceptional is the finding of Kim and co-workers, who found a ring-shaped heptamer to be the functional form of $ClpB_{Eco}$ [52].

Both binding of ADP and high salt concentrations lead to deoligomerization; under these conditions monomers and dimers are the prevailing species [32, 53]. The tendency of the thermophile $ClpB_{Tth}$ to form hexamers is enhanced at higher temperatures (55 °C) [51].

A comparison with *N*-ethylmaleimide-sensitive factor (NSF) led to the general assumption, that nucleotide binding to one of the two nucleotide binding domains (NBDs) of ClpB might serve simply to promote hexamerization, while the other NBD is responsible for ATP hydrolysis and enzymatic activity [54]. However, mutational studies demonstrated that in the case of Hsp104 from yeast, distinct functional roles of the two NBDs cannot be assigned unequivocally [55].

Temperature stability

DnaK

The thermal stability of $DnaK_{Tth}$ was investigated by CD in the absence of ADP and showed a transition at 96 °C [39]. The unfolding of $DnaK_{Tth}$ in the presence of 1 mM ADP was analysed by differential scanning calorimetry and gave a thermal transition midpoint at 100.7 °C [45]. The unfolding of $DnaK_{Tth}$ is completely irreversible after heating to 130 °C. In comparison, $DnaK_{Eco}$ has transitions at 41 °C without and 59 °C in the presence of nucleotide [56, 57], and three transitions were observed when GdnHCl was used as denaturant [58]. A second transition at 75 °C in the presence of nucleotide was reported recently using CD [59].

An interesting question concerns the structural basis for the increased temperature stability of $DnaK_{Tth}$. Since the sequence homology of $DnaK_{Eco}$ and $DnaK_{Tth}$ is 73% and the identity is 55%, the structures are expected to be similar. Yet the melting temperature of $DnaK_{Tth}$ is some 50 °C higher than that of $DnaK_{Eco}$. This supports the idea that thermostability can be achieved by only subtle structural

changes [60]. In this context it is important to remember, that $DnaK_{Tth}$ also forms a tight complex with $DnaJ_{Tth}$ and Daf_{Tth} [37, 46], supporting the notion that multimer formation is a strategy to increase thermostability [60].

DnaJ

$DnaJ_{Tth}$ also has an appreciable temperature stability with a T_m of 99.8–100.9 °C measured by differential scanning calorimetry [45]. After heating to 130 °C about 50% of the protein refolds. However, Yoshida and co-workers reported $DnaJ_{Tth}$ to be unstable beyond 65 °C [37]. In comparison, $DnaJ_{Eco}$ shows a single transition at 58 °C [59] as determined with CD: similar to $DnaK_{Tth}$, additional stabilization may be gained through $(DnaK_{Tth} \cdot DafA_{Tth} \cdot DnaJ_{Tth})_3$ complex formation.

$GrpE_{Tth}$

According to differential scanning calorimetry measurements (DSC), the dimeric GrpETth protein shows two distinct thermal transitions [45]. The first transition at 90 °C is independent of the protein concentration, while the second increases from 99.5 to 105 °C with increasing amounts of $GrpE_{Tth}$.

This behaviour differs from the folding properties that were reported for the majority of dimeric proteins. In most cases folding was described by a simple two-state-unfolding process, where unfolding of the protein was coupled to monomerization and only a single transition was observed with DSC [61–63]. Alternatively, two separate unfolding transitions were observed [64, 65] where the first transition (monomerization) was concentration dependent followed by the complete unfolding of monomers.

Further analysis revealed that the first transition can be assigned to the C-terminal domain of $GrpE_{Tth}$, which is mostly composed of β-sheets and loop structures [45, 66]. The structure of the $DnaK_{Eco} \cdot GrpE_{Tth}$ complex shows an interaction of the C-terminal domain with the ATPase domain of DnaK [40]. Accordingly, melting of this domain at the corresponding transition temperature of 90 °C for $GrpE_{Tth}$ ceases nucleotide exchange [45]. The second transition affects the long N-terminal α-helix of GrpE that mediates dimerization.

$GrpE_{Eco}$ also exhibits two separate transitions at 48 and 75 °C. Also here, at the temperature of the first thermal transition, the nucleotide exchange activity of $GrpE_{Eco}$ is reduced and deviates significantly from the hypothetical Arrhenius behaviour [59]. It is noteworthy that despite the high degree of homology, with $GrpE_{Eco}$ both temperature-induced transitions are coupled to a change of CD signal [59], whereas only the second transition of $GrpE_{Tth}$ results in a change of the CD signal [45].

