Biol. Chem., Vol. 381, pp. 877 – 885, September/October 2000

Review

Molecular Basis for Interactions of the DnaK Chaperone with Substrates

Matthias P. Mayer, Stefan Rüdiger and Bernd Bukau*

Institut für Biochemie und Molekularbiologie, Universität Freiburg, Hermann-Herder-Str. 7, D-79104 Freiburg, Germany

* Corresponding author

Hsp70 chaperones assist a large variety of protein folding processes in the cell by transient association with short peptide segments of proteins. The substrate binding and release cycle is driven by the switching between the low affinity ATP bound state and the high affinity ADP bound state of Hsp70. Considerable progress has been made recently by the identification of *in vivo* substrates for the *Escherichia coli* homolog, DnaK, and the molecular mechanisms which govern the DnaK-substrate interactions. Here we review the processes that generate DnaK substrates *in vivo* and the properties of these substrates, and we describe insights gained from structural and kinetic analysis of DnaK-substrate interaction.

Key words: Aggregation / Hsp70 / Nascent polypeptide chains / Refolding / Substrate association and dissociation / Thermolabile proteins.

Cellular Processes Generating DnaK Substrates

DnaK interacts with short hydrophobic segments of proteins which generally are accessible to the chaperone only in non-native conformers. Consequently, all cellular processes generating such conformers potentially generate DnaK substrates, including *de novo* protein synthesis (Deuerling *et al.,* 1999; Teter *et al.,* 1999), protein translocation (Wild *et al.,* 1996), assembly and disassembly of protein complexes (Alfano and McMacken 1989; Zylicz *et al.,* 1989), and protein misfolding, especially under heat stress conditions (Gragerov *et al.,* 1991; Mogk *et al.,* 1999). We describe here in more detail the roles of DnaK in two processes, the *de novo* folding of proteins and the refolding of misfolded proteins, since recent work elucidated specifically these two populations of DnaK substrates.

De Novo Folding

Under normal growth conditions at 30 °C DnaK is involved in the folding of an estimated 5 – 18 % of all newly synthesized proteins in *E. coli* (Deuerling *et al.,* 1999; Teter *et al.,* 1999). In this activity the DnaK system functionally cooperates with the ribosome-associated trigger factor, a protein with chaperone-like and peptidyl-prolyl-*cis*-*trans*-isomerase activities *in vitro* (Stoller *et al.,* 1995; Hesterkamp *et al.,* 1996; Deuerling *et al.,* 1999; Teter *et al.,* 1999). Due to its preferential location at the exit site for nascent chains, the trigger factor has the potential to assist the folding of newly synthesized proteins prior to DnaK, and the action of the trigger factor may make DnaK action dispensable in most cases as suggested by genetic analysis (Hesterkamp and Bukau, 1998). DnaK is dispensable for viability at 30 – 37 °C, unless the trigger factor is also absent due to deletion of the trigger factor-encoding gene *tig* (Hesterkamp and Bukau 1998; Deuerling *et al.,* 1999; Teter *et al.,* 1999). Depletion of DnaK in Δ*tig* cells causes massive aggregation of newly synthesized cytosolic proteins, amounting to 340 protein spots visible on two dimensional gels (E. Deuerling and B. Bukau, unpublished results; Deuerling *et al.,* 1999). These findings indicate that the DnaK system is able to compensate for the lack of ribosome-associated trigger factor, either by cotranslational or posttranslational action.

Protein Misfolding under Heat Shock Conditions

DnaK becomes essential for viability at high temperature (> 37 °C) due to its essential role in repair of denatured proteins (Figure 1; Kusukawa and Yura 1988; Skowyra *et al.,* 1990; Gragerov *et al.,* 1992; Schröder *et al.,* 1993; Tomoyasu *et al.,* 1998; Mogk *et al.,* 1999). This repair function relies in part on the ability of DnaK to prevent aggregation of misfolded proteins and allowing their refolding. In addition, DnaK appears to be capable of disaggregating aggregated proteins, as first shown by Zylicz and coworkers for aggregated RNA polymerase (Skowyra *et al.,* 1990; Ziemienowicz *et al.,* 1993). A recent study by Goloubinoff and coworkers provided further evidence for the potential of DnaK to disaggregate aggregated proteins (Diamant *et al.,* 2000). These authors succeeded in generating stable glucose-6-phosphate dehydrogenase aggregates of defined size which could be separated by size exclusion chromatography. They found that disaggregation efficiency and the stoichiometry of chaperone *versus* substrate

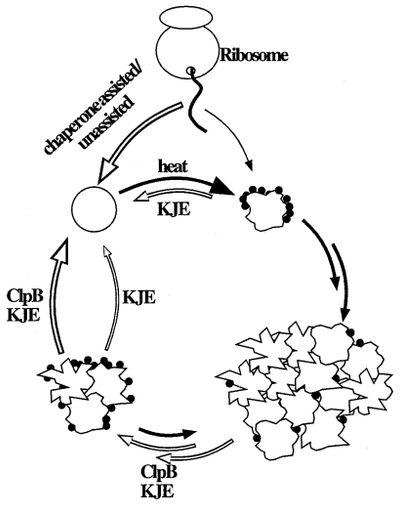

Fig. 1 Cycle of Protein Aggregation and Chaperone-Mediated Disaggregation.
Filled arrows indicate processes leading to misfolding and subsequent aggregate formation, transparent arrows indicate processes on the way towards the native state. Some of the newly synthesized proteins do not reach their native states and form misfolded species that expose hydrophobic patches (indicated as black dots) and are prone to aggregation. Native proteins may denature spontaneously or upon heat treatment into conformations which may differ from the misfolded species generated during *de novo* synthesis. Misfolded conformers can be refolded to the native states by the DnaK chaperone system. Denatured proteins associate to large aggregates, which bury a subset of the hydrophobic patches. Such aggregates are disentangled to smaller aggregates by the ClpB-DnaK bi-chaperone system (ClpB, DnaK, DnaJ, and GrpE), leading to an increasing exposure of hydrophobic patches. Such small aggregates are further dissolved and the proteins refolded to the native state by the ClpB-DnaK bi-chaperone system or to some extent by the DnaK system alone. KJE: DnaK, DnaJ, GrpE.

necessary for disaggregation depend on the size of the aggregates. Even large aggregates, causing the protein solution to become turbid, were disaggregated by a 10-fold excess of DnaK over the glucose-6-phosphate dehydrogenase protomers. Under limiting DnaK concentrations only small aggregates not detectable by light scattering were dissolved.