In summary, the GrpE protein is the only member of the DnaK system that exhibits a transition in the relevant temperature range of heat shock response both in *T. thermophilus* and *E. coli* [38, 59]. This gave rise to the attractive hypothesis that GrpE is the major temperature-controlled regulator of the DnaK ATPase cycle and, accordingly, its chaperone activity.

Functional properties

Peptides and substrate proteins

Hsp70 proteins function by binding and releasing extended polypeptides that are exposed by proteins in their nonnative state, in an ATP-dependent manner. Thus, Hsp70 recognizes structural features common to most nascent chains: exposed hydrophobic amino acid side chains flanked by positive charges with an accessible polypeptide backbone [67–71]. Two peptides that adhere to this motif and bind to $DnaK_{Eco}$, namely a 24mer derived from σ^{32} of *E. coli* [15, 39] and a 10mer derived from p53 [67], also bind to $DnaK_{Tth}$ [47].

Substrate proteins for the $DnaK_{Tth}$ system investigated so far included reduced carboxymethylated α-lactalbumin (RCMLA) [46], lactate dehydrogenase, glucose-6-phosphate dehydrogenase (G6PDH) and α-glucosidase [31]. Also, the widely used model substrate firefly luciferase is suitable [38]. Thus, at this point there is no reason to assume that the specificity of $DnaK_{Tth}$ and $DnaK_{Eco}$ differ substantially.

DnaK ATPase activity and nucleotide binding

ATPase activity of Hsp70 proteins is crucial for their biological function and thus an important indicator of enzymatic activity [72, 73]. Specifically, for an enzyme from a thermophile organism it is important to clarify that its mobility at ambient temperatures (e.g. 25 °C), where many functional measurements are performed, is not severely impaired [4, 74, 75]. $DnaK_{Tth}$ hydrolyses ATP in single turnover assays with 0.3×10^{-3} s^{-1} at 25 °C and with 1.0×10^{-3} s^{-1} at 75 °C, which is about threefold higher. An Arrhenius plot for ATPase activity between 25 and 95 °C is approximately linear, indicating no change of the rate limiting step [39]. From its slope an activation energy (E_a) of 25.7 kJ mol^{-1} was calculated, which is low in comparison to the rather pronounced temperature dependence of the ATPase activity of $DnaK_{Eco}$ [76] with an E_a of 150 kJ mol^{-1}. The $DnaK_{Tth} \cdot DnaJ_{Tth} \cdot Daf_{Tth}$ complex hydrolyses ATP with 5.0×10^{-3} s^{-1} at 80 °C [37], which indicates that even at 80°C the *T. thermophilus* complex is still substantially active. The absolute values of hydrolysis rates of $DnaK_{Tth}$ at 75 °C and $DnaK_{Eco}$ at 25 °C are thus comparable, supporting the concept of corresponding states [77].

Surprising, however, is the fact that the activity of $DnaK_{Tth}$ at 25 °C is only threefold lower than at 75 °C, which indicates a 'broad working range' for this enzyme. This might be a consequence of the wide temperature range *T. thermophilus* inhabits. Like other Hsp70/DnaK

chaperones, $DnaK_{Tth}$ requires potassium and magnesium ions for ATPase and chaperone activity [78].

$DnaK_{Tth}$ binds ADP with a dissociation constant (K_dADP) of 47 nM at 25 °C and 280 nM at 75 °C. [39]. The corresponding rate constant for dissociation (k_{off}ADP) are 0.08×10^{-3} s^{-1} (25 °C) and 1.7×10^{-3} s^{-1} (75 °C), respectively. In comparison, $DnaK_{Eco}$ has a K_dADP of 130 nM and k_{off}ADP of 35×10^{-3} s^{-1} [16]. It is thus evident that at 25 °C nucleotide binding and release are the rate-limiting steps in the thermophile DnaK system [39]. This is in sharp contrast to the $DnaK_{Eco}$ system, where ATP hydrolysis is rate limiting in the absence of cofactors [15, 16, 79].

ClpB ATPase/nucleotide binding

The Hsp104/ClpB proteins express both basal and protein-stimulated ATPase activity [30, 80]. ATP binding and hydrolysis is crucial for efficient functioning of the Clp proteins, as point mutations in the Walker motifs, which interfere with ATP binding or hydrolysis, eliminate the chaperone activity of Clp proteins [29, 32, 51, 53, 81].

The properties of the two NBDs regarding nucleotide binding and ATP hydrolysis are very different. In Hsp104 from yeast, NBD1 is a low-affinity site for ATP with a relatively high turnover ($k_{cat1} = 76$ min^{-1}, $K_{m1} = 170$ µM); the second site has much higher affinity and a 300-fold slower turnover at 30 °C ($k_{cat2} = 0.27$ min^{-1}, $K_{m2} = 4.7$ µM) [55, 82]. Both sites show positive cooperativity. $ClpB_{Tth}$ also exhibited positive cooperativity in ATP hydrolysis [$k_{cat} = 2.6$ min^{-1} at 25 °C, $K_m = 345$ µM, Hill coeffficient (n_h) = 3.1]. $ClpB_{Tth}$-mutants with defects in their WalkerA motifs of NBD1 and, -2, respectively, have lost this cooperativity [32]. It remains to be clarified, whether the cooperativity is based on homogeneous interactions through one NBD type exclusively or between NBD1 and NBD2 in the ring.

Some data for nucleotide affinity for NBD2 are also available. Notably, ADP is bound tighter by a factor of 10 compared to ATP, with dissociation constants of 2 µM for ADP and 30 µM for ATP for $ClpB_{Tth}$ [32, 51], and 9 µM and 69 µM for Hsp104 [55]. The mechanisms that link ATP binding and hydrolysis to disaggregation of protein substrates remain undefined at this point.

Regulation of ATP cycle and chaperone activity

A comparison of the regulatory features of the $DnaK_{Tth}$ and $DnaK_{Eco}$ system should specifically indicate potential differences that are connected to adaption of the thermophile organism. The DnaK system from *E. coli* is regulated by the two cohort proteins $DnaJ_{Eco}$ and $GrpE_{Eco}$. $DnaJ_{Eco}$ stimulates ATP hydrolysis thus populating the $DnaK_{Eco}$ · ADP state; and $GrpE_{Eco}$ acts as an antagonist of DnaJ and accelerates nucleotide exchange, thereby propagating the ATP state. For a more detailed description of the $DnaK_{Eco}$ chaperone cycle, see also [83, 84].

GrpE

The maximal stimulation of nucleotide exchange (k_{off}) by $GrpE_{Tth}$ is 80,000-fold, in comparison to a 5,000-fold stimulation by $GrpE_{Eco}$ [21]. Since the presence of $GrpE_{Tth}$ leads to an only moderate (10-fold) reduction of nucleotide affinity in the ternary complex, binding (k_{on}) and release (k_{off}) of nucleotides are accelerated to a similar extent [38]. As a consequence, $GrpE_{Tth}$ overcomes both rate-limiting steps of the $DnaK_{Tth}$ cycle – binding of ATP and release of ADP – and therefore causes a switch to the ATP state of $DnaK_{Tth}$. Kinetic experiments and the

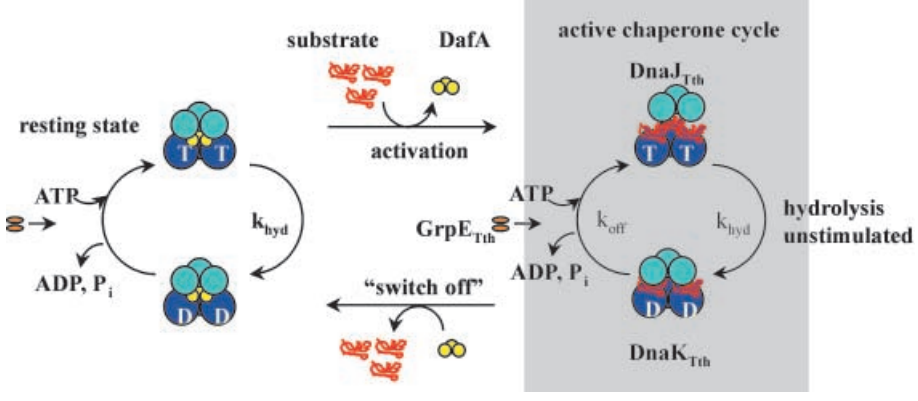

Figure 2. Model for the regulated chaperone cycle of DnaK from *Thermus thermophilus* ($DnaK_{Tth}$). Adapted from [47]. $DnaK_{Tth}$ (blue) and $DnaJ_{Tth}$ (cyan) form a heterohexameric complex that is assembled by the DnaK-DnaJ assembly factor $DafA_{Tth}$ (yellow). This complex has a slow intrinsic ATPase turnover that can only be moderately stimulated by the nucleotide exchange factor $GrpE_{Tth}$ (orange) since ATPase activity of $DnaK_{Tth}$ remains limiting. The binding of substrate protein (red) and $DafA_{Tth}$ is competitive, therefore, $DafA_{Tth}$ has to be released before protein can bind. The complex of $DnaK_{Tth}$, $DnaJ_{Tth}$ and substrate protein cycles between ATP and ADP states, comparable to DnaK from *E. coli*. Hydrolysis, however, remains unstimulated, which is in sharp contrast to $DnaK_{Eco}$, where hydrolysis of ATP is stimulated 10^3–10^4-fold in the presence of DnaJ and substrates.

structure of the $DnaK_{Eco}$-ATPase domain with $GrpE_{Eco}$ indicate that GrpE functions as nucleotide exchange factor by opening the nucleotide binding pocket [21, 40]. The kinetic data described for $GrpE_{Tth}$ imply that $GrpE_{Tth}$ applies the same mechanism – general principles of nucleotide exchange seem to be conserved [38].