DnaK's ability to disentangle aggregates is limited, but is dramatically extended by the Hsp100 homolog, ClpB (Figure 1). The ability of Hsp70 systems and Hsp100 chaperones to cooperate in the disaggregation of proteins was demonstrated first for the yeast homologs, Hsp104 (Hsp100 class) and Ssa1/Ydj1 (Hsp70 and Hsp40 classes) (Parsell *et al.,* 1994; Glover and Lindquist, 1998). ClpB is able to loosen up the aggregates, thereby increasing the number of hydrophobic sites on the surface and, together with the DnaK system, allowing to solubilize and refold substrate molecules (Figure 1; Goloubinoff *et al.,* 1999; Zolkiewski, 1999). The ClpB/DnaK bi-chaperone system is not restricted to any size of the aggregates and can efficiently dissolve heat-induced aggregates of almost any cellular protein *in vivo* (Mogk *et al.,* 1999).

Identification and Features of Cellular DnaK Substrates

Mogk *et al.* (1999) identified in a recent study thermolabile proteins of *E. coli* that require the activity of the DnaK system for preventing their aggregation at high temperature. In Δ*dnaK52* mutant cells, but not in wild-type cells, a heat shock by temperature upshift from 30 °C to 42 °C causes aggregation of about 10% of the total protein mass. Approximately 20 – 30% of about 800 soluble proteins visible on two-dimensional gels were affected. Mass spectrometric identification of 57 proteins revealed that these thermolabile proteins belong to unrelated classes of proteins which are involved in diverse cellular processes. The populations of thermolabile proteins aggregating under heat shock conditions in Δ*dnaK52* mutant cells, and the population of newly synthesized proteins which aggregate in DnaK depleted Δ*tig* cells at 37 °C, overlap to a large extent (E. Deuerling and B. Bukau, unpublished results). The thermolabile substrates of DnaK thus have also the need for chaperone assistance during *de novo* folding at regular growth temperature (37 °C).

The identification of thermolabile DnaK substrates by mass spectrometry allowed to elucidate some features of these proteins which may account for there aggregation propensity (Mogk *et al.,* 1999). They are not related in their folds, content of α-helices and β-strands, or pI. However, the fraction of thermolabile proteins within the total number of *E. coli* proteins increased with increasing molecular weight (Mogk *et al.,* 1999). While proteins smaller than 30 kDa were underrepresented, proteins larger than 70 kDa were 2 – 4-fold more frequent in the aggregated protein fraction of heat-treated cells than expected from the molecular weight distribution. 80% of all soluble proteins larger than 70 kDa aggregated upon heat shock in Δ*dnaK52* mutant cells. Large proteins that are organized in multiple domains are thus at higher risk to misfold and aggregate and to become DnaK substrates. This vulnerability may result from generation of folding intermediates with exposed hydrophobic domain contact sites which could nucleate intermolecular aggregation or chaperone binding. It may also be that larger proteins simply have a higher probability than smaller proteins to expose aggregation prone hydrophobic segments in folding intermediates generated during *de novo* folding and thermal unfolding.

The molecular weight spectrum of DnaK substrates differs from the spectrum of GroEL substrates that was determined by co-immunoprecipitation (Ewalt *et al.,* 1997; Houry *et al.,* 1999). For GroEL, an upper size limit for substrates of approx. 60 kDa seems to be defined by the dimensions of the binding cavity in the GroEL torus underneath the GroES dome. The substrate populations of both chaperones thus show considerable differences.

When comparing the population of thermolabile *in vivo* substrates for DnaK with thermoresistant proteins, a number of differences with respect to the occurrence of the DnaK binding motif was found (Mogk *et al.,* 1999). This motif had been determined earlier by scanning of cellu-

lose-bound peptide libraries (Rüdiger *et al.,* 1997b) and consists of a core of up to 5 consecutive hydrophobic residues that can be flanked by basic segments. A significantly higher fraction of the thermolabile DnaK substrates display DnaK binding sites on the surface, a property that is also shared by the thermolabile firefly luciferase (Rüdiger *et al.,* 1997b). Furthermore, the population of thermolabile proteins is enriched with proteins with a higher frequency of DnaK binding sites as compared to thermoresistant proteins (Mogk *et al.,* 1999). It is unclear at present to what extend these and additional features render a protein thermolabile and substrate for DnaK *in vivo*.

Mechanism of DnaK-Substrate Interactions

There is considerable progress being made in the understanding of the molecular mechanism by which DnaK interacts with substrates. In the following we will discuss the structural and kinetic basis for the DnaK-substrate interactions.

Topology of the Substrate Binding Cavity and Interactions Contributing to Substrate Binding

DnaK consists of the N-terminal ATPase domain of 45 kDa and the C-terminal substrate binding domain of 25 kDa. The DnaK substrate binding cavity within the C-terminal domain is formed by a β-sandwich of two times four antiparallel β-strands and four upward-protruding connecting loops (two inner and two outer loops, Figure 2; Zhu *et al.,* 1996). The α-helices A and B are packed onto the β-sandwich and the two inner loops. Helix B closes the substrate binding cavity without interacting directly with the bound substrate (Figures 2A and B). The distal part of helix B, starting at around amino acid 536, together with 3 additional helices (C, D, and E) builds up a hydrophobic helical core that constitutes a lid-like structure.

Several types of interactions contribute to substrate binding (Zhu *et al.,* 1996; Rüdiger *et al.,* 1997a). Hydrogen bonds formed primarily between the backbones of two pocket-forming loops and the peptide backbone mediate recognition of the extended peptide conformation. Van der Waals interactions of side chains lining the substrate binding cavity with peptide side chains mediate DnaK's preference for hydrophobic peptides. Hydrophobic contacts are possible to approx. five consecutive residues of the substrate. They are strongest at the central position (position 0) which builds up a deep pocket tailored to accommodate large hydrophobic side chains, in particular Leu (Zhu *et al.,* 1996). An additional structural element that contributes to peptide binding is an arch formed by residues Met^{404} and Ala^{429} of the two substrate contacting loops (Figure 1B), which encloses the peptide backbone at position 0. The flexibility of the arch-forming Met^{404} side chain is restricted by van der Waals contacts to substrate side chains adjacent to the central Leu. The architectural constraints imposed by the substrate binding pocket agree well with the observed substrate specificity of DnaK

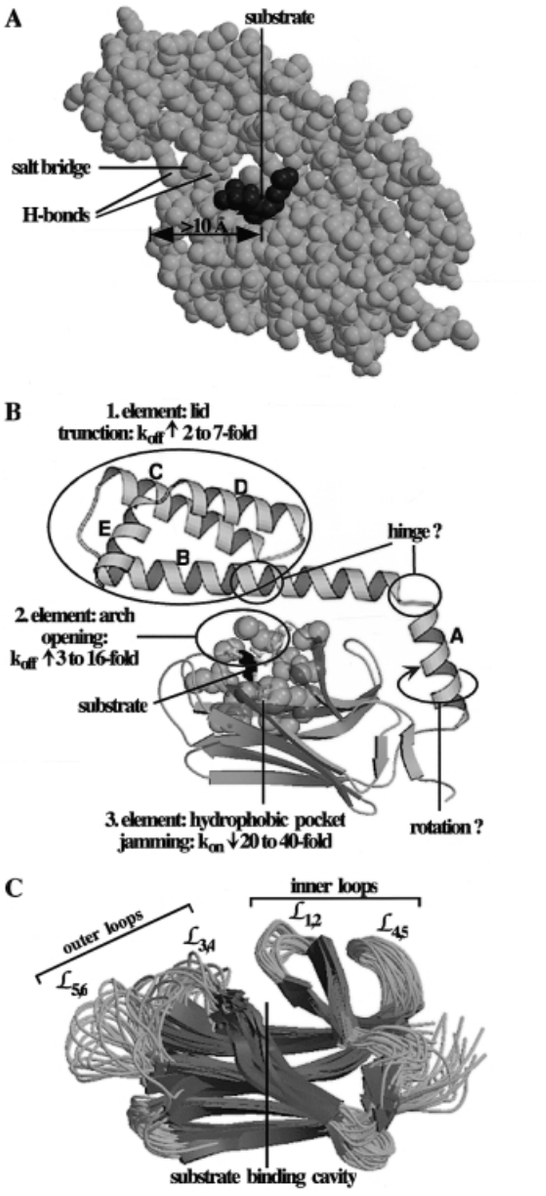

Fig. 2 Crystal Structure and NMR Structures of the Substrate Binding Domain of DnaK.
(A) Space filling representation of the crystal structure [DnaK(389-697)]. A bound polypeptide stretch represented by the co-crystallized peptide (dark gray) is completely enclosed by the substrate binding domain and at least 10 Å removed from other parts of the substrate protein. (B) Secondary structure representation of the substrate binding domain. The substrate interacting side chains are shown in transparent space filling representation. Three elements are indicated which have been shown to contribute to substrate binding and release: the helical lid, the arch, and the hydrophobic pocket. The major effects of alterations of these elements to DnaK-substrate interaction kinetic in the ADP state are listed. Three possible opening mechanisms for the lid are indicated as discussed in the text. (C) Secondary structure representation of the 20 energy-minimized DYANA conformations of the solution structure of residues 393 to 507 of DnaK. Drawings were made with MOLSCRIPT and RASTER3D (Kraulis, 1991; Merritt and Murphy, 1994).

as identified by peptide library screening (see above; Rüdiger *et al.,* 1997a, b).

Two NMR structures of the substrate binding domain fragments of DnaK and mammalian Hsc70 (Wang *et al.,* 1998; Morshauser *et al.,* 1999) revealed that the distal part of helix B becomes unstable in the absence of helices C to E and forms a loop which folds back and binds into the substrate binding cavity. In both structures a Leu of helix B is bound in the hydrophobic pocket which, however, may be a mere coincidence as there is no other hydrophobic amino acid available in the remaining distal part of helix B. The position of the proximal part of helix B differs between the two NMR structures and the crystal structure. While in the crystal structure helix B is exactly on the β-subdomain rim, it is behind the rim in the NMR structure of DnaK and in front of the rim in the NMR structure of Hsc70. This indicates that the proximal part of helix B is not as tightly associated to the β-subdomain as suggested by the crystal structure data. Furthermore, a recent NMR structure of DnaK's β-subdomain lacking helices A to E (Pellecchia *et al.,* 2000) demonstrated that the structural stability of the β-subdomain does not depend on the contacts to the two helices A and B suggesting that a tight packing of these helices onto the β-subdomain is not essential for stabilization and considerable conformational changes may be possible.