DnaJ

In contrast, the role of $DnaJ_{Tth}$ differs markedly in the ATPase cycle of $DnaK_{Eco}$, compared with $DnaK_{Tth}$. The ATP hydrolysis rate of $DnaK_{Tth}$ is not stimulated by $DnaJ_{Tth}$ [47]. $DnaJ_{Tth}$ also does not affect any other step of the cycle, neither ATP binding nor hydrolysis are noticeably changed. Thus, $DnaJ_{Tth}$ does not appear to exert a regulatory role in the $DnaK_{Tth}$ nucleotide cycle [38], which leaves regulation solely to $GrpE_{Tth}$.

Assistence of luciferase refolding

A direct comparison of chaperone activity of the *E. coli* and *T. thermophilus* DnaK systems was performed with GdmCl denatured firefly luciferase [38], whose folding properties are well characterized [85, 86].

The maximal yield of active luciferase in the presence of the $DnaK_{Tth}$ and $DnaK_{Eco}$ systems was found to be comparable, although the maximum is reached approximately three times faster with $DnaK_{Eco}$. Notably, the initial slopes of luciferase refolding with $DnaK_{Tth} \cdot GrpE_{Tth}$ and the heterologous $DnaK_{Eco} \cdot GrpE_{Tth}$ system are reported to be similar, as are the nucleotide exchange rates with 7 s^{-1} and 5.1 s^{-1} [38]. Thus, refolding kinetics appear to be at least partially determined by the nucleotide exchange rate of the respective system.

The dominant role of GrpE for regulation of overall luciferase folding kinetics is especially evident, if one considers that $DnaK_{Eco}$ possesses an ATPase activity of at least 1 s^{-1} in the presence of $DnaJ_{Eco}$, while the ATPase activity of $DnaK_{Tth}$ remains at 0.003 s^{-1} in the presence of $DnaJ_{Tth}$. Yet the heterologous $DnaK_{Eco} \cdot GrpE_{Tth}$ system displays the same luciferase folding kinetics as the $DnaK_{Tth} \cdot GrpE_{Tth}$ system [38].

DafA

As a key feature of the thermophilic system, the formation of the $DnaK_{Tth} \cdot DnaJ_{Tth} \cdot DafA_{Tth}$ complex interferes with substrate binding to $DnaK_{Tth}$ (fig. 2). Dissociation of $DafA_{Tth}$ from the ternary complex is therefore a prerequisite for chaperone function [47]. Since displacement of $DafA_{Tth}$ is only efficient with protein substrates but not peptides, a high specificity is mediated by $DafA_{Tth}$. In the *E. coli* DnaK system, this coupling is achieved by DnaJ stimulating the $DnaK_{Eco}$ ATPase efficiently in the presence of protein but not of peptide substrates [19]. Consequently, stimulation of the intrinsic ATPase may not be necessary for $DnaK_{Tth}$, as $DafA_{Tth}$ secures tight coupling.