For the understanding of DnaK's chaperone activity it is also important to know whether DnaK binds substrates in one N- to C-terminal orientation only or whether both orientations are possible. In both NMR structures of the substrate binding domain in which the distal part of helix B folds back and binds to the substrate binding cavity (Wang *et al.*, 1998; Morshauser *et al.*, 1999), the bound segment of helix B has the same N- to C-terminal orientation despite the different positions of the proximal part of helix B in the two structures. The orientation of the bound segments is also identical to the orientation of the peptide in the crystal structure. This indicates that substrates may bind to Hsp70s in one orientation only. Recent biochemical experiments lead to the same conclusion (unpublished data).

Structural Elements Determining the Kinetics of Substrate Binding and Release

As detailed above, the architecture of the DnaK substrate binding domain indicated three structural elements to crucially influence the binding and release of substrates, the α-helical lid, the arch, and the hydrophobic central pocket (Figure 1A). All three elements were recently shown to contribute to the stability of DnaK-substrate complexes in the ADP state.

Removal of the lid by truncation of helix B in its midpoint on top of the substrate binding cavity reduces affinity for protein and peptide substrates 3- to 7-fold, mostly by influencing the off-rates (Mayer *et al.,* 2000; Pellecchia *et al.,* 2000). These results are consistent with data obtained for the Hsp70 homolog of the endoplasmic reticulum (Misselwitz *et al.,* 1998).

Disturbing the formation of the arch by mutational alteration of Met404 to Ala or Ala429 to Trp also increases the dissociation constants by 2 to 5-fold, with predominant effect on the off-rates (Mayer *et al.,* 2000).

The most dramatic effect on substrate binding was achieved by blocking of the hydrophobic pocket by a Val436 to Phe exchange, which increased the dissociation equilibrium constants for protein and peptide substrates by 20- and 40-fold, respectively, solely by affecting the on-rates (Mayer *et al.,* 2000). The off-rates of the mutant protein were identical to those of wild type DnaK indicating that the substrate was bound to the mutant protein in a similar way as to wild type DnaK. The hydrophobic pocket may therefore have considerable flexibility to accommodate hydrophobic side chains even when association of substrates is hindered by the Val436 to Phe exchange. Such flexibility has already been detected in NMR structures of the substrate binding domain of DnaK. Here, the hydrophobic pocket was in a more open conformation as compared to the crystal structure, perhaps due to intramolecular steric constraints resulting from the binding of the distal part of helix B (Wang *et al.,* 1998; Morshauser *et al.,* 1999). Together, these data suggest that DnaK binds substrates in an induced fit like manner, with the major contribution to substrate affinity coming from the hydrophobic pocket.

Allosteric Opening Mechanism: Differences between ADP and ATP States

Based on an alternative crystal lattice where helix B was bend upwards by about 11°, Hendrickson and coworkers had proposed that the lid rotates upwards around a hinge point in the middle of helix B (residues 536 – 538) in response to the allosteric action of ATP (Figure 2B). In the closed conformation the distal part of helix B forms a salt bridge and two hydrogen bonds with the two outer loops of the β-subdomain which thereby may constitute a latch stabilizing the outer loops in the closed conformation. Upward rotation of the lid would break these bonds; the loops may become unstable and open the substrate binding cavity by a downward movement concomitantly with the upward movement of the lid.

This mechanism implies a major role for the lid in the allosteric control of substrate binding. Several lines of evidence, however, demonstrated that the lid is not solely responsible for mediating this control, but that it is rather exerted predominantly through the β-subdomain. The off-rates for the lidless DnaK mutant in the ADP state were only 5-fold increased as compared to wild-type DnaK in the ADP state, whereas ATP binding causes an off-rate increase of 440- to 2500-fold for wild-type DnaK (Schmid *et al.,* 1994; Mayer *et al.,* 2000). Furthermore, biochemical data from bovine Hsc70, yeast BiP, and *E. coli* DnaK clearly showed that lidless mutants retained the ability to re-

spond to ATP in a similar way as the wild type counterpart. Even the deletion of all helices does not convert DnaK into the completely open conformation (Ungewickell *et al.,* 1997; Misselwitz *et al.,* 1998; Mayer *et al.,* 2000; Pellecchia *et al.,* 2000).

New insights in the flexibility of the substrate binding domain with implications for the allosteric mechanism were gained by the NMR structure of the substrate free β-subdomain devoid of the helical parts (Pellecchia *et al.,* 2000). First, the outer loops showed a significantly higher mobility than in the earlier NMR structures containing helices A and B. This indicates that substrate binding causes their immobilization (Figure 2C), consistent with the interpretation of the crystal structure data (Zhu *et al.,* 1996). The inner loops, on the contrary, showed only very limited mobility. The interaction of these loops with the helices A and B are therefore less important for the overall structural stability as well as for the opening mechanism. The hinge for the upward movement of the lid might therefore be located at a position different from the one proposed by Hendrickson and coworkers (Zhu *et al.,* 1996), e.g. in between helix A and B (Figure 2B). Alternatively, the opening mechanism might involve a pivoting movement around helix A, as proposed by Zuiderweg and coworkers (Wang *et al.,* 1998), whereby the lid swings sideways away from the cavity (Figure 2B). Second, the 20 lowest energy structures also showed considerable movements of the residues forming the hydrophobic pocket. Notably, in some structures Phe426 moves into the hydrophobic pocket, leaving no space for a substrate side chain. Structural changes even in the hydrophobic pocket are therefore likely to participate in the allosteric control of substrate binding. This flexibility of the pocket explains why the DnaK-V436F mutant protein, designed to block this pocket, is still able to bind most substrates in a similar way as wild-type DnaK although with strongly reduced affinity due to decreased on-rates (see above; Mayer *et al.,* 2000; Rüdiger *et al.,* in preparation).

Surprising was the finding that the arch (Met404-Ala429) was closed in all 20 lowest energy NMR structures although the arch forming amino acids exhibited considerable mobility. It is difficult to imagine how a substrate could enter the substrate binding cavity under these conditions of a closed arch and the additional stabilizing interactions existing between helix B and the outer loops. From all available structures which are considered to represent the ADP state of the substrate binding domain it became clear that there is no space for substrates to enter the substrate binding cavity by a lateral sliding-in mechanism (see Figure 2A). Nevertheless, it has been shown for many substrate peptides and proteins that association with an appreciable rate is possible in the ADP state (Schmid *et al.*, 1994; McCarty *et al.*, 1996; Pierpaoli *et al.*, 1998a). It is therefore clear that there must be movements beyond those detected in the NMR structures which allow binding of substrates.

Christen and coworkers investigated the association of several related peptides to DnaK·ADP and found similar on-rates for all peptides (Pierpaoli *et al.*, 1998a). They concluded that the association to DnaK·ADP is limited by the opening rate of the substrate binding cavity. Furthermore, as bound substrate exchanges with free substrate at a considerable rate, the substrate binding cavity of DnaK·ADP including the arch and the helical lid must open occasionally even when substrate is bound (Schmid *et al.*, 1994; Gamer *et al.*, 1996). This conclusion was supported by the fact that mutational alterations in the arch increased the off-rate even in the presence of the helical lid. In addition, the combined alteration of the helical lid and the arch had additive rather than synergistic effects on the off-rates. On the basis of these results the terms closed and open conformation of DnaK, which were used synonymously to ADP and ATP state, respectively, had been redefined (Mayer *et al.*, 2000). The substrate binding cavity including the lid, the arch, the hydrophobic pocket, and further cavity forming residues of the β-subdomain, opens and closes continuously in the ADP and the ATP states of DnaK. Substrates bind only to the open conformation. The affinity of substrates is determined by both, the on- and off-rates of the DnaK-Substrate complexes in the open conformation and the fraction of time the substrate binding cavity stays in the closed conformation where association and dissociation is impossible. The difference between the ADP and ATP states of DnaK is primarily the frequency of transition from the closed to the open conformation which is dramatically higher in the ATP state (Figure 3; Mayer *et al.*, 2000).

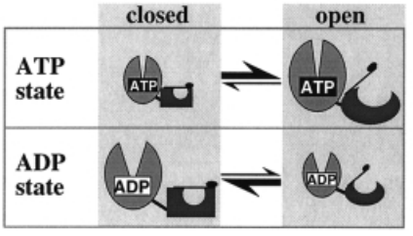

Fig. 3 Dynamic Model of the ATP and ADP State of DnaK. In both states the substrate binding domain opens and closes periodically. The principal differences between the ATP and ADP state are the rates of opening and closing of lid, arch, and loops and β-strands which constitute the substrate binding cavity. The size of the symbols represents the relative frequency of a given conformation within a population of DnaK proteins. Model modified from Mayer *et al.* (2000).

Structural Determinants of the Substrate Specificity of DnaK

A recent study investigated whether mutational alterations in key elements of the substrate binding domain (lid, pocket, arch) modulate the substrate specificity of DnaK (Rüdiger *et al.,* in preparation). Deletion of the lid (DnaK(2-538) causes no changes in the pattern of DnaK binding to libraries of cellulose-bound peptides. This finding is not surprising since the lid does not itself contact the bound

substrate. However, it allows concluding that the stabilization of the outer loops of the substrate binding cavity by the distal part of helix B is not important to control specificity. Blockage of the hydrophobic pocket (DnaK-V436F) strongly reduces the affinity of DnaK to all substrates but without affecting the binding pattern. In contrast, alterations in the arch residues (Met404, Ala429) conferred some changes to the substrate specificity of DnaK. This finding is interesting in view of the fact that the arch residues are the only substrate interacting amino acids which are subject to considerable evolutionary variation.

In particular, increasing the volume of the arch by replacement of the small Ala429 by the bulky Trp leads to disfavoring of a subset of peptides. These peptides are often characterized by consecutive large hydrophobic residues flanked by acidic residues. The options of such peptides in escaping potential steric hindrance at the arch are restricted since acidic residues are highly disfavored within the cavity. In addition, DnaK-A429W has higher affinity than wild type DnaK to a few peptides, but the principles behind this selective binding are unclear. It remains to be evaluated to which extent Hsp70s benefit from a modulation of their substrate specificity by alteration of the arch residues.

Relevance of Substrate Affinity in ADP and ATP States for Chaperone Activity of DnaK

Studies of the substrate specificity of DnaK and other Hsp70 chaperones have been conducted mostly with the high affinity ADP state of the chaperone (Flynn *et al.,* 1991; Blond-Elguindi *et al.,* 1993; Gragerov *et al.,* 1994; Rüdiger *et al.,* 1997b). However, the on-rates of substrates to DnaK·ADP are rather low (10^2 to 10^4 M^{-1} s^{-1}; Schmid *et al.,* 1994; Pierpaoli *et al.,* 1998a; Mayer *et al.,* 2000), resulting in half maximal association times of 1 to 100 min at concentrations of 1 μM DnaK and substrate. These on-rates are much too slow to compete with aggregation as shown experimentally *e.g.* for luciferase (Schröder *et al.,* 1993). Instead, in the functional chaperone cycle DnaK encounters the substrate in the ATP state where the on-rates are about two orders of magnitude higher. In addition, DnaK needs the simultaneous interaction with the substrate and the DnaJ cochaperone for efficient stimulation of ATP hydrolysis leading to the transition to the high affinity ADP state (Karzai and McMacken 1996; Misselwitz *et al.,* 1998; Laufen *et al.,* 1999; Mayer *et al.,* 1999). Therefore, the relevant state for substrates entering the chaperone cycle is the ATP state of DnaK. One could question whether the substrate specificities determined experimentally with the ADP state are relevant for the functional cycle of this chaperone. However, in a recent study it was found that the K_d values of DnaK substrate complex in the ADP state correlate well with the respective K_d values in the ATP state (Mayer *et al.,* 2000). This demonstrates the functional relevance of the K_d values determined for DnaK-substrate complexes in the ADP state.