1 Jaenicke R. (1990) Protein structure and function at low temperatures. Philos. Trans. R. Soc. Lond B Biol. Sci. **326:** 535–551
2 Rothschild L. J. and Mancinelli R. L. (2001) Life in extreme environments. Nature **409:** 1092–1101
3 Jaenicke R. and Böhm G. (1998) The stability of proteins in extreme environments. Curr. Opin. Struct. Biol. **8:** 738–748
4 Zavodszky P., Kardos J., Svingor A. and Petsko G. A. (1998) Adjustment of conformational flexibility is a key event in the thermal adaptation of proteins. Proc. Natl. Acad. Sci. USA **95:** 7406–7411
5 Hubbard T. J. P. and Sander C. (1991) The role of heat-shock and chaperone proteins in protein folding: possible molecular mechanisms. Protein Eng. **4:** 711–717
6 Schmid D., Baici A., Gehring H. and Christen P. (1994) Kinetics of molecular chaperone action. Science **263:** 97–973
7 Todd M. J., Viitanen P. V. and Lorimer G. H. (1994) Dynamics of the chaperonin ATPase cycle: implications for facilitated protein folding. Science **265:** 659–666
8 McKay D. B. (1993) Structure and mechanism of 70-kDa heat-shock-related proteins. Adv. Protein Chem. **44:** 67–98
9 Szabo A., Langer T., Schröder H., Flanagan J., Bukau B. and Hartl F. U. (1994) The ATP hydrolysis-dependent reaction cycle of the *Escherichia coli* Hsp70 system – DnaK, DnaJ and GrpE. Proc. Natl. Acad. Sci. USA **91:** 10345–10349
10 Gisler S. M., Pierpaoli E. V. and Christen P. (1998) Catapult mechanism renders the chaperone action of Hsp70 unidirectional. J. Mol. Biol. **279:** 833–840
11 Pierpaoli E. V., Sandmeier E., Baici A., Schönfeld H.-J., Gisler S. and Christen P. (1997) The power stroke of the DnaK/DnaJ/GrpE molecular chaperone system. J. Mol. Biol. **269:** 757–768
12 Shtilerman M., Lorimer G. H. and Englander S. W. (1999) Chaperonin function: folding by forced unfolding. Science **284:** 822–825
13 Voisine C., Craig E. A., Zufall N., von Ahsen O., Pfanner N. and Voos W. (1999) The protein import motor of mitochondria: unfolding and trapping of preproteins are distinct and separable functions of matrix Hsp70. Cell **97:** 565–574
14 Hartl F. U. (1996) Molecular chaperones in cellular protein folding. Nature **381:** 571–580
15 McCarty J. S., Buchberger A., Reinstein J. and Bukau B. (1995) The role of ATP in the functional cycle of the DnaK chaperone system. J. Mol. Biol. **249:** 126–137
16 Theyssen H., Schuster H.-P., Packschies L., Bukau B. and Reinstein J. (1996) The second step of ATP binding to DnaK induces peptide release. J. Mol. Biol. **263:** 657–670
17 Zylicz M., Ang D. and Georgopoulos C. (1987) The GrpE protein of *Escherichia coli*: purification and properties. J. Biol. Chem. **262:** 17437–17442
18 Liberek K., Georgopoulos C. and Zylicz M. (1988) Role of the *Escherichia coli* DnaK and DnaJ heat shock proteins in the initiation of bacteriophage λ DNA replication. Proc. Natl. Acad. Sci. USA **85:** 6632–6636
19 Laufen T., Mayer M. P., Beisel C., Klostermeier D., Mogk A., Reinstein J. et al. (1999) Mechanism of regulation of Hsp70 chaperones by DnaJ cochaperones. Proc. Natl. Acad. Sci. USA **96:** 5452–5457
20 Russell R., Karzai A. W., Mehl A. F. and McMacken R. (1999) DnaJ dramatically stimulates ATP hydrolysis by DnaK: insight into targeting of Hsp70 proteins to polypeptide substrates. Biochemistry **38:** 4165–4176
21 Packschies L., Theyssen H., Buchberger A., Bukau B., Goody R. S. and Reinstein J. (1997) GrpE accelerates nucleotide exchange of the molecular chaperone DnaK with an associative displacement mechanism. Biochemistry **36:** 3417–3422
22 Clarke A. K. (1996) Variations on a theme: combined molecular chaperone and proteolysis functions in Clp/HSP100 proteins. J. Biosci. **21:** 161–177

23 Pak M. and Wickner S. (1997) Mechanism of protein remodeling by ClpA chaperone. Proc. Natl. Acad. Sci. USA **94:** 4901–4906

24 Parsell D. A., Kowal A. S., Singer M. A. and Lindquist S. (1994) Protein disaggregation mediated by heat-shock protein Hsp104. Nature **372:** 475–478

25 Schirmer E. C., Glover J. R., Singer M. A. and Lindquist S. (1996) HSP100/Clp proteins: a common mechanism explains diverse functions. Trends Biochem. Sci. **21:** 289–296

26 Sanchez Y. and Lindquist S. L. (1990) Hsp104 required for induced thermotolerance. Science **248:** 1112–1115

27 Glover J. R. and Lindquist S. (1998) Hsp104, Hsp70, and Hsp40: a novel chaperone system that rescues previously aggregated proteins. Cell **94:** 73–82

28 Goloubinoff P., Mogk A., Zvi A. P., Tomoyasu T. and Bukau B. (1999) Sequential mechanism of solubilization and refolding of stable protein aggregates by a bichaperone network. Proc. Natl. Acad. Sci. USA **96:** 13732–13737

29 Mogk A., Tomoyasu T., Goloubinoff P., Rüdiger S., Röder D., Langen H. et al. (1999) Identification of thermolabile *Escherichia coli* proteins: prevention and reversion of aggregation by DnaK and ClpB. EMBO J. **18:** 6934–6949

30 Zolkiewski M. (1999) ClpB Cooperates with DnaK, DnaJ, and GrpE in suppressing protein aggregation: a novel multi-chaperone system from *Escherichia coli.* J. Biol. Chem. **274:** 28083–28086