Chaperone Activity of DnaK Requires Threshold Affinity for Substrates

It has been a challenging proposal by Rapoport and coworkers that Hsp70 chaperones in general act as unspecific traps which bind to almost any peptide provided that a DnaJ protein is in close proximity of the chaperone and its substrate (Misselwitz *et al.,* 1998; Matlack *et al.,* 1999). This proposal is based on surface plasmon resonance spectroscopic investigations in which the BiP homolog of *S. cerevisiae*, Kar2, was found to associate in an ATP-dependent manner with a large variety of peptides and proteins that were coimmobilized with the J-domain of Sec63, the membrane-bound ER homolog of DnaJ (Misselwitz *et al.,* 1998). In the absence of the J-domain an interaction was not observed for most of the substrates.

However, recent findings indicate that DnaK does not act as an unspecific trap (Mayer *et al.,* 2000). Using a range of DnaK mutant proteins with incrementally decreased affinities for a protein substrate, the *E. coli* heat shock transcription factor σ^{32}, it was shown that the efficiency by which σ^{32} stimulated the ATPase rate of DnaK in the presence of DnaJ tightly correlated with the affinity for σ^{32}. Below a certain threshold affinity for the substrate (K_d in the ADP state above approx. 7 μM; in the ATP state above 100 μM) the ATP hydrolysis step of the functional cycle of DnaK cannot be stimulated by the substrate and DnaJ. A similar correlation was found between the efficiency of luciferase refolding by the DnaK system and the affinity of DnaK for substrates. These data establish that the affinity of DnaK for the substrate is a key determinant for ATP hydrolysis and therefore the entire chaperone cycle.

Concerning the importance of high affinity sites within the substrates for the chaperone activity of eukaryotic Hsp70 proteins the situation is more complicated. The high conservation of the involved structures would argue for high similarities in all major aspects of the dynamics of the Hsp70 substrate binding domain and the Hsp70-substrate interactions. On the other hand, a conserved mechanism which relies on the high affinity interaction of the substrate binding cavity with hydrophobic substrate side chains would explain the high degree of evolutionary conservation of the substrate interacting amino acids forming the hydrophobic binding cavity (Rüdiger *et al.,* 1997a). This high conservation (except for the arch-forming residues) is difficult to rationalize if Hsp70 proteins would generally act as an unspecific trap. However, the individual threshold affinity may vary for different Hsp70 homologs. In particular, it is possible that Hsp70 homologs involved in protein translocation into the endoplasmic reticulum and mitochondria (BiP, Ssc-1) may have a lower threshold affinity. These Hsp70s are positioned through action of membrane bound cochaperones (Sec63, Tim44) in such close neighborhood to the incoming nascent chains that the local substrate concentration is extremely high.

Substrate Release and Chaperone Action

The cycle of substrate binding and release is completed by ADP dissociation and subsequent ATP binding, whereby DnaK is converted to the low-affinity state allowing substrate release. The ADP dissociation step is stimulated 5000-fold at saturating conditions by the nucleotide exchange factor, GrpE (Liberek *et al.,* 1991; Jordan and McMacken 1995; McCarty *et al.,* 1995; Packschies *et al.,* 1997). One of the intriguing questions is how the DnaK chaperone system prevents rebinding of the released substrate which is still in non-native conformation (Buchberger *et al.,* 1996). Such rapid rebinding may prevent the protein substrate to continue folding to the native state. There are at least three possible and not mutually exclusive mechanisms. First, in the course of the chaperone cycle, DnaJ dissociates from the DnaK-substrate complex before nucleotide exchange. Such 'catalytic' action of DnaJ is indicated by the requirement of only substoichiometric concentrations of DnaJ for DnaK-dependent folding reactions (Liberek *et al.,* 1995; Pierpaoli *et al.,* 1998b; Laufen *et al.,* 1999), the low DnaJ concentrations *in vivo* relative to the DnaK concentration (1/10 to 1/30; Tomoyasu *et al.,* 1998) and the low affinity of the J-domain for DnaK (10 to 30 μM; Wall *et al.,* 1994; Karzai and McMacken 1996; Greene *et al.,* 1998).

The second mechanism was proposed by Christen and coworkers and involves two intermediates of DnaK (Gisler *et al.,* 1998). The first intermediate releases the bound substrate upon ATP binding in a 'catapult-like' mechanism. The second intermediate results from subsequent slower conversion into a conformation which enables DnaK to rebind substrates. This hypothesis is based on the following observations. The off-rate constants of DnaK interacting with several different peptides determined under equilibrium conditions (by titrating preformed DnaK·ATP complexes to the peptide) revealed large differences between the peptides tested. On the contrary, off-rates determined under non-equilibrium conditions (by adding ATP to nucleotide-free DnaK-peptide complexes) were within the same range, disclosing for some peptides an up to 14 000-fold difference between the two rate determinations (Gisler *et al.,* 1998). The authors concluded that the DnaK·ATP·peptide complex formed from DnaK·ATP plus peptide differs from the complex formed by mixing DnaK·peptide with ATP.

The third hypothesis assumes that DnaK induces conformational changes in the substrates which prevent rapid rebinding. Before binding to DnaK the substrate is in a slow folding state or a kinetic folding trap. Upon interaction with DnaK the substrate conformation would be changed and the intermediate were able to fold much faster obscuring the binding sites of DnaK. Such DnaK-induced conformational transition would also provide an explanation for the ability of DnaK to actively promote the refolding and disaggregation of misfolded proteins (Pierpaoli *et al.,* 1997; Diamant *et al.,* 2000).

Despite the wealth of data describing the DnaK-substrate interaction, the basic question of how DnaK promotes refolding of misfolded proteins is still unanswered.

References

Alfano, C., and McMacken, R. (1989). Heat shock protein-mediated disassembly of nucleoprotein structures is required for the initiation of bacteriophage lambda DNA replication. J. Biol. Chem. *264*, 10709 – 10718.

Blond-Elguindi, S., Cwirla, S.E., Dower, W.J., Lipshutz, R.J., Sprang, S.R., Sambrook, J.F., and Gething, M.-J.H. (1993). Affinity panning of a library of peptides displayed on bacteriophages reveals the binding specificity of BiP. Cell *75*, 717 – 728.

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.

Deuerling, E., Schulze-Specking, A., Tomoyasu, T., Mogk, A., and Bukau, B. (1999). Trigger factor and DnaK cooperate in folding of newly synthesized proteins. Nature *400*, 693 – 696.

Diamant, S., Peres Ben-Zvi, A., Bukau, B., and Goloubinoff, P. (2000). Size-dependent disaggregation of stable protein aggregates by the DnaK chaperone machinery. J. Biol. Chem., in press.

Ewalt, K.L., Hendrick, J.P., Houry, W.A., and Hartl, F.U. (1997). *In vivo* observation of polypeptide flux through the bacterial chaperonin system. Cell *90*, 491 – 500.

Flynn, G.C., Pohl, J., Flocco, M.T., and Rothman, J.E. (1991). Peptide-binding specificity of the molecular chaperone BiP. Nature *353*, 726 – 730.

Gamer, J., Multhaup, G., Tomoyasu, T., McCarty, J.S., Rüdiger, S., Schönfeld, H.-J., Schirra, C., Bujard, H., and Bukau, B. (1996). A cycle of binding and release of the DnaK, DnaJ and GrpE chaperones regulates activity of the *E. coli* heat shock transcription factor σ^{32}. EMBO J. *15*, 607 – 617.

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.

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

Goloubinoff, P., Mogk, A., Peres Ben Zvi, A., 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.

Gragerov, A.I., Martin, E.S., Krupenko, M.A., Kashlev, M.V., and Nikiforov, V.G. (1991). Protein aggregation and inclusion body formation in *Escherichia coli rpoH* mutant defective in heat shock protein induction. FEBS Lett. *291*, 222 – 224.

Gragerov, A., Nudler, E., Komissarova, N., Gaitanaris, G., Gottesman, M., and Nikiforov, V. (1992). Cooperation of GroEL/GroES and DnaK/DnaJ heat shock proteins in preventing protein misfolding in Eschrichia coli. Proc. Natl. Acad. Sci. USA *89*, 10341 – 10344.

Gragerov, A., Zeng, L., Zhao, X., Burkholder, W., and Gottesman, M.E. (1994). Specificity of DnaK-peptide binding. J. Mol. Biol. *235*, 848 – 854.

Greene, M.K., Maskos, K., and Landry, S.J. (1998). Role of the J-domain in the cooperation of Hsp40 with Hsp70. Proc. Natl. Acad. Sci. USA *95*, 6108 – 6113.

Hesterkamp, T., Hauser, S., Lütcke, H., and Bukau, B. (1996). *Escherichia coli* trigger factor is a prolyl isomerase that associates with nascent polypeptide chains. Proc. Natl. Acad. Sci. USA *93*, 4437 – 4441.

Hesterkamp, T., and Bukau, B. (1998). Role of the DnaK and HscA homologs of Hsp70 chaperones in protein folding in *E. coli*. EMBO J. *17*, 4818 – 4828.

Houry, W.A., Frishman, D., Eckerskorn, C., Lottspeich, F., and Hartl, F.U. (1999). Identification of *in vivo* substrates of the chaperonin GroEL. Nature *402*, 147 – 154.

Jordan, R., and McMacken, R. (1995). Modulation of the ATPase activity of the molecular chaperone DnaK by peptides and the DnaJ and GrpE heat shock proteins. J. Biol. Chem. *270*, 4563 – 4569.

Karzai, A.W., and McMacken, R. (1996). A bipartite signaling mechanism involved in DnaJ-mediated activation of the *Escherichia coli* DnaK protein. J. Biol. Chem. *271*, 11236 – 11246.

Kraulis, P.J. (1991). MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures. J. Appl. Cryst. *24*, 946 – 950.

Kusukawa, N., and Yura, T. (1988). Heat shock protein GroE of *Escherichia coli*: key protective roles against thermal stress. Genes Dev. *2*, 874 – 882.

Laufen, T., Mayer, M.P., Beisel, C., Klostermeier, D., Reinstein, J., and Bukau, B. (1999). Mechanism of regulation of Hsp70 chaperones by DnaJ co-chaperones. Proc. Natl. Acad. Sci. USA *96*, 5452 – 5457.

Liberek, K., Marszalek, J., Ang, D., Georgopoulos, C., and Zylicz, M. (1991). *Escherichia coli* DnaJ and GrpE heat shock proteins jointly stimulate ATPase activity of DnaK. Proc. Natl. Acad. Sci. USA *88*, 2874 – 2878.

Liberek, K., Wall, D., and Georgopoulos, C. (1995). The DnaJ chaperone catalytically activates the DnaK chaperone to preferentially bind the σ^{32} heat shock transcriptional regulator. Proc. Natl. Acad. Sci. USA *92*, 6224 – 6228.

Matlack, K.E.S., Misselwitz, B., Plath, K., and Rapoport, T.A. (1999). BiP acts as a molecular ratchet during posttranslational transport of prepro-α factor across the ER membrane. Cell *97*, 553 – 564.

Mayer, M.P., Laufen, T., Paal, K., McCarty, J.S., and Bukau, B. (1999). Investigation of the interaction between DnaK and DnaJ by surface plasmon resonance. J. Mol. Biol. *289*, 1131 – 1144.

Mayer, M.P., Schröder, H., Rüdiger, S., Paal, K., Laufen, T., and Bukau, B. (2000). Multistep mechanism of substrate binding determines chaperone activity of Hsp70. Nature Struct. Biol. *7*, 586 – 593.