31 Motohashi K., Watanabe Y., Yohda M. and Yoshida M. (1999) Heat-inactivated proteins are rescued by the DnaKJ-GrpE set and ClpB chaperones. Proc. Natl. Acad. Sci. USA **96:** 7184–7189

32 Schlee S., Groemping Y., Herde P., Seidel R. and Reinstein J. (2001) The chaperone function of ClpB from *Thermus thermophilus* depends on allosteric interactions of its two ATP-binding sites. J. Mol. Biol. **306:** 889–899

33 Saraste M., Sibbald P. R. and Wittinghofer A. (1991) The P-loop – a common motif in ATP- and GTP-binding proteins. Trends Biochem. Sci. **15:** 430–434

34 Walker J. E., Saraste M., Runswick M. J. and Gay N. J. (1982) Distantly related sequences in the α- and β-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J. **1:** 945–951

35 Bardwell J. C. and Craig E. A. (1984) Major heat shock gene of Drosophila and the *Escherichia coli* heat-inducible dnaK gene are homologous. Proc. Natl. Acad. Sci. USA **81:** 848–852

36 Watanabe K. I. and Suzuki Y. (2000) Features of dnak operon genes of the obligate thermophile *Bacillus thermoglucosidasius* KP1006. Antonie Van Leeuwenhoek **77:** 241–250

37 Motohashi K., Yohda M., Endo I. and Yoshida M. (1996) A novel factor required for the assembly of the DnaK and DnaJ chaperones of *Thermus thermophilus*. J. Biol. Chem. **271:** 17343–17348

38 Groemping Y., Klostermeier D., Herrmann C., Veit T., Seidel R. and Reinstein J. (2001) Regulation of ATPase and chaperone cycle of DnaK from *Thermus thermophilus* by the nucleotide exchange factor GrpE. J. Mol. Biol. **305:** 1173–1183

39 Klostermeier D., Seidel R. and Reinstein J. (1998) Functional properties of the molecular chaperone DnaK from *Thermus thermophilus*. J. Mol. Biol. **279:** 841–853

40 Harrison C. J., Hayer-Hartl M., Di Liberto M., Hartl F. U. and Kuriyan J. (1997) Crystal-structure of the nucleotide exchange factor GrpE bound to the ATPase domain of the molecular chaperone DnaK. Science **276:** 431–435

41 Zhu X., Zhao X., Burkholder W. F., Gragerov A., Ogata C. M., Gottesman M. E. et al. (1996) Structural analysis of substrate binding by the molecular chaperone DnaK. Science **272:** 1606–1614

42 Pellecchia M., Szyperski T., Wall D., Georgopoulos C. and Wüthrich K. (1996) NMR structure of the j-domain and the gly/phe-rich region of the *Escherichia coli* DnaJ chaperone. J. Mol. Biol. **260:** 236–250

43 Schönfeld H.-J. and Behlke J. (1998) Molecular chaperones and their interactions investigated by analytical ultracentrifugation and other methodologies. Methods Enzymol. **290:** 269–296

44 King C., Eisenberg E. and Greene L. (1995) Polymerization of 70-kda heat-shock protein by yeast DnaJ in ATP. J. Biol. Chem. **270:** 22535–22540

45 Groemping Y. and Reinstein J. (2001) Folding properties of the nucleotide exchange factor GrpE from *Thermus thermophilus*: GrpE is a thermosensor that mediates heat shock response. J. Mol. Biol. **314:** 167–178

46 Motohashi K., Taguchi H., Ishii N. and Yoshida M. (1994) Isolation of the stable hexameric DnaK · DnaJ complex from *Thermus thermophilus*. J. Biol. Chem. **269:** 27074–27079

47 Klostermeier D., Seidel R. and Reinstein J. (1999) The functional cycle and regulation of the *Thermus thermophilus* DnaK chaperone system. J. Mol. Biol. **287:** 511–525

48 Krzewska J., Konopa G. and Liberek K. (2001) Importance of two ATP-binding sites for oligomerization, ATPase activity and chaperone function of mitochondrial Hsp78 protein. J. Mol. Biol. **314:** 901–910

49 Parsell D. A., Kowal A. S. and Lindquist S. (1994) *Saccharomyces cerevisiae* Hsp104 protein: purification and characterization of ATP-induced structural changes. J. Biol. Chem. **269:** 4480–4487

50 Zolkiewski M., Kessel M., Ginsburg A. and Maurizi M. R. (1999) Nucleotide-dependent oligomerization of ClpB from *Escherichia coli*. Protein Sci. **8:** 1899–1903

51 Watanabe Y. Y., Motohashi K. and Yoshida M. (2002) Roles of the two ATP binding sites of ClpB from *Thermus thermophilus*. J. Biol. Chem. **277:** 5804–5809

52 Kim K. I., Cheong G.-W., Park S.-C., Ha J.-S., Woo K. M., Choi S. J. et al. (2000) Heptameric ring structure of the heat-shock protein ClpB, a protein-activated ATPase in *Escherichia coli*. J. Mol. Biol. **303:** 655–666

53 Schirmer E. C., Ware D. M., Queitsch C., Kowal A. S. and Lindquist S. L. (2001) Subunit interactions influence the biochemical and biological properties of Hsp104. Proc. Natl. Acad. Sci. USA **98:** 914–919

54 May A. P., Whiteheart S. W. and Weis W. I. (2001) Unraveling the mechanism of the vesicle transport ATPase NSF, the N-ethylmaleimide-sensitive factor. J. Biol. Chem. **276:** 21991–21994

55 Hattendorf D. A. and Lindquist S. L. (2002) Cooperative kinetics of both Hsp104 ATPase domains and interdomain communication revealed by AAA sensor-1 mutants. EMBO J. **21:** 12–21

56 Palleros D. R., Reid K. L., McCarty J. S., Walker G. C. and Fink A. L. (1992) DnaK, Hsp73, and their molten globules. Two different ways heat shock proteins respond to heat. J. Biol. Chem. **267:** 5279–5285

57 Palleros D. R., Welch W. J. and Fink A. L. (1991) Interaction of hsp70 with unfolded proteins: effects of temperature and nucleotides on the kinetics of binding. Proc. Natl. Acad. Sci. USA **88:** 5719–5723

58 Palleros D. R., Shi L., Reid K. L. and Fink A. L. (1993) Three-state denaturation of DnaK induced by guanidine hydrochloride. Evidence for an expandable intermediate. Biochemistry **32:** 4314–4321

59 Grimshaw J. P., Jelesarov I., Schönfeld H. J. and Christen P. (2001) Reversible thermal transition in GrpE, the nucleotide exchange factor of the DnaK heat-shock system. J. Biol. Chem. **276:** 6098–6104

60 Jaenicke R., Schurig H., Beaucamp N. and Ostendorp R. (1996) Structure and stability of hyperstable proteins: glycolytic enyzmes from hypertherophilic bacterium *Thermotoga maritima*. Adv. Protein Chem. **48:** 181–269