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.

McCarty, J.S., Rüdiger, S., Schönfeld, H.-J., Schneider-Mergener, J., Nakahigashi, K., Yura, T., and Bukau, B. (1996). Regulatory region C of the *E. coli* heat shock transcription factor, σ^{32}, constitutes a DnaK binding site and is conserved among eubacteria. J. Mol. Biol. *256*, 829 – 837.

Merritt, E.A., and Murphy, M.E.P. (1994). Raster3D Version 2.0. A program for photorealistic molecular graphics. Acta Cryst. *D50*, 869 – 873.

Misselwitz, B., Staeck, O., and Rapoport, T.A. (1998). J proteins catalytically activate Hsp70 molecules to trap a wide range of peptide sequences. Mol. Cell *2*, 593 – 603.

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

Morshauser, R.C., Hu, W., Wang, H., Pang, Y., Flynn, G.C., and Zuiderweg, E.R.P. (1999). High-resolution solution structure of the 18 kDa substrate-binding domain of the mammalian chaperone protein Hsc70. J. Mol. Biol. *289*, 1387 – 1403.

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.

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.

Pellecchia, M., Montgomery, D.L., Stevens, S.Y., Vander Kooi, C.W., Feng, H., Gierasch, L.M., and Zuiderweg, E.R.P. (2000). Structural insights into substrate binding by the molecular chaperone DnaK. Nature Struct. Biol. *7*, 298 – 303.

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.

Pierpaoli, E.V., Gisler, S.M., and Christen, P. (1998a). Sequence-specific rates of interaction of target peptides with the molecular chaperones DnaK and DnaJ. Biochemistry *37*, 16741 – 16748.

Pierpaoli, E.V., Sandmeier, E., Schönfeld, H.-J., and Christen, P. (1998b). Control of the DnaK chaperone cycle by substoichiometric concentrations of the co-chaperones DnaJ and GrpE. J. Biol. Chem. *273*, 6643 – 6649.

Rüdiger, S., Buchberger, A., and Bukau, B. (1997a). Interaction of Hsp70 chaperones with substrates. Nature Struct. Biol. *4*, 342 – 349.

Rüdiger, S., Germeroth, L., Schneider-Mergener, J., and Bukau, B. (1997b). Substrate specificity of the DnaK chaperone determined by screening cellulose-bound peptide libraries. EMBO J. *16*, 1501 – 1507.

Schmid, D., Baici, A., Gehring, H., and Christen, P. (1994). Kinetics of molecular chaperone action. Science *263*, 971 – 973.

Schröder, H., Langer, T., Hartl, F.-U., and Bukau, B. (1993). DnaK, DnaJ, GrpE form a cellular chaperone machinery capable of repairing heat-induced protein damage. EMBO J. *12*, 4137 – 4144.

Skowyra, D., Georgopoulos, C., and Zylicz, M. (1990). The *E. coli dnaK* gene product, the Hsp70 homolog, can reactivate heat-inactivated RNA polymerase in an ATP hydrolysis-dependent manner. Cell *62*, 939 – 944.

Stoller, G., Ruecknagel, K.P., Nierhaus, K.H., Schmid, F.X., Fischer, G., and Rahfeld, J.-U. (1995). A ribosome-associated peptidyl-prolyl *cis/trans* isomerase identified as the trigger factor. EMBO J. *14*, 4939 – 4948.

Teter, S.A., Houry, W.A., Ang, D., Tradler, T., Rockabrand, D., Fischer, G., Blum, P., Georgopoulos, C., and Hartl, F.U. (1999). Polypeptide flux through bacterial Hsp70: DnaK cooperates with trigger factor in chaperoning nascent chains. Cell *97*, 755 – 765.

Tomoyasu, T., Ogura, T., Tatsuta, T., and Bukau, B. (1998). Levels of DnaK and DnaJ provide tight control of heat shock gene expression and protein repair in *E. coli*. Mol. Microbiol. *30*, 567 – 581.

Ungewickell, E., Ungewickell, H., and Holstein, S.E.H. (1997). Functional interaction of the auxilin J domain with the nucleotide- and substrate-binding modules of Hsc70. J. Biol. Chem. *272*, 19594 – 19600.

Wall, D., Zylicz, M., and Georgopoulos, C. (1994). The NH_2-terminal 108 amino acids of the *Escherichia coli* DnaJ protein stimulate the ATPase activity of DnaK and are sufficient for λ replication. J. Biol. Chem. *269*, 5446 – 5451.

Wang, H., Kurochkin, A.V., Pang, Y., Hu, W., Flynn, G.C., and Zuiderweg, E.R.P. (1998). NMR solution structure of the 21 kDa chaperone protein DnaK substrate binding domain: a preview of chaperone-protein interaction. Biochemistry *37*, 7929 – 7940.

Wild, J., Rossmeissl, P., Walter, W.A., and Gross, C.A. (1996). Involvement of the DnaK-DnaJ-GrpE chaperone team in protein secretion in *Escherichia coli*. J. Bacteriol. *178*, 3608 – 3613.

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

Ziemienowicz, A., Skowyra, D., Zeilstra-Ryalls, J., Fayet, O., Georgopoulos, C., and Zylicz, M. (1993). Both the *Escherichia coli* chaperone systems GroEL/Gro/ES and DnaK/DnaJ/GrpE, can activate heat treated RNA polymerase. Different mechanisms for the same Activity. J. Biol. Chem. *268*, 25425 – 25431.

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.

Zylicz, M., Ang, D., Liberek, K., and Georgopoulos, C. (1989). Initiation of λ DNA replication with purified host- and bacteriophage-encoded proteins: the role of the DnaK, DnaJ and GrpE heat shock proteins. EMBO J. *8*, 1601 – 1608.