61 Dams T. and Jaenicke R. (1999) Stability and folding of dihydrofolate reductase from the hyperthermophilic bacterium *Thermotoga maritima*. Biochemistry **38:** 9169–9178
62 Mandelman D., Schwarz F. P., Li H. Y. and Poulos T. L. (1998) The role of quaternary interactions on the stability and activity of ascorbate peroxidase. Protein Sci. **7:** 2089–2098
63 Todd M. J., Semo N. and Freire E. (1998) The structural stability of the HIV-1 protease. J. Mol. Biol. **283:** 475–488
64 Wieligmann K., Mayr E.-M. and Jaenicke R. (1999) Folding and self-assembly of the domains of βB2-crystallin from rat eye lens. J. Mol. Biol. **286:** 989–994
65 Conejerolara F. and Mateo P. L. (1996) Presence of a slow dimerization equilibrium on the thermal unfolding of the 205–316 thermolysin fragment at neutral pH. Biochemistry **35:** 3477–3486
66 Buchberger A., Schröder H., Hesterkamp T., Schönfeld H. J. and Bukau B. (1996) Substrate shuttling between the DnaK and GroEL systems indicates a chaperone network promoting protein-folding. J. Mol. Biol. **261:** 328–333
67 Fourie A. M., Sambrook J. F. and Gething M.-J. H. (1994) Common and divergent peptide binding specificities of Hsp70 molecular chaperones. J. Biol. Chem. **269:** 30470– 30478
68 Knarr G., Modrow S., Todd A., Gething M.-J. and Buchner J. (1999) BiP-binding sequences in HIV gp160: implications for the binding specificity of BiP. J. Biol. Chem. **274:** 29850–29857
69 Gething M. J., Blondelguindi S., Buchner J., Fourie A., Knarr G., Modrow S. et al. (1995) Binding-sites for hsp70 molecular chaperones in natural proteins. Cold Spring Harbor Symp. Quant. Biol. **60:** 417–428
70 Rüdiger S., Buchberger A. and Bukau B. (1997) Interaction of Hsp70 chaperones with substrates. Nat. Struct. Biol. **4:** 342–349
71 Rüdiger S., Germeroth L., Schneider-Mergener J. and Bukau B. (1997) Substrate-specificity of the DnaK chaperone determined by screening cellulose-bound peptide libraries. EMBO J. **16:** 1501–1507
72 Gething M.-J. and Sambrook J. (1992) Protein folding in the cell. Nature **355:** 33–45
73 Hartl F. U., Martin J. and Neupert W. (1992) Protein folding in the cell: the role of molecular chaperones Hsp70 and Hsp60. Annu. Rev. Biophys. Biomol. Struct. **21:** 293–322
74 Ang D., Liberek K., Skowyra D., Zylicz M. and Georgopoulos C. (1991) Biological role and regulation of the universally conserved heat shock proteins. J. Biol. Chem. **266:** 24233– 24236
75 Craig E. A., Gambill B. D. and Nelson R. J. (1993) Heat shock proteins: molecular chaperones of protein biogenesis. Microbiol. Rev. **57:** 402–414
76 McCarty J. S. and Walker G. C. (1991) DnaK as a thermometer: threonine-199 is site of autophosphorylation and is critical for ATPase activity. Proc. Natl. Acad. Sci. USA **88:** 9513–9517
77 Jaenicke R. and Zavodszky P. (1990) Proteins under extreme physical conditions. FEBS Lett. **268:** 344–349
78 Motohashi K., Yohda M., Odaka M. and Yoshida M. (1997) K^+ is an indispensable cofactor for GrpE stimulation of ATPase activity of DnaK.DnaJ complex from *Thermus thermophilus*. FEBS Lett. **412:** 633–636
79 Burkholder W. F., Panagiotidis C. A., Silverstein S. J., Cegielska A., Gottesman M. E. and Gaitanaris G. A. (1994) Isolation and characterization of an *Escherichia coli* DnaK mutant with impaired ATPase activity. J. Mol. Biol. **242:** 364–377
80 Woo K. M., Kim K. I., Goldberg A. L., Ha D. B. and Chung C. H. (1992) The heat-shock protein ClpB in *Escherichia coli* is a protein-activated ATPase. J. Biol. Chem. **267:** 20429–20434
81 Kim K. I., Woo K. M., Seong I. S., Lee Z.-W., Baek S. H. and Chung C. H. (1998) Mutational analysis of the two ATP-binding sites in ClpB, a heat shock protein with protein-activated ATPase activity in *Escherichia coli*. Biochem. J. **333:** 671–676
82 Hattendorf D. A. and Lindquist S. L. (2002) Analysis of the AAA sensor-2 motif in the C-terminal ATPase domain of Hsp104 with a site-specific fluorescent probe of nucleotide binding. Proc. Natl. Acad. Sci. USA
83 Bukau B. and Horwich A. L. (1998) The hsp70 and hsp60 chaperone machines. Cell **92:** 351–366
84 Buchberger A., Reinstein J. and Bukau B. (1999) The DnaK chaperone system. In: Molecular Chaperones and Folding Catalysts: Regulation, Cellular Functions and Mechanisms, pp. 573–635, Bukau B. (ed.), Harwood, Amsterdam
85 Herbst R., Schäfer U. and Seckler R. (1997) Equilibrium intermediates in the reversible unfolding of Firefly (*Photinus pyralis*) luciferase. J. Biol. Chem. **272:** 7099–7105
86 Herbst R., Gast K. and Seckler R. (1998) Folding of firefly (*Photinus pyralis*) luciferase: aggregation and reactivation of unfolding intermediates. Biochemistry **37:** 6586–6597
87 Brehmer D., Rüdiger S., Gassler C. S., Klostermeier D., Packschies L., Reinstein J. et al. (2001) Tuning of chaperone activity of Hsp70 proteins by modulation of nucleotide exchange. Nat. Struct. Biol. **8:** 427–432
88 Sondermann H., Scheufler C., Schneider C., Höhfeld J., Hartl F. U. and Moarefi I. (2001) Structure of a Bag/Hsc70 complex: convergent functional evolution of Hsp70 nucleotide exchange factors. Science **291:** 1553–1557
89 Wetzstein M., Volker U., Dedio J., Lobau S., Zuber U., Schiesswohl M. et al. (1992) Cloning, sequencing, and molecular analysis of the *dnaK* locus from *Bacillus subtilis*. J. Bacteriol. **174:** 3300– 3310
90 Osipiuk J. and Joachimiak A. (1997) Cloning, sequencing and expression of *dnaK*-operon proteins from the thermophilic bacterium *Thermus thermophilus*. Biochim. Biophys. Acta **1353:** 253–265
91 Hofman-Bang J., Lange M., Conway D. M., Macario A. J. and Ahring B. K. (1999) The genes coding for the Hsp70 (DnaK) molecular chaperone machine occur in the moderate thermophilic archaeon *Methanosarcina thermophila* TM-1. Gene **238:** 387–395